Lanthanide-Containing Microgels Intended for Bead-Based Bioassays by Mass Cytometry

By

Wanjuan Lin

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Chemistry
University of Toronto

© Copyright by Wanjuan Lin, 2013
Lanthanide-Containing Microgels Intended for Bead-Based Bioassays by Mass Cytometry

Wanjuan Lin
Doctor of Philosophy
Graduate Department of Chemistry
University of Toronto
2013

Abstract

This thesis describes the synthesis and characterization of functional polyelectrolyte copolymer microgels intended for bioassays based upon mass cytometry. The microgels are based upon copolymers of N-isopropylacrylamide (NIPAm), N-vinylcaprolactam (VCL) and methacrylic acid (MAA), crosslinked with methylene-bis-acrylamide, poly(NIPAm/VCL/MAA). The microgels were loaded with Ln(III) ions, which were then converted in situ to LnF$_3$ or LnPO$_4$ nanoparticles (NPs). Very specific conditions were required for confining the NPs to the core of the microgels. I used mass cytometry to measure the number and the particle-to-particle variation of Ln ions per microgel. By controlling the amount of LnCl$_3$ added to the neutralized microgels, we could vary the content of individual microgels from ca. $10^6$ to $10^7$ Ln atoms, either in the form of Ln$^{3+}$ ions or Ln NPs.
Leaching profiles of Ln ions from the Ln-containing microgels were measured by traditional ICP-MS. Under neutral or basic conditions, the leakage of Ln ions into the aqueous medium was not significant. In acidic buffer solutions, however, the leakage of metal from Ln$^{3+}$ ion-containing microgels was prominent. On subsequent exposures to buffers, the microgels underwent continuous loss of metals. For LnF$_3$-containing microgels, there was much smaller extent of metal leakage and very small of continuous loss of metal upon subsequent exposures to buffers. For LnPO$_4$-containing microgels, there was very little detectable leakage of metals to the acidic buffers.

In order to test the use of Ln nanoparticle as reagents of enhanced sensitivity in mass cytometry, we employed TmF$_3$ encoded microgels as model cells. In one sample, Tm-encoded poly(NIPAm/VCL/MAA) microgels were coated with streptavidin (SAv) and used as model biomarkers, in quantitative mass cytometric bioassays. The sensitivity of different reagents (including a biotinylated metal cheating polymer, containing 50 Tb$^{3+}$ ions per probe and a biotinylated NaHoF$_4$ NP, containing 15,000 Ho atoms per probe) was examined for detecting and quantifying the number of SAv per microgel by mass cytometry. By using the Bi-PAsp(Tb)$_{50}$ probes, a biomarker level at $\sim$10$^4$ per cell was detected. By using the Bi-NaHoF$_4$ NP probes, a biomarker level as low as $\sim$10$^2$ per cell was quantified.
Acknowledgments

I would like to express my gratitude and appreciation to my supervisor Professor Mitchell A. Winnik for his support, encouragement and guidance throughout this research work. I am also grateful for the opportunity he gave me to be a part of the exciting lanthanide tags project. I gratefully acknowledge and thank Professor Vladimir Baranov, Professor Mark Nitz, Dr. Olga Ornatsky and Professor Walter Richtering for the valuable suggestions, discussions and collaborations. I am also grateful to Professor Eugenia Kumacheva and Professor Dwight Seferos for being on my supervisory committee, and for their valuable insights and helpful discussions.

I would like to thank my friends and colleagues in the Winnik group. Particularly, I want to thank Professor Andrij Pich, Dr. Sheng Dai and Dr. Xiaomei Ma for giving me a lot of help when I started my research project. I owe a debt of gratitude to Dr. Yi Hou, Dr. Yijie Lu, Dr. Ahmed Abdelrahman, Dr. Jieshu Qian, Dr. Dirk Weinrich, and Ms. Yi (Sally) Liang for the fruitful collaborations. It was very enjoyable working with them. I especially thank Yi and Yijie for their important contribution to the success of our project and Dr. Chun Feng, and Mr. Guangyao Zhao for their time and valuable critique of my thesis. To my lab mates, Dr. Lin Jia, Dr. Gerald Guerin, Mr. Peng Liu, Mr. Meng Zhang, Mr. Lemuel Tong, Mr. Issacc Herrera, Mr. Graeme Cambridge and Mr. Hang Zhou for interesting discussions on science.

I feel a deep appreciation and gratefulness for my family members (my parents, my grandparents, and my parents-in-law) for their encouragement and unconditional love. This thesis was made possible by their full support and encouragement and is dedicated to them. I would like to express my love and gratitude to my husband Dr. Ruirui (Raymond) Huang, who has been my spiritual support through times of frustration and joy. Heartfelt thanks to my lovely son Alexander, whose smile and calling me “Mommy” simply make my world full of sunshine each and every day.
# Table of Contents

Abstract ............................................................................................................................. ii

Acknowledgements .......................................................................................................... iv

Table of Contents ............................................................................................................. v

List of Tables .................................................................................................................. xi

List of Figures and Schemes ............................................................................................ xiv

List of Appendices .......................................................................................................... xxviii

Abbreviations ................................................................................................................... xxix

## Chapter 1. General Introduction ................................................................................. 1

1.1 Multiplexed Bioassays .............................................................................................. 1

1.1.1 Cell-Based Assays ............................................................................................... 1

1.1.2 Bead-Based Assays ............................................................................................. 3

1.1.2.1 Bead-based Assays Based on Flow Cytometry ................................................. 3

1.1.2.2 Bead-based Assays Based on Mass Cytometry ................................................ 4

1.2 Introduction to Microgels ......................................................................................... 8

1.2.1 Definition of Microgels ....................................................................................... 8

1.2.2 Microgel Synthesis ............................................................................................. 11

1.2.2.1 Precipitation Polymerization ........................................................................ 11

1.2.2.2 Emulsification ............................................................................................... 13

1.2.2.3 Photolithography ........................................................................................... 14

1.2.2.4 Microfluidic Synthesis ................................................................................... 14

1.2.2.5 Micromolding ............................................................................................... 15

1.2.3 Functional Group Distribution in Polyelectrolyte Microgels ............................... 16

1.2.3.1 Microgels from Homogeneous Nucleation ..................................................... 17

1.2.3.2 Core-Shell Polymer Microgels ....................................................................... 19
1.2.4 Swelling Thermodynamics of Microgel Particles ........................................... 20
  1.2.4.1 Swelling in Neutral Gels .......................................................... 20
  1.2.4.2 Swelling in Ionic Gels .............................................................. 23
1.2.5 Microgel Containing Nanoparticles .......................................................... 25
  1.2.5.1 Growing Nanoparticles Using Microgels as Microreactor ..................... 25
  1.2.5.2 Growing Microgels in the Presence of Preformed Nanoparticles Acting as Seeds... 26
  1.2.5.3 Absorbing Nanoparticles into Microgels ..................................... 27
  1.2.5.4 Layer-by-Layer and Core-Shell Assembly .................................... 28
1.3 Lanthanide Properties .................................................................................. 29
  1.3.1 Lanthanide Complexes with Organic Ligands ...................................... 29
  1.3.2 Inorganic Lanthanide Nanoparticles (LnF₃, LnPO₄, LnVO₄, LnBO₃) ............ 31
1.4 Research Objectives .................................................................................... 33
1.5 Thesis Outline ............................................................................................ 33
Reference .......................................................................................................... 35

Chapter 2. Experimental: Materials, Protocols, Characterization and Data Analysis ........................................................................................................ 41

2.1 Materials .................................................................................................... 41
  2.1.1 Reagents for Particle Synthesis ......................................................... 41
  2.1.2 Reagents for Bioconjugation ............................................................ 42
  2.1.3 Reagents for Bead-Based Bioassays ................................................. 42
2.2 Synthesis of Lanthanide Encoded Microgels as Classifier Beads ............... 42
  2.2.1 Synthesis of Polyelectrolyte Microgels by Precipitation Polymerization .... 42
    2.2.1.1 Poly(NIPAm/VCL/MAA) Microgels ........................................... 43
    2.2.1.2 Poly(NIPAm/MAA/PEGMA) Microgels ................................... 44
  2.2.2 Lanthanide Encoded Microgels ......................................................... 45
    2.2.2.1 Ln³⁺ Ions-Containing Microgels .............................................. 45
    2.2.2.2 LnF₃ Nanoparticle-Containing Microgels ................................ 49
    2.2.2.3 LnPO₄ Nanoparticle-Containing Microgels ............................... 51
2.3 Characterization Methods and Data Analysis ............................................. 53
  2.3.1 Titration ........................................................................................... 53
  2.3.2 Light scattering ................................................................................. 55
2.3.2.1 Dynamic Light Scattering ................................................................. 55
2.3.2.2 Static Light Scattering ................................................................. 59
2.3.3 Mass Cytometry .............................................................................. 61
2.3.4 ICP-MS ......................................................................................... 62
2.3.5 Electron Microscopy ...................................................................... 63
2.3.6 Energy-Dispersive X-Ray Spectroscopy .......................................... 63
2.3.7 UV-Vis Spectrometry ...................................................................... 63
2.3.8 Zeta Potential (ζ) and Electrophoretic Mobility (μe) ......................... 65
2.3.9 NMR .............................................................................................. 66
Reference ................................................................................................. 67

Chapter 3. Ln(III) ions and LnF3 Nanoparticle Containing Microgels as
Classifier Beads for Mass Cytometric Analysis .................................... 68
3.1 Introduction ....................................................................................... 68
3.2 Experimental Procedures ............................................................... 71
3.3 Results and Discussions .................................................................. 72
  3.3.1 Polyelectrolyte Microgels ............................................................. 72
    3.3.1.1 Synthesis of Polyelectrolyte Microgels ................................. 72
    3.3.1.2 Microgel Characterization ................................................. 74
    3.3.1.3 Polyelectrolyte Properties of Copolymer Microgels .......... 77
  3.3.2 Ln (III) Ions- and LnF3 Nanoparticle-Containing Microgels ................ 80
    3.3.2.1 Incorporation of Ln Elements into Microgels via One-Stage in-situ Precipitation... 80
    3.3.2.2 Incorporation of Ln Elements into Microgels via Two-Stage in-situ Precipitation... 88
  3.3.3 Quantitative Determination of Metal Incorporation by Mass Cytometry .......... 90
    3.3.3.1 Singly-Labeled Microgels ................................................. 91
    3.3.3.2 Binary-Labeled Microgels ................................................. 98
  3.4 Conclusions ....................................................................................... 100
Reference ................................................................................................. 103

Chapter 4. Lanthanide Phosphate-Containing Microgels as Classifier Beads
for Mass Cytometric Analysis ................................................................. 105
4.1 Introduction ....................................................................................... 105
6.3.1.1 CdSe-Containing Microgels (MG-CdSe)..........................................................150
6.3.1.2 NaHoF₄-Containing Microgels (MG-NaHoF₄).................................................153
6.3.2 Estimation on Average Mesh Size of Microgels.................................................155
6.3.3 Quantification of Hybrid Microgels by Mass Cytometric Analysis.....................157
  6.3.3.1 CdSe-Containing Microgels (MG-CdSe).......................................................157
  6.3.3.2 NaHoF₄ Nanoparticle-Containing Microgels (MG-NaHoF₄).........................159
6.4 Conclusions...........................................................................................................160
Reference...................................................................................................................162
Appendix to Chapter 6..............................................................................................163

Chapter 7. A High-Sensitivity Lanthanide Nanoparticle Reporter for Mass
Cytometry: Tests on a Model Cell.................................................................164
7.1 Introduction..........................................................................................................165
7.2 Experimental Procedures....................................................................................169
  7.2.1 Materials.........................................................................................................169
  7.2.2 Synthesis.........................................................................................................169
    7.2.2.1 Synthesis of Tm-Encoded Poly(NIPAm/VCL/MAA) Microgels...............169
    7.2.2.2 Synthesis of Streptavidin-Coated Microgels via Covalent Attachment (SAv-
          MG(Tm))........................................................................................................170
    7.2.2.3 Synthesis of BSA-Coated Microgels via Covalent Attachment (BSA-MG(Tm))...171
    7.2.2.4 Synthesis of Biotinylated Microgels (Bi-MG(Tm)).................................171
    7.2.2.5 Synthesis of Streptavidin-Coated Microgels via Formation of a Biotin-SAv
          Complex: SAv-Bi-MG(Tm)........................................................................172
    7.2.2.6 Synthesis of BSA-Coated Microgels via Passive Absorption (BSA/MG(Tm))....172
    7.2.2.7 Synthesis of Biotinylated NaHoF₄ Nanoparticle Probes (Bi-NaHoF₄).........173
    7.2.2.8 Synthesis of a Biotinylated Metal Chelating Polymer Probe (Bi-PAsp(Tb)₅₀)....173
  7.2.3 Mass Cytometric Bioassays.............................................................................174
    7.2.3.1 Streptavidin-Biotin Coupling Assays Employing Bi-PAsp(Tb)₅₀...............174
    7.2.3.2 Biotin-Streptavidin-Biotin Sandwich Assays...........................................174
7.3 Results and Discussions.....................................................................................175
  7.3.1 Covalent Attachment of Streptavidin and BSA to Microgels: SAv-MG(Tm) and BSA-
          MG(Tm)......................................................................................................177
7.3.2 Streptavidin-Coated Microgels via Formation of a Biotin-SA\textsuperscript{v} Complex: SAv-Bi-MG(Tm)……………………………………………………………………………………………………179
7.3.3 Reagents for Biotin-Streptavidin Coupling Bioassays……………………………………180
7.3.4 Biotin-Streptavidin Coupling Bioassays……………………………………………………183
7.3.5 Biotin-SA\textsuperscript{v}-Biotin Sandwich Bioassays………………………………………………189
7.4 Summary and Conclusions……………………………………………………………………195
Reference……………………………………………………………………………………………197
Appendix to Chapter 7………………………………………………………………………………200

Chapter 8 Summary and Future Outlook…………………………………………………………235
8.1 Summary…………………………………………………………………………………………235
8.2 Future Outlook……………………………………………………………………………………238
List of Tables

Chapter 2……………………………………………………………………………………………………………………………………………..41

Table 2-1. Recipes for precipitation polymerization of poly(NIPAm/VCL/MAA) microgels....44
Table 2-2. Recipes for precipitation polymerization of poly(NIPAm/MAA/PEGMA) microgels ...........................................................................................................................................45
Table 2-3. Recipes for synthesis of Eu$^{3+}$ ions encoded poly(NIPAm/VCL/MAA) and poly(NIPAm/MAA/PEGMA) microgel...........................................................................................................................................46
Table 2-4. Synthesis of microgel-Ln composites with different microgel to Ln feed ratios.....47
Table 2-5. Recipes for different Ln-encoded-microgels.........................................................48
Table 2-6. Recipes for microgels containing both Eu$^{3+}$ and Tb$^{3+}$....................................48
Table 2-7. Recipes for in-situ synthesis of EuF$_3$ nanoparticles using poly(NIPAm/VCL/MAA) and poly(NIPAm/MAA/PEGMA) microgel templates...........................................................................................................................................49
Table 2-8. Recipes for different Ln-encoded-microgels.........................................................50
Table 2-9. Recipes for Ln-encoded-microgels prepared by two-stage in-situ precipitation......51
Table 2-10. Recipes for synthesis of EuPO$_4$ nanoparticles using poly(NIPAm/VCL/MAA) microgel templates...........................................................................................................................................52
Table 2-11. Recipes for different Ln-encoded-microgels.....................................................53

Chapter 3…………………………………………………………………………………………………………………………………………68

Table 3-1. Recipes and dimensions of model poly(NIPAm/VCL) microgels prepared under surfactant-free conditions..............................................................................................................................................73
Table 3-2. Copolymer microgels: conversion (8h), $R_h$, PDI, solids content (SC) and acid content ..................................................................................................................................................74
Table 3-3. Hydrodynamic radii of the microgels determined by DLS..................................87
Table 3-4. Eu content of the microgels..................................................................................94
Table 3-5. Characteristics of different Ln-encoded-microgels...........................................97
Table 3-6. Characteristics of Eu-encoded-microgels prepared by multi-step in-situ precipitation. ..................................................................................................................................................98
Table 3-7. Characteristics of different Eu/Tb-encoded-microgels determined by mass cytometry

Chapter 4.................................................................................................................................................105

Table 4-1. Average diffusion coefficients and hydrodynamic radii values of microgels obtained by multi-angular dynamic light scattering..................................................................................118

Table 4-2. Characteristics of different Ln-encoded-microgels.................................................................122

Chapter 5..................................................................................................................................................128

Table 5-1. Characteristics of sedimented microgel particles after ageing in buffer solutions for 200 min.................................................................134

Table 5-2. Tb release from microgels into acidic buffer solutions after repeated centrifugation and redispersion with fresh buffer.............................................................135

Table 5-3. Characteristics of sedimented microgel particles after aging in buffer solutions for one week.................................................................................142

Table 5-4. Stability of lanthanide-containing microgels towards metal leaching..................145

Chapter 7.................................................................................................................................................164

Table 7-1. Synthesis of biotinylated Tm-encoded microgels .................................................172

Appendix to Chapter 7..........................................................................................................................200

Table A7-1. UV-Vis absorbance of HABA/Avidin and HABA/Avidin/Biotin at 500 nm and conversion of biotinylation reaction on microgels.................................................................220

Table A7-2. Average diffusion coefficient and hydrodynamic radii values of microgels obtained from multi-angular dynamic light scattering............................................................224

Table A7-3. Estimated polymer volume fraction ($\alpha^3$) and average mesh size ($\zeta$) within hybrid microgels..................................................................................................................228
Table A7-4. Apparent zeta potentials and electrophoretic mobilities for proteins and microgel composites

.................................................................................................................................................229
List of Figures and Schemes

Chapter 1......................................................................................................................1

Figure 1-1. A schematic illustration of mass cytometry analysis on beads or cells.................2
Figure 1-2. Comparison of fluorescence spectra from 8 Alex dyes with ICP-MS spectra from 30
enriched stable isotopes from lanthanides.................................................................3
Figure 1-3. A schematic representation of analyte detection by a lanthanide barcoded bead and a
metal tag containing detection reporter.................................................................7
Figure 1-4. Structures of crosslinking monomers.............................................................10
Scheme 1-1. Mechanism for the preparation of PNIPAm-based microgels via the surfactant-free
radical precipitation polymerization.........................................................................12
Figure 1-5. Schematic representation of microgel preparation by radical crosslinking
polymerization in (inverse) miniemulsion [45]: (a) emulsification and homogenization, (b)
polymerization, (c) removal of excess surfactant by washing/dialysis and subsequent freeze-
drying and (d) redispersion of microgels in a good solvent for the network-forming polymer
by swelling. Reproduced by permission from [45]; copyright 2012 by Elsevier............13
Figure 1-6. Fabrication process of a 2D monolayer of gel beads by the double-template
polymerization method. The optical micrograph illustrates the product gel beads. Reproduced
by permission from Ref. [49]; copyright 2005 by the American Chemical Society.........14
Figure 1-7. Production of monodisperse pNIPAm microgels from macromolecular precursors
using a PDMS microfluidic device.(A) A cross-junction channel serves to form
monodisperse pre-microgel droplets from a semidilute precursor solution, which are exposed
to strong UV light as they flow through a basin channel to solidify them. (B) Monodisperse
microgels obtained from the experiment in Panel A. All scalebars denote 200 μm.
Reproduced from Ref. [54] by permission of Elsevier 2010......................................15
Figure 1-8. Schematic diagram of the HA micromolding process. PDMS molds were used to
mold a layer of HA into the void regions of the stamp. The polymer was then cured with
exposure to UV light to fabricate HA microstructures. To fabricate HA microstructures
without cells, a thin polymer film on the substrate was molded (A). To fabricate HA
microstructures that encapsulated cells (B) the HA solution was transferred from the PDMS
mold onto the substrate and subsequently crosslinked. Reproduced from Ref. [55] by permission of John Wiley and Sons. 

**Figure 1-9.** A schematic diagram of different approaches for preparing microgels containing nanoparticles. Reproduced by permission from Ref. [88,89,91]. 

**Figure 1-10.** Ligand structure for Lanthanide chelates. Notation: EGTA: Ethylene bis (oxyethylenenitrilo)tetraacetic acid [115]; DTPA: Diethylenetriaminopentaacetic acid [115]; OA: Oleic acid [118]; DOTA: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid [119]; EDTA: Ethylenediaminotetraacetic acid [113-114]; BPTA: N,N,N’,N’-[2,6-bis(3’-aminomethyl-1’-pyrazolyl)-4-phenylpyridine] tetrakis(acetic acid) [120]; DHDA: N,N’-bis(2,4-dihydroxobenzylidene) 1,2-diaminobenzene [121]; FSA: 5-Fluorosalicylic acid [122]; CA: Citric acid [123]; BCPDA: 4,7-Bis(chlorosulfophenyl)-1,10 penanthroline-2,9-dicarboxylic acid [124]; ADDP: Ammonium di-n-octadecyldithiophosphate [106; EGMAP: Ethyleneglycol methacrylate phosphate [125]. 

**Chapter 2.** 

**Figure 2-1.** Functions and structures of reactants. 

**Figure 2-2.** Potentiometric and conductivity titration curves of V27 microgel solution. 

**Figure 2-3.** Plots of decay rates ($\gamma$) (ms$^{-1}$) vs scattering vector squared ($q^2$) (nm$^{-2}$), where the straight line in each plot represents the linear fitting through scattering data from 30°, 45° and 60° toward the origin. The average diffusion coefficient can be obtained from the slopes of the linear regression lines. 

**Figure 2-4.** CONTIN plots of V27-TmF$_3$ microgels from multi-angular dynamic light scattering. 

**Figure 2-5.** Log-normal distribution of molecular weight, reproduced from Ref. [9] by permission of John Wiley & Sons. 

**Figure 2-6.** A cartoon illustration of microgel structure with density decrease with increasing distance to the core. 

**Figure 2-7.** Cartoon schematic of the mass cytometry profiling of immune cells. The cells are nebulized into single-cell droplets, and an elemental mass spectrum is acquired for each.
Reproduced from Ref. [13] by permission of the American Association for the Advancement of Science

**Figure 2-8.** A cartoon reaction scheme between biotinylated protein and HABA/Avidin complex reproduced from Ref. [15].

**Figure 2-9.** Schematic representation of zeta potential, reproduced from Ref. [17].

**Chapter 3**

**Figure 3-1.** Kinetics study: formation of Poly(NIPAm/VCL/MAA) microgels with MAA feed ratio ranging from 10 mol % to 38 mol %. The notation indicates the comonomer (V = VCL) and the number indicates the mole fraction of MAA in the reaction.

**Figure 3-2.** CONTIN plots of the DLS data: (a) poly(NIPAm/VCL/MAA) microgels with MAA feed ratio ranging from 10 mol % to 38 mol %. (b) poly(NIPAm/MAA/PEGMA) microgels with MAA feed ratio ranging from 3 mol% to 23 mol %. The notation indicates the comonomer (V = VCL, PG = PEGMA) and the number indicates the mole fraction of MAA in the reaction.

**Figure 3-3.** pH and conductometric titration curves for poly(NIPAm/VCL/MAA) microgel solutions.

**Figure 3-4.** pH and conductometric titration curves for poly(NIPAm/MAA/PEGMA) microgel solutions.

**Figure 3-5.** Electrophoretic mobility ($\mu_e$) vs pH for microgel particles.

**Figure 3-6.** Plots of the degree of ionization ($\alpha_d$) vs pH for: A) poly(NIPAm/VCL/MAA) microgels; B) poly(NIPAm/MAA/PEGMA) microgels. The red dashed lines indicate the degree of ionization for microgels at pH 6.

**Figure 3-7.** Plots of $pK_a$ vs degree of ionization ($\alpha_d$) for microgels: a) poly(NIPAm/VCL/MAA) microgels; b) poly(NIPAm/MAA/PEG) microgels. The red dashed arrows represent the largest $pK_a$ change in each type of microgel over the titration range from 10% to 90%.

**Scheme 3-1.** Concept for loading of Ln nanoparticles into carboxylated microgels containing significant levels of methacrylic acid (MAA) as a comonomer.

**Figure 3-8.** TEM images of EuF$_3$ NPs generated with microgel templates under different pH conditions: EuF$_3$ obtained from pH 6 solution (A and D), bis-tris buffer (B and E) and pH 9.
solution (C and F). A) PG17 microgel solution was neutralized to pH 6 by 0.1 M NaOH solution. B) PG17 microgel solution was combined with 10 molar eq. of pH 6.28 bis-tris buffer solution. C) PG17 microgel solution was pre-treated with 1 eq. of NaOH according to the amount of MAA determined by titration. D) V27 microgel solution was neutralized to pH 6 by 0.1 M NaOH solution. E) V27 microgel solution was combined with 10 molar eq. of pH 6.28 bis-tris buffer solution. F) V27 microgel solution was pre-treated with 1 eq. of NaOH according to the amount of MAA determined by titration.

Figure 3-9. Elemental analysis by EDX. A) Linear scan of two PG17 microgels containing EuF₃. B) Linear scan of two V27 microgels containing EuF₃. The microgel sample was treated with 1 eq. of NaOH per –COOH, and then with 1/3 molar eq. of EuCl₃ solution. Subsequently, 3 eq. of a NaF solution per Eu³⁺ was injected to precipitate EuF₃.

Figure 3-10. CONTIN plots of the hybrid microgels: (A) sample V27: top to bottom: at pH 7, at pH 9, after addition of Eu³⁺ (1 Eu²⁺ for every 3 –COO⁻), after addition of NaF (3 F⁻ for every Eu³⁺). (B) similar data for microgel sample PG17.

Scheme 3-2. Concept for loading of LnF₃ nanoparticles into carboxylated microgels containing significant levels of methacrylic acid (MAA) as a comonomer by two-stage in-situ precipitation process.

Figure 3-11. DLS CONTIN plots from measurements on microgel solutions from each step of the two-stage in-situ precipitation process to prepare EuF₃-labeled samples. 0-a: as-prepared microgels at pH 7 before neutralization with NaOH; 0-b: microgels after neutralization with 1 eq. NaOH; I-a: ion exchanged microgels after treatment with EuCl₃; I-b: EuF₃ nanoparticle-containing microgels after the first stage treatment with NaF; II-a: ion exchanged microgels obtained by second stage treatment with EuCl₃ solution; II-b: EuF₃ nanoparticle-containing microgels from the second stage.

Figure 3-12. A mass cytometry screen capture for V27-EuCl₃ microgels. The x-axis corresponds to m/z of ions reaching the detector. The y-axis reports successive mass spectra. Each horizontal bar highlights one microgel event.

Figure 3-13. Histograms of ¹⁵¹Eu distribution from Eu³⁺ ions and EuF₃ nanoparticle encoded microgels.

Figure 3-14. Box-and-whisker plots displaying the distribution of Eu signal intensity for hybrid microgels determined by mass cytometry. Data obtained from V27 microgels are presented in the first three columns; data from PG17 microgels are presented in the last three columns.
PG17-Eu and V27-Eu refer to the microgels at pH 9 to which EuCl$_3$ was added. PG17-EuF$_3$ (NaF) and V27-EuF$_3$ (NaF) refer to Eu-containing microgels to which NaF was added. PG17-EuF$_3$ (NH$_4$F) and V27-EuF$_3$ (NH$_4$F) refer to microgel samples in which NH$_4$F was added. Values of the number of Eu atoms per microgel were calculated using the mass cytometry transmission efficiency for Eu ions of $7.91 \times 10^{-5}$.

**Figure 3-15.** Box-and-whisker plots of mass cytometry distribution of Eu signal intensity for hybrid V27 microgels synthesized with increasing Eu : COOH feed ratios. The transmission efficiency of mass cytometry for the Eu ions was $7.91 \times 10^{-5}$. Using this value, the atoms per microgel were calculated and are presented on the right-hand y-axis.

**Figure 3-16.** Box-and-whisker plots distribution of number of Ln atoms per hybrid microgel containing different Ln elemental tags, as determined by mass cytometry. On the x-axis, the elements (La, Nd, Eu, Tb, Ho and Tm) refer to metal containing microgels obtained by ion exchange, whereas LaF$_3$, NdF$_3$, EuF$_3$, TbF$_3$, HoF$_3$ and TmF$_3$ refer to microgels containing LnF$_3$ nanoparticles. The mass cytometry transmission efficiencies for La ions was $1.85 \times 10^{-5}$; for Nd ions, $2.71 \times 10^{-5}$; for Eu ions, $3.83 \times 10^{-5}$; for Tb ions, $6.24 \times 10^{-5}$; for Ho ions, $5.69 \times 10^{-5}$, and for Tm ions, $4.84 \times 10^{-5}$.

**Figure 3-17.** Box-and-whisker plots of the distribution of number of Eu atoms per microgel prepared by two-stage in-situ precipitation as determined by mass cytometry.

**Figure 3-18.** Box-and-whisker plots of mass cytometry distribution of Eu (upper plots) and Tb (lower plots) atoms per binary encoded microgels prepared with different Eu/Tb feed ratios.

**Chapter 4**

**Scheme 4-1.** Concept for loading of LnF$_3$ or LnPO$_4$ nanoparticles into carboxylated microgels containing methacrylic acid (MAA) as a comonomer by in-situ precipitation.

**Figure 4-1.** TEM images of EuPO$_4$ nanostructures generated with microgel templates under different conditions. A) The microgel solution was pretreated with 1 eq. of NaOH, then with 1/3 molar eq. of EuCl$_3$ solution, i.e. formation of V27(Eu). Subsequently, 1/3 molar eq. of NaH$_2$PO$_4$ was injected to precipitate EuPO$_4$; B) The microgel solution was first mixed with 10 mol% succinic acid before adding 1 eq. of NaOH per MAA. Then 1/3 molar eq. of EuCl$_3$
solution was added. Subsequently, 1/3 molar eq. of NaH$_2$PO$_4$ was injected to precipitate EuPO$_4$; C) The microgel solution was first mixed with 10 mol% citric acid before adding 1 eq. of NaOH per MAA, then with 1/3 molar eq. of EuCl$_3$ solution. Subsequently, 1/3 molar eq. of NaH$_2$PO$_4$ was injected to precipitate EuPO$_4$; D) To a solution of V27(Eu), 1/3 molar eq. of Na$_2$HPO$_4$ was injected to precipitate EuPO$_4$.

Figure 4-2. TEM images of EuPO$_4$ containing microgels generated using PBS buffer as the precipitating reagent (V27-EuPO$_4$). A: a dark field TEM image; B: an enlarged image of a hybrid microgel under bright field TEM; C: an enlarged image of a different hybrid microgel under dark field TEM; D: a high magnification image of the selected area in C. A few of the many electron-rich nanostructures are marked with red arrows.

Figure 4-3. Electron diffraction X-ray (EDX) analysis of EuPO$_4$ containing microgels: V27-EuPO$_4$. Elemental profiles of Europium, Phosphorus and Lanthanum were obtained by recording the EDX signal along the yellow solid line as shown in the upper left figure.

Figure 4-4. CONTIN plots of the hybrid microgels determined by dynamic light scattering at 45° scattering angle at 295 K. V27-COOH: microgel solution was adjusted with 0.1 M NaOH to pH 7. V27-COO(-): microgel solution was fully neutralized with 1 eq. of NaOH solution. V27(Eu): microgel solution was first neutralized with 1 eq. of NaOH, and then ion exchanged with 1 eq. of Eu$^{3+}$ for every 3 eq. of –COO$^-$. V27-EuPO$_4$: microgel solution was first neutralized with 1 eq. of NaOH, and then ion exchanged with 1 eq. of Eu$^{3+}$ for every 3 eq. of –COO$^-$, after that incubated with 10 eq. of PBS buffer (0.1 M, pH 7.0) overnight. The concentration of microgels was ca. 0.02 mg/mL.

Figure 4-5. Plots of the autocorrelation (first cumulant) decay rates (ms$^{-1}$) vs $q^2$(nm$^{-2}$), where the straight line in each plot represents the linear fit of the scattering data from 30°, 45° and 60° toward the origin. The scattering data at 90° was not included in the fitting. These data points are considered to be unreliable, because of the low value of the form factor at this scattering angle. The average diffusion coefficient was obtained from the slopes of the linear regression lines.

Figure 4-6. TEM images of different LnPO$_4$-containing microgels. A: V27-LaPO$_4$; B: V27-NdPO$_4$; C: V27-EuPO$_4$; D: V27-TbPO$_4$; E: V27-HoPO$_4$; F: V27-TmPO$_4$. The scale bars are 800 nm. In C, D, and F, there appears to be a small amount of LnPO$_4$ outside the confines of the microgel.
Figure 4-7. Mass cytometry screen captures for different V27-LnPO$_4$ microgels (Ln = La, Nd, Tb, Eu, Tm, Ho). The Ln$^{3+}$ ions in the ionexchanged microgel samples were converted into LnPO$_4$ using 10 eq. of PBS buffer solution as a precipitant.........................121

Figure 4-8. Box-and-whisker plots distribution of number of Ln atoms per hybrid microgel containing different Ln elemental tags, as determined by mass cytometry. On the x-axis, the elements (La, Nd, Eu, Tb, Ho and Tm) refer to LnPO$_4$ containing microgels obtained with PBS buffer. The mass cytometry transmission efficiencies for La ions was 2.14 x 10$^{-5}$; for Nd ions, 2.58 x 10$^{-5}$; for Eu ions, 3.48 x 10$^{-5}$; for Tb ions, 5.49 x 10$^{-5}$; for Ho ions, 3.78 x 10$^{-5}$, and for Tm ions, 3.19 x 10$^{-5}$...............................122

Appendix to Chapter 4..................................................126

Scheme 4A-1 Structure of succinic acid and citric acid.................................126

Figure 4A-1. Elemental analysis by EDX. A linear scan of Eu containing microgels with the presence of 10 mol% of succinic acid before adding phosphorous precipitant. The microgel was first combined with 10 mol% of succinic acid before pretreated with 1 molar eq. of NaOH solution, and then 1/3 molar eq. of EuCl$_3$ was combined.........................126

Figure 4A-2. Elemental analysis by EDX. A map scan of Eu containing microgels with the presence of 10 mol% of citric acid before adding phosphorous precipitant. The microgel was first combined with 10 mol% of citric acid before pretreated with 1 molar eq. of NaOH solution, and then 1/3 molar eq. of EuCl$_3$ was combined...............................127

Chapter 5.................................................................128

Figure 5-1. Ion release profiles of Ln$^{3+}$ ions from MG-Ln particles at 0.005 wt% concentration into different buffer media: MES buffer (0.1 M, pH 4.7), Bis-tris buffer (0.03 M, pH 6.1), HEPES buffer (0.01 M, pH 7.4), PBS buffer (0.01 M, pH 7.4), AmAc buffer (0.01 M, pH 9.0), and DI water. Samples were centrifuged as described in the man text to sediment the microgels, and then the supernatant was analyzed by ICP-MS...........................................132

Figure 5-2. Leaching of Ln$^{3+}$ ions from MG-Ln into different buffer solutions after stirring for 200 min, as determined by ICP-MS. Buffer media include: MES buffer (0.1 M, pH 4.7), Bis-tris buffer (0.03 M, pH 6.1), HEPES buffer (0.01 M, pH 7.4), PBS buffer (0.01 M, pH 7.4), ammonium acetate buffer (AmAc, 0.01 M, pH 9.0), and DI water.......................133
Figure 5-3. Leaching profile of Tb$^{3+}$ ions from MG-Tb microgels contained within dialysis bags under different pH conditions: pH 4: $10^{-4}$ M HCl; pH 7: DI water; pH 10: $10^{-4}$ M NaOH solution. The concentrations of Tb ions in the solutions outside the dialysis bags were measured by ICP-MS.................................................................136

Figure 5-4. Ion release profiles of Ln$^{3+}$ ions from MG-LnF$_3$ particles at 0.005 wt% concentration into different buffer media: MES buffer (0.1 M, pH 4.7), Bis-tris buffer (0.03 M, pH 6.1), HEPES buffer (0.01 M, pH 7.4), PBS buffer (0.01 M, pH 7.4), Ammonium acetate buffer (AmAc, 0.01 M, pH 9.0), and DI water. Samples were centrifuged as described in the man text to sediment the microgels, and then the supernatant was analyzed by ICP-MS........137

Figure 5-5. Leaching of Ln atoms from MG-LnF$_3$ into different buffers after stirring in buffer for 200 min, as determined by ICP-MS. Buffer media include: MES (0.1 M, pH 4.7), Bis-tris (0.03 M, pH 6.1), HEPES (0.01 M, pH 7.4), PBS (0.01 M, pH 7.4) Ammonium acetate (AmAc, 0.01 M, pH 9.0), and DI water.................................................................138

Figure 5-6. Leaching profile of Tb$^{3+}$ ions from MG-TbF$_3$ microgels contained within dialysis bags under different pH conditions: pH 4: $10^{-4}$ M HCl; pH 7: DI water; pH 10: $10^{-4}$ M NaOH solution. The concentrations of Tb ions in the solutions outside the dialysis bags were measured by ICP-MS.................................................................139

Figure 5-7. Ion release profiles of Ln$^{3+}$ ions from MG-LnPO$_4$ particles at 0.005 wt% concentration into different buffer media: MES buffer (0.1 M, pH 4.7), Bis-tris buffer (0.03 M, pH 6.1), HEPES buffer (0.01 M, pH 7.4), PBS buffer (0.01 M, pH 7.4), Ammonium acetate buffer (AmAc, 0.01 M, pH 9.0), and DI water. Samples were centrifuged at 5000 rpm for 30 min to sediment the microgels, and then the supernatant was analyzed by ICP-MS..141

Figure 5-8. Box-and-whisker plots of mass cytometry distribution of number of Tb atoms per microgels for sedimented MG-Tb microgels from MES buffer (4.7), bis-tris buffer (6.1) and DI water. The microgels were incubated with 0.01 M PBS buffer at pH 7.4 for 200 min and washed with DI water before being resuspended into MES buffer, Bis-tris buffer and DI water........................................................................................................142

Figure 5-9. EDX analysis on Tb-containing microgels. The MG-TbF$_3$ microgels were incubated with PBS buffer (0.01 M, pH 7.4) with stirring for 2 hours then redispersed with DI water. Titanium signals were chosen as an internal blank for the EDX analysis..................144

Chapter 6.................................................................147
Scheme 6-1. Incorporation of oleic acid-capped nanoparticles into MAA functionalized microgels via ligand exchange process

Figure 6-1. Dark field TEM images of CdSe-encoded microgels. The CdSe NPs are with diameter of $d = 4.6$ nm. (A) CdSe-encoded microgels from THF solution (B) CdSe-encoded microgels from H$_2$O solution, (C) CdSe-encoded microgels from dilute H$_2$O solution. This sample is a dilution of the sample used to prepare the grid in (B), (D) a magnified image of a single CdSe-encoded microgel from H$_2$O.

Figure 6-2. Elemental analysis of a CdSe encoded microgel by EDX: linear scan of CdSe encoded microgel from dilute aqueous solution.

Figure 6-3. Confocal images of CdSe encoded microgels, the CdSe NPs are with diameter of $d = 4.6$ nm (A) in water, (B) in THF. The excitation wavelength was set at $\lambda_{\text{ex}} = 488$ nm. The emission peak from CdSe encoded microgels was at $\lambda_{\text{em}} = 594$ nm in THF, and $\lambda_{\text{em}} = 602$ nm in water.

Figure 6-4. TEM images of NaHoF$_4$ nanoparticles and microgels, to which the NaHoF$_4$ nanoparticles are attached. The histograms of NaHoF$_4$ nanoparticle size distribution corresponding to the TEM image of the NPS to the left of the histogram. (A) Hybrid microgels obtained with 33.5 nm NaHoF$_4$/OA nanoparticles after transfer to water; (B) 33.5 nm NaHoF$_4$/OA nanoparticles from hexane; (C) histogram of 33.5 nm NaHoF$_4$/OA nanoparticle size distribution; (D) hybrid microgels obtained with 10.2 nm NaHoF$_4$/OA nanoparticles after transfer to water; (E) 10.2 nm NaHoF$_4$/OA nanoparticles from hexane; (F) histogram of 10.2 nm NaHoF$_4$/OA nanoparticle size distribution; (G) hybrid microgels obtained with 5.1 nm NaHoF$_4$/OA nanoparticles after transfer to water; (H) 5.1 nm NaHoF$_4$/OA nanoparticles from hexane; (I) histogram of 5.1 nm NaHoF$_4$/OA nanoparticle size distribution.

Figure 6-5. A) Isotopic $^{112}$Cd-$^{114}$Cd dot-dot plot of mass cytometry measurements on MG-CdSe hybrid microgels prepared from CdSe QDs with $d = 4.6$ nm; B) Histograms of $^{112}$Cd signal distribution among microgels determined by mass cytometry; C) Histograms of $^{114}$Cd signal distribution among microgels determined by mass cytometry. The data collection was gated to exclude “cell” debris and microgel aggregates. At least 10,000 microgels were analyzed per sample.
Figure 6-6. Box-and-whisker plots displaying the distribution of $^{112}$Cd and $^{114}$Cd signal intensity for hybrid microgels determined by mass cytometry……………………………………………….158

Figure 6-7. A) Histograms of $^{165}$Ho signal distribution of mass cytometry measurement of MG-NaHoF$_4$ hybrid microgels prepared from NaHoF$_4$ NPs with $d = 5.1$ nm. B) Box-and-whisker plots displaying the distribution of $^{165}$Ho signal intensity for NaHoF$_4$ encoded microgels determined by mass cytometry. Data collection was gated to exclude cell debris and cell aggregates. Approximately 10,000 microgels were analyzed……………………………………………….159

Appendix to Chapter 6………………………………………………………………………………………………………163

Figure A6-1. A normalized absorption spectrum of CdSe/OA QDs in toluene solution. The first exitonic absorption peak is at 600 nm………………………………………………………………163

Figure A6-2. A normalized emission spectrum of CdSe encoded microgels in THF and aqueous solutions. The excitation wavelength of 488 nm was used. The emission peak was at 594 nm in THF and 602 nm in water………………………………………………..163

Chapter 7………………………………………………………………………………………………………………….164

Scheme 7-1. Covalent attachment of Bi-NH$_2$ to microgels via DMTMM coupling………………..177

Figure 7-1. TEM images for the functional microgels described in the text. A) MG(Tm); B) BSA-MG(Tm) C) SAv-MG(Tm). The microgels are characterized by: $d_{MG(TM)} = 350 \pm 20$ nm, $d_{BSA-MG(TM)} = 330 \pm 20$ nm, $d_{SAv-MG(TM)} = 230 \pm 20$ nm. Scale bars are 500 nm………………178

Figure 7-2. A) TEM images of streptavidin-coated microgels (SAv-MG(Tm)) after treatment with a biotinylated metal chelating polymer (Bi-PAsp(Tb)$_{50}$). $d_{Bi-PAsp(Tb)SAv-MG(TM)} = 180 \pm 10$ nm. B) EDX linear scan through assembly of streptavidin-coated microgels (SAv-MG(Tm)) with a biotinylated metal chelating polymer (Bi-PAsp(Tb)$_{50}$). The strong Tb signal indicates the success of biotin-SAv coupling. C) A cartoon scheme shows the assembly between SAv-MG(Tm) with Bi-PAsp(Tb)$_{50}$. D) Histograms of Tm, Tb and La distributions among assemblies between SAv-MG(Tm) with Bi-PAsp(Tb)$_{50}$, where the La signal is an internal blank as compared to the signals from Tm and Tb………………………………………………………181

Figure 7-3. TEM images: A) bright field image of biotinylated NaHoF$_4$ nanoparticles (Bi-NaHoF$_4$); B) dark field TEM image of microgels with BSA adsorbed non-covalently
(BSA/MG(Tm)); C) dark field TEM image of biotinylated microgels treated successively with BSA, then SAv, and finally with Bi-NaHoF$_4$.

**Figure 7-4.** EDX Linear scans through biotinylated microgel, streptavidin, biotinylated NaHoF$_4$ NP (biotin-SAv-biotin) sandwich assembly samples.

**Scheme 7-2.** Biotin-Streptavidin coupling bioassays.

**Figure 7-5.** Isotopic Tb-Tm dot-dot plots (upper panel), histograms of Tb content distribution (middle panel) and histograms of Tm content distribution (lower panel) from biotin-SAv coupling assays. Part A: SAv-MG(Tm) (100 µL, containing ca. 2.5 x 10$^9$ microgels in total) were incubated with different amounts of Bi-PAsp(Tb)$_{50}$ solution (3.3 µmol/L: S1, 20 µL; S2, 40 µL; S3, 60 µL; S4, 80µL). Part B: BSA-MG(Tm) (100 µL, containing ca. 2.5 x 10$^9$ microgels in total) were incubated with different amounts of Bi-PAsp(Tb)$_{50}$ solution (3.3 µmol/L: S1, 20 µL; S2, 40 µL; S3, 60 µL; S4, 80µL). Part C: SAv-MG(Tm) microgel solution. Data collection was gated to exclude cell debris and cell aggregates. At least 10,000 microgels were analyzed per sample.

**Figure 7-6.** Tb and Tm content per microgel determined by mass cytometry from biotin-SAv coupling assays. Numbers of Tb and Tm atoms per cell were calculated using the mass cytometry transmission coefficient for Tb ions of 9.88 x 10$^{-5}$ and for Tm ions of 7.30 x 10$^{-5}$. Microgel samples contained 100 µL solution in DI water containing 0.132 µmol total MAA functional groups within microgel network. For SAv-MG(Tm) and BSA-MG(Tm) samples, the amounts of Bi-PAsp(Tb)$_{50}$ employed are indicated on the x-axis. The SAv-MG(Tm) samples not treated with metal chelating polymer are indicated by the cross-hatched bars in the histograms. A. Tb content per microgel. The redline indicates a saturation level for SAv-MG(Tm) + Bi-PAsp(Tb)$_{50}$ of 4.0 x 10$^6$ Tb atoms per cell microgel. B. Tm content per microgel. The red line indicates a mean value of 1.1 x10$^7$ Tm atoms per microgel for all samples, with a cell-to-cell CV of 30%. The error bars indicate the cell-by-cell coefficient of variation of lanthanide ion content ($CV_{Ln}$) determined from gated mass cytometry data.

**Scheme 7-3.** Biotin-streptavidin-biotin sandwich assays.

**Figure 7-7.** Isotopic Ho-Tm dot-dot plots (upper panel), histograms of Ho content distribution (middle panel) and histograms of Tm content distribution (lower panel) from biotin-SAv-biotin sandwich assays. Part A: SAv-Bi-MG(Tm) microgel solution in PBS buffer (100 µL, containing 2.5 x 10$^9$ microgels) were incubated with excess of Bi-NaHoF$_4$ NPs (0.015 µmol...
in 10 μL DI water). Part B: BSA/MG(Tm) solution in PBS buffer (100 μL, containing ca. 2.5 x 10⁹ microgels) were incubated with SAv solution (500 nmol/L, 10 μL, 0.005 nmol), then with excess of Bi-NaHoF₄ NPs (0.015 μmol in 10 μL DI water). Part C: SAv-Bi-MG(Tm) microgels. Data collection was gated to exclude cell debris and cell aggregates. At least 10,000 cells were analysed per sample.

**Figure 7-8.** Ho and Tm intensities for cells from biotin-SAv-biotin sandwich assays. Numbers of Tm and Ho atoms per cell were calculated using the mass cytometry transmission coefficient for Tm ions of 1.11 x 10⁻⁴ and for Ho ions of 1.23 x 10⁻⁴. A) Ho content per microgel. B) Tm content per microgel. The red line indicates a mean value of 1.1 x 10⁷ Tm atoms per microgel for all samples, with a cell-to-cell CV of 30%. The error bars indicate the cell-by-cell coefficient of variation of lanthanide ion content (CVₐₙ) determined from gated mass cytometry data.

**Appendix to Chapter 7**

**Figure A7-1.** The TEM image of NaHoF₄ nanoparticles: A) Oleic acid capped NaHoF₄ nanoparticles (NaHoF₄-OA) dispersed in hexane, B) NaHoF₄-COOH nanoparticles obtained by ozonolysis followed by H₂O₂ oxidation from a solution in DI water.

**Figure A7-2.** Particle size distribution for NaHoF₄ nanocrystals determined by TEM: A) NaHoF₄-OA NPs before ozonolysis; B) NaHoF₄-COOH NPs obtained after the ozonolysis reaction; C) Bi-NaHoF₄ NPs.

**Figure A7-3.** Hydrodynamic radius of NaHoF₄ nanoparticles determined by DLS. A) NaHoF₄-OA nanoparticle dispersed in cyclohexane; B) NaHoF₄-COOH nanoparticles dispersed in DI water; C) Bi-NaHoF₄ nanoparticles dispersed in DI water. PDI values are shown in parentheses in the plots.

**Figure A7-4.** ¹H NMR spectrum (CDCl₃) of methyl biotinate.

**Figure A7-5.** ¹H NMR spectrum (D₂O) of Bi-NH₂.

**Scheme A7-1.** Synthesis of Bi-PAsp(DTPA)₅₀ polymer.

**Figure A7-6.** The pH and conductometric titrations of the biotinylated NaHoF₄ (Bi-NaHoF₄) nanoparticles and the carboxylated nanoparticles obtained from the ozonolysis reaction (NaHoF₄-COOH). Each titration involved 10 mg (dry weight) nanoparticles, which were neutralized with a small excess of NaOH. The concentration of the HCl titrant solution was 0.025 mol/L.
**Figure A7-7.** pH and conductometric titration curves of poly(NIPAm/VCL/MAA) microgels. 2.435 g of a purified microgel solution was adjusted to ca. pH 10.5 with 0.1 M NaOH and then titrated with HCl (0.100 M) to determine the amount of MAA functional groups per g of microgel solution. The limits of the titration are denoted $V_{\text{HCl,1}}$ and $V_{\text{HCl,2}}$.

**Figure A7-8.** A) A schematic description of nanoparticle attachment to streptavidin surface. Point O, A, B, C represent four bioin binding sites within streptavidin. Point N is the mass center of streptavidin molecule. Point M is the mass center of nanoparticle. OA = OB = OC = AB = AC = BC = 2.4 nm, $r_{\text{SAv}}$ = 3.4 nm, $c$ = 1.9 nm, $h_1$ = 3.9 nm, $h = r_{\text{NP}} - h_1$, $R = c + r_{\text{NP}}$, $r = (R^2 - h^2)^{1/2}$. B) Maximum distance between mass centers ($M_1$, $M_2$, $M_3$) in three nanoparticle packing model. $r = (R^2 - h^2)^{1/2}$, $D_{\text{max}} = 1.73r + a$. C) Maximum distance between mass centers ($M_1$, $M_2$) in two nanoparticle packing model $D_{\text{max}} = 2r + a$.

**Figure A7-9.** Mass cytometry screen capture. A) SAv-Bi-MG(Tm)-3 (100 μL, equivalent to a total of ca. 2.5 x 10^9 microgels) was incubated with Bi-NaHoF_4 solution (10 μL, containing 0.015 μmol NPs). B) BSA/MG(Tm) (100 μL, equivalent to a total of ca. 2.5 x 10^9 microgels) was incubated with 6-ACA (0.03 μmol in 3 μL DI water solution), followed by SAv solution (0.005 nmol in 10 μL PBS buffer solution), and then with Bi-NaHoF_4 solution (10 μL, containing 0.015 μmol NPs). C) SAv-Bi-MG(Tm)-3 solution (100 μL, containing a total of ca. 2.5 x 10^9 microgels).

**Figure A7-10.** CONTIN plots showing the angular dependence of $R_h$ of microgels at 296K in PBS buffer (pH 7.4, 0.01 M). MG(Tm) stands for TmF_3-containing poly(NIPAm/VCL/MAA) microgels.

**Figure A7-11.** Plots of decay rates (ms^{-1}) vs scattering form factor squared (nm^{-2}), where the straight line in each plot represents the linear fitting through scattering data from 30°, 45° and 60° toward the origin. The average diffusion coefficient can be obtained from the slopes of the linear regression lines. MG(Tm) stands for TmF_3-containing poly(NIPAm/VCL/MAA) microgels.

**Figure A7-12.** Experimental static light scattering data (squares) from dilute solution of MG(Tm) (0.018 mg/mL) in PBS buffer (0.01 M, pH 7.4), form factor as a function of the scattering vector and fits with a monodisperse homogeneous sphere model (blue line) and a polydisperse inhomogeneous sphere model (black line). The minimum of $P(q)$ corresponds to
a scattering angle at 98°, \( R = 228 \text{ nm and } R_{\text{q min}} = 4.493 \). MG(Tm) stands for TmF\(_3\)-containing poly(NIPAm/VCL/MAA) microgels.

**Figure A7-13.** Isotopic Tb-Tm dot-dot plots (upper panel), histograms of Tm content distribution (middle panel) and histograms of Tb content distribution (lower panel) from biotin-SAv-biotin sandwich assays using Bi-PAsp(Tb)\(_{50}\) as a detection reagent. Part A: SAv-Bi-MG(Tm) solution (100 \( \mu \text{L, containing } 2.5 \times 10^{9} \text{ microgels in total, each microgels carrying a total of } 5.6 \times 10^{5} \text{ biotin molecules) were incubated with different amounts of Bi-PAsp(Tb)\(_{50}\) solution (0.33 \( \mu \text{mol/L: S1, 10 } \mu \text{L; S2, 20 } \mu \text{L; S3, 40 } \mu \text{L; S4, 80 } \mu \text{L). Part B: BSA/MG(Tm) solution (100 } \mu \text{L, containing } 2.5 \times 10^{9} \text{ microgels in total) were first treated with 6-ACA (0.03 } \mu \text{mol in 3 } \mu \text{L DI water), then with SAv solution (0.005 nmol in 10 } \mu \text{L PBS buffer), and finally combined with different amounts of Bi-PAsp(Tb)\(_{50}\) solution (0.33 } \mu \text{mol/L: NC1, 10 } \mu \text{L; NC2, 20 } \mu \text{L; NC3, 40 } \mu \text{L; NC4, 80 } \mu \text{L). Part C: SAv-Bi-MG(Tm) solution (100 } \mu \text{L, containing } 2.5 \times 10^{9} \text{ microgels in total, each microgels carrying a total of } 5.6 \times 10^{5} \text{ biotin molecules). Data collection was gated to exclude cell debris and cell aggregations. At least 10,000 microgels were analyzed per sample. The mass cytometry transmission coefficient for Tb ions was } 3.60 \times 10^{-5} \text{, and for Tm ions was } 3.02 \times 10^{-5} \text{.}

**Figure A7-14.** Tb and Tm content per microgel determined by mass cytometry from biotin-SAv-biotin sandwich assays using Bi-PAsp(Tb)\(_{50}\) as the detecting reagent. Numbers of Tb and Tm atoms per cell were calculated using the mass cytometry transmission coefficient for Tb ions of 3.60 \( \times 10^{-5} \) and for Tm ions of 3.02 \( \times 10^{-5} \). Microgel samples contained 100 \( \mu \text{L solution in DI water containing } 2.5 \times 10^{9} \text{ microgels in total. The SAv-Bi-MG(Tm) sample was prepared by coupling with biotinylated microgels (Bi-MG(Tm)-3, from which each microgel contained } 5.6 \times 10^{5} \text{ biotin moieties). For SAv-Bi-MG(Tm) and BSA/MG(Tm) samples, the amounts of Bi-PAsp(Tb)\(_{50}\) employed are indicated on the x-axis. The Tb levels (background) detected for SAv-Bi-MG(Tm) samples not treated with metal chelating polymer are indicated by the cross-hatched bars in the histograms. A. Tb atom content per microgel. B. Tm atom content per microgel. The red line indicates a mean value of 1.1 \( \times 10^{7} \text{ Tm atoms per microgel for all samples, with a cell-to-cell CV of 30\%}. \text{ The error bars indicate the cell-by-cell coefficient of variation of lanthanide ion content (CV}_{\text{Ln}} \) determined from gated mass cytometry data.
List of Appendices

Chapter 4 ....................................................................................................................... 105
Appendix to Chapter 4 .................................................................................................... 126

Chapter 6 ....................................................................................................................... 147
Appendix to Chapter 6 .................................................................................................... 163

Chapter 7 ....................................................................................................................... 164
Appendix to Chapter 7 .................................................................................................... 200
Abbreviations

2-DisP  two-stage dispersion polymerization
3-DisP  three-stage dispersion polymerization
AA     acrylic acid
Ab     antibody
ACVA   azo-bis-(cyanvaleric acid)
ADDP   ammonium di-n-octadecyldithiophosphate
AM     acrylamide
AmAc   ammonium acetate
BCPDA  4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9 dicarboxylic acid
Bi     biotin
Bi-MG(Tm)  biotinylated thulium-encoded microgels
Bi-NaHoF_{4}  biotinylated NaHoF_{4} nanoparticle probes
Bi-NH_{2}  N-biotinyl-3,6-dioaoctane-1,8-diamine
Bi-PAsp(DET)_{50}  biotin-end-capped polyaspartamide with diethylenetriamine on each of the 50 repeat units
Bi-PAsp(DTPA)_{50}  biotin-end-capped polyaspartamide with diethylenetriaminepentaacetic acid chelators on each of the 50 repeat units
Bi-PAsp(Tb)_{50}  biotinylated metal chelating polymer probe
Bi-PBLA  biotin-end-capped poly((\beta\text{-}benzyl-L-aspartate)
BIS     N,N\prime-methylenebis(acrylamide)
Bi-SAv-Bi  biotin-streptavidin-biotin
Bis-tris  bis(2-hydroxyethyl) amino-tris(hydroxymethyl)methane
BLA     \(\beta\text{-}benzyl-L\text{-}aspartate\)
BLA-NCA  \(\beta\text{-}benzyl-L\text{-}aspartate-N\text{-}carboyanhydride\)
BPTA   N,N,N\prime,N\prime\prime-2,6-bis(3\prime\text{-}aminomethyl-1\prime\text{-}pyrazolyl)-4-phenylpyridine]
tetrakis(acetic acid)

BSA albumin from bovine serum

BSA/MG(Tm) BSA-coated thulium-encoded microgels via passive absorption

BSA-MG(Tm) BSA-coated thulium-encoded microgels via covalent attachment

CA citric acid

CEA 2-carboxylethyl acrylate

CV coefficient of variance

CV\textsubscript{d} coefficient of variance of particle diameter

coefficient of variance of particle molecular weight (or volume)
determined from dynamic light scattering

CV\textsubscript{DLS} coefficient of variance

CV\textsubscript{Ln} coefficient of variance of metal content of particle

coefficient of variance of particle molecular weight (or volume)
determined from electron microscopy

CV\textsubscript{TEM} coefficient of variance of particle molecular weight (or volume)
determined from electron microscopy

CV\textsubscript{V} coefficient of variance of particle volume

DCM dichloromethane

DET diethylenetriamine

DHDA N,N\textsuperscript{\prime}-bis(2,4-dihydroxobenzylidene) 1,2-diaminobenzene

DI water deionized water

DLS dynamic light scattering

DMF N,N-dimethylformamide

DMTMM 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride

DOTA 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid

DTPA diethylenetriaminopentaacetic acid

EDTA ethylenediaminetetraacetic acid

EDX energy-dispersive X-ray spectroscopy

EGMAP ethyleneglycol methacrylate phosphate

EGTA ethylenbis(oxyethylenenitrilo) tetraacetic acid
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA</td>
<td>fumaric acid</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FSA</td>
<td>5-fluorosalicylic acid</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronic acid</td>
</tr>
<tr>
<td>HABA</td>
<td>4’-hydroxyazobenzene-2-carboxylic acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-2-hydroxyethyl-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HRP</td>
<td>horseadish peroxidase</td>
</tr>
<tr>
<td>ICP</td>
<td>inductively coupled plasma</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>inductively coupled plasma mass spectrometer</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>ITC</td>
<td>isothermal titration calorimetry</td>
</tr>
<tr>
<td>KPS</td>
<td>potassium peroxodisulfate</td>
</tr>
<tr>
<td>LCST</td>
<td>lower critical solution temperature</td>
</tr>
<tr>
<td>Ln</td>
<td>lanthanide</td>
</tr>
<tr>
<td>MA</td>
<td>maleic acid</td>
</tr>
<tr>
<td>MAA</td>
<td>methacrylic acid</td>
</tr>
<tr>
<td>MCB</td>
<td>mass-tag cellular barcoding</td>
</tr>
<tr>
<td>MCP</td>
<td>metal chelating polymer</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MG</td>
<td>microgel</td>
</tr>
<tr>
<td>MG-CdSe</td>
<td>CdSe nanoparticle-containing microgel</td>
</tr>
<tr>
<td>MG-Ln</td>
<td>microgel containing Ln^{3+} ions</td>
</tr>
<tr>
<td>MG-LnF_{3}</td>
<td>microgel containing LnF_{3} NPs</td>
</tr>
<tr>
<td>MG-LnPO_{4}</td>
<td>microgel containing LnPO_{4} NPs</td>
</tr>
<tr>
<td>MG-NaHoF_{4}</td>
<td>NaHoF_{4} nanoparticle containing microgel</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------------------------------------------------------------</td>
</tr>
<tr>
<td>MG(Tm)</td>
<td>thulium-encoded microgel</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometer</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut-off</td>
</tr>
<tr>
<td>NC</td>
<td>nanocrystal</td>
</tr>
<tr>
<td>NP</td>
<td>nanoparticle</td>
</tr>
<tr>
<td>OA</td>
<td>oleic acid</td>
</tr>
<tr>
<td>ODE</td>
<td>1-octadecene</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDB</td>
<td>protein data base</td>
</tr>
<tr>
<td>PDI</td>
<td>polydispersity index</td>
</tr>
<tr>
<td>PDMS</td>
<td>poly(dimethyl siloxane)</td>
</tr>
<tr>
<td>PEGMA</td>
<td>poly(ethyleneglycol methacrylate)</td>
</tr>
<tr>
<td>PG</td>
<td>polyacrylamide gel</td>
</tr>
<tr>
<td>PNIPAm</td>
<td>poly(N-isopropylacrylamide)</td>
</tr>
<tr>
<td>PS</td>
<td>polystyrene</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
</tr>
<tr>
<td>QD</td>
<td>quantum dot</td>
</tr>
<tr>
<td>SANS</td>
<td>small-angle neutron scattering</td>
</tr>
<tr>
<td>SAv</td>
<td>streptavidin from streptomyces avidinii</td>
</tr>
<tr>
<td>SAv-Bi-MG(Tm)</td>
<td>streptavidin-coated thulium-encoded microgels via formation of a biotin-streptavidin complex</td>
</tr>
<tr>
<td>SAv-MG(Tm)</td>
<td>streptavidin-coated thulium-encoded microgels via covalent attachment</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SFEP</td>
<td>surfactant-free emulsion polymerization</td>
</tr>
<tr>
<td>SG</td>
<td>sol-gel process</td>
</tr>
<tr>
<td>SLS</td>
<td>static light scattering</td>
</tr>
</tbody>
</table>

xxxii
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR</td>
<td>solid state reaction</td>
</tr>
<tr>
<td>TBAm</td>
<td>N-t-butyl acrylamide</td>
</tr>
<tr>
<td>TEGA</td>
<td>triethylene glycol acrylate</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TOP</td>
<td>trioctylphosphine</td>
</tr>
<tr>
<td>TOPO</td>
<td>trioctylphosphine oxide</td>
</tr>
<tr>
<td>VAA</td>
<td>vinylacetic acid</td>
</tr>
<tr>
<td>VCL</td>
<td>N-vinylcaprolactam</td>
</tr>
<tr>
<td>VPTT</td>
<td>volume phase transition temperature</td>
</tr>
<tr>
<td>WP</td>
<td>wet process</td>
</tr>
<tr>
<td>ZP</td>
<td>zeta potential</td>
</tr>
</tbody>
</table>
Chapter 1. General Introduction

This chapter introduces the analytical method of mass cytometry and mass cytometric bead-based bioassays in order to understand the necessary criteria that lanthanide-containing microgel must satisfy for applications in bead-based bioassay. Then I introduce microgels and provide an overview of their preparation and some aspects of microgel properties. Subsequently I review the different strategies for the synthesis of microgel-metal nanocomposites. Additionally the properties of lanthanide metals and their complexes will be covered due to their applicability as metal tags in this project.

1.1 Multiplexed Bioassays

To obtain the correct molecular “signature” of a disease, it is rarely sufficient to determine the presence and amount of one or even a few biomarkers. One needs a robust analytical technology capable of providing a simultaneous assay for a broad constellation of proteins, small molecules and gene transcripts, which translates into the information required to understand, diagnose, and treat human diseases such as cancer, metabolic disorders, and heart disease. Assays which provide simultaneous information about the presence and amount of multiple biomarkers in a single sample are referred to as “multiplexed” bioassays. With the recent emergence of new high-throughput technologies, the field of biomarker discovery, development and application has been the subject of intense interest and activity.

1.1.1 Cell-Based Assays

One of the goals of modern bioanalytical chemistry is the simultaneous (multiparameter) detection of multiple biomarkers in individual cells. Flow cytometry is a technique that permits rapid measurements on particles or cells as they flow in a fluid stream one-by-one (in a cytometric way) through a sensing point [1]. The important feature of flow cytometric analysis is that measurements are made separately on each particle within the suspension in turn, and not just as average values for the whole population.

In traditional fluorescence-based flow cytometry, antibodies designed to tag cell surface markers are color-coded with fluorescent dyes or quantum dots (QDs), which passed in single
file across the path of a laser that causes the dye molecules or QDs to be selectively excited and individually detected, allowing rapid cell-by-cell analysis of multiple biomarkers. One of the limitations of flow cytometry is the breadth of the emission bands of the luminescent species used as Ab labels. The spillover of overlapping emissions requires compensation and restricts the number of species that can be detected simultaneously for each cell. The Roederer group has shown that 18-color flow cytometry is possible [2], but this level of multiplexing is not routine.

Mass cytometry [3] transcends this technical plateau by labeling cells with heavy metal isotopes as tags coupled with atomic mass spectrometry as the detection tool. In mass cytometry (Figure 1-1), cells or beads are introduced individually but stochastically into the torch by an inductively coupled plasma mass spectrometer equipped with time of flight detection (CyTOF®). In the plasma, the cells or beads are vaporized, atomized and ionized. Then the ion clouds from individual cell are collected and detected by the TOF detector based on mass spectrometry.

Many more biomarkers can be used in combination to assay single cells without the inherent spillover between fluorescence spectra that is inherent in optical fluorescence systems (Figure 1-2). For example, in mass cytometry experiments, Bendall et al. [4] labeled cells with a cocktail of 31 epitope-specific antibodies, each conjugated to different isotopes of transition elements through metal-chelating coupling reagents. Three additional parameters (DNA content, cell viability and cell length) were also measured, allowing for a total of 34 parameters to be identified simultaneously for each cell in the sample. A recent advance of mass-tag cellular barcoding (MCB), an analogous to fluorescent cell barcoding, improves sample throughput and
enables sample arrays in the a 96-well format to be multiplexed and analyzed in one run through a mass cytometer [5].

**Figure 1-2.** Comparison of fluorescence spectra from 8 Alex dyes with ICP-MS spectra from 30 enriched stable isotopes from lanthanides.

### 1.1.2 Bead-Based Assays

In biomarker detection, a highly multiplexed assay enables the extraction of large amounts of data from small sample volumes with high efficiency. The requirements for increased throughput in biomarker detection led to the development of a variety of different formats, including multi-well microtiter plates, modified polymer surfaces (chips), and micrometer-sized polymer beads. Multiplexed bead-based arrays are an attractive option for supporting surface chemistries of immunoassays [6] and gene expression assays [7].

#### 1.1.2.1 Bead-based Assays Based on Flow Cytometry

For example, the commercially available Luminex 100-plex bead sets were produced by loading two different encoding fluorescent dyes at nine different concentrations into different polymer classifier particles. The variability (VR) of the bead sets, defined as the number of distinguishable encoded particles, depends upon two variables: the number of encoding elements (fluorescent dyes, \( N \)) that can be loaded into the particles and the number of concentration levels
(K) of each fluorescent dye that can be distinguished by the fluorescence detector [8]. The variability (VR) can be calculated as:

\[
VR = K^N - 1 = 10^2 - 1 = 99
\]

In addition to signals from the classifier beads, one needs a reporter signal to indicate which beads recognized a target (i.e. which antibody formed complexes with their target antigen). This can be done via a sandwich assay [9]. Briefly, antibodies (Abs) to specific target biomarkers (e.g. proteins) are immobilized at a capture site on bar-coded beads. The sample mixed with diluent is brought into contact with the beads to facilitate the binding of target biomarkers to the immobilized Abs. This is followed with a wash step to remove unbound materials. Next, secondary Abs labeled with a reporter dye flow through the capture site to bind with the now immobilized target proteins. Finally, the unbound secondary Abs are washed away. The beads are then read on a dual laser fluorescent flow-based detection instrument. One laser excites the internal dyes that identify each bead that is being detected. The second laser excites the reporter dye that captured during the assay. The magnitude of the reporter signal is in direct proportion to the amount of bound analyte. Many readings are made on each bead set, further validating the results. Luminex claims that, in this way, multiplexing of up to 100 unique assays within a single sample was is possible.

1.1.2.2 Bead-based Assays Based on Mass Cytometry

Classifier polymer beads for mass cytometry. Polymer particles for bead-based mass cytometric bioassays must satisfy several important criteria. First, the particles must have a narrow particle size distribution, in order to minimize variability during analysis. Second, the particles must possess a surface that permits bioconjugation to biomolecules such as proteins and oligopeptides, with a minimum amount of non-specific interaction. These criteria are universal for bioassays, however for the mass cytometric based bioassays, additional two criteria must be satisfied: the particles must be large enough to be easily injected into the mass cytometer on a bead-by-bead basis, but small enough to guarantee complete burning and ionization in the ICP torch. Particles with diameters (d) in the range of 0.5 to 3.0 μm satisfy these requirements. Additionally, related to the need for a monodisperse particle size distribution, is the need for the lanthanide content distribution (denoted as CV_{La}) to be narrow on a particle-by-particle basis.
The Ln metals loaded into these particles serve as classifier ions, which encode the beads [10]. A minimal bead-to-bead variability in Ln ion content allows for a greater ability to perform a multiplexed analysis. Given the precision of ICP-MS measurements, not only can different types of Ln ions be used to distinguish one set of particles from another, but different concentrations of the same Ln ion can also be used. This expands the capability and size of the multiplexed assay, but is only possible if the bead-to-bead variability of the Ln ion content is small. For a high quality MS signal, it is desirable to load particles with a significant quantity of Ln, e.g. more than $10^5$ ions per particle. These criteria must all be met with respect to a successful and reproducible bead-based assay system.

Many possible options are available for the synthesis of particles with these characteristics, such as dispersion polymerization, emulsion polymerization, miniemulsion polymerization and layer-by-layer assembly. For example in one approach, Abdelrahman in our group synthesized polystyrene (PS) beads (with $d = 2 \mu m$, $CV_d = 2\%$) by dispersion polymerization of styrene in the presence of polyvinylpyrrolidone (PVP) as a steric stabilizer, acrylic acid (2 wt%), and various LnCl$_3$ salts (0.02 to 0.1 wt% based on styrene) [10]. He was able to vary the Ln ion content from $10^5$ to $10^8$ atoms per particle and showed that metal ion content could be measured quantitatively by mass cytometry for particles containing $10^6$ to $3 \times 10^7$ Ln ions, but higher amounts saturated the detector. By varying the amount of LnCl$_3$ in the synthesis, he obtained particles from which he could resolve 4 different finite concentrations of 4 different Ln elements. Since zero is an encoding level, these beads offer a variability of $(5^4 - 1) = 624$ discrete identifiable classifier beads.

These particles suffer two significant problems. First, the initial particles synthesized showed a large bead-to-bead variability in Ln content. Examples of $CV_{Ln}$ ranged from 25% to 54% for particles with very narrow size distribution $CV_V < 10\%$. This problem could be partially overcome by manipulation of the polymerization reaction conditions, by adding additional acrylic acid and a crosslinking agent (ethylene glycol dimethacrylate) after about 60% styrene conversion. A second problem is that the PVP corona surrounding the particles interfered with biofunctionalization of the particles, so that the extent to which antibodies could be covalently attached to these particles was much smaller than with typical commercial PS beads.

In a second approach, Thickett in our group synthesized PS particles by surfactant-free emulsion polymerization (SFEP) [11]. These seed particles were swollen with a mixture of
additional styrene plus a styrene-soluble chelated lanthanide in the presence of methyl-β-cyclodextrin as a phase-transfer agent. Polymerization was initiated with azo-bis-(cyanovaleric acid) (ACVA) to install –COOH groups at the particle surface. This approach yielded \( d = 0.8 \, \mu m \) particles with a narrow size distribution and a controllable number of Ln ions per particle (from \( 10^6 \) to \( 10^7 \)). Model studies with IgG showed that these particles could be functionalized to a high level with antibodies. Two other problems made it difficult to use these SFEP particles in mass cytometry bioassays. The first was an unfortunate tendency for the particles to aggregate in solution. In the mass cytometer, the particles gave doublet and triplet signals in addition to signals from individual particles. In addition, they suffered from broad values of \( CV_{\text{Ln}} \), on the order or 40%, in spite of their narrow particle size distribution (\( CV_V \approx 10\% \)).

Mass cytometric bead-based assays. Figure 1-3 gives a schematic representation of one type of mass cytometric bead-based sandwich assay. This example describes analyte detection by lanthanide-encoded beads and a lanthanide-containing reporter. In the assay, each type of lanthanide encoded bead is precoated with a specific type of bioaffinity agent (e.g. an antibody). The antibodies in the bead capture their target analytes (e.g. a protein or antigen). After washing the analyte-coated beads, an analyte-specific detecting agent (e.g. a primary antibody) was added and incubated with the beads. During this incubation, the analyte-specific detecting agent binds to the target analytes. After removal of excess detecting agents from the beads, a given secondary antibody that can be used to targets all the detecting primary antibodies is used as a common reporter. The reporter also bears a metal chelating polymer labeled with a specific common lanthanide ions (e.g. Pr). When a mixture of beads is incubated with the reporter, the detecting primary antibodies on the beads react with the reporter. After removing excess unbound reporters from the beads, a flow of beads can be injected into the mass cytometer. Simultaneous detection of the internal metal signals from the bead together with the metal signal from the reporter indicates the presence of the target analyte. The intensity of the metal signals from the reporter signals from each type of bead is directly proportional to the concentration of the target analyte.
The initial attempts towards mass cytometric bead-based assays were carried out by Dr. Abdelrahman from our group [10], where the authors used the mass cytometric classifier beads (lanthanide containing polystyrene beads) as platforms for a proof-of-principle model bioassay involving conjugation of mouse IgG to the surface of La and Tm containing PS particles and its detection by an antimouse IgG bearing a metal-chelating polymer with Pr.

As a test for protein binding capabilities of these particles, the authors conjugated three different proteins to aliquots of one type of PS beads (sample AA105), which was encoded with $^{139}$La and $^{169}$Tm. FITC-avidin, BSA, and mouse IgG. The model bioassay was constructed by choosing as an analyte antimouse IgG labeled with a $^{141}$Pr containing metal chelating polymer. In mass cytometry experiments, a strong signal for Pr accompanied the signals for La and Tm associated with mouse IgG conjugated particle. The Pr signal was 2 orders of magnitude stronger than that due to nonspecific adsorption of the metal chelating polymer onto the AA105 beads themselves and an order of magnitude stronger than that from nonspecific adsorption to the BSA-functionalized beads.

As mentioned previously, PS particles prepared in presence of PVP stabilizer had a limited ability to be biofunctionalized. To demonstrate the utilization of the PS based classifier beads for attachments to biomolecules, the authors also carried out oligonucleotide assays [12]. In this work, the beads were first coated with a silica shell. Then silica-coated particles were further coated with 3-(Aminopropyl)triethoxysilane (APTS) to provide -NH$_2$ functionality on the surface. After that SAy-coated particles were generated from amine-functionalized silica-coated particles via a biotin linker or covalent binding.
To construct the assays, the SAv-coated particles were functionalized with either biotinylated oligonucleotide A (5'-Biotin-AGCGGATAACAATTTCACACAGGA-3') or biotinylated oligonucleotide B (5'-Biotin-CTGAGGTAGGTAGATCAGTTGAGGT-3'). Then the ability of these oligonucleotide functionalized particles to capture fluorescence-labeled oligonucleotide probes was examined. Using a Cy5-labeled anti-A probe (5'-Cy5-TCCTGTGTGAAATTGTTATCCGCT-3') against oligonucleotide A and a Cy3-labeled anti-B probe (5'-Cy3-ACCTCAAGTGATCTACCTACCTCAG-3') against oligonucleotide B, strong specific binding and relatively low non-specific binding were observed with confocal fluorescence microscopy. These initial experiments demonstrate the utilization of the polystyrene based classifier beads for attachments to biomolecules, however didn’t utilize the mass cytometry techniques as an analytical tool for bioassays. A mass cytometry-based oligonucleotide assay employs lanthanide encoded oligonucleotide probes.

Another prototype sandwich assays were based on Thickett’s PS beads [11]. The authors first exposed particle-Neutravidin conjugates to biotin-labelled mouse IgG. Then they incubated these conjugates with goat anti-mouse-IgG that was prelabeled with a MCP carrying copies of \(^{159}\)Tb. The entire system was then analyzed by mass cytometry. In principle, they should be able to simultaneously detect the metal in the particle core (Eu), on the Neutravidin (Tm) and on the antibody (Tb). However accurate determination of the number of copies of each ion attached to the Neutravidin and the anti-IgG was not allowed due to the broad and multimodal distribution of Eu and Tm signals.

My research goal was to develop a new platform of classifier beads for mass cytometric bioassays based on polymeric microgels. Examples in the literature show that functional groups in the microgel surface allow the attachment of bioaffinity agents to the microgel. [13-15]

**1.2 Introduction to Microgels**

**1.2.1 Definition of Microgels**

Microgels are defined as intramolecularly cross-linked molecules [16]. Although microgels have been known for more than 70 years [17-18], their investigation has attracted a greatly
expanding interest since Pelton and Chibante discovered in 1986 that poly(N-isopropylacrylamide) (PNIPAm) based microgels could be prepared [19]. From an operational perspective, microgels can be thought of as cross-linked polymer particles which are subsequently swollen in a solvent. Depending upon the composition and preparation conditions, microgels range in size from tens of nanometers in diameter to micrometers. Microgels with diameters less than about 200 nm are sometimes referred to as nanogels. Microgels in organic media are often used in solvent-borne coatings compositions, where they serve as rheology modifiers. Most of the contemporary interest is in aqueous microgels (hydrogels).

Based on the types of crosslinks, microgels can be classified into two classes: chemically cross-linked microgels and physically crosslinked microgels. Chemically crosslinked microgels refer to the class of microgels where the polymer components are crosslinked into a framework with covalent bonds [20]. The microgels as formed have a permanent stable structure unless labile bonds have been intentionally added to the microgel network [21-28]. The incorporation of labile bonds enables the release of trapped payload from the microgel network in response to stimuli such as (light [21-23], reduction/oxidation [24-26], or the presence of enzymes [27-28]). Chemically crosslinked microgels are typically prepared in presence of a multifunctional crosslinking comonomer. The commonly used crosslinking monomer includes: N,N'-methylenebisacrylamide [19], N,N’-(1,2-Dihydroxyethylene)bisacrylamide) [24], Tetraethylene glycol dimethacrylate [29], 1,4-Butanedioldimethacrylate [29], 1,3-Butanediol dimethacrylate [29], polyethylene glycol dimethacrylate [30], 1,3-Divinylimidazolid-2-one [31], 1,4-Butanediol diacrylate [32], and N,N’-Bis(acryloyl)cystamine [33-34].
Physically crosslinked microgels refer to the class of microgels where the polymer components are crosslinked into a framework with noncovalent interactions such as ionic interaction and hydrogen bonding. These microgels lead to a structural transition from a three dimensional framework to a polymer solution under stimuli such as change of ionic strength of medium, pH condition of solution, temperature, as well as the polymer concentration. One example of crosslinking by ionic interaction is alginate, a polysaccharide composed of mannnuronic acid and gluconic acid crosslinked by divalent calcium ions, which can be dissolved by using a chelating agent that binds to calcium ions [35]. Noncovalent crosslinks can also be formed from linear polymers. For example, poly(acrylic acid) and poly(methacrylic acid) form hydrogen bonds with poly(ethylene glycol), which results in formation of hydrogels. The hydrogen bond is formed between the oxygen of the poly(ethylene glycol) and carboxylic group of the polyacids. The hydrogen bonds are formed only when the acid groups are protonated, hence the hydrogel formation is pH dependent [36-37].
1.2.2 Microgel Synthesis

In this work my objective is to make microgel-based metal carriers for mass cytometry analysis. There are several prerequisites for the microgel carriers: firstly, the diameter of the microgel particles must be large enough to be easily injected into the mass cytometer on a bead-by-bead basis, but small enough to guarantee complete burning and ionization in the ICP torch. Particles with diameters \( d \) in the range of 0.5 to 3.0 μm satisfy these requirements. Secondly, the material should be capable of imbibing high contents of metals in the interior, which falls in the dynamic detection region of the mass cytometer instrument. Thirdly, the materials should be biocompatible and there should be surface functional groups which can be used to covalently attach biomolecules or to immobilize cells on the surface of microcarriers. Therefore the goals of microgel synthesis include: controlling the particle size and the colloidal stability, incorporation of high contents of metal species via specific functional groups such as charged groups, and creating reactive centers for further chemical derivatization.

In following section, I summarize some recent results from the literature on polymer microgel synthesis. This overview, particularly focused on microgels with micron and submicron meter dimensions, covers five strategies: 1) precipitation polymerization, 2) emulsion polymerization, 3) photolithography, 4) microfluidic synthesis, 5) micromolding.

1.2.2.1 Precipitation Polymerization

Precipitation polymerization is the most common technique in producing pH-, salinity- and temperature-sensitive microgel particles. In the preparation of PNIPAm based microgels, all the monomers, NIPAm and the crosslinker are dissolved in water. The solution is purged with N₂ and heated to a temperature above the LCST of PNIPAm (at ca. 70 °C), then an initiator is added. The polymerization is carried out at high temperature, at which radical initiator forms and initiates the polymerization. After initiation, the monomer is attacked by the radical initiator and then follows radical propagation and chain growth. Once the chain reaches a critical length, it collapses upon itself producing precursor particles. The chain collapses because the polymerization temperature is higher than the LCST of the polymer, hence the name precipitation polymerization. The precursor particles grow by aggregation with the other precursor particles by being captured by existing particles, growing oligoradicals and monomer
addition. The charge imparted by the initiator stabilizes the microgels once they have reached a critical size. To synthesize smaller microgels, the precursor particles must be stabilized earlier in the reaction. Since there is not enough charge provided by the initiator, additional ionic surfactant can provide enhanced colloidal stability to the smaller precursor particles. Therefore one can control the size of the microgels obtained by varying the amount of surfactant in the reaction [38-40].

The microgels generated by this method are formed by homogeneous nucleation. A key requirement for discrete microgel particle formation is that the polymer formed must be insoluble under the polymerization conditions; monomer giving soluble polymers under the polymerization conditions will form a macrogel. For example, PNIPAm microgels are readily formed when the monomer is polymerized in water at 70 °C because PNIPAm is water insoluble at high temperature. In contrast, acrylamide, a common monomer with a similar chemical structure of PNIPAm, gives a water soluble polymer at all temperatures, so polyacrylamide microgels cannot be prepared by homogeneous polymerization in water.

Scheme 1-1. Mechanism for the preparation of PNIPAm-based microgels via the surfactant-free radical precipitation polymerization.
1.2.2.2 Emulsification

Emulsification is a widely used method for fabricating microgels. In this process an aqueous solution of monomers was dispersed into oil phase to form a multi-phase mixture. Then the mixture is stirred to generate small aqueous droplets of the hydrogel precursors within an organic phase, i.e. to form a water-in-oil emulsion. The size of the droplets can be controlled by the degree of mechanical agitation, viscosity of each phase, as well as the presence of surfactants that can modify the surface tension between the two phases. The resulting droplets can be gelled using a variety of crosslinking mechanisms to generate spherical microgels. This process can be used to fabricate microgels made from a variety of materials including agarose [41], alginate and collagen [42]. By adding cells to the aqueous phase, cell-laden microgels can be fabricated for applications such as immuno-isolation, as carriers within bioreactors or for analyzing stem cell biology [43-44].

The major advantage of emulsification is the ease with which it can be used to generate microgels. Depending on the process conditions, the size distribution in the gels can be minimized. Although emulsification is a relatively simple process, it does have a number of potential limitations. For example, the shape of the resulting gels is usually limited to spheres, and despite the ability to control the resulting sizes, there will always be some degree of heterogeneity in the resulting spherical gels.

Figure 1-5. Schematic representation of microgel preparation by radical crosslinking polymerization in (inverse) miniemulsion [45]: (a) emulsification and homogenization, (b) polymerization, (c) removal
of excess surfactant by washing/dialysis and subsequent freeze-drying and (d) redispersion of microgels in a good solvent for the network-forming polymer by swelling. Reproduced by permission from [45]; copyright 2012 by Elsevier.

1.2.2.3 Photolithography

Photolithography can be used to create microstructured hydrogel scaffolds. In the photolithographic processes a thin film of a polymer is exposed to UV light through a mask. When the light reaches the photosensitive polymer through the transparent regions of a mask, it causes a photoreaction that crosslinks the prepolymer to form hydrogels [46-48]. The resolution that can be achieved by using photolithography ranges from the submicron scale to millimeters. Potential disadvantages with photolithography include the need for photocrosslinkable materials, as well as the effects of UV light on cell function. Furthermore, since photolithography is inherently a 2D process it forms structures that may require further assembly to generate 3D objects.

![Photolithography process](image)

**Figure 1-6.** Fabrication process of a 2D monolayer of gel beads by the double-template polymerization method. The optical micrograph illustrates the product gel beads. Reproduced by permission from Ref. [49]; copyright 2009 by Springer.

1.2.2.4 Microfluidic Synthesis

Microfluidics has emerged as a potentially powerful method for generating microengineered hydrogels. The unique flow properties within microfluidic channels and the precision with which these devices can be fabricated present a unique opportunity for generating hydrogels with
controlled features. In microfluidic synthesis, a common method to generate microparticles is by using multiphase systems [50]. In these approaches the viscous and surface tension forces are used to create uniform particles that can be crosslinked to form microscale hydrogels. A range of particle sizes and shapes can be created by generating the properly designed microfluidic channels. For example, by changing the dimensions of the microchannels, the flow rates and the droplet shapes it is possible to create hydrogels in the form of spheres, disks and rods [51].

The advantage of microfluidic fabrication of microgels is that the spatial properties of hydrogels can be controlled. For example, by using a microfluidic device that can create concentration gradients of two or more inlets it is possible to create hydrogels with controlled gradients of signaling molecules or material properties embedded in the hydrogel [52]. In addition, it is possible to generate Janus particles (i.e. particles in two or more distinct sides) by flowing multiple streams and generating droplets of the two or more regions [53].

![Figure 1-7](image)

**Figure 1-7.** Production of monodisperse PNIPAm microgels from macromolecular precursors using a PDMS microfluidic device.(A) A cross-junction channel serves to form monodisperse pre-microgel droplets from a semidilute precursor solution, which are exposed to strong UV light as they flow through a basin channel to solidify them. (B) Monodisperse microgels obtained from the experiment in Panel A. All scalebars denote 200 μm. Reproduced from Ref. [54] by permission of the Elsevier 2010.

### 1.2.2.5 Micromolding

Micromolding is another technique that is capable of generating 3D hydrogels microstructures with controlled features. The technique can be accomplished by first using a sacrificial poly(dimethyl siloxane) (PDMS) template around the region hydrogels can be formed. The precursor polymers are initially molded and subsequently gelled to generate structures of a
variety of shapes and sizes. Micromolding has been used to generate microengineered hydrogels from a variety of materials including hyaluronic acid (HA) [55-56], chitosan [57] and PEG [58].

Until recently micromolding techniques were unable to fabricate microengineered hydrogels of controlled shapes and sizes from a class of materials that require the addition of gelling agents such as divalent cations. These materials encompass a large class of hydrogels including alginate and fibrin. To alleviate this limitation hydrogel micromolds have been developed that deliver the crosslinking agent in a controlled manner. Therefore, these molds enable molding the hydrogel precursors and subsequently delivering the curing agent in a desired manner [59].

![Figure 1-8. Schematic diagram of the HA micromolding process. PDMS molds were used to mold a layer of HA into the void regions of the stamp. The polymer was then cured with exposure to UV light to fabricate HA microstructures. To fabricate HA microstructures without cells, a thin polymer film on the substrate was molded (A). To fabricate HA microstructures that encapsulated cells (B) the HA solution was transferred from the PDMS mold onto the substrate and subsequently crosslinked. Reproduced from Ref. [55] by permission of John Wiley and Sons.](image)

1.2.3 Functional Group Distribution in Polyelectrolyte Microgels

Recent studies have pointed to the importance of understanding and characterizing functional group distributions for designing polyelectrolyte microgels with optimized physical
properties as well as application performance. In this section we discuss the functional group
distribution within the microgel framework focusing on two topics: microgels prepared by
homogeneous nucleation and core-shell microgels.

1.2.3.1 Microgels from Homogeneous Nucleation

Microgels composed of NIPAm and vinylacetic acid (VAA) were synthesized by Hoare et
al. using radical emulsion polymerization. These microgels exhibited novel swelling responses
compared to microgels with similar -COOH group contents prepared using more conventional
acrylic acid (AA) or methacrylic acid (MAA) comonomers [60]. Ionization induced a much
larger swelling (three times) response in VAA-NIPAm microgels than in the conventional MAA-
NIPAm. These highly responsive and tunable ionization and swelling profiles observed for
VAA-NIPAm were consistent with the tendency of VAA to act as a chain transfer agent,
resulting in the incorporation of a large number of well-separated VAA units on highly mobile
chain ends at or near the microgel surface. Both copolymerization kinetics and experimental
observations indicated that AA and MAA tended to form blocks within the NIPAm-rich polymer
chains comprising the microgel [61]. This unique NIPAm-rich core/carboxyl-terminated
oligomer shell morphology allowed VAA-NIPAm microgels to ionize over a much narrower pH
range. When fully protonated, VAA-NIPAm copolymer microgels possessed sharp and highly
responsive thermal deswelling profiles that were similar to those of non-functionalized PNIPAm
microgels. However, when fully ionized, the volume phase transitions in VAA-NIPAm
copolymeric microgels were dramatically shifted to higher temperatures by even small amounts
of comonomer, where a relatively low amount (6.5 mol %) VAA increased the volume phase
transition temperature (VPTT) by at least 40 °C.

Copolymers containing PNIPAm and polyacrylamide were synthesized by Hoare and Pelton
[62-65] and compared with the NIPAm-PAA system. Acrylamide-containing microgels
hydrolyzed below the VPTT, and they possessed (a) broad particle size versus temperature
profiles, (b) relatively low electrophoretic mobilities at basic pH, and (c) time-dependent base
titration profiles, suggesting the presence of internal functional groups where the acid-base
neutralization was diffusion-controlled. Methacrylic acid containing microgels exhibited sharper
particle size versus temperature profiles, higher electrophoretic mobilities at basic pH, and time-
independent base titration profiles, suggesting the presence of a “core-shell” structure with primarily surface functionalization. Similar results were obtained when acrylamide-containing microgels were hydrolyzed at temperatures above the VPTT. For systems with MAA/NIPAm ratios < 10%, Zhou and Chu proposed a “core-shell” microstructure, where the NIPAm-rich core was surrounded by a MAA-rich shell [66]. At AA/NIPAm ratios greater than 30 %, the bulk of charges did not reside on the particle surface [67]. Hydrolysis of acrylamide/NIPAm copolymer microgels at temperatures below the volume phase transition temperature produced microgels with a significant number of carboxylic acid groups (more than 35%) located within the bulk of microgel.

The functional group distribution in hydrolyzed Am-NIPAm copolymer microgels can be influenced by hydrolysis temperature. Conducting the hydrolysis at temperatures above the volume phase transition temperature appeared to produce predominantly surface functionalized microgels. MAA/NIPAm copolymer microgels exhibited a core-shell morphology in which most carboxylic acid groups [acrylic acid (AA), methacrylic acid (MAA), vinylacetic acid (VAA), fumaric acid (FA), and maleic acid (MA)] resided at or near the microgel surface [63]. The effect of chain distributions of -COOH groups in five microgels prepared using different -COOH functionalized monomers suggested that the distribution of acidic groups could affect their $pK_a$.

In general, pH swelling responses were enhanced and thermal swelling responses were suppressed as functional groups were localized closer to the microgel surface and delocalized within the individual sub-chains of the microgel. Furthermore, both the radial and intrachain functional group distributions had significant impact on the behavior of the microgels in applications. Microgels with near-surface, well-isolated functional groups permitted the conjugation of nearly double the amount of small molecule grafts compared to microgels with clustered, interior functional groups. Alternately, microgels with core-localized carboxylic acid groups could adsorb and bind more cationic drug than microgels with surface-localized functional groups [68]. Hence, the development of methods to both control and characterize functional group distributions in microgels is a key step towards advancing both the science and technology of microgels.

In Hoare and Pelton’s work, several titration approaches were employed to investigate the functional group distributions within the microgels. Potentiometric and conductometric titrations
for COOH functionalized microgels generate the excess Gibbs free energy of ionization and the apparent pKa versus degree of ionization profiles. The total changes in the apparent pKa and the excess Gibbs free energy during the ionization process both increase systematically as the copolymerization kinetics-predicted probability of functional monomer block formation increases. Isothermal titration calorimetry (ITC) was useful for quantifying the total charge content of microgels containing the same general functional group distribution using a very small sample volume. These authors found that the heat released upon functional group ionization (measured by ITC) increased as the probability of functional monomer block formation increased for monoacid-functionalized microgels. Diacid-functionalized microgels exhibited proportionately lower heat releases, most likely due to the break-up of intramolecular hydrogen bond ring complexes upon functional group ionization. Differences in pH-induced swelling responses for microgels with similar radial functional group distributions could be correlated with the chain distributions of functional groups in the different microgels. The more delocalized the chain functional group distribution, the larger the pH-swelling response.

### 1.2.3.2 Core-Shell Polymer Microgels

In this section I describe core-shell microgel particles where both the core and the shell are made of a hydrogel-like material. Jones and Lyon were the first authors to report the synthesis of NIPAm-core plus AA/NIPAm-shell gels and inverse gels by two-stage precipitation polymerization. These microgels contained acrylic acid only in the particle core or only in the particle shell, giving the authors *a priori* knowledge of the radial functional group distribution [69]. The NIPAm-shell, AA/NIPAm-core microgel were found to deswell in two steps, as anticipated given the distinct differences between volume phase transition temperatures of the shell and the core; however, the AA/NIPAm-shell, NIPAm-core microgel did not deswell until temperatures well above the phase transition temperature of non-functionalized NIPAm-core. Jones and Lyon attributed this effect to the high charge density in the shell which elastically restricts deswelling of the NIPAm-rich core. To visualize the local functional group distribution within the microgels by transmission electron microscopy (TEM), Jones and Lyon stained their core-shell poly(NIPAm)/poly(NIPAm-co-acrylic acid) microgels with uranyl acetate to illustrate the localization of ionic carboxylic acid functional groups in the outer shell of the microgel [70].
The structure of temperature-sensitive core-shell microgels was also investigated by means of small-angle neutron scattering (SANS) [71-72]. To study the swollen state morphology of PNIPAm based microgels, Stieger et al. generated a core-shell form factor expression for PNIPAm-based microgels featuring a uniform chain density in the microgel core and gradual decay in the segment density toward the surface of the microgel [71]. This was accomplished using the fitting strategy, which compensates for the non-uniform internal density (crosslinker) distributions in microgels as well as microgel polydispersity and liquid-like reorientations of the polymer sub-chains within the microgel. Stieger et al. were able to describe both the overall particle form and the internal microstructure using this approach. Increases in crosslinker concentrations resulted in an increase in the volume fraction in the core and a less significant decrease in the local segment density toward the surface of the particles. In addition, smaller microgel particles prepared in the presence of SDS surfactant had higher local polymer volume fractions in the swollen state than particles prepared in the absence of surfactant, hypothesized to correlate with an increase in N, N-methylenebisacrylamide crosslinker polymerization kinetics in the presence of SDS.

1.2.4 Swelling Thermodynamics of Microgel Particles

Over the past 10 years, there has been an intense and growing interest in stimuli-responsive aqueous microgels. These microgels undergo a change in volume in response to a change in the environment such as temperature [73], pH [74], pressure [75], electric field [76], ionic strength [77], magnetic field, and specific biomolecules. These types of microgels are often called “smart” materials. Multiresponsive microgels are responsive to several of these stimuli. Some authors envision application of these microgels as materials for tissue engineering, biomedical implants, bionanotechnology and drug delivery vehicles [46, 78-82].

1.2.4.1 Swelling in Neutral Gels

Flory and Rehner's [83] theory of network swelling has been used to describe the swelling of macroscopic gels. In their theory, the free energy change on swelling of gels by solvent consists of two contributions, the free energy of mixing $\Delta G_{\text{mix}}$, and the free energy of elastic deformation $\Delta G_{\text{elastic}}$, which are assumed to be separable and additive.
\[ \Delta G = \Delta G_{\text{mix}} + \Delta G_{\text{elastic}} \]  \hfill (1.1)

The change of free energy of mixing is described as:
\[ \Delta G_{\text{mix}} = k_B T \left[ n_1 \ln \nu_{1,s} + \chi_1 n_1 \nu_{2,s} \right] , \]  \hfill (1.2)

where \( k_B \) is the Boltzmann constant, \( T \) is the absolute temperature in Kelvin, \( n_1 \) is the molecules of solvent, \( \nu_{1,s} \) is the volume fraction of the solvent in the swollen state, \( \nu_{2,s} \) is the volume fraction of polymer in the swollen state and \( \chi_1 \) is the Flory-Huggins polymer-solvent interaction parameter.

For the elastic component, if we assume the change of internal energy of the network structure during the deformation of the gel is 0, i.e. \( \Delta H_{\text{elastic}} = 0 \), the elastic free energy, \( \Delta G_{\text{elastic}} \), is related to the entropy change associated with the network configuration. Thus we may write:
\[ \Delta G_{\text{elastic}} = -T \Delta S_{\text{elastic}} \quad \text{(assuming } \Delta H_{\text{elastic}} = 0) \]  \hfill (1.3)

According to the entropy change involved in deformation:
\[ \Delta S = -k_B \nu_e / 2 \left[ \alpha_x^2 + \alpha_y^2 + \alpha_z^2 - 3 - \ln(\alpha_x \alpha_y \alpha_z) \right] \]  \hfill (1.4)

where \( \nu_e \) is the effective number of polymer chains in the gel volume. If \( \alpha \) represents the linear deformation factor, then by assuming isotropic swelling there is:
\[ \alpha_x = \alpha_y = \alpha_z = \alpha , \]  \hfill (1.5)

noted that:
\[ \alpha^3 = \frac{V}{V_0} = \frac{V_p + n_1 V_1}{V_0} = \frac{1}{\nu_{2,s}} , \]  \hfill (1.6)

Here \( V \) is the volume of the swollen gel, \( V_0 \) is the volume of the relaxed network, i.e., the volume occupied by the polymer when the crosslinks were introduced into the random system, \( V_p \) is the volume of the unswollen polymer, and \( V_1 \) is the molar volume of solvent (for water \( V_1 = 1.8 \times 10^{-5} \text{ m}^3 \)).
According to equation 1.4 and 1.5, there is:

$$\Delta G_{\text{elastic}} = \left(k_B T \nu_c / 2\right) \left(3\alpha^2 - 3 - \ln \alpha^3\right) \quad (1.7)$$

At the equilibrium state, the total osmotic pressure is equal to zero, i.e.

$$\Delta G = \Delta G_{\text{mix}} + \Delta G_{\text{elastic}} = 0. \quad (1.8)$$

By differentiating equation 1.2 and 1.7, there are:

$$\frac{\delta \Delta G_{\text{mix}}}{\delta n_1} = k_B T \left[ \ln(1 - \nu_{2,s}) + \chi \nu_{2,s}^2 \right] \quad (1.9)$$

$$\frac{\delta \Delta G_{\text{elastic}}}{\delta n_1} = k_B T v_c \frac{V_1}{V_0} \left[ \frac{\nu_{2,s}^{1/3} - \nu_{2,s}}{2} \right] \quad (1.10)$$

By Flory there is:

$$\nu_c = \nu \left(1 - 2 \frac{M_c}{M_n}\right) = \frac{V_p}{\nu M_c} \left(1 - 2 \frac{M_c}{M_n}\right) \quad (1.11)$$

where $\nu$ is the total number of subchains, and $\bar{\nu}$ is the specific volume of polymer.

Substituting equation 1.8, 1.9 and 1.10 and rearranging equation 1.11 yields the average molecular weight between crosslinks $\bar{M}_c$ in gels crosslinked during polymerization as [84]:

$$\frac{1}{\bar{M}_c} = \frac{2}{M_n} - \frac{\left(\frac{\bar{\nu}}{V_1}\right) \left[ \ln(1 - \nu_{2,s}) + \nu_{2,s} + \chi \nu_{2,s}^2 \right]}{\nu_{2,s}^{1/3} - \nu_{2,s}} \quad (1.12)$$

Here $\bar{M}_n$ is the average molecular weight of the linear polymer chains before crosslinking; $\bar{\nu}$ is the specific volume of polymer, i.e. the reciprocal of its amorphous density; $V_1$ is the molar volume of swelling agent (water); $\chi_1$ is the polymer-water interaction parameter; $\nu_{2,s}$ is the polymer volume fraction in the equilibrium-swollen polymer gel.
An extended model [84], which takes into account of the non-Gaussian distribution of macromolecular chains which is observed at high crosslinking ratios, yields the expression:

\[
\frac{1}{M_c} = \frac{2}{M_n} - \frac{\left(\frac{\nu_1}{V_1}\right) \left[\ln\left(1 - \nu_{2,s}\right) + \nu_{2,s} + \chi \nu_{2,s}^2\right]}{(\nu_{2,s}^{1/3} - \frac{\nu_{2,s}}{2})[1 + \frac{1}{N} \nu_{2,s}^{\frac{1}{3}}]^2}
\]

(1.13)

where \( N \) is the number of bonds between two consecutive crosslinks. \( N \) can be calculated as:

\[
N = \frac{\lambda M_c}{M_r}
\]

(1.14)

where \( \lambda \) is the number of bonds per repeating units and \( M_r \) is the molecular weight of repeating unit.

1.2.4.2 Swelling in Ionic Gels

In pH responsive microgels, the extent of pH sensitive swelling generally increases with the increase of microgel’s hydrophilicity and extent of ionization. In addition, the swelling equilibrium of pH sensitive microgels is also influenced by the ionic strength and the type of ions present in the swelling medium. In such a system, the free energy change has three contributions: the free energy of mixing \( \Delta G_{\text{mix}} \), the free energy of elastic deformation \( \Delta G_{\text{elastic}} \) and the ionic free energy from translation entropic contribution of counterions \( \Delta G_{\text{Donnan}} \) [85, 86].

\[
\Delta G = \Delta G_{\text{mix}} + \Delta G_{\text{elastic}} + \Delta G_{\text{Donnan}},
\]

(1.15)

The ionic free energy can be described as:

\[
\Delta G_{\text{Donnan}} = \frac{N_A k_B T \phi_0 f}{V_s N_s} \left( \frac{\phi}{\phi_0} \right) = \frac{N_c k_B T f}{V_0} \left( \frac{\phi}{\phi_0} \right),
\]

(1.16)

where \( N_A \) is the Avogadro number, \( k_B \) is the Boltzmann constant, \( V_s = 1.81 \times 10^{-5} \text{ m}^3 \) is the molar volume of the solvent (water), \( \phi \) is the polymer volume fraction in the particle in the swollen state, \( \phi_0 \) is the volume fraction of polymer in the reference state, \( N_c \) is the effective
number of polymer chains in the gel volume, $V_0$ is the volume of the gel in the reference state, and $f$ is the average number of ionic groups present between crosslinks. 

Authors obtain an expression for the chemical potential of the system by taking the derivatives from each term in equation 1.15 [84]:

$$\mu_i - \mu_i^0 = (\Delta \mu_i)_{\text{mix}} + (\Delta \mu_i)_{\text{elastic}} + (\Delta \mu_i)_{\text{Donnan}}$$

(1.17)

where $\mu_i$ is the chemical potential of solvent (water) in swollen polymer, $\mu_i^0$ is the chemical potential of pure solvent. At swelling equilibrium, the chemical potential of the solvent, $\mu_i$, is equal to the chemical potential of the solvent in the solution surrounding the polymer $\mu_i^*$. 

The mixing contribution $(\Delta \mu_i)_{\text{mix}}$ is calculated as [84]:

$$(\Delta \mu_i)_{\text{mix}} = -RT[\phi + \ln(1-\phi) + \chi\phi^2]$$

(1.18)

The elastic contribution can be calculated by [84]:

$$(\Delta \mu_i)_{\text{elastic}} = RT \left[ \frac{V_1}{\bar{v}M_c} \left[ 1 - \frac{2M_c}{M_n} \right] \left[ (\frac{\phi}{\phi_0})^{\chi} - (\frac{\phi}{2\phi_0}) \right] \right]$$

(1.19)

The contribution from ionic free energy change can be given by [84]:

$$(\Delta \mu_i^*)_{\text{Donnan}} - (\Delta \mu_i)_{\text{Donnan}} = V_1 \left( f^* \frac{\phi}{z - \bar{v}} \right)^2 \left( \frac{K_a}{10^{-\phi_\text{pH}} + K_a} \right)^2$$

(1.20)

where $\mu_i$ is the chemical potential of solvent (water) in swollen polymer, $\mu_i^0$ is the chemical potential of pure solvent, $V_1$ is the molar volume of the swelling agent (water), $f^*$ is the mole fraction of ionizable groups, $\bar{v}$ is the specific volume of polymer, $z$ is the negative charge on polymer, $\nu_{2,s}$ is the polymer volume fraction in equilibrium swollen polymer gel, and $K_a$ is the dissociation constant of ionic groups in polymer.
1.2.5 Microgels Containing Nanoparticles

The incorporation of metal nanoparticles into microgel networks not only results in improved mechanical properties, but also imparts superior physio-chemical properties. For example, for polymer microgels containing gold nanoparticles, gold absorbs light, converts it to heat and triggers a phase transition of microgels under physiological conditions [87]. Magnetic microgels are attractive for diagnostic applications because they can be easily isolated from aqueous suspensions. In the following section, we review different approaches for preparing microgels containing nanoparticles.

Figure 1-9. A schematic diagram of different approaches for preparing microgels containing nanoparticles. Reproduced by permission from Ref. [88,89,91].

1.2.5.1 Growing Nanoparticles Using Microgels as Microreactor

The microgels can be used as microreactors for synthesizing the inorganic nanoparticles \textit{in situ}. The first examples, from the Kumacheva laboratory [88], described a general approach for the preparation of Au NPs, CdS semiconductor NPs and magnetic Fe$_3$O$_4$ NPs using polymeric microgel as templates. The particle syntheses were performed in two steps: polymeric microgels
functionalized with carboxyl groups were first generated using precipitation polymerization, and then *in-situ* coprecipitation was used to grow nanoparticles at these carboxyl sites. The carboxyl groups in the microgels act as nucleation sites for the nanoparticle formation. The facile transport of small molecules into the gel structures offers the possibility of high nanoparticle loading. Subsequent examples reported the use of microgels for the synthesis of a variety of different hybrid structures containing, for example, Pd [92] or Pt [93] metal NPs, CdTe [94], SiO$_2$ [95], CaCO$_3$ [96-98] and ZnO NPs [99].

Doyle and Hotten [100] reported nonspherical magnetic microparticles synthesized by successive growth of magnetic nanoparticles within a microgel template using stop-flow lithography in a microfluid device. The authors found that after coprecipitation of the magnetic nanoparticles, they were able to reload the template microgels with additional Fe$^{3+}$/Fe$^{2+}$ cations, followed by a second coprecipitation of magnetic nanoparticles by a second reaction with NH$_4$OH. After several growth cycles, they obtained polymeric particles with saturation magnetization of up to 42 emu/g microparticle. They found that the particle size was limited by the physical constraint of the effective mesh within the hosting microgel template.

In this approach, the nanoparticle distribution was predominantly determined by the distribution of the functional nucleation sites. In another study, the authors created PNIPAm microgels with a carboxylic group enriched shell by feeding functional sodium acrylate monomers at a latter stage of polymerization [101]. Using the microgels as a template, iron oxide nanoparticles were prepared by *in-situ* coprecipitation. The resulting microgels were covered with iron oxide nanoparticles preferentially distributed on the surface of the microgels, and this strongly affected the thermoresponsive properties of the PNIPAm microgels.

**1.2.5.2 Growing Microgels in the Presence of Preformed Nanoparticles Acting as Seeds.**

Another approach to incorporate nanoparticles into microgels is to conduct an emulsion-polymerization in the presence of surface-functionalized nanoparticles. If the nanoparticles do not exceed a certain size, and if the surface functionalization provides free double bonds, this process can lead to well-defined hybrids with a core-shell structure. The hydrophobicity of the nanoparticles plays also an important role. Nanoparticle loading by this method is limited by the original nanoparticle concentration, which is usually low.
Singh and Lyon [102] reported the synthesis of gold-filled PNIPAm microgels, where the metal template was prepared by adsorption of a layer of amino-terminated PNIPAm onto the pre-formed Au nanoparticles. The initial formation of hydrophobic polymer nuclei acted as seeds for the precipitation of the growing polymer chain. When the polymerization condition were maintained above the polymer’s LCST, the growing polymer chains phase separated and collapsed onto the hydrophobic nuclei to form a cross-linked polymer-shell around the nanoparticle-core. It was envisioned that the use of a small metal nanoparticle based nucleus can facilitate the formation of a polymer-shell with nanometer dimensions.

In another example, silica-coated FITC-labeled magnetic particles were coated with PNIPAm to give a core-shell microgel [89]. The silica coating on the magnetite particles not only protected the particles from dissolving in harsh conditions, but also provided a silica-like surface for subsequent grafting or coating with the polymer shell. When labeled with FITC, magnetic PNIPAm microspheres could be used as tracers for targeted drug controlled delivery under the guidance of a magnetic field.

Kondo et al. prepared polystyrene particles loaded with magnetic particles, following which PNIPAm was grafted onto the latex surface [103]. The minimum NaCl concentration for flocculation of these magnetic latex particles decreased with increasing temperature. At a certain NaCl concentration, some of the magnetic latex particles showed a reversible transition between flocculation and dispersion by controlling the temperature, and the thermo-flocculated magnetic latex particles were separated quickly in a magnetic field. These thermo-sensitive magnetic immunomicrospheres were effective for separation and purification of antibodies.

The recent surge of interest in developing Pickering emulsions has led to a new approach to prepare hydrogel-nanoparticle hybrid structures. Inorganic nanoparticles are used to stabilize emulsion droplets (instead of surfactants or polymeric dispersants) to formulate inverse emulsions (water in oil emulsions). Hydrogel beads produced with shells of partially fused colloidal particles have been called colloidosomes. Applications have included the formulation of magnetic colloidosomes using shells stabilized with Fe$_3$O$_4$ [104]. The Weitz group has suggested that these colloidosomes have high potential for controlled release and drug delivery applications in addition to food and cosmetic applications [105].
1.2.5.3 Absorbing Nanoparticles into Microgels

The two main criteria for inserting nanoparticles into preformed microgels are that the nanoparticles must adhere to the microgels and that they must be small enough to penetrate the gel. The adhesion is usually driven by electrostatic interactions, however, for charge-stabilized microgels, there is a tendency to destabilize the gel by adsorbing oppositely charged particles. Examples of this approach have been published for gold-loaded microgels [106-107], for magnetic microgels [101] and for quantum-dot-loaded microgels [90].

Hellweg and Liz-Marzan [106] reported gold nanorod-coated PNIPAm microgels, where on one hand, the PNIPAm microgels carried a negative surface charge due to the ionic radical initiator. On the other hand, the gold nanorods were initially stabilized with the cationic surfactant cetyltrimethyl ammonium bromide (CTAB) were coated with two oppositely charged polyelectrolyte bilayers (PSS+PAH) so as to enhance colloidal stability and achieve a positively charged surface. By simply mixing the microgel dispersion with polyelectrolyte-coated gold nanorods (Au-rods@PSS@PAH) in water, the authors were able to prepare nanorod-coated microgel spheres. The collapse of the microgel core led to an increase in surface coverage and hence a decrease in the gold nanorod interparticle distance. As a consequence the plasmon band of the gold nanorods could be used to follow the temperature-induced volume phase transition optically.

Another example was given by Stamm and co-workers, who adsorbed the fluorescent CdTe quantum dots on a thermoresponsive microgel [108], where the temperature-induced volume phase transition of the microgel induced a steep variation in the nanocrystals’ photoluminescence intensity.

1.2.5.4 Layer-by-Layer and Core-Shell Assembly

Decher has promoted the generation of multilayer films on macroscopic surfaces by exposing them sequentially to solutions of cationic and anionic polyelectrolytes with interstage washing [109]. Microgels can replace one of the polyelectrolytes to give microgel layers on a surface [110].
Richtering et al. covered PNIPAm microgels with positively or negatively charged magnetic particles by a layer-by-layer technique [91]. In their protocol, they first modified the PNIPAm microgel surface using a polyelectrolyte. Then they deposited magnetic nanoparticles on the polyelectrolyte, and in a third step confined the magnetic nanoparticles with another layer of polyelectrolyte. The authors suggested that the resulting hybrid core–shell structure retains both thermoresponsive and magnetic properties and the nanoparticles do not get detached after the phase transition.

In another example, Sauzedde et al. describe an elegant procedure in which anionic ~10 nm iron particles are adsorbed onto cationic polystyrene-core PNIPAm-shell microgels [111-112]. In the second step, a carboxylated PNIPAm encapsulating shell is coated to encapsulate the iron oxide, giving an overall content of iron oxide up to 23 wt%.

1.3 Lanthanide Properties

The lanthanide series comprises the fifteen elements with atomic numbers 57 through 71, from lanthanum to lutetium. All lanthanides are f-block elements, corresponding to the filling of the 4f electron shell, except for lutetium which is a d-block lanthanide. Lanthanides are chemically similar to each other. The ionic radii of the lanthanides decrease through the period - the so-called lanthanide contraction. Due to their specific electronic configurations, lanthanide atoms tend to lose three electrons, usually 5d and 6s, to attain their most stable oxidation state as trivalent ions. Of the 15 lanthanide metal ions, only four (Sm³⁺, Eu³⁺, Tb³⁺, Dy³⁺) are fluorescent. When excited by UV radiation of appropriate energy, each ion emits characteristic radiation in the visible region. Although the fluorescence of simple inorganic salts of these ions is weak, the fluorescence is dramatically enhanced when the metal ion forms a chelate with appropriate organic ligands. The ligand serves as an antenna to enhance the light absorption cross section followed by energy transfer to the lanthanide ion.

1.3.1 Lanthanide Complexes with Organic Ligands

Figure 1-10 summarizes the chemical structures of organic ligands commonly used for chelating Ln (III) ions. Various carboxylic acids such as ethylenediaminetetraacetic acid (EDTA) [113 - 114], ethylene bis(oxyethylenenitrilo) tetraacetic acid (EGTA), and diethylene triaminopentaacetic acid (DTPA) complex with Eu³⁺ [115]. These complexes are very stable,
with stability constants in the range of $10^{16}$ to $10^{22}$ L / mol, for the 1:1 metal-chelate adducts, and are useful for carrying Eu\(^{3+}\) in a noncovalent manner when the polycarboxylic acids are linked covalently to antibodies. Eu\(^{3+}\) can be dissociated completely from such ligands by lowering the pH because the concentration of the complexing anionic species is diminished. Wullens et al. described the process of chelating Ln (Ln=La or Pr) with H\(_4\)EDTA, H\(_5\)DTPA and H\(_4\)EGTA \[114\]. They dissolved La\(_2\)O\(_3\) or Pr\(_2\)(CO\(_3\))\(_3\) in aqueous solutions of the corresponding ligand. The molar ratio of metal to ligand was always 1:1. Only in the case of H\(_4\)EDTA, it was necessary to add NH\(_3\) to raise the pH in order to achieve the formation of the La–EDTA complex.

A different class of Eu\(^{3+}\) chelates is the β-diketones \[116\]. The maximum number of ligands per metal ion is normally three. The β-diketones have two oxygen atoms available for coordination so that a total of six oxygens are coordinated around the metal ion. The Eu\(^{3+}\) ion, however, prefers eight to nine oxygen molecules in its coordination sphere, the balance usually being made available by coordinated water molecules. An "insulating sheath" can be created around an Eu\(^{3+}\) ion if a synergistic agent having an oxygen atom available for coordination can be found, thereby excluding water. Trioctylphosphine oxide (TOPO) has a lone oxygen atom suitable for this purpose and a Eu(β-diketone)\(_3\) (TOPO) complex can be formed.

The Eu chelates of the carboxylic acids are stable but do not exhibit strong fluorescence. The Eu chelates of the diketones exhibit strong fluorescence but do not form very stable complexes. There are problems of aqueous solubility and stability for ligands such as thenoyltrifluoroacetone. A new chelating agent, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9 dicarboxylic acid, BCPDA, meeting the both criteria is used as a tracer in the CyberFluor system \[117\]. The molecule has two sulfonylchloride groups that can react covalently under mild conditions with available amino groups of protein molecules. The chelating site consists of two heteroaromatic nitrogens and two carboxyl groups.
3.2 Inorganic Lanthanide Nanoparticles (LnF₃, LnPO₄, LnVO₄, LnBO₃)

As background to my synthesis (see Chapter 3 and 4) of LnF₃ and LnPO₄ nanoparticles in the interior of microgels, I briefly review selected examples from the literature for synthesis of LnF₃, LnPO₄, LnVO₄ and LnBO₃ nanoparticles. These lanthanide nanoparticles are commonly synthesized by a sequence of reactions involving high temperature in an organic medium containing monofunctional chelating agents, and yield nanoparticle products that are coated with a layer of surface ligands. The surfactant-like surface ligands provide colloidal stability in nonpolar solvents.

Figure 1-10. Ligand structure for Lanthanide chelates. Notation: EGTA: Ethylene bis(oxyethylenenitrilo)tetraacetic acid [115]; DTPA: Diethylenetriaminopentaacetic acid [115]; OA: Oleic acid [118]; DOTA: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid [119]; EDTA: Ethylenediaminetetraacetic acid [113-114]; BPTA: N,N,N',N'-[2,6-bis(3'-aminomethyl-1'-pyrazolyl)-4-phenylpyridine] tetrakis(acetic acid) [120]; DHDA: N,N'-bis(2,4-dihydroxobenzylidene) 1,2-diaminobenzene [121]; FSA: 5-Fluorosalicylic acid [122]; CA: Citric acid [118]; BCPDA: 4,7-Bis(chlorosulfophenyl)-1,10 penanthroline-2,9-dicarboxylic acid [123]; ADDP: Ammonium di-n-octadecylthiophosphate [124]; EGMAP: Ethyleneglycol methacrylate phosphate [125].
The synthesis of pyridinium di-n-hexaoctyldithiophosphate ligand-capped LaF₃ NCs was first reported by Dang and coworkers [126]. Then, van Veggel et al. used ammonium di-n-octadecyldithiophosphate as a surface modification agent to obtain redispersible LaF₃:Er and LaF₃:Er,Yb NCs [124,127-128]. These LaF₃ nanoparticles were prepared by heating a solution of ammonium di-n-octadecyldithiophosphate and NaF in ethanol/water at 75 °C. Then a solution of La(NO₃)₃·6H₂O and Eu(NO₃)₃·6H₂O in water was added dropwise, and the solution was stirred at 75 °C for 2 h and then cooled to room temperature. The precipitate was purified by centrifugation and was washed with water and ethanol. After washing, the particles were dried in a vacuum over P₂O₅ for 2 days. The resulting nanoparticles are soluble in nonpolar solvents such as chloroform, dichloromethane, and toluene.

Riwotzki et al. reported the preparation of LaPO₄: Ce, Tb nanocrystals [129]. Tris(ethylhexyl) phosphate was purged with dry nitrogen and a methanol solution of LaCl₃, CeCl₃ and TbCl₃ was added. Then, water and methanol were distilled off by heating the solution in vacuum. A freshly prepared solution of crystalline phosphoric acid dissolved in a mixture of trioctylamine and tris(ethylhexyl)phosphate was added. The solution was subsequently heated at 200 °C with nitrogen purge. During heating, some of the phosphoric acid ester was cleaved and the boiling point of the colloid decreased slowly. Heating was stopped when the temperature had dropped to about 170-175 °C (after about 30 to 40 h). After the mixture had been cooled to room temperature, a fourfold excess of methanol was added to the transparent colloid, resulting in precipitation of the nanocrystals.

Zhang et al. synthesized nanocrystalline YVO₄: Eu via the polyacrylamide gel (PG) method [130]. Y₂O₃, Eu₂O₃ and NH₄VO₃ were taken as the starting chemicals. Citric acid was used as the chelating agent. Acrylamide, BIS, and AIBN were used as polymerization agents for polymerization process. In the PG process Y₂O₃, Eu₂O₃ and NH₄VO₃ were dissolved in dilute HNO₃ to prepare the solution. After dissolution, the Y, Eu elements were complexed by the addition of citric acid, and the pH was adjusted to 6-7. Subsequently, the monomers of acrylamide and BIS were added into the clear solution. The resulting solution was heated to 80 °C, when the AIBN initiator was added to the solution and polymerization occurred quickly and a transparent polymeric resin was obtained without any precipitation.
Boyer et al reported the synthesis of YBO$_3$: Eu$^{3+}$ through three synthetic routes: a solid state reaction (SR), a wet process (WP) and a sol-gel process (SG) [131]. In the solid state reaction, polycrystalline YBO$_3$: Eu$^{3+}$ was obtained by direct reaction of the mixture of yttrium oxide, europium oxide and boric acid in a furnace. The wet process involved the reaction between Y(NO$_3$)$_3$, Eu(NO$_3$)$_3$ and H$_3$BO$_3$ solutions, while an ammonia solution was added to adjust the pH to 7. After reaction, the product was dried at 120 °C for 24 hours to yield a xerogel, and finally this xerogel was heated in a furnace to obtain YBO$_3$: Eu$^{3+}$ nanoparticles. In sol-gel process, boron yttrium and boron europium heterometallic alkoxides was first prepared from lanthanide salts, after which the conventional steps of the sol–gel process were used (hydrolysis and condensation). The gel obtained was submitted to several thermal treatments.

### 1.4 Research Objectives

The objectives of my research were:

1. To investigate the details of the synthesis of micron size gels, which carry functional groups preferably distributed in the core of microgels to allow lanthanide metal ion incorporation, and also functional groups in the vicinity of surface to allow for bioconjugation.
2. To synthesize lanthanide encapsulated microgels, using the functional microgels as a template.
3. To investigate the stability of lanthanide encoded microgels and their ability to maintain their lanthanide content.
4. To functionalize the surface of microgels with biomolecules and establish bioassays based on mass cytometry.

### 1.5 Thesis Outline

I present my thesis in 8 chapters. Chapter 2 describes the general experimental methods used in my research. Chapter 3 presents the detailed studies of the synthesis of functional microgels and preparation of lanthanide fluoride nanoparticles within the microgel network using an in-situ coprecipitation approach. Chapter 4 describes the synthesis of lanthanide phosphate containing microgels as classifier beads for mass cytometric analysis. Chapter 5 examines the stabilities of different types of lanthanide encoded microgels and their ability to maintain the
lanthanide ion contents within the microgels. Chapter 6 reports hybrid microgels prepared by a ligand exchange approach. Chapter 7 reports the functionalization of lanthanide encoded microgels with biomolecules and use of these microgels as model cells in quantifying number of biomarkers that can be detected by mass cytometry. The following list contains manuscripts that were submitted or are in preparation based on my thesis work.


Reference


Chapter 2. Experimental: Materials, Protocols, Characterization and Data Analysis

In this chapter, I describe the experimental details including materials, synthetic protocols for polyelectrolyte microgels and lanthanide encoded microgels, characterization techniques and data analysis methods employed in this thesis. Unless otherwise stated, all of the synthesis, experiments and characterizations presented in this thesis were done by me. Light scattering experiments were done by Dr. Jieshu Qian and Dr. Yijie Lu. Poly(NIPAm/MAA/PEGMA) microgels were synthesized by precipitation polymerization by Dr. Xiaomei Ma.

2.1 Materials

2.1.1 Reagents for Particle Synthesis

Monomers N-isopropylacrylamide (NIPAm, 97%, Aldrich), N-vinylcaprolactam (VCL, 98%, Aldrich), polyethylene glycol methacrylate (PEGMA, Mn = 360, Aldrich) and methacrylic acid (MAA, 99%, Aldrich), initiator potassium peroxodisulfate (KPS, 99%, Aldrich), cross-linker N,N’-methylenebis(acrylamide) (BIS, 99+, Aldrich), and the buffer sodium bicarbonate (NaHCO₃) were used as received.

Lanthanide salts: LaCl₃.7H₂O (99.9%), NdCl₃.6H₂O (99.9%), EuCl₃.6H₂O (99.9%), TbCl₃.6H₂O (99.999%), HoCl₃.6H₂O (99.9%), TmCl₃.6H₂O (99.99%) and precipitant NaF (99.99%), NH₄F, NaH₂PO₄, citric acid (99%), succinic acid (99.0%), phosphate buffer saline (PBS) were purchased from Aldrich and used without purification.

2-(N-morpholino)ethanesulfonic acid (MES, Aldrich, 0.1 M, pH 4.7), ammonium acetate (AmAc, Aldrich, 0.01M, pH 9.0), bis(2-hydroxyethyl) amino-tris(hydroxymethyl)methane (Bis-tris, Aldrich, 0.03 M, pH 6.1) and 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES, BioShop, 0.01 M, pH 7.4) were prepared as aqueous solutions. High purity HNO₃ for ICP-MS analysis was purchased from Seastar Chemical Inc. All solutions were prepared with deionized water (Elix/Gradientwater purification system, Millipore).
2.1.2 Reagents for Bioconjugation

4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, Acros Organics, 99+, from Fisher Science, Canada), 2,2’-(ethylenedioxy)bis-(ethylamine) (Aldrich, 98%), Amberlite IR-120 resin, biotin (> 99%, Nanjing Tianzun Zezhong Inc., China), streptavidin from Streptomyces avidinii (SAv, salt free lyophilized powder, Sigma), Albumin from bovine serum (BSA, lyophilized powder, 96+, Sigma) were used as received. Phosphate buffered saline (PBS, Aldrich, 10 mM, pH 7.4, containing 0.0027 M KCl, 0.137 M NaCl), phosphate buffer (PB, 200mM, pH 8.5), phosphate buffer (PB, 10 mM, pH 7.0, containing 0.0027 M KCl, 0.137 M NaCl) were prepared as aqueous solutions with deionized water (Elix/Gradientwater purification system, Millipore). Pierce biotin quantitation kit (containing HABA/Avidin premix and biotinylated horseradish peroxidase positive control) was purchased from Thermo Scientific.

2.1.3 Reagents for Bead-Based Bioassays

Oleic acid (OA, 99%), 1-octadecene (ODE, 97%), acetic acid (CH₃COOH, 99.7%), hydrogen peroxide solution (H₂O₂, 30%, w/w), diethylene triamine pentaacetic acid (DTPA, 98%), tetrahydrofuran (THF, anhydrous), β-benzyl-L-aspartate (BLA), diethylenetriamine (DET), phosgene solution (~ 20% in toluene) were purchased from Sigma-Aldrich and used without purification. N-Biotinyl-3, 6-dioxaoctane-1,8-diamine (Bi-NH₂) was synthesized in our lab which is also commercially available from Pierce Biotechnology. All other chemical reagents with analytical grade were used directly without further purification. Water was purified through a MilliQ water purification system (18 MΩcm).

2.2 Synthesis of Lanthanide Encoded Microgels as Classifier Beads

2.2.1 Synthesis of Polyelectrolyte Microgels by Precipitation Polymerization

In this section, I describe the synthesis protocols for two types of polyelectrolyte microgels with different chemical compositions, which are both based upon poly(N-isopropyl acrylamide) (PNIPAm) and both containing large quantities of methacrylic acid (MAA) as a functional comonomer [1]. One microgel composition contained N-vinylcaprolactam (VCL) as a
comonomer, with 10-38 mol % of MAA. The other contained poly(ethylene glycol methacrylate) (PEGMA) as a comonomer [2], with 3-23 mol % of MAA. Both compositions are interesting to us because of our observations that the synthesis of these microgels by free-radical polymerization in water in the absence of surfactant led to stable polymer particles at temperatures above the LCST of the base polymer [3-4]. Upon cooling, these particles expanded to yield solvent-swollen microgels with micrometer diameters. Figure 2-1 shows the functions and structures of the reactants used in microgel synthesis in this work.

![Figure 2-1. Functions and structures of reactants](image)

### 2.2.1.1 Poly(NIPAm/VCL/MAA) Microgels

A series of poly(NIPAm/VCL/MAA) microgels were synthesized by surfactant-free, free radical precipitation polymerization in aqueous solution at 70 °C. Table 2-1 provides the recipes. In general, each reaction mixture contained a total of 70 mmol of comonomers in water, consisting of different MAA/NIPAm/VCL mole ratios. Each reaction also contained 2.1 mmol (3.0 mol % of the total monomer content) of BIS, 0.7 mmol (1.0 mol % of the total monomer content) of NaHCO₃. Comonomers, crosslinker and NaHCO₃ were mixed with 495 mL of
distilled water and added to a 1 L three-neck round bottom flask equipped with a N₂ inlet, a reflux condenser and a mechanical stirring paddle. To remove oxygen, the solution was heated to 70 °C in an oil bath and stirred at 200 rpm for 1 hour while being purged with N₂. Then 0.7 mmol (1.0 mol % of the total monomer content) of KPS dissolved in 5 mL of water was injected to the reaction mixture to start the polymerization. The reaction mixture turned white after ca. 20 min. The reaction mixture was stirred at 70 °C for 8 hours, leading to formation of a homogenous microgel solution. This microgel solution was purified by dialysis against Milli-Q water for 10 days using a Spectra/pro dialysis membrane with a molecular weight cut-off of 50,000 g/mol. The Milli-Q water was changed twice a day. After dialysis, we concentrated the microgel solution by centrifugation (Sorvall Evolution RC superspeed centrifuge, Kendro, USA) at 20500 rpm, 4 °C for 10 min. We examined the supernatant to monitor the completeness of purification. The conductivity of the supernatant was below 10 μs/cm after purification. The conversion of monomer to polymer was determined by gravimetry. During the reaction about 3 g of aliquots were removed from the reaction mixture at different time intervals. The reaction was quenched by adding 10 μL of quinoline solution (100 mM) and then the solvent and free monomers were removed by freeze-drying under vacuum overnight and the dry products were weighed.

Table 2-1. Recipes for precipitation polymerization of poly(NIPAm/VCL/MAA) microgels

<table>
<thead>
<tr>
<th>Microgels</th>
<th>NIPAm (mmol)</th>
<th>VCL (mmol)</th>
<th>MAA (mmol)</th>
<th>BIS (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V10</td>
<td>50.4</td>
<td>12.6</td>
<td>7.0</td>
<td>2.1</td>
</tr>
<tr>
<td>V19</td>
<td>44.8</td>
<td>11.0</td>
<td>14.1</td>
<td>2.1</td>
</tr>
<tr>
<td>V27</td>
<td>40.0</td>
<td>10.0</td>
<td>20.0</td>
<td>2.1</td>
</tr>
<tr>
<td>V38</td>
<td>33.6</td>
<td>8.4</td>
<td>28.0</td>
<td>2.1</td>
</tr>
</tbody>
</table>

a. To inhibit the hydrolysis of VCL monomer under acidic condition, 0.7 mmol of NaHCO₃ was combined with the monomer mixture. The polymerization was initiated with 0.7 mmol of KPS solution at 70 °C. The reaction was carried out in 500 mL of deionized water. The notation indicates the comonomer (V = VCL) and the number indicates the mole fraction of MAA in the reaction.

2.2.1.2 Poly(NIPAm/MAA/PEGMA) Microgels.

A series of poly(NIPAm/MAA/PEGMA) microgels with varying MAA content were synthesized in a similar manner. Recipes are presented in Table 2-2. In general, the reaction mixtures contained a total of 12 mmol of co-monomers in water, consisting of different mole

Table 2-2. Recipes for precipitation polymerization of poly(NIPAm/MAA/PEGMA) microgels

<table>
<thead>
<tr>
<th>Microgels</th>
<th>NIPAm (mmol)</th>
<th>MAA (mmol)</th>
<th>PEGMA (mmol)</th>
<th>BIS (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W10</td>
<td>48.4</td>
<td>12.0</td>
<td>5.0</td>
<td>2.1</td>
</tr>
<tr>
<td>W19</td>
<td>42.8</td>
<td>10.0</td>
<td>9.0</td>
<td>2.1</td>
</tr>
<tr>
<td>W27</td>
<td>38.0</td>
<td>8.0</td>
<td>12.0</td>
<td>2.1</td>
</tr>
<tr>
<td>W38</td>
<td>33.2</td>
<td>6.4</td>
<td>15.4</td>
<td>2.1</td>
</tr>
</tbody>
</table>

...
ratios of NIPAm and MAA, 2 mol % of PEGMA, 4 mol % BIS crosslinker and 2 mol % KPS initiator. Comonomers and cross-linker were mixed with distilled water and added to a 250 mL three-neck round bottom flask equipped with a N₂ inlet, a reflux condenser and a mechanical stirring paddle. To remove oxygen, the solution was heated to 75 °C in the oil bath and stirred at 200 rpm for 1 hour while being purged with N₂. Then 0.2 mmol (2 mol % of the total monomer content) of KPS dissolved in 10 mL of deionized water was injected to the reaction mixture to start the polymerization. The reaction mixture was stirred at 75 °C for 6 hours, leading to formation of a homogenous microgel solution. This microgel solution was purified by dialysis against Milli-Q water for 10 days using a Spectra/pro dialysis membrane with a molecular weight cut-off of 50,000 g/mol. The Milli-Q water was changed twice a day. After dialysis, the microgel solutions were concentrated by centrifugation as described above, and conversion was monitored by gravimetry.

**Table 2-2. Recipes for precipitation polymerization of poly(NIPAm/MAA/PEGMA) microgels**

<table>
<thead>
<tr>
<th>Microgels</th>
<th>NIPAm (mmol)</th>
<th>MAA (mmol)</th>
<th>PEGMA (mmol)</th>
<th>BIS (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG3</td>
<td>7.8</td>
<td>0.29</td>
<td>0.17</td>
<td>0.34</td>
</tr>
<tr>
<td>PG6</td>
<td>10.2</td>
<td>0.67</td>
<td>0.22</td>
<td>0.46</td>
</tr>
<tr>
<td>PG11</td>
<td>9.6</td>
<td>1.36</td>
<td>0.22</td>
<td>0.46</td>
</tr>
<tr>
<td>PG17</td>
<td>9.0</td>
<td>2.10</td>
<td>0.23</td>
<td>0.47</td>
</tr>
<tr>
<td>PG23ᵇ</td>
<td>8.5</td>
<td>2.83</td>
<td>0.24</td>
<td>0.47</td>
</tr>
</tbody>
</table>

a. NIPAm, PEGMA, MAA and BIS were dissolved in water and the solution was heated to 75 °C. The polymerization was initiated by addition of KPS. The amounts of water added were adjusted to achieve a constant solids content of 1.2% w/w. The notation indicates the comonomer (PG = PEG methacrylate) and the number indicates the mole fraction of MAA in the reaction.

b. PG23 microgels precipitated from solution during storage.

### 2.2.2 Lanthanide Encoded Microgels

#### 2.2.2.1 Ln³⁺ Ions-Containing Microgels

We began with a number of tests with Eu³⁺ ions and microgels to investigate proper reaction conditions for incorporating Ln³⁺ ions into the polyelectrolyte microgels. In all the experiments, the microgel solutions were neutralized with NaOH or bis-tris buffer solution before addition of Eu salts. In the first set of experiments, the pH value of microgel solutions was
adjusted to pH 6 by adding 0.1 M NaOH and following the change in pH with a pH meter. Alternatively, microgel solutions were mixed with 10 molar equivalents of pH 6.3 bis-tris buffer solution compared to the MAA content of the microgel solutions. The microgel solution remained at pH 6. In the third set of experiments, microgel solutions were neutralized with 1 molar equivalents of NaOH solution according to the amount of MAA determined by pH titration. The microgel solutions were at pH 9 prior to the addition of EuCl₃. Eu incorporation was performed by injecting a solution of EuCl₃ in water (0.002 M) into the microgel solution at a –COOH: Eu³⁺ mole ratio of 3:1. The addition of the EuCl₃ caused an increase in the turbidity of the solution as well as a decrease in pH. The dispersion was stirred overnight to promote ion exchange between the Na⁺ and Eu³⁺ ions. The recipes are shown in Table 2-3.

**Table 2-3.** Recipes for synthesis of Eu³⁺ ions encoded poly(NIPAm/VCL/MAA) and poly(NIPAm/MAA/PEGMA) microgel

<table>
<thead>
<tr>
<th>Sample</th>
<th>MAA ⁵ (mmol)</th>
<th>pH</th>
<th>EuCl₃ ⁶ (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V27-Eu-pH6</td>
<td>0.030</td>
<td>6</td>
<td>0.010</td>
</tr>
<tr>
<td>V27-Eu-pH6 buffer</td>
<td>0.030</td>
<td>6 b</td>
<td>0.010</td>
</tr>
<tr>
<td>V27-Eu</td>
<td>0.030</td>
<td>9</td>
<td>0.010</td>
</tr>
<tr>
<td>PG17-Eu-pH6</td>
<td>0.030</td>
<td>6</td>
<td>0.010</td>
</tr>
<tr>
<td>PG17-Eu-pH6 buffer</td>
<td>0.030</td>
<td>6 b</td>
<td>0.010</td>
</tr>
<tr>
<td>PG17-Eu</td>
<td>0.030</td>
<td>9</td>
<td>0.010</td>
</tr>
</tbody>
</table>

a. The amount of MAA was determined by pH and conductometric titration. A concentrated solution containing 0.030 mmol of MAA was diluted to 5 mL. The solids content for V27-Eu and PG17-Eu microgels was 0.3 wt %.
b. The pH was adjusted by 10 molar equivalents of pH 6.3 bis-tris buffer.

We also examined the relationship between the MAA content of the microgels and the amount of Eu³⁺ ions it could bind by preparing a series of Eu-encoded microgel samples with different amounts of EuCl₃ added to the V27 microgel. Compositions for these experiments are presented in Table 2-4. The samples were prepared as follows: the V27 microgel samples were first treated with 1 molar eq. of 0.1 M NaOH solution. Then different amounts of EuCl₃, (ranging from 0.1 / 3 to 10 / 3 in mole ratio of Eu/MAA) were injected into the solution. The mixtures were stirred continuously overnight. To purify microgels from free Eu³⁺ ion, the solutions were
sedimented by centrifugation (5000 rpm, 30 min) and redispersed in deionized water (repeated 3 times).

**Table 2-4.** Synthesis of microgel-Ln composites with different microgel to Ln feed ratios

<table>
<thead>
<tr>
<th>Sample</th>
<th>MAA (^c) mmol</th>
<th>EuCl(^3) (^d) mmol</th>
<th>Eu : MAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank (^b)</td>
<td>0.030</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V27-Eu-1</td>
<td>0.030</td>
<td>0.001</td>
<td>0.1 : 3</td>
</tr>
<tr>
<td>V27-Eu-2</td>
<td>0.030</td>
<td>0.002</td>
<td>0.2 : 3</td>
</tr>
<tr>
<td>V27-Eu-3</td>
<td>0.030</td>
<td>0.004</td>
<td>0.4 : 3</td>
</tr>
<tr>
<td>V27-Eu-4</td>
<td>0.030</td>
<td>0.008</td>
<td>0.8 : 3</td>
</tr>
<tr>
<td>V27-Eu-5</td>
<td>0.030</td>
<td>0.010</td>
<td>1 : 3</td>
</tr>
<tr>
<td>V27-Eu-6</td>
<td>0.030</td>
<td>0.020</td>
<td>2 : 3</td>
</tr>
<tr>
<td>V27-Eu-7</td>
<td>0.030</td>
<td>0.050</td>
<td>5 : 3</td>
</tr>
<tr>
<td>V27-Eu-8</td>
<td>0.030</td>
<td>0.100</td>
<td>10 : 3</td>
</tr>
</tbody>
</table>

a. The pH was adjusted with 1 molar eq. of NaOH solution before adding Eu\(^3+\) ions.
b. As a negative control, a sample containing microgel was treated with 1 molar eq. of NaOH solution but no Eu\(^3+\) ions were added.
c. The amount of MAA in solution was determined by pH and conductometric titrations. The total volume of V27 microgel solution before introducing EuCl\(^3\) was 5 mL.
d. The EuCl\(^3\) solutions were prepared by adding different amounts for EuCl\(^3\) solids into 1 mL of deionized water.

The characterization of the products are described in the Chapter 3, from which we determined that the optimal condition for preparing Eu\(^3+\) ions encoded microgels is by pre-neutralizing the microgels with 1 molar eqv. of NaOH before ion exchange with Eu\(^3+\) ions. The number of Eu\(^3+\) that could bind to microgels reaches its maximum value when the added amount of Eu\(^3+\) equals to ca. 1/3 of the MAA content determined by titration. Following this general protocol, we prepared microgels labeled with other metal ions from the lanthanide series [5]. Table 2-5 lists the reaction recipes for preparing different Ln-encoded microgels (Ln = La, Nd, Tb, Ho, Tm).
Table 2-5. Recipes for different Ln-encoded-microgels

<table>
<thead>
<tr>
<th>Samples(^a)</th>
<th>MAA(^b) (\text{mmol})</th>
<th>LaCl(_3) (\text{mmol})</th>
<th>NdCl(_3) (\text{mmol})</th>
<th>TbCl(_3) (\text{mmol})</th>
<th>HoCl(_3) (\text{mmol})</th>
<th>TmCl(_3) (\text{mmol})</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG-La</td>
<td>0.030</td>
<td>0.010</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MG-Nd</td>
<td>0.030</td>
<td>-</td>
<td>0.010</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MG-Tb</td>
<td>0.030</td>
<td>-</td>
<td>-</td>
<td>0.010</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MG-Ho</td>
<td>0.030</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.010</td>
<td>-</td>
</tr>
<tr>
<td>MG-Tm</td>
<td>0.030</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.010</td>
</tr>
</tbody>
</table>

\(\text{a. MG-Ln: microgels containing Ln}^{3+}\) ions by ion exchange. \\
\(\text{b. The amount of MAA groups as determined by pH and conductometric titration. A}
\text{concentrated solution containing 0.030 mmol of MAA was diluted to 5 mL. The solids content of}
\text{microgels before adding NaF was 0.3 wt%}.

We also performed experiments to prepare microgels tagged with multiple lanthanide species with mixture of Ln ions. As above, the carboxylated microgel solutions were pre-neutralized with 1 eqv NaOH per MAA groups. Then mixtures of EuCl\(_3\) and TbCl\(_3\) in aqueous solution were added to the microgels and allowed to ion exchange. Keeping the overall Ln amount constant at a –COOH: Ln\(^{3+}\) mole ratio of 3:1, different feed ratios of Eu/Tb were added to create different binary encoded microgels. Table 2-6 summarizes the recipes for the binary encoded microgels.

Table 2-6. Recipes for microgels containing both Eu\(^{3+}\) and Tb\(^{3+}\)

<table>
<thead>
<tr>
<th>Samples</th>
<th>MAA(^a) (\text{mmol})</th>
<th>EuCl(_3) (\text{mmol})</th>
<th>TbCl(_3) (\text{mmol})</th>
<th>Eu/Tb</th>
</tr>
</thead>
<tbody>
<tr>
<td>V27-Eu/Tb-1</td>
<td>0.030</td>
<td>0</td>
<td>0.010</td>
<td>0/100</td>
</tr>
<tr>
<td>V27-Eu/Tb-2</td>
<td>0.030</td>
<td>0.0017</td>
<td>0.0083</td>
<td>17/83</td>
</tr>
<tr>
<td>V27-Eu/Tb-3</td>
<td>0.030</td>
<td>0.0050</td>
<td>0.0050</td>
<td>50/50</td>
</tr>
<tr>
<td>V27-Eu/Tb-4</td>
<td>0.030</td>
<td>0.0080</td>
<td>0.0020</td>
<td>80/20</td>
</tr>
<tr>
<td>V27-Eu/Tb-5</td>
<td>0.030</td>
<td>0.010</td>
<td>0</td>
<td>100/0</td>
</tr>
</tbody>
</table>

\(\text{a. The amount of MAA groups was determined by pH and conductometric titration. A}
\text{concentrated solution containing 0.030 mmol of MAA was diluted to 5 mL.}
2.2.2.2 LnF$_3$ Nanoparticle- Containing Microgels

**Single-stage in-situ precipitation.** Here we define single-stage in-situ precipitation as one cycle of loading Ln$^{3+}$ ions through ion exchange followed by in-situ precipitation of LnF$_3$ nanoparticles. To investigate the reaction conditions for preparing LnF$_3$ nanoparticles within microgels, we carried out a series of model experiments with EuF$_3$. The conditions applied to prepare Eu$^{3+}$-encoded microgels were the same as in the previous section. The microgel solutions were neutralized with NaOH or bis-tris buffer solution before addition of Eu salts. Eu incorporation was performed by injecting a solution of EuCl$_3$ in water (0.002 M) into the microgel solution at a –COOH: Eu$^{3+}$ mole ratio of 3:1. After stirring overnight to promote ion exchange between the Na$^+$ and Eu$^{3+}$ ions, F$^-$ precipitant was added dropwise using a lab pump (Fluid Metering Inc, Model QG 50) followed by stirring overnight. After the reaction, any free metal ions were removed by three successive centrifugation–redispersion cycles, and the microgels were redispersed in deionized water. Different precipitants (NH$_4$F and NaF) were studied to check the effect of counterions on EuF$_3$ co-precipitation within the microgels. Table 2-7 provides the recipes for EuF$_3$ nanoparticle containing microgels prepared under different condition.

**Table 2-7.** Recipes for in-situ synthesis of EuF$_3$ nanoparticles using poly(NIPAm/VCL/MAA) and poly(NIPAm/MAA/PEGMA) microgel templates

<table>
<thead>
<tr>
<th>Sample</th>
<th>MAA a (mmol)</th>
<th>pH</th>
<th>EuCl$_3$ (mmol)</th>
<th>NaF e (mmol)</th>
<th>NH$_4$F e (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V27-EuF$_3$-pH6-1</td>
<td>0.030</td>
<td>6 b</td>
<td>0.010</td>
<td>0.030</td>
<td>-</td>
</tr>
<tr>
<td>V27-EuF$_3$-pH6-2</td>
<td>0.030</td>
<td>6 b</td>
<td>0.010</td>
<td>-</td>
<td>0.030</td>
</tr>
<tr>
<td>V27-EuF$_3$-pH6 buffer-1</td>
<td>0.030</td>
<td>6 c</td>
<td>0.010</td>
<td>0.030</td>
<td>-</td>
</tr>
<tr>
<td>V27-EuF$_3$-pH6 buffer-2</td>
<td>0.030</td>
<td>6 c</td>
<td>0.010</td>
<td>-</td>
<td>0.030</td>
</tr>
<tr>
<td>V27-EuF$_3$-1</td>
<td>0.030</td>
<td>9 d</td>
<td>0.010</td>
<td>0.030</td>
<td>-</td>
</tr>
<tr>
<td>V27-EuF$_3$-2</td>
<td>0.030</td>
<td>9 d</td>
<td>0.010</td>
<td>-</td>
<td>0.030</td>
</tr>
<tr>
<td>PG17-EuF$_3$-pH6-1</td>
<td>0.030</td>
<td>6 b</td>
<td>0.010</td>
<td>0.030</td>
<td>-</td>
</tr>
<tr>
<td>PG17-EuF$_3$-pH6-2</td>
<td>0.030</td>
<td>6 b</td>
<td>0.010</td>
<td>-</td>
<td>0.030</td>
</tr>
<tr>
<td>PG17-EuF$_3$-pH6 buffer-1</td>
<td>0.030</td>
<td>6 c</td>
<td>0.010</td>
<td>0.030</td>
<td>-</td>
</tr>
<tr>
<td>PG17-EuF$_3$-pH6 buffer-2</td>
<td>0.030</td>
<td>6 c</td>
<td>0.010</td>
<td>-</td>
<td>0.030</td>
</tr>
<tr>
<td>PG17-EuF$_3$-1</td>
<td>0.030</td>
<td>9 d</td>
<td>0.010</td>
<td>0.030</td>
<td>-</td>
</tr>
<tr>
<td>PG17-EuF$_3$-2</td>
<td>0.030</td>
<td>9 d</td>
<td>0.010</td>
<td>-</td>
<td>0.030</td>
</tr>
</tbody>
</table>
a. The amount of MAA was determined by pH and conductometric titration. A concentrated solution containing 0.030 mmol of MAA was diluted to 5 mL. The solids content for V27-Eu and PG17-Eu microgels before adding NaF or NH₄F was 0.3 wt %.

b. Before adding EuCl₃, the pH of microgel solution was adjusted by 0.1 M NaOH solution.

c. Before adding EuCl₃, the pH of microgel solution was adjusted with 10 mol eqv. of bis-tris buffer at pH 6.3.

d. Before adding EuCl₃, the pH of microgel solutions were adjusted by 1 molar eqv. of 0.1 M NaOH solution.

e. NaF or NH₄F was dissolved in 10 mL of water.

The characterization of the products are described in the Chapter 3, from which we determined that the optimal condition for preparing EuF₃ nanoparticles within a microgel network is by pre-neutralizing the microgels with 1 molar eqv. of NaOH before ion exchange with Eu³⁺ ions. There is no difference in EuF₃ nanoparticles formation when a different F⁻ source was used. Table 2-8 shows the recipes for preparing different Ln-encoded-microgels using NaF as a precipitant.

### Table 2-8. Recipes for different Ln-encoded-microgels

<table>
<thead>
<tr>
<th>Samplesᵃ</th>
<th>MAAᵇ (mmol)</th>
<th>LaCl₃ (mmol)</th>
<th>NdCl₃ (mmol)</th>
<th>EuCl₃ (mmol)</th>
<th>TbCl₃ (mmol)</th>
<th>HoCl₃ (mmol)</th>
<th>TmCl₃ (mmol)</th>
<th>NaFᶜ (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG-LaF₃</td>
<td>0.030</td>
<td>0.010</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.030</td>
</tr>
<tr>
<td>MG-NdF₃</td>
<td>0.030</td>
<td>-</td>
<td>0.010</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.030</td>
</tr>
<tr>
<td>MG-EuF₃</td>
<td>0.030</td>
<td>-</td>
<td>-</td>
<td>0.010</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.030</td>
</tr>
<tr>
<td>MG-TbF₃</td>
<td>0.030</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.010</td>
<td>-</td>
<td>-</td>
<td>0.030</td>
</tr>
<tr>
<td>MG-HoF₃</td>
<td>0.030</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.010</td>
<td>-</td>
<td>0.030</td>
</tr>
<tr>
<td>MG-TmF₃</td>
<td>0.030</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.010</td>
<td>0.030</td>
</tr>
</tbody>
</table>

a. MG-LnF₃: microgels in which F⁻ was added to convert the Ln salts to LnF₃ nanoparticles.

b. The amount of MAA groups as determined by pH and conductometric titration. A concentrated solution containing 0.030 mmol of MAA was diluted to 5 mL. The solids content of microgels before adding NaF was 0.3 wt%.

c. NaF was dissolved in 10 mL of water.

**Two-stage in-situ precipitation.** The two-stage in-situ precipitation process entails two cycles of Ln³⁺ ion incorporation and in-situ precipitation of LnF₃ nanoparticles. The microgel
solutions were first neutralized with 1 eqv NaOH solution per MAA groups as determined by pH titration. Then a solution of LnCl$_3$ in water was injected into the microgel solution at a –COOH : Ln$^{3+}$ ratio of 3 : 1. After that, LnF$_3$ nanoparticles were formed inside the microgels upon addition of an NaF solution dropwise using an FMI Lab pump, and the solution was stirred overnight. After the reaction, any free metal ions were removed by centrifugation and redispersion in deionized water. Then we injected another 1/3 molar eqv of Ln$^{3+}$ per MAA group into the purified LnF$_3$-containing microgel solution. The hybrid microgels were separated from any excess Ln salt by centrifugation and redispersion in deionized water. After that 3 eqv NaF per Ln$^{3+}$ was added dropwise via the FMI Lab pump. After the mixture was stirred overnight, the microgels were purified by centrifugation and redispersion in deionized water. We denote the products from the first stage, hybrid microgels containing Ln$^{3+}$ ions as I-a, and the microgels containing LnF$_3$ nanoparticles as I-b. The products we received from the second stage, hybrid microgels before addition of NaF precipitant as II-a, and those after addition of NaF as II-b. The quantities of reagents employed in these experiments are presented in Table 2-9.

<table>
<thead>
<tr>
<th>Samples</th>
<th>MAA$^a$ (mmol)</th>
<th>1$^{st}$ addition of EuCl$_3$ (mmol)</th>
<th>1$^{st}$ addition of NaF (mmol)</th>
<th>2$^{nd}$ addition of EuCl$_3$ (mmol)</th>
<th>2$^{nd}$ addition of NaF (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-a</td>
<td>0.030</td>
<td>0.010</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>I-b</td>
<td>0.030</td>
<td>0.010</td>
<td>0.030</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II-a</td>
<td>0.030</td>
<td>0.010</td>
<td>0.030</td>
<td>0.010</td>
<td>-</td>
</tr>
<tr>
<td>II-b</td>
<td>0.030</td>
<td>0.010</td>
<td>0.030</td>
<td>0.010</td>
<td>0.030</td>
</tr>
</tbody>
</table>

a. The amount of MAA groups was determined by pH and conductometric titration. A concentrated solution containing 0.030 mmol of MAA was diluted to 5 mL.

2.2.2.3 LnPO$_4$ Nanoparticle-Containing Microgels

To investigate the reaction condition for preparing LnPO$_4$ nanoparticles within microgels, we carried out a series of model experiments to generate EuPO$_4$-containing microgels. In all the experiments, the microgel solutions were first neutralized with NaOH or bis-tris buffer solution before addition of Eu salts at a –COOH: Eu$^{3+}$ mole ratio of 3:1. The dispersion was stirred overnight to promote ion exchange between the Na$^+$ and Eu$^{3+}$ ions, following which phosphate
ions as a precipitant was combined in order to generate EuPO\(_4\) nanoparticles within the microgels. In the first set of experiments, we used NaH\(_2\)PO\(_4\) as a common precipitating agent for Eu\(^{3+}\) ions, while the microgel solutions were adjusted to different pH conditions by 0.1 M of NaOH or bis-tris (pH 6.3) solution before Eu\(^{3+}\) ions were injected. In the second set of experiment, succinic acid and citric acid were employed. The acid (10 mol\% according to total amount of MAA from the microgels) was first added to the microgel solution, and then the microgel solution was pre-neutralized with 1 molar eqv. of NaOH solution, after the Eu\(^{3+}\) incorporation, NaH\(_2\)PO\(_4\) solution was added dropwise to precipitate the Eu\(^{3+}\) ions from the Eu\(^{3+}\)-containing microgels. In the third set of experiment, I first prepared Eu\(^{3+}\)-containing microgels by treating the microgel solution with 1 eq. of NaOH, then with 1/3 eq. of EuCl\(_3\). Then different types of phosphate precipitant was combined including a) 1 eq. of Na\(_2\)HPO\(_4\) and b) 10 molar eq. of 0.1 M PBS buffer solution of pH 7.0 (containing 84% NaCl, 12% Na\(_2\)HPO\(_4\), 2% KH\(_2\)PO\(_4\) and 2% KCl). Table 2-10 shows the recipes for preparing EuPO\(_4\) nanoparticles under different conditions using poly(NIPAm/VCL/MAA) microgel as templates.

**Table 2-10.** Recipes for synthesis of EuPO\(_4\) nanoparticles using poly(NIPAm/VCL/MAA) microgel templates

<table>
<thead>
<tr>
<th>Sample</th>
<th>MAA (^{a}) (mmol)</th>
<th>NaOH (mmol)</th>
<th>EuCl(_3) (mmol)</th>
<th>NaH(_2)PO(_4) (mmol)</th>
<th>Na(_2)HPO(_4) (mmol)</th>
<th>PBS (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V27-EuPO(_4)-pH6 (NaH(_2)PO(_4))</td>
<td>0.030</td>
<td>(~ 0.006)(^{b})</td>
<td>0.010</td>
<td>0.010</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V27-EuPO(_4)-pH6 buffer (NaH(_2)PO(_4))(^{c})</td>
<td>0.030</td>
<td>-</td>
<td>0.010</td>
<td>0.010</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V27-EuPO(_4)-(NaH(_2)PO(_4))</td>
<td>0.030</td>
<td>0.030</td>
<td>0.010</td>
<td>0.010</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V27-EuPO(_4)-succinic acid (NaH(_2)PO(_4))(^{d})</td>
<td>0.030</td>
<td>0.030</td>
<td>0.010</td>
<td>0.010</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V27-EuPO(_4)-citric acid(NaH(_2)PO(_4))(^{e})</td>
<td>0.030</td>
<td>0.030</td>
<td>0.010</td>
<td>0.010</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V27-EuPO(_4)-(Na(_2)HPO(_4))</td>
<td>0.030</td>
<td>0.030</td>
<td>0.010</td>
<td>-</td>
<td>0.010</td>
<td>-</td>
</tr>
<tr>
<td>V27-EuPO(_4)</td>
<td>0.030</td>
<td>0.030</td>
<td>0.010</td>
<td>-</td>
<td>-</td>
<td>0.300</td>
</tr>
</tbody>
</table>

\(^{a}\) The amount of MAA groups as determined by pH and conductometric titration. A concentrated solution containing 0.030 mmol of MAA was diluted to 5 mL. The solids content of microgels before adding PO\(_4\)^{3-} was 0.3 wt\%.

\(^{b}\) Before adding EuCl\(_3\), the pH of microgel solution was adjusted to pH 6 with NaOH solution (0.1 M).
c. Before adding EuCl₃, the pH of microgel solution was adjusted with 10 mol eqv. of bis-tris buffer at pH 6.3.

d. 0.003 mmol of succinic acid was first combined with the microgels.

e. 0.003 mmol of citric acid was first combined with the microgels.

The characterization of the products are described in the Chapter 3, based on which we determined that the optimal condition for preparing EuPO₄ nanoparticles within microgel network was by pre-neutralizing the microgels with 1 molar eqv. of NaOH before ion exchange with Eu³⁺ ions. EuPO₄ nanoparticles formed when 10 eq. of PBS buffer (0.1 M, pH 7.0) was used as a precipitating agent. Therefore, we prepared a series of LnPO₄ nanoparticle encoded microgels based on this protocol. The recipes are listed in Table 2-11.

<table>
<thead>
<tr>
<th>Samples a</th>
<th>MAA b mmol</th>
<th>LaCl₃ mmol</th>
<th>NdCl₃ mmol</th>
<th>TbCl₃ mmol</th>
<th>HoCl₃ mmol</th>
<th>TmCl₃ mmol</th>
<th>PBS mmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>V27-LaPO₄</td>
<td>0.030</td>
<td>0.010</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.300</td>
</tr>
<tr>
<td>V27-NdPO₄</td>
<td>0.030</td>
<td>-</td>
<td>0.010</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.300</td>
</tr>
<tr>
<td>V27-TbPO₄</td>
<td>0.030</td>
<td>-</td>
<td>-</td>
<td>0.010</td>
<td>-</td>
<td>-</td>
<td>0.300</td>
</tr>
<tr>
<td>V27-HoPO₄</td>
<td>0.030</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.010</td>
<td>-</td>
<td>0.300</td>
</tr>
<tr>
<td>V27-TmPO₄</td>
<td>0.030</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.010</td>
<td>0.300</td>
</tr>
</tbody>
</table>

a. V27-LnPO₄: microgels in which PO₄³⁻ was added to convert the Ln salts to LnPO₄ nanoparticles.

b. The amount of MAA groups as determined by pH and conductometric titration. A concentrated solution containing 0.030 mmol of MAA was diluted to 5 mL. The solids content of microgels before adding PO₄³⁻ was 0.3 wt%.

2.3 Characterization Methods and Data Analysis

2.3.1 Titration

Potentiometric and conductometric titrations were conducted using Sevenmulti™ benchtop pH and conductivity meters (Mettler Toledo). Each microgel was suspended at ca. 2 mg/mL in 50 mL of deionized water. At the beginning of the titration, the pH of microgel solution was manually adjusted to ca. pH 10.5 by 0.1 M NaOH to ensure that all the MAA groups were in
their charged state. Then the solution was titrated with 0.1 M HCl solution under a N₂ purge. A wait time of 1 min between injections was used to ensure ionic equilibration between the solution and microgel phase. Quantitative information was acquired from the titration plots using the standard extrapolation/intersection method to determine the titration endpoints.

Figure 2-2 illustrates an example of the potentiometric and conductometric titrations for microgel sample V27. Three regions were observed in the conductivity titration curves of poly(NIPAm/VCL/MAA) microgels, plotted as the variation in conductivity of the system versus volume of titrant. In the first region, the conductivity of the microgel solution decreased with the neutralization of excess OH⁻ ions that were present due to pre-alkalization. In the second region, ionization of basic groups in the microgel polymer caused an increase in conductivity, and hence the slope of curve conductivity vs volume of titrant. In the third region, the conductivity of the solution increased with the increase of excess titrant (HCl) with a slope equal to the theoretical conductivity of the titrant (HCl). The intersection of the extrapolated lines drawn as tangents to the titration curve in the first and the third regions yielded the equivalence point and provided the moles of H⁺ consumed by the polymer:

\[
\text{Moles of } H^+ \text{ consumed by polymer} = C_{HCl} \times (V_{HCl,2} - V_{HCl,1}), \quad (2.1)
\]

The MAA content in poly(NIPAm/VCL/MAA) microgels was determined as:

\[
C_{MAA} \text{ (mol/g dry microgel)} = \frac{\text{Moles of } H^+ \text{ consumed by polymer}}{W \times S.C.}, \quad (2.2)
\]

Where \( W \) is the mass of the wet microgel solution (in g) in a dialysed dispersion and \( S.C. \) is the solids content of the microgel solution after dialysis.

![Figure 2-2. Potentiometric and conductivity titration curves of V27 microgel solution.](image-url)
2.3.2 Light Scattering

Static (SLS) and dynamic light scattering (DLS) measurements were performed using a wide-angle light scattering photometer from ALV-Laser GmbH, Deutschland. The light source is a JDS Uniphase He-Ne laser ($\lambda = 632.8$ nm, 35 mW) emitting vertically polarized light. The sample cell was placed into the ALV/DLS/SLS-5000 Compact Goniometer System. The cell was surrounded by thermostated toluene, which matches the refractive index of the glass cell. The angular range of the goniometer is 30-90°. The scattered light was detected by a Dual ALV-High Q.E. APD avalanche photodiode module. This detector is interfaced to the ALV-5000/EPP multiple tau digital correlator. All measurements were performed at ca. 20.0 °C. Autocorrelation functions were analyzed by the ALV-Correlator Software v3.0.

2.3.2.1 Dynamic Light Scattering

Hydrodynamic radius $R_h$ determined from multi-angular dynamic light scattering. In dynamic light scattering, the intensity-intensity time correlation function $G^{(2)}(t,q)$ can be related to the normalized first-order electric field time correlation function $|g^{(1)}(\tau,q)|$ as [6, 7]:

$$G^{(2)}(\tau,q) = \langle I(t,q)I(t+\tau,q) \rangle = \langle I(0,q)I(\tau,q) \rangle = A[1 + \beta|g^{(1)}(\tau,q)|^2], \quad (2.3)$$

where $A$ is a measured base line, $\beta$ is a parameter depending on the coherence of the detection, $\tau$ is the delay time and $q$ is the scattering vector.

The scattering vector, $q$, can be calculated as:

$$q = \frac{4\pi n}{\lambda}\sin\frac{\theta}{2} \quad (2.4)$$

where $n$ is the reflective index of solution, $\lambda$ is the wavelength of incident light and $q$ is the scattering angle.

For a polydispersed sample, $|g^{(1)}(\tau,q)|$ is related to the line-width distribution $G(\Gamma)$ as:

$$|g^{(1)}(\tau,q)| = \langle E(0,q)E^*(0,q) \rangle = \int_0^{\infty} G(\Gamma)e^{-\Gamma\tau}d\Gamma \quad (2.5)$$
where \( \Gamma \), the decay rate, is a function of both \( C \) and \( q \). \( G(\Gamma) \) can be calculated from the Laplace inversion of \( G^{(2)}(\tau, q) \).

From a dilute solution at a small scattering angle, the translational diffusion coefficient \( (D) \) can be calculated as:

\[
D = \frac{\Gamma}{q^2},
\]

(2.6)

The hydrodynamic radius \( R_h \) can be determined by the Stokes-Einstein equation as:

\[
R_h = \frac{k_B T}{6 \pi \eta D}
\]

(2.7)

Here \( k_B \) is Boltzmann’s constant, \( T \) is the absolute temperature in Kelvin, \( \eta \) is the solvent viscosity.

To determine the translational diffusion coefficient \( (D) \), we plotted the autocorrelation function of the decay rate \( (\Gamma) \) of microgel solution as a function of scattering vector squared \( (q^2) \). An example is shown in Figure 2-3. The mean diffusion coefficient of microgels can be obtained from the slope of the linear regression lines as \( D_{\text{ave}}(V27-TmF_3) = 658 \text{ ms}^{-1}\text{nm}^2 \), and the hydrodynamic radius can be calculated according to Stokes-Einstein equation 2.7, as \( R_h(V27-TmF_3) = 351 \text{ nm} \).

The hydrodynamic radius distribution of samples was obtained by a CONTIN analysis [8] on the dynamic light scattering data. Figure 2-4 illustrate CONTIN plots of V27-TmF₃ microgels in PBS buffer solution (0.01M, pH 7.4) determined from different scattering angles, where the concentration of microgels solution is 0.018 mg/mL, and the temperature is at 296 K.

![Graph showing autocorrelation function](image_url)
Figure 2-3. Plots of decay rates \( (\Gamma) \) (ms\(^{-1}\)) vs scattering vector squared \( (q^2) \) (nm\(^{-2}\)), where the straight line in each plot represents the linear fitting through scattering data from 30\(^\circ\), 45\(^\circ\) and 60\(^\circ\) toward the origin. The average diffusion coefficient can be obtained from the slopes of the linear regression lines.

![Figure 2-3](image)

Figure 2-4. CONTIN plots of V27-TmF\(_3\) microgels from multi-angular dynamic light scattering.

**Polydispersity index (PDI\(_{DLS}\)) and coefficient of variance (CV\(_{DLS}\)) for polymer molecular weight.** In dynamic light scattering, the analysis of the cumulant expansion of the correlation function, \( \ln|g_1(\tau)| \), is performed by fitting a polynomial of \( \tau \) as\([9]\):

\[
\ln|g_1(\tau)| = -\langle \Gamma \rangle \tau + \frac{1}{2} \langle \Delta \Gamma^2 \rangle \tau^2 - \frac{1}{6} \langle \Delta \Gamma^3 \rangle \tau^3 + ... \tag{2.8}
\]

where the first cumulant represents the mean of decay rate, the second cumulant represents the variance of \( \Gamma \).

The polydispersity index determined by DLS (PDI\(_{DLS}\)) are calculated from the second cumulant according to the following formula:

\[
PDI_{DLS} = \frac{\langle \Delta \Gamma^2 \rangle}{\langle \Gamma \rangle^2} = \frac{\langle \Delta D^2 \rangle}{\langle D \rangle^2} \quad \text{(noting that } \Gamma = D q^2) \tag{2.9}
\]
In log-normal distribution of molecular weight as in Figure 2-5, \( \bar{M} \) is the peak molecular weight on the log scale and \( \sigma^2 \) is the variance.

Figure 2-5. Log-normal distribution of molecular weight, reproduced from Ref. [9] by permission of John Wiley & Sons.

Polydispersity index of molecular weight (PDI) in log-normal distribution is calculated as [9]:

\[
PDI = \frac{M_w}{M_n} = \text{Exp}\left(\sigma^2\right),
\]

(2.10)

At \( \sigma^2 << 1 \), there is

\[
PDI = \text{Exp}\left(\sigma^2\right) \approx 1 + \sigma^2
\]

(2.11)

Also, the diffusion coefficient \( D \) can be related to molecular weight, \( M \), by

\[
D = \bar{D}(M / \bar{M})^{-\nu}
\]

(2.12)

where \( \nu \) is the scaling relationship, \( \bar{D} \) is the value of \( D \) for \( M = \bar{M} \) there is [9]:

\[
\langle \Delta D^2 \rangle / \langle D \rangle^2 = \nu^2 \sigma^2
\]

(2.13)

Combining equation 2.9, 2.11, 2.13, the polydispersity index can be calculated as:
\[
PDI = \text{Exp}(\sigma^2) \approx 1 + \sigma^2 = 1 + \frac{\text{PDI}_{\text{DLS}}}{\nu^2}
\]  
(2.14)

The coefficient of variance for molecular weight of particles, \(CV_{\text{DLS}}\) can be calculated as:

\[
CV_{\text{DLS}} = \left( \frac{M_w}{M_n} - 1 \right)^\nu = (\text{PDI} - 1)^\nu \approx \left( \frac{\text{PDI}_{\text{DLS}}}{\nu^2} \right)^\nu
\]  
(2.15)

For spheres, the scaling relationship \(\nu\) equals to 1/3, therefore there is the relationship between \(CV_{\text{DLS}}\) and \(\text{PDI}_{\text{DLS}}\) as following:

\[
CV_{\text{DLS}} \approx \left( \frac{\text{PDI}_{\text{DLS}}}{\nu^2} \right)^\nu = 3\sqrt{\text{PDI}_{\text{DLS}}}
\]  
(2.16)

### 2.3.2.2 Static Light Scattering

In Static Light Scattering (SLS), one obtains structural and dimensional information about the scattering objects by measuring the angular dependence of the excess absolute scattering intensity (the Rayleigh ratio, \(\Delta R_0\)). For dilute solutions, \(\Delta R_0\) is related to the optical constant \(K\), the concentration of the scattering particles \(C\), the second virial coefficient \(A_2\), the weight-averaged molecular weight \(M_w\) of the scattering particles, and the form factor \(P(q)\) through the expression:

\[
\left( \frac{KC}{\Delta R_0} \right) = \frac{1}{P(q)} \left[ \frac{1}{M_w} + 2A_2C + 3A_3C^2 + \ldots \right].
\]  
(2.17)

The optical constant \(K\) is given by:

\[
K = \frac{1}{N_{Av}} \left( \frac{2\pi n}{\lambda^2} \frac{dn}{dc} \right)^2
\]  
(2.18)
The particle form factor $P(q)$ describes angular dependence of the scattering intensity, where $q$ is the scattering vector as given by equation 2.4. At $C \to 0$, the form factor $P(q)$ can be experimentally obtained by:

$$P(q) = \frac{\Delta R_w}{KCM_w}.$$ (2.19)

For homogeneous spheres, the form factor is given as [10]:

$$P_{\text{hom}}(q) = \left(3\left[\sin(qR) - qR \cos(qR)\right]\right)^2. \quad (2.20)$$

Microgel particles have an inhomogenous structure, normally with a higher density in the core and a lower density in the surface. To describe an inhomogeneous system, the form factor is rewritten as [11]:

$$P_{\text{inhom}}(q) = \left[3\left[\sin(qR) - qR \cos(qR)\right]\exp\left(-\left(\frac{\sigma_{\text{surf}}q}{2}\right)^2\right)\right]^2, \quad (2.21)$$

where $\sigma_{\text{surf}}$ represents the width of the smeared particle surface and $R$ denotes the radius of the dense core (Figure 2-6).

![Figure 2-6](image)

**Figure 2-6.** A cartoon illustration of microgel structure with density decrease with increasing distance to the core.

To consider size polydispersity of the particles, the number distribution $f$ with respect to the particle radius $R$ can be described as a Gaussian function [11]:
\[ f(R, \langle R \rangle, \sigma_{\text{poly}}) = \frac{1}{\sqrt{2\pi \sigma_{\text{poly}}^2 \langle R \rangle^2}} \exp \left( -\frac{(R - \langle R \rangle)^2}{2\sigma_{\text{poly}}^2 \langle R \rangle^2} \right), \]  

(2.22)

where \( \langle R \rangle \) denotes the average particle radius and \( \sigma_{\text{poly}} \) describes the relative particle size polydispersity. The form factor for polydisperse inhomogeneous spheres is rewritten as:

\[ P_{\text{poly,inhom}}(q) = \int_0^\infty P_{\text{inhom}}(q, R, \sigma_{\text{surf}}) f(R, \langle R \rangle, \sigma_{\text{poly}}) dR \]  

(2.23)

From fitting the angular dependent form factor of polydisperse inhomogeneous spheres to the experimental data, terms in equation 2.23 (\( \sigma_{\text{surf}} \), \( \langle R \rangle \), \( \sigma_{\text{poly}} \)) can be determined.

The overall size of particle obtained by static light scattering is given by [11]:

\[ R_{\text{SLS}} = \langle R \rangle + 2\sigma_{\text{surf}}. \]  

(2.24)

### 2.3.3 Mass Cytometry

Mass cytometry experiments were carried out using a model C2 instrument (CyTOF™) from DVS Sciences (Markham, ON Canada, www.dvssciences.com) [12]. In this technique, beads or cells are delivered microgels individually but stochastically into the inductively coupled plasma torch. The high temperature of the plasma was sufficient to vaporize, atomize and then ionize the microgels and the Ln ions embedded in them. The ion stream was then introduced into the time-of-flight mass analyzer. The transient signals corresponding to each microgel ionization event were recorded by the detector and stored. Samples were examined at a rate of ca. 1000 microgels per sec.

![Cartoon schematic of the mass cytometry profiling of immune cells. The cells are nebulized into single-cell droplets, and an elemental mass spectrum is acquired for each.](image)
The figures of merit of the instrument are measured under standard ICP operating conditions (< 3% oxide ratio). At mass resolution (full width at half maximum) for m/z = 159 M/ΔM > 900, sensitivity with a standard sample aspiration is $1.4 \times 10^8$ ion counts per second per mgL$^{-1}$ of Tb typically, and abundance sensitivity $6 \times 10^{-4} – 1.4 \times 10^{-3}$ (trailing and leading masses, respectively). The mass range (variable, but fixed at m/z = 125 – 215 for this work) and the abundance sensitivity are sufficient for elemental encoding with up to 60 distinct isotopes. The ion signals are collected by dual-counting, the combination of digital counting and analog modes of ion detection, which allows a much wider range of ion signal (simultaneous detection of very small and very large signals). The data was collected in FCS 3.0 format and was processed by FlowJo$^\text{TM}$ software.

From mass cytometry, metal variation from bead-to-bead was evaluated by the magnitude of the coefficient of variation of metal content $CV_{Ln}$ as:

$$CV_{Ln} = \frac{\sigma_{Ln}}{Ln} = \sqrt{\frac{1}{n-1} \sum_{i=1}^{n} \left( Ln_i - \bar{Ln} \right)^2} / Ln,$$

where $\bar{Ln}$ is the number average Ln ion content of all microgels, $Ln_i$ is the metal content of the $i^{th}$ microgel, and $n$ is the total number of microgels counted in the analysis.

### 2.3.4 ICP-MS

The lanthanide ion content of solution samples were measured on an ELAN DRCPlus$^\text{TM}$ (PerkinElmer SCIEX) ICP-MS instrument operated under normal plasma conditions. The sample uptake rate was adjusted depending on the particular experiment and sample size, typically 100µL/min. A MicroFlow PFA-ST concentric nebulizer (Elemental Scientific, Inc) was used in all instances. Experiments were performed using an autosampler (Perkin Elmer AS91) modified for operation with Eppendorf 1.5 ml tubes. Sample size varied from 150-300 µl. Standards were prepared from 1000 μg/mL PE Pure single-element standard solutions (PerkinELmer, Shelton, CT) by sequential dilution with high purity deionized water produced using a Elix/Gradient (Millipore, Bedford, MA) water purification system. Analyte signals were normalized to the signal of an internal standard (1 ppb Ir) added during sample preparation.
2.3.5 Electron Microscopy

Electron microscopy (TEM) images were obtained with a Hitachi HD 2000 instrument operating at 200 kV. Diluted microgel dispersions were placed onto carbon-coated copper grids and dried at room temperature. TEM image analysis was done by ImageJ software. A particle-size histogram was constructed from measurements of at least 200 individual particles for each sample. If we assume that all the particles are spherical and have the same density, then the mass of particle should be proportional to its volume. The polydispersity index, $\text{PDI}_{\text{TEM}}$, is described by:

\[
\text{PDI}_{\text{TEM}} = \frac{M_w}{M_n} = \left( \frac{d_{w}}{d_{n}} \right)^3 = \left[ \frac{\sum_{i=1}^{n} n_i d_i^2 (\sum_{i=1}^{n} n_i)}{\left( \sum_{i=1}^{n} n_i d_i \right) (\sum_{i=1}^{n} n_i)} \right]^3 = \left[ \frac{\sum_{i=1}^{n} n_i d_i^2 / \sum_{i=1}^{n} n_i}{\left( \sum_{i=1}^{n} n_i d_i / \sum_{i=1}^{n} n_i \right)^2} \right]^3 = \left( \frac{\overline{d^2}}{\overline{d}} \right)^3
\]

where $d_i$ is the diameter of the particle, $n_i$ is the number of particles with diameter of $d_i$, $\overline{d^2}$ is the mean squared diameter of the particles and $\overline{d}$ is the mean diameter of particles.

The coefficient of variance of particle molecular weight determined by electron microscopy, $\text{CV}_{\text{TEM}}$, can be calculated as:

\[
\text{CV}_{\text{TEM}} = \left( \frac{M_w}{M_n} - 1 \right)^{1/2} = (\text{PDI}_{\text{TEM}} - 1)^{1/2}
\]  

(2.27)

2.3.6 Energy-Dispersive X-Ray Spectroscopy

Using the Energy-Dispersive X-ray Spectroscopy (EDX) attachment (Inca, Oxford Instruments), composition-based line scans were performed on relevant particles detected on the sample grid. The accelerating voltage for EDX measurements was 20 kV and the current 20 mA. Data were collected over a period of ten minutes. The signal-to-noise ratio of the obtained signal was determined by considering the signal of an element known not to be present in the sample (e.g. La). EDX was used to determine incorporation of Ln ions into the particle interior.

2.3.7 UV-Vis Spectrometry

UV-Vis spectrometry was used to detect free biotin in solution with a Piece Biotin Quantitation Kit (Thermo Scientific). Absorption spectra were recorded at room temperature on
a Perkin Elmer Lambda 35 UV/Vis spectrometer using 1.5 mL semimicro cuvettes with light path length of 1 cm. Values of the absorbance of the solution at 500 nm were recorded once the value remained constant for at least 15 seconds.

The Pierce Biotin Quantitation Kit contains a premix of HABA/Avidin and a biotinylated horseradish peroxidase (HRP) positive control. When a solution containing biotin is added to a mixture of HABA/Avidin, because of its higher affinity for binding, biotin displaces the HABA and an absorbance at 500 nm (from HABA/Avidin) decreases proportionately [14]. The change in absorbance relates to the amounts of biotin in solution by the extinction coefficient of the HABA/Avidin complex (Figure 2-8).

![Figure 2-8. A cartoon reaction scheme between biotinylated protein and HABA/Avidin complex reproduced from Ref. [15].](image)

The HABA/Avidin premix was first equilibrated to room temperature before mixed with 100 µL of ultrapure water in one microtube. 800 µL of PB buffer (containing 100 mM sodium phosphate, 150 mM NaCl, pH 7.2) was pipetted into a 1 mL cuvette, and used to zero the UV-Vis spectrometer. Then the 100 µL HABA/Avidin premix solution was pipetted into the previous cuvette, and the absorbance of the solution at 500 nm was recorded as \( A_{500} \) (HABA/Avidin). The solutions containing free biotin were diluted (by a factor \( f \)) with ultrapure water before usage. We added 100 µL of diluted solution to the cuvette containing HABA/Avidin and mixed the solution by inversion. The absorbance of the solution at 500 nm was recorded as \( A_{500} \) (HABA/Avidin/biotin).

The concentration of biotin in the original solutions was evaluated based on the Beer Lambert Law:
\[ A_\lambda = \varepsilon_\lambda bC \]  

(2.28)

where \( A_\lambda \) is the absorbance of the sample at wavelength \( \lambda \) (\( \lambda = 500 \text{ nm} \) for this assay); \( \varepsilon_\lambda \) is the extinction coefficient for HABA/Avidin sample at wavelength \( \lambda \). For HABA/Avidin samples at pH 7, the extinction coefficient was given by the manufacturer to be \( \varepsilon_{500\text{nm}} = 34,000 \text{ M}^{-1}\text{cm}^{-1} \). \( C \) is the molar concentration of the solution.

The change in absorbance at 500 nm is calculated as [16]:

\[ \Delta A_{500} = 0.9 \times A_{500}(\text{HABA/Avidin}) - A_{500}(\text{HABA/Avidin/Biotin}) \]  

(2.29)

where the 0.9 correction factor is used to adjust the dilution for the HABA/Avidin mixture. The concentration of biotin in solution was calculated as:

\[ C_{\text{biotin}} = \frac{f \Delta A_{500}}{34000 \text{ M}^{-1}\text{cm}^{-1} \times 1 \text{ cm} \times 0.1 \text{ mL}} , \]  

(2.30)

where 1 cm is the light path length of the solution in UV-Vis spectrometer, the correction factor \( f \) is used to adjust the dilution of the supernatant solution.

### 2.3.8 Zeta Potential (\( \zeta \)) and Electrophoretic Mobility (\( \mu_e \)).

For a charged hard sphere, the liquid layer surrounding the particle exists as two parts; an inner region (Stern layer) where the ions are strongly bound and an outer (diffuse) region where they are less firmly associated. Within the diffuse layer there is a notional boundary inside which the ions and particles form a stable entity. When a particle moves (e.g. due to gravity), ions within the boundary move with it. Those ions beyond the boundary remain with the bulk dispersant. The potential at this boundary (surface of hydrodynamic shear) is defined as the zeta potential (\( \zeta \)) (Figure 2-9) [17]. For microgels, the surface of the particle contains fuzzy structures; therefore it is hard to define the boundary of layers. The apparent zeta potentials (\( \zeta_{\text{app}} \)) were sometimes calculated from electrophoretic mobility (\( \mu_e \)) for microgels with the understanding that there is no firm theoretical meaning.

Measurements of electrophoretic mobility (\( \mu_e \)) of the samples were conducted using a Zetasizer Nano ZS (Malvern instruments, U.K.). Prior to data collection, each sample was equilibrated for 10 min at 25 °C. A suspension of microgels or protein sample (20 μg, 1 μL, solid content 20 mg/mL) was diluted in phosphate buffer (0.01 M, pH 7.0, containing 0.0027 M KCl, 0.137 M NaCl) in H\(_2\)O (1 mL), vortexed, and transferred into a 1 mL clear zeta potential cuvette.
(DTS1061, Malvern). The electrophoretic mobility ($\mu_e$) of the sample was converted into the apparent zeta potential ($\zeta_{app}$) by applying the Smoluchowski model [18].

$$\mu_e = \frac{2 \cdot \varepsilon \cdot \zeta_{app} \cdot f(k_a)}{3\eta} = \frac{\varepsilon \cdot \zeta_{app}}{\eta} \quad (f(k_a) = 1.5)$$

(2.31)

where $\varepsilon$ is the dielectric constant of the sample, $f(k_a)$ is Henry’s function, and $\eta$ is the viscosity of the liquid phase.

Figure 2-9. Schematic representation of zeta potential, reproduced from Ref. [17].

2.3.9 NMR

$^1$H NMR (400 MHz) spectra were recorded on a Varian Mercury 400 spectrometer. All spectra were collected as 64 transients with a delay time of 10 seconds.
Reference


[16] Pierce Biotin Quantitation Kit Instructions, Thermoscientific.


Chapter 3. Ln (III) Ions and LnF₃ Nanoparticle Containing Microgels as Classifier Beads for Mass Cytometric Analysis

In this chapter, I first describe the synthesis and characterization of two series of functional polyelectrolyte copolymer microgels designed as carrying vehicles for Ln(III) ions and LnF₃ nanoparticles. Both series of microgels are based upon copolymers of N-isopropylacrylamide (NIPAm) and methacrylic acid (MAA), poly(NIPAm/VCL/MAA) (VCL = N-vinylcaprolactam, V-series) and poly(NIPAm/MAA/PEGMA) (PEGMA = poly(ethylene glycol)methacrylate, PG-series). The microgels were first loaded with Ln (III) ions, which were then converted in situ to LnF₃ nanoparticles. Different conditions were studied in order to confine the lanthanide elements to the core of the microgels. We used mass cytometry to measure the number and the particle-to-particle variation of Ln ions per microgel. Most of the results in this chapter were presented in ref [1] and ref [2].

3.1 Introduction

Microgel particles designed as lanthanide metal carriers for bead-based mass cytometric bioassays must satisfy four important criteria:

1- Narrow particle size distribution.

2- Narrow lanthanide contents distribution.

3- Diameters in the range of 0.5-3.0 µm.

4- Surface functionality for bioconjugation.

5- Large numbers of functional groups in the core to confine lanthanide metals to the core of microgels.

First, the particles must have a narrow particle size distribution, in order to minimize variability during analysis. In the solution state, the particle size distribution can be characterized by the polydispersity index (PDI). The coefficient of variance for molecular weight (or volume) of particles (CV_DLS), can be related to PDI as:
\[ CV_{\text{DLS}} \approx 3\sqrt{\text{PDI}} \] (3.1).

I also characterize the particle size of microgels in their dry state by analysis of the TEM images. If we assume that all the dry microgels have a spherical shape, the coefficient of variance of particle molecular weight (\( CV_{\text{TEM}} \)) can be calculated as:

\[
CV_{\text{TEM}} = \left( \frac{M_w}{M_n} - 1 \right)^2 = \left( \frac{d^2}{\bar{d}^2} \right)^2 - 1
\]

(3.2)

where \( \bar{d}^2 \) is the mean-squared diameter of the particles and \( \bar{d} \) is the mean diameter of particles.

Related to the need for a monodisperse particle size distribution, is the need for the lanthanide content distribution (denoted as \( CV_{\text{Ln}} \)) to be narrow on a particle-by-particle basis. The Ln metals loaded into these particles serve as classifier ions which encode the beads [3]. A narrow bead-to-bead variability in Ln ion content allows for a greater ability to perform a multiplexed analysis. From mass cytometry, metal variation from bead-to-bead was evaluated by the magnitude of the coefficient of variation of metal content \( CV_{\text{Ln}} \) as:

\[
CV_{\text{Ln}} = \frac{\sigma_{\text{Ln}}}{\bar{\text{Ln}}} = \sqrt{\frac{1}{n-1} \sum_{i=1}^{n} \left( \text{Ln}_i - \bar{\text{Ln}} \right)^2 / \bar{\text{Ln}}},
\]

(3.3)

where \( \bar{\text{Ln}} \) is the number average Ln ion content of all microgels, \( \text{Ln}_i \) is the metal content of the \( i \)th microgel, and \( n \) is the total number of microgels counted in the analysis. Given the precision of ICP-MS measurements, not only can different types of Ln ions be used to distinguish one set of particles from another, but different concentrations of the same Ln ion can also be used. This expands the capability and size of the multiplexed assay, but is only possible if the bead-to-bead variability of the Ln ion content is small.

In addition, the particles must be large enough to be easily injected into the mass cytometer on a bead-by-bead basis, but small enough to guarantee complete burning and ionization in the ICP torch. Particles with diameters (\( d \)) in the range of 0.5 to 3.0 μm satisfy these requirements. Particles of this size are also convenient to manipulate in terms of washing and re-dispersing by centrifugation.

Furthermore, the particles must possess a surface that permits bioconjugation to biomolecules such as proteins and oligopeptides, with a minimum amount of non-specific interaction. Carboxylic acid group facilitates a variety of bioconjugation reactions that can be
employed in order to attach biomolecules to the surface of the particles [4-6]. Many NIPAm based microgels have been synthesized with acrylate or methacrylate as functional comonomers that can provide the carboxylic acid groups at the particle surface [7-8].

Finally, the microgel particles must bear large number of functional groups in the core to allow a sufficient number of lanthanide metal ions for our target range of codes. For a high quality MS signal, it is desirable to load microgel particles with a significant quantity of Ln, e.g. more than $10^6$ ions per particle. The distribution of functional components in microgels dominates the distribution of metals ions within the microgels. The distribution of carboxylic acid groups in PNIPAm microgels has been examined in detail by Hoare and Pelton [7, 9-11] for methacrylic acid, acrylic acid (AA), and vinyl acetic acid. Because of the reactivity ratio mismatch between MAA and NIPAm ($r_{\text{NIPAm}} = 0.20$, $r_{\text{MAA}} = 2.8$), one expects MAA to be consumed more rapidly than NIPAm. The product $r_1r_2$ also provides useful information, since values of $r_1r_2 > 1$ indicate a tendency for block formation. Based on these guidelines, MAA should have a much higher tendency to form blocks than AA ($r_{\text{NIPAm}} = 0.57$, $r_{\text{AA}} = 0.32$), whose reactivity is similar to that of NIPAm. VAA incorporation occurs even more slowly and very few monomer blocks are predicted to be formed. Khokhlov et al. [12] studied the copolymerization rates of VCL and MAA and determined that $r_{\text{VCL}} = 0.03 \pm 0.02$ and $r_{\text{MAA}} = 0.7 \pm 0.2$ under their conditions, which indicates that the reactivity of MAA is also much higher than that of VCL. Based upon these results, we chose MAA as the carboxylic acid source, inferring that it would tend to react faster and to become concentrated in the center of the microgels. We hypothesized that preferential localization of the $\text{–COOH}$ groups within the microgel would favor deposition of Ln nanoparticles in the interior of the microgel.

We note that prior to the Hoare and Pelton publications, Zhou and Chu [13] examined P(NIPAm-\text{-co-MAA}) microgels for a broad range of MAA compositions. For MAA/NIPAm ratios $< 10$ mol %, these authors proposed a core-shell microstructure model in which a MAA-enriched shell surrounds a NIPAm-enriched core. Note that this description is different from what one would infer from the Hoare and Pelton analysis. Zhou and Chu also observed significant changes in the copolymerization kinetics when the degree of MAA incorporation exceeded 25 mol %. Kokufuta et al, [14-15] carried out similar investigations on an acrylic acid/NIPAm copolymer microgels with high (30%) acrylic acid content, and concluded that the bulk of charges did not reside on the particle surface.
In this chapter, I will show the basic features of the synthesis and characterization of carboxylated microgels and their use as microreactors for the deposition of Ln(III) ions and LnF$_3$ nanoparticles. I will compare two types of microgels with different chemical compositions, both based upon poly(N-isopropyl acrylamide) (PNIPAm) and both containing large quantities of methacrylic acid (MAA) as a functional comonomer. One microgel composition contains N-vinylcaprolactam (VCL) as a comonomer, with 10-38 mol % of MAA. The other contains poly(ethylene glycol methacrylate) (PEGMA) as a comonomer [16], with 3-23 mol % of MAA. Both compositions are interesting to us because of our observations that the synthesis of these microgels by free-radical polymerization in water in the absence of surfactant led to stable polymer particles at temperatures above the LCST of the base polymer [17-18]. Upon cooling, these particles expanded to yield solvent-swollen microgels with micrometer diameters. A detailed study on the stability of lanthanide encapsulated microgels and their ability to maintain their lanthanide content will be covered in Chapter 5. The employment of lanthanide encoded microgels in bead-based bioassays will be discussed in Chapter 7.

3.2 Experimental Procedures

In this work, the typical synthesis of microgels uses free-radical surfactant free precipitation polymerization of monomers crosslinked with N,N-methylenebisacrylamide (BIS) [19]. The crosslinker is vital because it prevents the microgel from dissolving in water at low temperatures [20]. In the preparation of microgels, all the monomers, NIPAm and the crosslinker are dissolved in water. The solution is purged with N$_2$ and heated to a temperature above the LCST of PNIPAm (at ca. 70 °C), then an initiator is added. During the synthesis no additional ionic surfactant was added. Therefore colloidal stability of microgels is maintained by electrostatic repulsion from the charge imparted by the initiator and steric repulsion from comonomer such as PEGMA. A description of the synthetic procedure for polyelectrolyte microgels is provided in Chapter 2.

To prepare lanthanide containing microgels as mass cytometric classifier beads, I incorporated Ln(III) ions into the polyelectrolyte microgels, and these ions were then converted in situ to LnF$_3$ nanoparticles. I first studied the conditions to confine the Ln(III) ions or LnF$_3$ nanoparticles in the core of polyelectrolyte microgels by carrying a series of model experiments
with Eu$^{3+}$. Then I extended the *in-situ* precipitation protocol to different elements from the lanthanide series with special focus on one microgel formulation poly(N-isopropyl acrylamide/N-vinylcaprolactam/methacrylic acid), poly(NIPAm/VCL/MAA), with 27 mol% MAA based on total monomer in the synthesis. The lanthanide ion content of the microgels and the particle-to-particle variation of Ln atoms per microgel were investigated based on quantitative mass cytometric analysis. A description of the synthetic procedure for Ln(III) ions and LnF$_3$ nanoparticle containing polyelectrolyte microgels is provided in Chapter 2.

**3.3 Results and Discussions**

**3.3.1 Polyelectrolyte Microgels**

In the sections below, I will first describe the synthesis and characterization of the microgels themselves, including a careful analysis of titrations curves for the microgels following the approach developed by Pelton and Hoare [21]. Information about the distribution of $pK_a$ values for the carboxyl groups helps one understand why some protocols for introducing Ln NPs into the microgels do not work.

**3.3.1.1 Synthesis of Polyelectrolyte Microgels**

One type of microgel that I examined in this study comprised a copolymer of NIPAm and VCL. As model poly(NIPAm/VCL) microgels synthesized under surfactant-free conditions with NIPAm/VCL ratios ranging from 1:10 to 4:1 were colloidally stable, both at the reaction temperature and at room temperature, and were narrow in size distribution. This is in contrast to PNIPAm homopolymer microgels prepared until similar conditions, which formed aggregates during the synthesis. The V-series of microgels contain MAA as a comonomer, in which the mole ratio of NIPAm/VCL was fixed at 4:1. VCL itself is sensitive to acid-catalyzed hydrolysis from the decomposition product of persulfate. Following the suggestion of Forcada et al. [22], I carried out these reactions in the presence of 1 molar equivalent of sodium bicarbonate to neutralize HSO$_4^-$ as it was formed. Microgels were synthesized with 10 to 38 mol % MAA based on total monomer in the reaction. The conversion of monomer to polymer was followed by gravimetry. As shown in Figure 3-1, polymerization of the V-series of microgels was initially rapid, and the typical final conversion reached 70 - 80%.
Table 3-1. Recipes and dimensions of model poly(NIPAm/VCL) microgels prepared under surfactant-free conditions

<table>
<thead>
<tr>
<th>Poly(NIPAm/VCL) microgels</th>
<th>NIPAm mmol</th>
<th>VCL mmol</th>
<th>BIS mmol</th>
<th>NIPAm/VCL R</th>
<th>( R_b / 20 \degree C ) nm</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.55</td>
<td>5.45</td>
<td>0.18</td>
<td>1 : 10</td>
<td>620</td>
<td>0.05</td>
</tr>
<tr>
<td>2</td>
<td>1.24</td>
<td>4.50</td>
<td>0.18</td>
<td>1 : 4</td>
<td>470</td>
<td>0.11</td>
</tr>
<tr>
<td>3</td>
<td>2.00</td>
<td>4.00</td>
<td>0.18</td>
<td>1 : 2</td>
<td>470</td>
<td>0.18</td>
</tr>
<tr>
<td>4</td>
<td>3.00</td>
<td>3.00</td>
<td>0.18</td>
<td>1 : 1</td>
<td>470</td>
<td>0.09</td>
</tr>
<tr>
<td>5</td>
<td>4.00</td>
<td>2.00</td>
<td>0.18</td>
<td>2 : 1</td>
<td>480</td>
<td>0.07</td>
</tr>
<tr>
<td>6</td>
<td>4.80</td>
<td>1.20</td>
<td>0.18</td>
<td>4 : 1</td>
<td>410</td>
<td>0.01</td>
</tr>
</tbody>
</table>

a. NIPAm, VCL and BIS were dissolved in 48 mL of DI water. The mixture was kept in a 100 mL three-necked flask and stirred under N\(_2\) flow for 30 min. The polymerization was initiated with 60 mmol of V-50 (in 2 mL of DI water) at 70 \(\degree\)C. The reaction mixture became turbid, and the reaction was continued for 8 h.

Figure 3-1. Kinetics study: formation of Poly(NIPAm/VCL/MAA) microgels with MAA feed ratio ranging from 10 mol % to 38 mol %. The notation indicates the comonomer (V = VCL) and the number indicates the mole fraction of MAA in the reaction.

A second set of microgels were prepared (by Dr. Xiaomei Ma) using PEG methacrylate (\(M_n\approx 360\)) as an alternative approach to suppressing aggregation during the surfactant-free
polymerization reaction. For the PG-series of microgels, the amount of MAA in the reaction ranged from 3 to 23 mol % based on total monomer. The final conversion was above 70%.

### 3.3.1.2 Microgel Characterization

In our microgel syntheses, the microgel size (the apparent hydrodynamic radii, $R_h$) at pH 7 was determined by dynamic light scattering at a 90° observation angle. Values of $R_h$ were calculated from the first cumulant ($I$) and did not change upon further dilution of the samples. CONTIN plots [23] (in Figure 3-2) showed monomodal distributions. The polydispersity, PDI, was calculated from $(\mu^2/I)$, where $\mu$ is the second cumulant. For the V-series microgels, all microgels were narrowly distributed, with diameters ranging from 700 to 1000 nm. Microgels containing different MAA fractions show a general trend that the size decreased when the MAA fraction increased. The PG-series of microgels were similar, with monomodal CONTIN plots, with hydrodynamic diameters ranging from 800 nm to 1000 nm, and with a decrease in size with increase in MAA content.

**Table 3-2.** Copolymer microgels: conversion (8h), $R_h$, PDI, solids content (SC) and acid content

<table>
<thead>
<tr>
<th>Microgels&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Conversion (8 h)</th>
<th>$R_h$&lt;sup&gt;b&lt;/sup&gt; (nm)</th>
<th>PDI&lt;sup&gt;c&lt;/sup&gt;</th>
<th>SC&lt;sup&gt;d&lt;/sup&gt; (%)</th>
<th>acid group content&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>V10</td>
<td>82%</td>
<td>530</td>
<td>0.02</td>
<td>1.31</td>
<td>0.87</td>
</tr>
<tr>
<td>V19</td>
<td>83%</td>
<td>420</td>
<td>0.02</td>
<td>1.16</td>
<td>1.64</td>
</tr>
<tr>
<td>V27</td>
<td>73%</td>
<td>390</td>
<td>0.01</td>
<td>0.83</td>
<td>2.66</td>
</tr>
<tr>
<td>V38</td>
<td>81%</td>
<td>390</td>
<td>0.01</td>
<td>1.06</td>
<td>3.25</td>
</tr>
<tr>
<td>PG3</td>
<td>83%</td>
<td>470</td>
<td>0.22</td>
<td>0.99</td>
<td>0.43</td>
</tr>
<tr>
<td>PG6</td>
<td>79%</td>
<td>480</td>
<td>0.08</td>
<td>0.95</td>
<td>0.60</td>
</tr>
<tr>
<td>PG12</td>
<td>73%</td>
<td>480</td>
<td>0.03</td>
<td>0.87</td>
<td>1.05</td>
</tr>
<tr>
<td>PG17</td>
<td>77%</td>
<td>480</td>
<td>0.01</td>
<td>0.92</td>
<td>1.53</td>
</tr>
<tr>
<td>PG23</td>
<td>78%</td>
<td>430</td>
<td>0.07</td>
<td>0.94</td>
<td>2.11</td>
</tr>
</tbody>
</table>

---

<sup>a</sup> The notation indicates the comonomer (V = VCL, PG = PEG methacrylate) and the number indicates the mole fraction of MAA in the reaction.

<sup>b</sup> Calculated from the first cumulant $I$ of the autocorrelation decay.

<sup>c</sup> Calculated from $(\mu^2/I)$, where $\mu$ is the second cumulant.

<sup>d</sup> SC: solids contents of the microgel solutions after dialysis.

<sup>e</sup> mmol COOH / g dry microgel.
Figure 3-2. CONTIN plots of the DLS data: (a) poly(NIPAm/VCL/MAA) microgels with MAA feed ratio ranging from 10 mol % to 38 mol %. (b) poly(NIPAm/MAA/PEGMA) microgels with MAA feed ratio ranging from 3 mol% to 23 mol %. The notation indicates the comonomer (V = VCL, PG = PEGMA) and the number indicates the mole fraction of MAA in the reaction.

The MAA content in the copolymer microgels was determined by pH and conductometric titration. Microgels were neutralized with a small excess of NaOH and then back titrated with 0.10 M HCl. Figure 3-3 and Figure 3-4 shows the titration curves for both series of microgels.
**Figure 3-3.** pH and conductometric titration curves for poly(NIPAm/VCL/MAA) microgel solutions.

![Graphs showing pH and conductometric titration curves for poly(NIPAm/VCL/MAA) microgel solutions.](image)

**Figure 3-4.** pH and conductometric titration curves for poly(NIPAm/MAA/PEGMA) microgel solutions.

Electrophoretic mobility ($\mu_e$) measurements were carried out as a function of pH for two representative microgels, V27 and PG17. The electrophoretic mobility of microgel is dominated by the contribution of charges localized within the outermost “soft” particle shell of the gel particles [24]. For both solutions (see Figure 3-5), $\mu_e$ became more negative as the pH increased, with the sharpest decrease at ca. pH 5. This inflection occurs in the range of $pK_a$ of MAA (4.65) [25] and provides evidence for the presence of at least some –COOH groups in the surface region of the microgels.
3.3.1.3 Polyelectrolyte Properties of Copolymer Microgels

Degree of ionization (αd) vs pH. The fractional degree of ionization (αd) for a microgel solution is determined by the moles of OH- added compared to the –COOH content of the sample. These values as a function of the measured pH of the microgel solutions are presented for all of the samples in Figure 3-6. The transition from initial ionization to full ionization occurs over a broad range of pH (ca. 5 pH units). The red dotted lines show intersections of αd vs pH curves at pH 6. Clear differences are observed between microgels containing different amounts of functional groups. For the V-series (Figure 3-6 A), the microgels containing 10 mol% –COOH groups show the largest value of αd (0.36) at pH 6. For the microgels containing higher amounts of MAA, the degree of ionization curves overlap, with a common αd value of ca. 0.25 at pH 6. Similarly, in the PG-series of microgels (Figure 3-6 B), the degree of ionization curves are well separated for microgels containing relatively small amounts of –COOH groups, while with higher MAA contents, the curves tend to overlap. At pH 6, the PG3 microgel shows largest value of αd (0.45). One important conclusion from these plots is that microgels with increasing MAA content require higher pH to be converted from the uncharged state to the fully neutralized state.
Figure 3-6. Plots of the degree of ionization ($\alpha_d$) vs pH for: A) poly(NIPAm/VCL/MAA) microgels; B) poly(NIPAm/MAA/PEGMA) microgels. The red dashed lines indicate the degree of ionization for microgels at pH 6.

**Apparent pK$_a$ values.** From the plots in Figure 3-6, one can calculate the distribution of pK$_a$ values as a function of the degree of ionization of the carboxylic acids.

$$pK_a = pH - \log \left( \frac{\alpha_d}{1 - \alpha_d} \right)$$  \hspace{1cm} (3.4)

Plots of these calculated pK$_a$ versus $\alpha_d$ are shown in Figure 3-7.
Figure 3-7. Plots of $pK_a$ vs degree of ionization ($\alpha_d$) for microgels: a) poly(NIPAm/VCL/MAA) microgels; b) poly(NIPAm/MAA/PEG) microgels. The red dashed arrows represent the largest $pK_a$ change in each type of microgel over the titration range from 10% to 90%.

These plots are sensitive to the underlying functional group distributions. For the V-series of microgels, the $pK_a$ profiles for the microgel with 10% of MAA are separated from all the other microgels in this series. At 90% of titration, V10 microgels show a highest $pK_a$ of ca. 7.3, while all the other microgels in the same series show peak $pK_a$ values close to 8. The overall $pK_a$ changes for this series of microgels are up to ca. 2 units. For the PG-series of microgels, since this series contains more microgels with lower MAA contents, the $pK_a$ profiles show less overlap. PG3 microgels show a longer flat $pK_a$ region over about 60% of titration. Then the $pK_a$ starts to
increase with $0.60 < \alpha_d < 0.90$. At $\alpha_d = 0.90$, the $pK_a$ of the PG3 microgels reached 7. With the increase of MAA content in the microgel samples, the initial flat $pK_a$ regions became shorter. At $\alpha_d = 0.90$, the highest $pK_a$ for all the other microgels in this series reach ca. 7.5, which is three units higher than the $pK_a$ of MAA itself.

The large change in apparent $pK_a$ over the pH titration is a typical “polyelectrolyte” effect, arising from the fact that $–COOH$ groups in the presence of the negatively charged field of surrounding $–COO($-) groups are harder to deprotonate. One explanation for the origin of this polyelectrolyte effect is that there is blockiness to the distribution of acid groups along the polymer backbone. This is consistent with the reactivity ratio mismatch between MAA and NIPAm, which was a feature of our experimental design. I imagine that close proximity of $–COO($-) groups will enhance the ability of these groups to complex Eu$^{3+}$ or other lanthanide ions.

3.3.2 Ln(III) Ions- and LnF$_3$ Nanoparticle-Containing Microgels

3.3.2.1 Incorporation of Ln Elements into Microgels via One-Stage in-situ Precipitation

The synthesis of uniform lanthanide nanoparticles in water is a difficult challenge. The exception is LaF$_3$, which van Veggel [26] prepared by the addition of NaF in water to an aqueous solution of LaCl$_3$ in the presence of aminoethyl phosphate. In this way they obtained uniform particles with diameters that could be varied from about 4 to 6 nm. Pich and Berger in our group have modified this synthesis to introduce a polymerizable group into the particle surface and used these modified particles as reactants in the synthesis of small VCL-based microgels (nanogels) [27-28]. These LaF$_3$ nanoparticles can be prepared in the presence of other lanthanide ions to obtain doped particles containing as much as 12 atom % dopant ion.

Attempts to extend this synthesis to other lanthanides have met with failure. Reports suggest that larger GdF$_3$ nanoparticles can be prepared but with a broad sized distribution [29]. Efforts in our laboratory to prepare uniform colloidal EuF$_3$ nanoparticles in the absence of microgels were all unsuccessful. It is important to recall these difficulties when considering the templated syntheses described below.

The design of experiments in this section is presented in Scheme 3-1. The fundamental idea was to prepare carboxylated microgel particles with diameters on the order of 1 μm, convert the
carboxyl groups to the corresponding Ln\(^{3+}\) carboxylate, and then add a source of fluoride anion to precipitate Ln nanoparticles (NPs) in the interior of the microgels.

**Scheme 3-1.** Concept for loading of Ln nanoparticles into carboxylated microgels containing significant levels of methacrylic acid (MAA) as a comonomer.

*Singly-labeled microgels.* In the sections below, I first describe a number of tests with Eu\(^{3+}\) ions and microgels to investigate proper reaction conditions for the incorporating Ln\(^{3+}\) ions into the polyelectrolyte microgels. As I show below, this process is not as straight forward as it might at first seem. Successful incorporation of EuF\(_3\) NPs into the microgels required a specific protocol of carboxyl group neutralization, followed by ion exchange with Eu\(^{3+}\).

Our first experiments were carried out prior to the careful analysis of the microgel titrations described above. Initial experiments were carried out on microgel solutions neutralized with NaOH to pH 6. Our concern at that point was that more basic solutions might lead to precipitation of Eu(OH)\(_3\) outside the microgels. For the known value of the solubility product of Eu(OH)\(_3\) (\(K_{sp}\text{Eu(OH)3} = 9.4 \times 10^{-27}\)) [30], the calculated upper value of the pH for the onset of precipitation is 5.1. At pH 6, however, there was no observable precipitate. Then 1 eq. of Eu\(^{3+}\) for 3 eq. of –COOH groups was added in the microgel sample. To obtain EuF\(_3\), 3 eq. of F\(^-\) (as NaF or NH\(_4\)F) for each Eu that had been added to the microgel solution was combined. While
these results were unsuccessful in meeting our goal, the outcome is fascinating. Transmission electron microscopy (TEM) images for this experiment are shown in Figure 3-8. For both samples PG17 (Figure 3-8A) and sample V27 (Figure 3-8D), large EuF₃ nanocrystals were obtained. In Figure 3-8D the EuF₃ nanoparticles are uniformly shaped like bacteria, ca. 200 nm in length and ca. 100 nm in width. This is a novel morphology and an unprecedented result that merits further investigation. Since these structures appear to be on the outside of the microgels, rather than in the interior, I have deferred this study to a later time.

In a second attempt, a buffer (10 molar equivalents of bis-tris buffer) was used to maintain the pH at 6 throughout the reaction. Under these circumstances (c.f. TEM images in Figures 3-8 B and E) large aggregates were obtained, with most of the material located outside of the microgels.

In our third attempt, I added 1 molar equivalent of NaOH solution to microgel samples PG17 and to V27 to convert all MAA functional groups to their sodium salts. These solutions were at pH 9. Then the EuCl₃ solution was added. No precipitation was observed, and over the course of the addition of the Eu salt, the pH of the solution decreased to about 7. The addition of the Eu salt caused an increase in the turbidity of the solution, which can be attributed to the increase in the effective index of refraction of the microgels relative to the solvent once they incorporate large numbers of Eu ions. There were two contributors to the dimension change of microgels at this stage: the first one is the cross-linking of multiple -COO(-) groups by the Eu salts and the other is the osmotic deswelling of microgels due to reduced concentration of counter-ions inside the microgel particles. Upon drop wise injection of NaF, small Ln NPs were generated inside the microgel particles, as shown in Figure 3-8C (PG17-EuF₃) and Figure 3-8 F (V27-EuF₃). There was little indication of Eu nanoparticles being formed outside the microgels. I also prepared samples with NH₄F as precipitating reagent and obtained very similar TEM images. This is the result I was looking for.

Samples 3-8A, 3-8B, 3-8D and 3-8E were accompanied by some precipitation, presumably due to the formation of microgel/NPs aggregates. In contrast, samples 3-8C and 3-8F retained their colloidal stability. I interpret this result to indicate that there is a swollen microgel corona on each microgel particle that prevents interparticle aggregation between hybrid microgels in solution.
The presence of Eu and F ions within the microgel was confirmed by energy dispersive X-ray (EDX) analysis. Figure 3-9 shows linear scan profiles across hybrid PG17-EuF₃ microgels on the grid used to obtain the TEM image in Figure 3-8C and V27-EuF₃ microgels on the grid used to obtain the TEM image in Figure 3-8F. While the signal from Eu and F are not as pronounced as that from carbon, they are much higher than that of La, chosen as a negative control, i.e., as a lanthanide element not expected to be present in the sample, except as an impurity. EDX profiles (not shown) for other hybrid V27 microgels on this grid and of the V27 microgels prepared at pH 6 (Figure 3-8D) also support our interpretation that EuF₃ nanoparticles form inside of the microgels only at a high degree of ionization.
Figure 3-9. Elemental analysis by EDX. A) Linear scan of two PG17 microgels containing EuF$_3$. B) Linear scan of two V27 microgels containing EuF$_3$. The microgel sample was treated with 1 eq. of NaOH per –COOH, and then with 1/3 molar eq. of EuCl$_3$ solution. Subsequently, 3 eq. of a NaF solution per Eu$^{3+}$ was injected to precipitate EuF$_5$. 
The hydrodynamic radius of microgels in aqueous solution were characterized by DLS. Figure 3-10 compares CONTIN plots of the apparent hydrodynamic radius distribution of the microgels at pH 9, before and after addition of Eu$^{3+}$, and after adding F. For both V27 (Figure 3-10A) and PG17 (Figure 3-10B) microgels, the evolution of hydrodynamic radius followed the same trend. Upon addition of 1 molar eq. of NaOH, the microgels underwent a large extent of swelling, an increase of 70 nm in V27 microgels and 60 nm in PG17 microgels in $R_h$ upon deprotonation. The swelling of microgel particles originates from the increase in osmotic pressure caused by the relatively high concentration of solvated counter cations within the microgel particles due to the presence of the $\text{COO}(-)$ groups [31]. The osmotic swelling helps to overcome the internal Donnan potential. Upon adding EuCl$_3$, the $R_h$ values decreased by 180 nm in V27 and 110 nm in PG17 microgels. At this stage cross-linking of multiple $\text{COO}(-)$ groups by the Eu salts reduced the internal concentration of counter cations, which, in turn, led to an osmotic deswelling of microgel particles. Subsequent addition of F$^-$ led to an additional small contraction in the radius. Here the osmotic deswelling was less pronounced than in the previous case, probably due to the partial release of the initially bound $\text{COO}(-)$ groups upon formation of EuF$_3$ nanoparticles. The results are summarized in Table 3-3.
Figure 3-10. CONTIN plots of the hybrid microgels: (A) sample V27: top to bottom: at pH 7, at pH 9, after addition of Eu\(^{3+}\) (1 Eu\(^{3+}\) for every 3 -COO-), after addition of NaF (3 F\(^-\) for every Eu\(^{3+}\)). (B) similar data for microgel sample PG17.
Table 3-3. Hydrodynamic radii of the microgels determined by DLS

<table>
<thead>
<tr>
<th>microgels</th>
<th>$R_h$ (nm)</th>
<th>PDI</th>
<th>microgels</th>
<th>$R_h$ (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>V27-pH7</td>
<td>390</td>
<td>0.010</td>
<td>PG17-pH7</td>
<td>480</td>
<td>0.008</td>
</tr>
<tr>
<td>V27-pH9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>460</td>
<td>0.002</td>
<td>PG17-pH9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>540</td>
<td>0.032</td>
</tr>
<tr>
<td>V27-EuCl&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>280</td>
<td>0.13</td>
<td>PG17-EuCl&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>430</td>
<td>0.046</td>
</tr>
<tr>
<td>V27-EuF&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>310</td>
<td>0.020</td>
<td>PG17-EuF&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>440</td>
<td>0.031</td>
</tr>
</tbody>
</table>

a. after addition of 1 eq. of NaOH.
b. after addition of 1 eq. of Eu<sup>3+</sup> for every 3 eq. of –COO⁻.
c. after addition of 3 eq. of NaF for every eq. of Eu<sup>3+</sup>.

Quantitative analysis of all the metal containing microgels were carried out by mass cytometry and described in detail in the next section. From the mass cytometric results, I determined that the optimal condition for preparing Eu<sup>3+</sup> ion-encoded microgels is by pre-neutralizing the microgels with 1 molar eq. of NaOH before ion exchange with Eu<sup>3+</sup> ions. The number of Eu<sup>3+</sup> that could bind to microgels reached its maximum value when the added amount of Eu<sup>3+</sup> equaled ca. 1/3 of the MAA content as determined by titration. Following this general protocol, microgels labeled with different metal ions from the lanthanide series were prepared. Metal loading began with addition of 1 eq. NaOH per –COOH group to a microgel solution initially at 0.25 wt% concentration. Then 1/3 molar eq. Ln<sup>3+</sup> ions per –COOH group was introduced into the microgel solution and allowed to undergo counterion exchange with the microgels. During ion exchange, the pH of microgel solution dropped from pH 9 to pH 6.5. This type of microgel containing Ln<sup>3+</sup> ions as their carboxylate salt is denoted MG-Ln.

Samples of these microgels were then treated with a solution of NaF (1 eq. F⁻ per original –COOH group) to form LnF₃ nanoparticles (NPs) trapped within the microgel. After addition of the F⁻ ions, the pH of microgel solutions increased from 6.5 to 7. Microgels carrying entrapped LnF₃ NPs are denoted MG-LnF₃.

**Binary-labeled microgels.** Some experiments were carried out with mixtures of Ln ions, so that one can design microgels tagged with more than one metal species. As above, the carboxylated microgel solutions were pre-neutralized with 1 eq. NaOH per MAA groups. Then
mixtures of EuCl$_3$ and TbCl$_3$ in aqueous solution were added to the microgels and allowed to ion exchange. Keeping the overall Ln amount constant at a −COOH: Ln$^{3+}$ mole ratio of 3:1, different feed ratios of Eu/Tb were added to create different binary encoded microgels. From mass cytometric analysis I examined how the metal-ion composition of the microgels varied with the composition of the metal salt mixture.

3.3.2.2 Incorporation of Ln Elements into Microgels via Two-Stage in-situ Precipitation

The idea of two-stage in-situ ion exchange and precipitation outlined in Scheme 3-2 is based on an approach reported by Moffitt et al. [32] for the synthesis of CdS nanoparticles within reverse micelles of polystyrene-b-poly(cadmium acrylate). These authors treated the micelles with H$_2$S to precipitate CdS and simultaneously regenerate −COOH groups. They were able to reload the micelles with additional cadmium acetate, followed by a second reaction with H$_2$S. By this method, CdS clusters of 3.7 nm in diameter were enlarged to 5.6 nm in two steps.

Here I tested the hypothesis that when NaF is added to generate LnF$_3$ NPs, some of the MAA functional groups initially bound to the Ln$^{3+}$ will be released. I used the MG-EuF$_3$ microgels described above in this test. After purifying the sample by sedimentation and redispersion, 1/3 molar eq. of Eu$^{3+}$ per total MAA were added into the MG-EuF$_3$ microgel solution. This sample was also purified by sedimentation and redispersion in DI water. Then 3 eq. of NaF per Eu$^{3+}$ was added for secondary precipitation of EuF$_3$ inside of microgel. In a two-stage process, an increase in metal content in the microgels was anticipated.

![Scheme 3-2](image)

Scheme 3-2. Concept for loading of LnF$_3$ nanoparticles into carboxylated microgels containing significant levels of methacrylic acid (MAA) as a comonomer by two-stage in-situ precipitation process.
This is a useful point to examine the size and size distribution of the microgels themselves, as monitored by right-angle dynamic light scattering, as a function of the ion exchange and nanoprecipitation processes. Figure 3-11 shows the CONTIN plots for the microgel solutions at each stage of the two-stage in-situ precipitation process. Values of the apparent hydrodynamic radii, $R_h$, were calculated from the first cumulant ($I$). The polydispersity, PDI, was calculated from $\mu^2/I$, where $\mu$ is the second cumulant. The results are summarized in Table 3-4. From CONTIN plots, one sees that the microgel particles maintain monomodal and narrow size distributions throughout the ion exchange and precipitation steps. This result indicates that the increase in metal content per microgel in the second stage does not originate from aggregation caused by added salt.

The value for $R_h$ of the as-prepared V27 microgel was 390 nm. This increased to 460 nm following neutralization with NaOH. This increase in microgel size is associated with an increase in the osmotic swelling of the microgels by the counterions associated with the -COO(-) groups that help to overcome the internal Donnan potential [31]. Ion exchange with EuCl$_3$ led to a contraction in size to $R_h = 277$ nm. Here, most of the -COO(-) groups were cross-linked by Eu$^{3+}$ ions, leading to a substantial decrease in the amount of free counter cations in the microgels. Upon the first addition of NaF, the microgels underwent a small extent of osmotic swelling, with a 30 nm increase in $R_h$, likely related to the increased solvated counterion concentration inside the microgels, when the initially bound -COO(-) groups were partially released during the formation of EuF$_3$ nanoparticles. In the second stage, similar behavior was observed. After the microgels were treated with additional Eu$^{3+}$ ions, $R_h$ decreased to 279 nm, associated with additional cross-linking by Eu$^{3+}$. Subsequent addition of NaF caused a small increase in swelling (20 nm increase in $R_h$) reflecting a slight increase of solvated counterions inside microgels. The changes seen in Figure 5 for the Eu$^{3+}$-containing microgels are much smaller that those associated with neutralization of the as-prepared microgels and the initial cross-linking with Eu$^{3+}$ ions.
3.3.3 Quantitative Determination of Metal Incorporation by Mass Cytometry

The lanthanide loading efficiency and distribution among microgels were investigated by mass cytometry. In this technique, particles (i.e., microgels) are injected individually but stochastically into the torch of an inductively coupled plasma mass spectrometer (ICP-MS) at a rate of about 1000 beads per second. The high temperature of the plasma vaporizes, atomizes, and ionizes each bead, generating an ion cloud. During the transit time of the ion cloud through the instrument (ca. 200 µs) 20 to 30 time-of-flight mass spectra are taken in the mass range of m/z 110 to 200.
3.3.3.1 Singly-Labeled Microgels

Figure 3-12 shows a representative screen capture from mass cytometry measurement on a sample of V27-EuCl₃ microgels. The x-axis corresponds to m/z for the ions reaching the detector. The y-axis reports successive mass spectra. Each horizontal grey-blue bar highlights a series of signals for one microgel event. In the figure, three microgel events were captured during the time of acquisition.

![Time of flight (m/Z)](image)

**Figure 3-12.** A mass cytomtery screen capture for V27-EuCl₃ microgels. The x-axis corresponds to m/z of ions reaching the detector. The y-axis reports successive mass spectra. Each horizontal bar highlights one microgel event.

In Figure 3-13, I compare the lanthanide atoms distributions among microgels before and after adding F⁻ by histograms of $^{151}$Eu distributions from Eu encoded microgels. In Figure 3-14, the Eu signal intensity and Eu contents per microgels were displayed in box-and-whisker plots [33]. The small box in the middle of each plot shows the mean value of Eu signal intensity (left axis) or average number of atoms per microgel (right axis). The big box includes the middle one half of the data, in which the ends of the box are the upper and lower quartiles. The bar near the middle of the box is the median. The two lines outside the box are from the upper and lower 5$^{th}$ percentile of data. The two ‘×’s show the upper and lower 99$^{th}$ percentile of data. The upper outlier is the maximum and the lower outlier is the minimum value recorded. Two sets of data are presented, for microgel samples V27 and PG17. The left-most plots in each set describe the distribution of Eu atoms per microgel before adding F⁻. The middle plots display the Eu atom distribution of microgels after adding F⁻ in the form of an NaF solution. The right hand plots
represent a second set of microgels obtained using NH$_4$F as the source of fluoride ion. The first feature of these data to notice is that the PG17 microgels, which have a lower MAA content than the V27 microgels, also have a lower Eu content after neutralization with NaOH followed by ion exchange with Eu$^{3+}$. The second important feature is that precipitation of the Eu$^{3+}$ ions with added fluoride largely conserves the number of Eu atoms per microgel. The same result was obtained if F$^-$ was added as sodium fluoride or ammonium fluoride. And thirdly, the lower and upper extremes of mass cytometry signal intensity indicates that some microgel breakage and flocculation occurred during the detection process.

The transmission efficiency of mass cytometry is on the order of 10$^{-4}$, which means that about 1 in every 10$^4$ ions generated in the plasma reaches the detector. The mean number of metal ion per microgel can be calculated from the intensity value measured by the mass cytometry detector according to the equation:

$$N = \frac{I}{T} \quad (3.5)$$

$I$ is the mean intensity measured by mass cytometry detector, and $T$ is the transmission coefficient of the detector. Generally the transmission coefficient is on the order of 10$^{-4}$ to 10$^{-5}$, which means that about 1 in every 10$^4$ to 10$^5$ ions generated in the plasma reaches the detector. Values of $T$ are obtained by daily instrument calibration with tuning solutions, and one should note that early Ln elements are characterized by lower values of $T$ than the later Ln elements.

Values of the mean number of Eu atoms per hybrid microgel and the calculated coefficient of variation (CV) for these samples are collected in Table 3-4. Understanding these results remains a challenge, because this type of information, about the distribution of species in a sample of polymer particles, has never before been available.
Figure 3-13. Histograms of $^{151}\text{Eu}$ distribution from Eu$^{3+}$ ions and EuF$_3$ nanoparticle encoded microgels.

Figure 3-14. Box-and-whisker plots displaying the distribution of Eu signal intensity for hybrid microgels determined by mass cytometry. Data obtained from V27 microgels are presented in the first three columns; data from PG17 microgels are presented in the last three columns. PG17-Eu and V27-Eu refer to the microgels at pH 9 to which EuCl$_3$ was added. PG17-EuF$_3$ (NaF) and V27-EuF$_3$ (NaF) refer to Eu-containing microgels to which NaF was added. PG17-EuF$_3$ (NH$_4$F) and V27-EuF$_3$ (NH$_4$F) refer to Eu-containing microgels to which NH$_4$F was added.
(NH₄F) refer to microgel samples in which NH₄F was added. Values of the number of Eu atoms per microgel were calculated using the mass cytometry transmission efficiency for Eu ions of $7.91 \times 10^{-5}$.

**Table 3-4.** Eu content of the microgels

<table>
<thead>
<tr>
<th>samples</th>
<th>Eu atoms per microgel (^a)</th>
<th>CV (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V27-Eu</td>
<td>$1.0 \times 10^7$</td>
<td>33%</td>
</tr>
<tr>
<td>V27-EuF(_3) (NaF)</td>
<td>$1.1 \times 10^7$</td>
<td>37%</td>
</tr>
<tr>
<td>V27-EuF(_3) (NH₄F)</td>
<td>$9.5 \times 10^6$</td>
<td>34%</td>
</tr>
<tr>
<td>PG17-Eu</td>
<td>$5.0 \times 10^6$</td>
<td>36%</td>
</tr>
<tr>
<td>PG17-EuF(_3) (NaF)</td>
<td>$5.0 \times 10^6$</td>
<td>35%</td>
</tr>
<tr>
<td>PG17-EuF(_3) (NH₄F)</td>
<td>$5.0 \times 10^6$</td>
<td>37%</td>
</tr>
</tbody>
</table>

\(^a\) Mean number of Eu atoms per microgel determined by mass cytometry.

\(^b\) Standard deviation of the number of Eu atoms per microgel determined by mass cytometry, expressed as CV in percent.

**Figure 3-15.** Box-and-whisker plots of mass cytometry distribution of Eu signal intensity for hybrid V27 microgels synthesized with increasing Eu : COOH feed ratios. The transmission efficiency of mass cytometry for the Eu ions was $7.91 \times 10^{-5}$. Using this value, the atoms per microgel were calculated and are presented on the right-hand y-axis.

I also varied the Eu to −COOH molar ratios and used mass cytometry to followed changes in the number of Eu atoms incorporated per microgel. Figure 3-15 shows box-and-whisker plots of the Eu distribution in this series of microgels. In experiments, in which the Eu to −COOH
molar ratio was low (0.1/3), each microgel contained about $10^6$ Eu atoms. As this ratio of reactants was increased, the average number of Eu atoms per microgel increased, reaching a plateau for a reactant ratio of one Eu per 3 $\text{COOH}$ groups, corresponding to $10^7$ Eu atoms/microgel. Further increases of the Eu feed did not lead to an increase in the Eu content of the microgels, and the distribution of Eu intensity remained similar. As a control experiment, a sample of V27 microgels was treated with 1 molar eq. of NaOH, but no EuCl$_3$ was added. Here only 2 counts were detected as the Eu intensity, which is more than 2 orders of magnitude below the other samples in Figure 3-15. These results indicate that it takes 3 $\text{COOH}$ groups in the microgel to bind each Eu ion.

Now I will discuss microgels containing different Ln elements, including metals from early (La$^{3+}$) to late (Tm$^{3+}$) elements. In Figure 3-16, the lanthanide content and atom distributions among microgels is presented by box-and-whisker plots [33]. On the x-axis, the elements (La, Nd, Eu, Tb, Ho and Tm) refer to Ln$^{3+}$ ion containing microgels obtained by ion exchange, whereas the corresponding fluorides (LaF$_3$, NdF$_3$, EuF$_3$, TbF$_3$, HoF$_3$ and TmF$_3$) refer to LnF$_3$ nanoparticle containing microgels. The values of the mean number of Ln atoms per microgel and the calculated coefficient of variation (CV) for these samples are collected in Table 3-5.

For the ion exchanged microgels (MG-Ln), the number of metals per microgel increases slightly from early lanthanides (La and Nd) to the middle lanthanides (Eu and Tb) and the late lanthanides (Ho and Tm). For instance, the smallest Ln contents were found in MG-La, containing $0.6 \times 10^7$ Ln per microgel, followed by MG-Nd with $0.8 \times 10^7$ Ln per microgel. Both MG-Eu and MG-Tb contain $1.0 \times 10^7$ Ln per particle, while slightly higher levels of metal content per particle were found in MG-Ho ($1.1 \times 10^7$) and MG-Tm with ($1.2 \times 10^7$). Along with lower intensities for early-Ln-encoded microgels, the data also show larger bead-to-bead distributions of the Ln ions, which corresponds well with the statistical theory that the counting errors decreases with the increase of number of counts. For LnF$_3$ nanoparticle containing microgels, the number of Ln metals per microgels remains similar to that of the precursor ion-exchanged microgels. This result indicates that adding fluoride largely conserves the number of Ln atoms per microgel.
Figure 3-16. Box-and-whisker plots distribution of number of Ln atoms per hybrid microgel containing different Ln elemental tags, as determined by mass cytometry. On the x-axis, the elements (La, Nd, Eu, Tb, Ho and Tm) refer to metal containing microgels obtained by ion exchange, whereas LaF\textsubscript{3}, NdF\textsubscript{3}, EuF\textsubscript{3}, TbF\textsubscript{3}, HoF\textsubscript{3} and TmF\textsubscript{3} refer to microgels containing LnF\textsubscript{3} nanoparticles. The mass cytometry transmission efficiencies for La ions was 1.85 x 10\textsuperscript{-5}; for Nd ions, 2.71 x 10\textsuperscript{-5}; for Eu ions, 3.83 x 10\textsuperscript{-5}; for Tb ions, 6.24 x 10\textsuperscript{-5}; for Ho ions, 5.69 x 10\textsuperscript{-5}, and for Tm ions, 4.84 x 10\textsuperscript{-5}.

Table 3-5. Characteristics of different Ln-encoded-microgels

<table>
<thead>
<tr>
<th>Samples</th>
<th>(I_{Ln}^a) counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG-La</td>
<td>112.9</td>
</tr>
<tr>
<td>MG-LaF\textsubscript{3}</td>
<td>136.9</td>
</tr>
<tr>
<td>MG-Nd</td>
<td>216.8</td>
</tr>
<tr>
<td>MG-NdF\textsubscript{3}</td>
<td>216.8</td>
</tr>
<tr>
<td>MG-Eu</td>
<td>386.8</td>
</tr>
<tr>
<td>MG-EuF\textsubscript{3}</td>
<td>417.5</td>
</tr>
<tr>
<td>MG-Tb</td>
<td>624.0</td>
</tr>
<tr>
<td>MG-TbF\textsubscript{3}</td>
<td>624.0</td>
</tr>
<tr>
<td>MG-Ho</td>
<td>625.9</td>
</tr>
<tr>
<td>MG-HoF\textsubscript{3}</td>
<td>603.1</td>
</tr>
<tr>
<td>MG-Tm</td>
<td>580.8</td>
</tr>
<tr>
<td>MG-TmF\textsubscript{3}</td>
<td>556.6</td>
</tr>
</tbody>
</table>

\(I_{Ln}^a\): the mean Ln intensity measured by the mass cytometry detector.

\(T_{Ln}^b\): the transmission coefficient of the detector for each Ln element.

\(N_{Ln}^c\): the mean number of Ln atoms per microgel calculated with equation (3.5).
d. \(CV_{Ln}\): the coefficient of variation for the number of Ln atoms per microgel.

Figure 3-17 presents mass cytometry results on the number of Eu atoms per microgels prepared from the two-stage *in-situ* precipitation process. Table 3-6 lists the mean value of Eu content per microgel and the \(CV\) of these values. In these experiments, \textbf{I-a} refers to the NaOH-neutralized microgel to which Eu\(^{3+}\) ions were added (1 eq. Eu/-COO\(^{-}\)); \textbf{I-b} refers to the sample after the first addition of NaF (3F/Eu); \textbf{II-a} refers to the subsequent addition of the same amount of EuCl\(_3\) as in the first step; and \textbf{II-b} refers to addition of NaF to this dispersion. In each instance the samples were washed by successive sedimentation and redispersion in DI water prior to the next addition step. The data shows that the precipitation of the Eu\(^{3+}\) ions with added fluoride largely conserves the number of Ln atoms per microgel from each stage, as the mean values of Eu atoms per microgel are similar before and after fluoride was added. In the first stage, as described above, ca. \(1 \times 10^7\) Eu\(^{3+}\) were incorporated into the microgels, with \(CV\) values of 33 to 34%. In the second stage, the number of Eu\(^{3+}\) ions increased to \(1.5 \times 10^7\), accompanied by a small increase in \(CV\) to 40%.

\[\text{Figure 3-17.} \text{ Box-and-whisker plots of the distribution of number of Eu atoms per microgel prepared by two-stage *in-situ* precipitation as determined by mass cytometry.}\]
Table 3-6. Characteristics of Eu-encoded-microgels prepared by multi-step in-situ precipitation.

<table>
<thead>
<tr>
<th>Samples</th>
<th>$I_{Eu}$</th>
<th>$N_{Eu}^a$ (10$^7$)</th>
<th>$CV_{Eu}^b$ (%)</th>
<th>$R_h^c$ (nm)</th>
<th>PDId</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>390</td>
<td>0.01</td>
</tr>
<tr>
<td>0-b</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>460</td>
<td>0.01</td>
</tr>
<tr>
<td>I-a</td>
<td>387</td>
<td>1.0</td>
<td>33</td>
<td>277</td>
<td>0.02</td>
</tr>
<tr>
<td>I-b</td>
<td>418</td>
<td>1.1</td>
<td>34</td>
<td>308</td>
<td>0.01</td>
</tr>
<tr>
<td>II-a</td>
<td>575</td>
<td>1.5</td>
<td>41</td>
<td>279</td>
<td>0.02</td>
</tr>
<tr>
<td>II-b</td>
<td>578</td>
<td>1.5</td>
<td>40</td>
<td>301</td>
<td>0.02</td>
</tr>
</tbody>
</table>

a. $N_{Eu}$: mean number of Ln atoms per microgel determined by mass cytometry. The transmission efficiency for the Eu ions was 3.83 x 10$^{-5}$.
b. $CV_{Eu}$: the coefficient of variation for the number of Ln atoms per microgel.
c. Calculated from the first cumulant $\Gamma$ of the autocorrelation decay.
d. Calculated from $(\mu^2/\Gamma)$, where $\mu$ is the second cumulant.

3.3.3.2 Binary-Labeled Microgels

In this section, I describe experiments on microgels ion exchanged with mixtures of EuCl$_3$ and TbCl$_3$ to examine how the metal-ion composition of the microgels varies with the composition of the metal salt mixture. I kept the overall salt concentration at 1/3 molar eq. Ln$^{3+}$/MAA and varied the ratio of Eu/Tb to obtain different binary-encoded poly(NIPAm/VCL/MAA) microgels.

Figure 3-18 presents box-and-whisker plots of mass cytometry distribution of the binary labeled microgels. For each Eu/Tb feed ratio, the upper plots report the Eu atom distributions among microgels, while the lower plots are the corresponding Tb atom distributions. The y-axis values refer to the numbers of atoms per microgels calculated using equation (3.5). The values of the mean number of each Ln elements per microgel and the calculated CV for samples are collected in Table 3-7. There are two main observations from these experiments. The first is that the Tb/Eu ratio in the microgel is always slightly higher than that in the feed. The second is that the particle-to-particle variation in ion content is large enough that large ion ratios (i.e., a factor of 2 or more) would be needed for encoding particles by variation of ion content for an individual element.
### Table 3-7. Characteristics of different Eu/Tb-encoded-microgels determined by mass cytometry

<table>
<thead>
<tr>
<th>Samples</th>
<th>(N_{\text{Eu}}^{a}) (\times 10^6)</th>
<th>(CV_{\text{Eu}}^{b})</th>
<th>(N_{\text{Tb}}^{c}) (\times 10^6)</th>
<th>(CV_{\text{Tb}}^{d})</th>
<th>(N_{\text{total}}^{e}) (\times 10^6)</th>
<th>(\frac{n_{\text{Eu}}}{n_{\text{total}}})</th>
<th>(\frac{n_{\text{Tb}}}{n_{\text{total}}})</th>
<th>(\frac{N_{\text{Eu}}}{N_{\text{total}}})</th>
<th>(\frac{N_{\text{Tb}}}{N_{\text{total}}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG-Eu/Tb-1</td>
<td>0</td>
<td>-</td>
<td>1.0</td>
<td>34</td>
<td>10</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>MG-Eu/Tb-2</td>
<td>1.4</td>
<td>39</td>
<td>8.5</td>
<td>26</td>
<td>9.9</td>
<td>0.17</td>
<td>0.83</td>
<td>0.14</td>
<td>0.86</td>
</tr>
<tr>
<td>MG-Eu/Tb-3</td>
<td>4.4</td>
<td>30</td>
<td>5.7</td>
<td>31</td>
<td>10</td>
<td>0.50</td>
<td>0.50</td>
<td>0.44</td>
<td>0.56</td>
</tr>
<tr>
<td>MG-Eu/Tb-4</td>
<td>8.0</td>
<td>28</td>
<td>2.4</td>
<td>58</td>
<td>10</td>
<td>0.80</td>
<td>0.20</td>
<td>0.77</td>
<td>0.23</td>
</tr>
<tr>
<td>MG-Eu/Tb-5</td>
<td>10.0</td>
<td>33</td>
<td>0</td>
<td>-</td>
<td>10</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

a. \(N_{\text{Eu}}\): mean number of Eu atoms per microgel. The transmission efficiency for Eu ions was 3.80 \(\times 10^5\).

b. \(CV_{\text{Eu}}\): coefficient of variation for the number of Eu atoms per microgel.

c. \(N_{\text{Tb}}\): mean number of Tb atoms per microgel. The transmission efficiency for Tb ions was 5.90 \(\times 10^5\).

d. \(CV_{\text{Tb}}\): coefficient of variation for the number of Tb atoms per microgel.

e. \(N_{\text{total}}\): total number of Ln atoms per microgel.

f. \(n_{\text{Eu}}/n_{\text{total}}\) is the mole fraction of EuCl\(_3\) in the feed mixture in the ion exchange step; \(n_{\text{Tb}}/n_{\text{total}}\) is the corresponding mole fraction of TbCl\(_3\).

g. \(N_{\text{Eu}}/N_{\text{total}}\) is the measured mole fraction of Eu ions in the microgel determined by mass cytometry; \(N_{\text{Tb}}/N_{\text{total}}\) is the corresponding mole fraction of Tb ions.
In this Chapter, I reported the synthesis of highly carboxylated microgels, based upon a poly(N-isopropylacrylamide) copolymer framework, with diameters on the order of 1 µm. These microgels were then used as a template for incorporating Ln$^{3+}$ ions. The long-term goal of these experiments is to employ these metal-labeled microgel hybrids in bead-based bioaffinity assays using mass cytometry for sample detection. These experiments show that by adjusting the Eu$^{3+}$/–COOH group ratio, the Eu content of the microgels could be controlled within the range of $10^6$ to $10^7$ Eu ions per microgel. The Eu-containing microgels prepared by exchanging Na$^+$ ions for Eu$^{3+}$ had the same Eu content after the microgels were subsequently treated with F$^-$ to convert the Eu$^{3+}$ ions to EuF$_3$ nanoparticles. Following the general protocols, a series of Ln-encoded microgels was prepared. The lanthanide content per microgel and their distributions were determined by mass cytometry. It is shown that there was a small variation in Ln ion uptake.
during the ion exchange, favoring late Ln ions (Ho, Tm) over early Ln elements (La, Nd). Conversion of the Ln carboxylates to the corresponding LnF$_3$ NPs conserved the number and distribution of Ln ions per microgel. We also examined a two-stage *in-situ* precipitation process, in which MG-EuF$_3$ microgels were treated with additional EuCl$_3$ followed, after washing, by addition of NaF. This led to a 50% increase in the Eu content to ca. $1.5 \times 10^7$ Eu ions per microgel.

The most important factor that controls the number of Eu atoms per microgel as measured by mass cytometry is the number of MAA groups per microgel. I was able to demonstrate that each Eu ion bound to the microgel required 3 –COO(-) ions. The number of MAA groups per microgel depends upon the amount of MAA in the reaction mixture (27 mol % in V27, 17 mol % in PG17), its conversion in the microgel synthesis, and the size of the microgels obtained in the reaction. Table 3-2 shows that all of the microgels had a narrow size distribution, but that the mean diameter decreased as the amount of MAA in the reaction was increased. It would be very interesting to understand the role played by the distribution of –COOH groups within the microgel on metal ion content and its distribution between microgels. Hoare and Pelton found a tendency toward formation of blocks of MAA functional groups in P(NIPAm/MAA) copolymer microgels containing 6.5 mol % MAA [9]. It is expected that with the larger amounts of MAA employed here, much of the MAA present in the microgel network may be in the form of carboxyl-rich blocks.

Other factors can play a role. Over a longer term, leaching of metal ions from the microgel will decrease the metal ion content, and might lead to a broadening of the particle-to-particle variation in metal ion content. I will discuss the stability of different types of lanthanide encoded microgels against leaching in Chapter 5. Some factors operate at the level of the mass cytometry measurement itself. Microgel dimers, formed as the aqueous solution was nebulized prior to entering the plasma torch, or microgel aggregates, would burn as a single entity and appear as a particle with a much larger Eu content. Any microgel fragments formed as they entered the plasma torch would appear as particles with a reduced metal ion content. The box and whiskers plots presented in Figures 9 and 10 show some evidence for “outliers”, small sets of particles with metal-ion content relatively far from the mean. The values at the extremes of the distribution contribute to the magnitude of the CV$_{Ln}$ of the microgel-to-microgel content of Eu ($CV_{Eu} = 33\% - 37\%$ for V27-Eu and V27-EuF$_3$), which is slightly larger than the bead-to-bead
variation in microgel volume inferred from DLS measurements ($CV_{\text{DLS}} \approx 3\sqrt{\text{PDI}} = 30\%$ for V27), however, much higher than the coefficient of variance of particle molecular weight ($CV_{\text{TEM}}$) calculated from dry state analysis of microgel particles from TEM image ($CV_{\text{TEM}} = \left(\left(\frac{d^2}{d'}\right)^3 - 1\right)^{\frac{1}{2}} = 11\%$ for V27, where $d^2 = 150041 \text{ nm}^2$ and $d = 387 \text{ nm}$). The DLS measurements also showed small extents of microgel aggregation for samples stored in DI water for two months. These aggregates were easily disrupted by mild sonication. Mild sonication of samples immediately prior to mass cytometry analysis had little effect on the signals obtained.

My goal is to use these microgels for multiplexed bioassays based upon mass cytometry. For this application I need to be able to control the level of lanthanide ion incorporation into the microgels, to vary the type of lanthanide ion incorporated, and to attach bioaffinity agents to the microgel surface. In the work described here, I showed that I can vary and control the level of Ln incorporation into these two types of microgels. Experiments to attach bioaffinity agents to the microgels will be described in Chapter 7.
Reference


Chapter 4. Lanthanide Phosphate-Containing Microgels as Classifier Beads for Mass Cytometric Analysis

In this chapter, I describe the synthesis and characterization of LnPO$_4$ containing poly(N-isopropyl acrylamide-co-N-vinylcaprolactam-co-methacrylic acid) [poly(NIPAm/VCL/MAA)] microgels. The synthetic methodology was based on in-situ precipitation of LnPO$_4$ using functional microgels as soft templates. The main challenge was to find appropriate conditions to confine lanthanide phosphate precipitate to the interior of the microgels. Various sources of phosphate ions led to precipitation of LnPO$_4$ on the exterior of the microgels. One approach worked; it involved three steps: neutralization of the microgels with NaOH, ion exchange with a lanthanide salt, followed by treatment with a large excess of PBS buffer at pH 7. In this way I obtained microgels containing ca. $10^7$ Ln atoms per microgel (Ln = La, Nd, Eu, Tb, Ho, Tm). I presume that PBS acted both as a precipitating reagent for Ln$^{3+}$ ions as well as a buffering reagent to neutralize protons produced during the formation of LnPO$_4$. The LnPO$_4$ containing microgels were studied in an attempt to prepare microgels that meet metal-content and surface functionality requirements and, more importantly, to compare the stabilities of different types of lanthanide metal containing microgels (i.e. Ln$^{3+}$ ions-, LnF$_3$- and LnPO$_4$-containing microgels) during storage as well as in experimental assays. A detailed study on the stability of microgels will be discussed in the next Chapter.

4.1 Introduction

We are interested in metal-encoded polymer microbeads for applications in multiplexed immunoassays based on mass cytometry [1]. In mass cytometry, beads (or cells) are injected individually but stochastically into the plasma torch of an inductively coupled plasma mass spectrometer (ICP-MS) equipped with time-of-flight detection. The cells or beads are vaporized, atomized and ionized to yield an ion cloud with a transient duration of ca. 200 µs, during which 20 to 30 mass spectra are taken. In bead-based assays, the beads are encoded as a signature of the bioaffinity agent such an antibody (Ab) attached to the bead surface. In flow cytometry beads are encoded with fluorescent dyes. For mass cytometry, the beads should be encoded with well-defined levels of metal ions in the range of detection of the ICP-MS. For mass cytometry, lanthanide ions are a useful set of encoding elements.
Initial experiments in our laboratory focused on polystyrene (PS) microbeads, but more recently we turned our attention to microgels. Aqueous microgels [2] are cross-linked polymer particles which swollen in water. Microgels offer an advantage over PS beads in the ease of attaching biomolecules such as antibodies to the surface. We recently reported a synthesis of aqueous microgels in which we were able to deposit EuF$_3$ and other LnF$_3$ salts specifically in the core [3]. A specific protocol involving ion exchange of the microgels with lanthanide ions prior to adding fluoride was necessary to confine the precipitate to the core of the microgels, but the overall synthesis was successful. For the case of Europium, microgel samples could be prepared with levels of Eu ranging from $10^6$ to $10^7$ ions per microgel. One of the disadvantages of EuF$_3$ as the labeling reagent is that it is somewhat soluble at acidic pH, and can leach from the microgels on storage or during applications.

Lanthanide phosphates are highly insoluble soluble salts with solubility products ranging from $10^{-25}$ to $10^{-27}$ M$^2$ [4], much lower than the solubility products of the corresponding fluorides, which range from $10^{-15}$ to $10^{-19}$ M$^4$ [5,6]. Our hypothesis in undertaking this work is that LnPO$_4$ is more stable toward etching or leaching in acidic buffers than LnF$_3$. This Chapter reports my efforts to synthesize corresponding microgels with LnPO$_4$ NPs confined to the core.

My basic design for the synthesis of LnF$_3$-containing microgels is depicted in Scheme 4-1. While a number of different approaches were attempted and are described in ref [3], only the process shown in Scheme 4-1 was successful in confining the LnF$_3$ precipitate within the microgels. In the first step, the –COOH groups in the microgels were neutralized with 1 equiv of NaOH. Then this solution was treated with an aqueous solution of LnCl$_3$. Mass cytometry experiments showed that this ion exchange reaction saturated (i.e., the Ln ion content of the microgel saturated) at a ratio of one Ln$^{3+}$ ion per 3 carboxylate ions. Addition of fluoride (NaF, NH$_4$F) in the ratio of 3 F/Ln ion led to LnF$_3$ precipitation within the microgel. By mass cytometry, I learned that this step preserved the number of Ln ions per microgel.
I used a similar approach in attempts to prepare microgels embedded with LnPO₄. I used the same poly(N-isopropylacrylamide-vinylcaprolactam-methacrylic acid) [poly(NIPAm/VCL/MAA)] microgel sample as that denoted V27 in ref [3]. These microgels have a diameter on the order of 700 nm and contain 27 mol % methacrylic acid as a comonomer. As we will see below, it is also necessary to ion-exchange the microgels to the corresponding Ln³⁺ carboxylates to obtain LnPO₄ incorporation into the microgels, but this itself is not sufficient. Finding appropriate conditions in which the LnPO₄ precipitate was confined within the microgels was much more challenging. In order to put my work in context, I first provide a brief review of previous attempts to prepare LnPO₄ nanoparticles.

Lanthanide phosphates can be obtained by precipitation from aqueous solutions of their salts using, for example, sodium or ammonium phosphates or by crystallization from boiling phosphoric acid solution [7]. In practice, when lanthanide phosphates are precipitated from phosphoric acid solution, Na₂CO₃ or NH₄OH are used as coprecipitants [8-11]. The precipitate obtained tends to be gelatinous, containing undefined amounts of water and is contaminated with foreign ions derived from the precipitants. Under hydrothermal conditions, Haase et al. [12,13] prepared lanthanide-doped phosphate nanocrystals by reacting the corresponding metal chlorides, phosphoric acid, and a base at 200 °C in the coordinating solvent tris(ethylhexyl) phosphate; while Ito et al. [14] precipitated lanthanide phosphates using the hydrothermal reaction of a Ln-EDTA chelate precursor in the presence of PO₄³⁻ ions. Kijkowska [15] developed a simplified preparation method for LnPO₄·nH₂O (n ≈ 1) crystals based on crystallization of pure crystalline
lanthanide phosphates from boiling phosphoric acid solution with an initial concentration 2 mol/L of H$_3$PO$_4$ and 0.02 mol/L of Ln.

Many of the experiments described in the previous paragraph involved heating the samples to high temperature. In my synthesis design, I wanted to avoid heating the samples, particularly after addition of phosphate ions. The microgel polymer has a lower critical solution temperature (LCST) in water in the range of 40 – 50 °C [3]. Phase separation takes place at temperatures above its LCST, accompanied by aggregation and loss of colloidal stability. The aim of the present work was to prepare LnPO$_4$ containing microgels suitable for mass cytometry experiments. For this purpose, it is not necessary that the LnPO$_4$ be in the form of discrete nanocrystals. What is important is that the precipitated salt has to be confined to the interior of the microgel. To circumvent the difficulties associated with elevated temperatures, I attempted to prepare LnPO$_4$-containing microgels via in-situ co-precipitation at room temperature using the same basic approach described in Scheme 4-1 for the preparation of the LnF$_3$ containing microgels. Unfortunately, all the protocols that I tested initially for adding a phosphate source as a precipitant led to solid formation outside of the microgel.

One can imagine two sources of differences between precipitating LnF$_3$ within the confines of the microgel, and precipitating LnPO$_4$. First, as mentioned above, lanthanide phosphates have much lower solubility products than the corresponding fluorides. Second, the conjugate acids of fluoride and phosphate have different dissociation constants in water. Hydrofluoric acid is monoprotic, with a pK$_a$ of 3.17 [16]. At neutral pH, the hydrolysis of fluoride ions is suppressed to less than 1%. Phosphoric acid, however, is triprotic. At 25 °C, the corresponding pK$_a$ values are: pK$_{a1}$ = 2.12, pK$_{a2}$ = 7.21, and pK$_{a3}$ = 12.67 [17]. Over a wide pH range, a phosphate solution contains a mixture of different phosphate species (i.e. H$_3$PO$_4$, H$_2$PO$_4^-$, HPO$_4^{2-}$ and PO$_4^{3-}$). For example, at pH 7.0, H$_2$PO$_4^-$ and HPO$_4^{2-}$ are present in significant amounts: 62% H$_2$PO$_4^-$ and 38% of HPO$_4^{2-}$. For a given phosphate precipitating reagent, both the pH of the solution and the phosphate composition may have an impact on the formation of LnPO$_4$ in the presence of microgels. One of the variables that I examined was the nature of the phosphate species added as a precipitant to the Ln-containing microgels.

Another factor that I examined was the impact of low molecular weight organic acids on the formation of LnPO$_4$ containing microgels. In agriculture and in environmental science, it is well
accepted that low molecular weight organic acids can chelate or form complexes with polyvalent cations; and these complexes, in turn, play an important role in the dissolution and transport of these elements in soil formation and in plant nutrition [18,19]. There is experimental evidence to support the role of organic acids in mineral phosphate solubilization [20]. One of my hypotheses was that these low molecular weight acids (citric acid, succinic acid), through their binding affinity to the Ln$^{3+}$ ions, could be used to achieve a local thermodynamic equilibrium between the metal species inside and outside of microgel network, and therefore have an impact on the formation of LnPO$_4$-containing microgels. This approach also did not work.

The only method I found for confining the precipitated LnPO$_4$ within the microgel involved treating the Ln carboxylate microgel with phosphate buffer, more specifically phosphate-buffered saline (PBS). This worked remarkably well. I examined the particle size and particle size distribution of these particles by dynamic light scattering (DLS) and electron microscopy (TEM). The metal content of these particles was measured by mass cytometry, and their stability was monitored in different aqueous buffer media over different time intervals (reported in the next Chapter). These results were evaluated with respect to satisfying the some of the key criteria necessary for reproducible bead-based assays as measured by mass cytometry.

4.2 Experimental Procedure

4.2.1 Synthesis of Ln-containing microgels by ion exchange

The microgels employed here are the same poly(NIPAm/VCL/MAA) microgel sample as that denoted V27 in ref [3]. The samples were repurified by sedimentation-redispersion in deionized water. Acid group concentration was determined by simultaneous conductometric and potentiometric titration. Microgel concentration was estimated from mass cytometry measurements of Ln-containing microgels using the result that these microgels contain one Ln ion per 3 carboxylate groups.

4.2.2 Synthesis of LnPO$_4$-containing microgels

The microgel solutions were first neutralized with 1 molar eq. of NaOH (0.1 M) before addition of Ln salts at a –COOH: Ln$^{3+}$ mole ratio of 3:1. The dispersion was stirred overnight to
promote ion exchange between the \( \text{Na}^+ \) and \( \text{Ln}^{3+} \) ions, following which 10 molar eq. of PBS buffer solution at pH 7.0 (0.1 M, containing 84% NaCl, 12% \( \text{Na}_2\text{HPO}_4 \), 2% \( \text{KH}_2\text{PO}_4 \) and 2% KCl) as a precipitant was combined in order to generate \( \text{LnPO}_4 \) nanoparticles within the microgels. Based on this general protocol, I prepared a series of \( \text{LnPO}_4 \)-containing microgels with different lanthanide metals (\( \text{Ln} = \text{La}, \text{Nd}, \text{Eu}, \text{Tb}, \text{Ho}, \text{Tm} \)), denoted as V27-LnPO4. The recipes are listed in Table 2-11. To determine the metal content of the microgels, aliquots of the purified microgel solutions were taken for analysis by mass cytometry (see below). After purification, the solutions were diluted to ca. \( 10^6 \) microgels/mL and nebulized into the Ar plasma by the sample introduction system of the mass cytometer.

### 4.3 Results and Discussions

The starting poly(NIPAm/VCL/MAA) microgels, as described in ref [3], were synthesized by the precipitation copolymerization of NIPAM, VCL, MAA and the cross-linker methylene-bis-acrylamide (mole ratio 56:14:27:3). These microgels had a narrow size distribution and a mean hydrodynamic diameter \( d_h = 680 \) nm (at pH 7), as characterized by multiangle dynamic light scattering (DLS). Based on the work of Hoare and Pelton [21,22], one expects, because of the reactivity ratio mismatch in the polymerization reaction, that most of the –COOH groups will be located in the core of the microgel. The –COOH content of the entire sample was determined by acid-base titration, which showed that the carboxylic acid groups of the microgels were characterized by a distribution of \( pK_a \) values ranging from 4.5 to 7.5 [3].

All experiments reported here follow the overall synthetic strategy outlined in Scheme 4-1. To illustrate my various approaches, I describe experiments with Eu\(^{3+} \) ions. When the microgel solution was neutralized with 1 eqv of NaOH, the measured pH of the microgel solution was 9. During the ion exchange with EuCl\(_3\), the pH decreased, reaching a value of 7 for addition of 1 Eu\(^{3+} \) for 3 carboxylate ions. I refer to this sample as V27(Eu). This was the starting point for attempts to precipitate EuPO\(_4\) in the microgels. I begin with a description of results that were unsuccessful from the perspective of my desire to prepare microgels with EuPO\(_4\) confined to the core, but are still interesting from the broader perspective of factors that affect LnPO\(_4\) formation.
4.3.1 Reactions that did not work

In the first set of experiments, I added NaH$_2$PO$_4$ to a solution of V27(Eu). Upon dropwise injection of a solution of NaH$_2$PO$_4$, the pH of the microgel solution dropped to ca. pH 6. Bunches of long needle-like structures were observed on the surface of the microgels or in the space between microgels (as shown in Figure 4-1A). These objects had lengths ranging from 100 to 200 nm. To my surprise, even with Eu$^{3+}$ initially bound to the interior of the microgels, I did not find the EuPO$_4$ within the microgels. What I learn from this experiment is that the Eu$^{3+}$ ions are mobile. During addition of the phosphate source, Eu$^{3+}$ ions can dissociate from the carboxyl groups, diffuse outside the microgel, and rapidly form a precipitate of EuPO$_4$. It is also possible that the protons generated during the formation of EuPO$_4$ affected the confinement of Eu$^{3+}$ ions within the microgels. To test these ideas, I designed two rather different sets of experiments. First, I introduced low molecular weight organic acids (citric acid, succinic acid) as competitive ligands for Eu$^{3+}$ ions. In the presence of this ligands, the rate of EuPO$_4$ precipitation may be reduced. Second, I used different phosphate reagents to examine the influence of proton release on the formation of EuPO$_4$-containing microgels.

![Figure 4-1. TEM images of EuPO$_4$ nanostructures generated with microgel templates under different conditions. A) The microgel solution was pretreated with 1 eq. of NaOH, then with 1/3 molar eq. of EuCl$_3$ solution, i.e. formation of V27(Eu). Subsequently, 1/3 molar eq. of NaH$_2$PO$_4$ was injected to precipitate EuPO$_4$; B) The microgel solution was first mixed with 10 mol% succinic acid before...](image-url)
adding 1 eq. of NaOH per MAA. Then 1/3 molar eq. of EuCl₃ solution was added. Subsequently, 1/3 molar eq. of NaH₂PO₄ was injected to precipitate EuPO₄. C) The microgel solution was first mixed with 10 mol% citric acid before adding 1 eq. of NaOH per MAA, then with 1/3 molar eq. of EuCl₃ solution. Subsequently, 1/3 molar eq. of NaH₂PO₄ was injected to precipitate EuPO₄; D) To a solution of V27(Eu), 1/3 molar eq. of Na₂HPO₄ was injected to precipitate EuPO₄.

Effect of competitive ligands. Succinic acid and citric acid were employed as competitive ligands for the Ln³⁺ ions. A relatively small amount of organic acid (10 mol % based on –COOH group content of the microgel) was added to the microgel solution, before neutralization with NaOH. In these experiments, the amount of base added was equal to the –COOH group content of the microgel, so that some of the carboxyl groups in the solution remained protonated. At this point, the microgel solution was at pH 8. Upon addition 1/3 molar eq. of EuCl₃ (based on microgel –COOH groups), the pH of microgel solution dropped to pH 6. I used TEM and energy dispersive X-ray (EDX) analysis to examine these ion exchanged microgels. From EDX traces shown in Figures 4A-1 and 4A-2, I found that the Eu signals only associated with the microgels.

Then I added dropwise 1 molar eq. of NaH₂PO₄ solution (based on Eu) to precipitate the Eu³⁺ ions from the Eu³⁺-containing microgels. In presence of succinic acid, upon addition of NaH₂PO₄, the pH of microgel solution remained at pH 6. In TEM images, I found large bright spherical structures that grew on the outside of the microgels, as shown in Figure 1B. For microgels prepared in presence of citric acid, upon injecting NaH₂PO₄ solution, the pH of microgel solution also remained at pH 6. Here by TEM, I observed bright signals from the microgels as well as small nanoparticles outside of the microgels, as shown in TEM (Figure 4-1C). To conclude, the addition of relatively small amounts (10 mol% based on the MAA content of the microgels) of low molecular weight organic acid did change the growth of EuPO₄ in the system. The impact of citric acid was different than that of succinic acid, and I do not understand the origin of this difference. The important point, however, is that I could not obtain microgels in this way with EuPO₄-nanoparticles confined to the interior.

Other phosphate sources. In this section, I first consider the consequences of the protons released in the reaction of Eu³⁺ with NaH₂PO₄ or Na₂HPO₄ solution to form EuPO₄. The processes can be described by the chemical reactions:
\[ \text{Eu}^{3+} + \text{H}_2\text{PO}_4^- \rightarrow \text{EuPO}_4 \downarrow + 2\text{H}^+ \] (4.1)

\[ \text{Eu}^{3+} + \text{HPO}_4^{2-} \rightarrow \text{EuPO}_4 \downarrow + \text{H}^+ \] (4.2)

I briefly examined the pH of Eu\(^{3+}\)-containing solutions (in the absence of microgels) before and after the addition of different sources of phosphate ions. I injected 1 molar eq. of NaH\(_2\)PO\(_4\) (0.1 M at pH 4.3) or Na\(_2\)HPO\(_4\) (0.1 M at pH 8.4) into a 5 mL aqueous solution containing 0.01 mmol EuCl\(_3\). In both cases, white precipitates formed. Upon addition of NaH\(_2\)PO\(_4\), the pH of the Eu solution dropped from pH 5.7 to pH 2.8. After injecting Na\(_2\)HPO\(_4\) into EuCl\(_3\) solution, the pH of Eu solution dropped from pH 5.7 to pH 3.3. These experiments confirmed that protons were produced in the formation of EuPO\(_4\).

To test the impact of newly formed protons on EuPO\(_4\)-containing microgels, I first used 1 eq. of Na\(_2\)HPO\(_4\) solution as a precipitating reagent for Eu\(^{3+}\) ions in V27(Eu) microgels. Upon dropwise addition of Na\(_2\)HPO\(_4\), the pH of microgel solution increased slightly from 6.9 to 7.4. From TEM images, I saw that the EuPO\(_4\) structures were formed outside of the microgels, as shown in Figure 4-1D.

### 4.3.2 Reactions that worked

The key to success in generating EuPO\(_4\) nanoparticles confined to the core of the microgels was to treat the ion-exchanged microgels with an excess of phosphate buffer at pH 7. For this purpose I use a commercial sample of PBS buffer (phosphate buffered saline containing 84% NaCl, 12% Na\(_2\)HPO\(_4\), 2% KH\(_2\)PO\(_4\) and 2% KCl). For example, I incubated the Eu\(^{3+}\) containing-microgels with 10 molar eq. of 0.1 M PBS buffer solution of pH 7.0 and stirred the solution overnight. The resultant microgel solution maintained its pH at 7. Figure 4-2A shows a dark field TEM image of hybrid microgels obtained in this way. In the image, I observe bright signals from the metal ions in the microgels. Figure 4-2B and 4-2C provide enlarged images of a hybrid microgel obtained by bright field TEM and dark field TEM respectively, from the same grid. In Figure 4-2D, a portion of the microgel surface (as indicated by the dashed rectangle in Figure 4-2C) is shown at even higher magnification. Here one can see large number of bright structures embedded in the interior of the microgel, while a few of these nanostructures can be clearly seen.
on dangling ends of the polymer protruding from the surface of microgels. I believe that these bright nanostructures correspond to the EuPO$_4$ nanoparticles in the hybrid system. Some of these nanostructures are marked with red arrows in Figure 4-2D. These individual structures are small, with diameters less than 5 nm.

![Figure 4-2](image-url)

**Figure 4-2.** TEM images of EuPO$_4$ containing microgels generated using PBS buffer as the precipitating reagent (V27-EuPO$_4$). A: a dark field TEM image; B: an enlarged image of a hybrid microgel under bright field TEM; C: an enlarged image of a different hybrid microgel under dark field TEM; D: a high magnification image of the selected area in C. A few of the many electron-rich nanostructures are marked with red arrows.

The presence of Eu and P elements from EuPO$_4$-containing microgels was confirmed by EDX analysis. Figure 4-3 shows linear scan profiles across three EuPO$_4$ containing microgels on the grid used to obtain the TEM images in Figure 4-2. While the signals from Eu and P are not as pronounced as that from carbon, they are much higher than that of La, chosen as a negative control, i.e., as a lanthanide element not expected to be present in the sample, except as an impurity. Both TEM and EDX results confirm that essentially all of the EuPO$_4$ is located in the interior of microgels. This is the result I were looking for.
Figure 4-3. Electron diffraction X-ray (EDX) analysis of EuPO$_4$ containing microgels: V27-EuPO$_4$. Elemental profiles of Europium, Phosphorus and Lanthanum were obtained by recording the EDX signal along the yellow solid line as shown in the upper left figure.

4.3.3 Characterization of EuPO$_4$-containing microgels

I used dynamic light scattering (DLS) to determine microgel diameters in solution Figure 4-4 presents DLS CONTIN plots measured at a $45^\circ$ scattering angle. While these CONTIN plots provide evidence for the narrow size distribution of the microgels in solution, it is difficult to have confidence in the values of the apparent hydrodynamic radii obtained from these data. The problem is that spherical particles of this size (i.e., with diameters on the order of 1 $\mu$m) have a form factor that decreases strongly with increasing angle in the range of angles at which static and dynamic light scattering measurements are carried out. Other technical problems become important because of the difference in refractive index between water and the glass vial containing the solution.
**Figure 4-4.** CONTIN plots of the hybrid microgels determined by dynamic light scattering at 45° scattering angle at 295 K. V27-COOH: microgel solution was adjusted with 0.1 M NaOH to pH 7. V27-COO(-): microgel solution was fully neutralized with 1 eq. of NaOH solution. V27(Eu): microgel solution was first neutralized with 1 eq. of NaOH, and then ion exchanged with 1 eq. of Eu³⁺ for every 3 eq. of –COO⁻. V27-EuPO₄: microgel solution was first neutralized with 1 eq. of NaOH, and then ion exchanged with 1 eq. of Eu³⁺ for every 3 eq. of –COO⁻, after that incubated with 10 eq. of PBS buffer (0.1 M, pH 7.0) overnight. The concentration of microgels was ca. 0.02 mg / mL.

To obtain measuring translational diffusion of these microgels, I carried out measurements over a range of angles and assume that the solutions are sufficiently dilute that I can ignore possible concentration effects on the magnitude of the measured diffusion coefficient. For translational diffusion in dilute solution, the diffusion coefficient \(D\) varies with scattering vector \(q = (4\pi n/\lambda) \sin(\theta/2)\) as:

\[
D = \Gamma / q^2
\]  

(4.3)

where \(n\) is the reflective index of solution, \(\lambda\) is the wavelength of incident light and \(\theta\) is the scattering angle. \(\Gamma\) is the decay rate of the autocorrelation in ms⁻¹. The hydrodynamic radius \(R_h\) can be determined by the Stokes-Einstein equation as:
\[
R_h = \frac{k_B T}{6\pi\eta D}
\]

(4.4)

where \(k_B\) is Boltzmann’s constant, \(T\) is the absolute temperature in Kelvin, and \(\eta\) is the solvent viscosity.

To determine \(D\), I plotted values of \(\Gamma\), evaluated as the first cumulant, as a function of \(q^2\), for each of the microgel solutions as shown in Figure 4-5. Values of \(D\) (in \(\text{nm}^2\text{ms}^{-1}\)) for each microgel were obtained from the slope of the linear regression lines, and values of the hydrodynamic radius (\(R_h\) in nm) were calculated with eq (4.4), as. These results are listed in Table 4-1.

For the original microgel solution at pH 7, I found \(R_h = 330\) nm, PDI = 0.01 (\(d_{\text{h.app}} = 2R_h\)). Upon addition of 1 molar eq. of NaOH, the pH of microgel solution increased to pH 9, accompanied by an increase in size (\(R_h \approx 440\) nm, PDI = 0.08). The swelling of microgel particles originates from the increase in osmotic pressure caused by the relatively high concentration of solvated counter cations within the microgel particles due to the presence of the \(–\text{COO(-)}\) groups [23]. Upon adding EuCl\(_3\), a corresponding decrease in \(R_h\) was observed (\(R_h \approx 290\) nm, PDI = 0.01). One can explain this change in terms of a reduced internal concentration of counter cations upon cross-linking of multiple \(–\text{COO(-)}\) groups by the Eu\(^{3+}\) ions. Subsequent addition of PBS buffer led to an increase in the radius (\(R_h \approx 325\) nm, PDI = 0.01) from the Eu\(^{3+}\) containing microgels. Here the osmotic swelling was less pronounced than the initial neutralization of the microgels, probably due to the partial release of the initially bound \(–\text{COO(-)}\) groups upon formation of EuPO\(_4\) nanoparticles. These results are quite similar to those found in the preparation of EuF\(_3\)-containing microgels.\(^3\)
Figure 4-5. Plots of the autocorrelation (first cumulant) decay rates (ms\(^{-1}\)) vs \(q^2\) (nm\(^{-2}\)), where the straight line in each plot represents the linear fit of the scattering data from 30°, 45° and 60° toward the origin. The scattering data at 90° was not included in the fitting. These data points are considered to be unreliable, because of the low value of the form factor at this scattering angle. The average diffusion coefficient was obtained from the slopes of the linear regression lines.

Table 4-1. Average diffusion coefficients and hydrodynamic radii values of microgels obtained by multi-angular dynamic light scattering.

<table>
<thead>
<tr>
<th></th>
<th>(D^a)</th>
<th>(R_h^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V27-COOH</td>
<td>695 (\text{nm}^2\text{ms}^{-1})</td>
<td>330 nm</td>
</tr>
<tr>
<td>V27-COO(-)</td>
<td>518 (\text{nm}^2\text{ms}^{-1})</td>
<td>444 nm</td>
</tr>
<tr>
<td>V27(Eu)</td>
<td>756 (\text{nm}^2\text{ms}^{-1})</td>
<td>289 nm</td>
</tr>
<tr>
<td>V27-EuPO(_4)</td>
<td>697 (\text{nm}^2\text{ms}^{-1})</td>
<td>324 nm</td>
</tr>
</tbody>
</table>

a. \(D\) values were obtained from the slopes of the linear regression plots of \(\Gamma\) vs \(q^2\) in Figure 4-5.

b. The hydrodynamic radius was obtained by the Stokes-Einstein equation, eq (4.4).
4.3.4 Characterization of V27-LnPO$_4$ by TEM

Based on my successful preparation of microgels with EuPO$_4$ confined within the microgels, I carried out new experiments to prepare other microgel samples carrying different LnPO$_4$ salts. Table 2-11 shows the recipes for preparing these different LnPO$_4$-containing microgels. The microgel solutions were first neutralized with 1 molar eq. of NaOH solution based on the MAA content of the microgels. Ion exchange of Ln$^{3+}$ ions was performed by injecting a solution of LnCl$_3$ in water into each microgel solution at a –COO(-) : Ln$^{3+}$ ratio of 3:1 to form samples that I can refer to as V27(Ln). LnPO$_4$ nanoparticles were precipitated inside the microgels upon addition of an excess of PBS buffer at pH 7.0 followed by overnight stirring. In our notation, microgels carrying entrapped LnPO$_4$ NPs are denoted as V27-LnPO$_4$. Figure 4-6 shows TEM images of six hybrid microgels containing different LnPO$_4$ NPs. In each of the images, I observed bright signals from the metal ions in the microgels. These LnPO$_4$-containing microgels all have an average diameter of ca. 220 to 230 nm in the dry state.

Figure 4-6. TEM images of different LnPO$_4$-containing microgels. A: V27-LaPO$_4$; B: V27-NdPO$_4$; C: V27-EuPO$_4$; D: V27-TbPO$_4$; E: V27-HoPO$_4$; F: V27-TmPO$_4$. The scale bars are 800 nm. In C, D, and F, there appears to be a small amount of LnPO$_4$ outside the confines of the microgel.
4.3.5 Quantitative Determination of the Metal Content of V27-LnPO₄ Samples by Mass Cytometry

The lanthanide content per microgel and the bead-to-bead variation in the Ln-ion content were investigated by mass cytometry. In this analysis, a dilute slurry (ca. 10⁶ microgels/mL) was nebulized into the mass cytometer, directly delivering particles into the inductively coupled plasma torch. The transient signals corresponding to each bead ionization event were recorded by the detector and stored. The raw data (i.e. the intensity of metal signals per microgel) were converted to the number of metals per microgel with the equation:

\[ N_{Ln} = \frac{I_{Ln}}{T_{Ln}} \]  

(5)

where \( N_{Ln} \) is the number of metal atoms per microgel, \( I_{Ln} \) is the intensity measured by mass cytometry detector, and \( T_{Ln} \) is the transmission coefficient of the detector. Generally the transmission coefficient is on the order of \( 10^{-4} \) to \( 10^{-5} \), which means that about 1 in every \( 10^4 \) to \( 10^5 \) ions generated in the plasma reaches the detector. Values of \( T_{Ln} \) are obtained by daily instrument calibration with tuning solutions, and one should note that early Ln elements are characterized by lower values of \( T_{Ln} \) than the later Ln elements.

Figure 4-7 shows screen capture images from mass cytometry measurement on six different samples of LnPO₄-containing microgels (V27-LnPO₄, Ln= La, Nd, Tb, Eu, Ho and Tm), synthesized with PBS buffer (pH 7.0) as a precipitating reagent for the Ln³⁺ ions. The microgel events were captured during the time of acquisition. In Figure 4-7, the x-axis corresponds to m/Z for the ions reaching the detector. The y-axis reports successive mass spectra. Each microgel event appears as an intense series of dark spots in an m/Z channel, representing a detectable Ln signal. These are highlighted in the figure with horizontal grey bars.
Figure 4-7. Mass cytometry screen captures for different V27-LnPO₄ microgels (Ln = La, Nd, Tb, Eu, Tm, Ho). The Ln³⁺ ions in the ionexchanged microgel samples were converted into LnPO₄ using 10 eq. of PBS buffer solution as a precipitant.

In Figure 4-8, the lanthanide contents and atom distributions among microgels are presented as box-and-whisker plots [24]. On the x-axis, the elements (La, Nd, Eu, Tb, Ho and Tm) refer to LnPO₄-containing microgels obtained with PBS buffer as a precipitating reagent. The values of the mean number of Ln atoms per microgel and the calculated coefficient of variation (CV) for these samples are collected in Table 4-2.

For the V27-LnPO₄, the number of metal atoms per microgel increased from the early lanthanides (La and Nd) to the middle lanthanides (Eu and Tb) to the late lanthanides (Ho and Tm). However, the differences are small. For instance, the smallest Ln content found for V27-LaPO₄, containing an average of 0.85 x 10⁷ Ln ions per microgel, and for V27-NdPO₄, containing an average of 0.86 x 10⁷ Ln per microgel. Both V27-EuPO₄ and V27-TbPO₄ were found to contain ca. 0.9 x 10⁷ Ln per microgel, while slightly higher levels of metal content per
particle were found in V27-HoPO$_4$ (1.05 x 10$^7$ Ho per microgel) and V27-TmPO$_4$ (1.15 x 10$^7$ Tm per microgel). Along with lower intensities for early-Ln-encoded microgels, the data also show larger bead-to-bead distributions of the Ln ions, which corresponds well with the statistical theory that the counting errors decreases with the increase of number of counts. These results are quite similar to what I found for the Eu$^{3+}$ ions in ionexchanged microgels and for the EuF$_3$-containing microgels. These results indicate that when a proper protocol is followed for adding a precipitant, this process largely conserves the number of Ln atoms per microgel.

![Figure 4-8](image)

**Figure 4-8.** Box-and-whisker plots distribution of number of Ln atoms per hybrid microgel containing different Ln elemental tags, as determined by mass cytometry. On the x-axis, the elements (La, Nd, Eu, Tb, Ho and Tm) refer to LnPO$_4$ containing microgels obtained with PBS buffer. The mass cytometry transmission efficiencies for La ions was 2.14 x 10$^{-5}$; for Nd ions, 2.58 x 10$^{-5}$; for Eu ions, 3.48 x 10$^{-5}$; for Tb ions, 5.49 x 10$^{-5}$; for Ho ions, 3.78 x 10$^{-5}$, and for Tm ions, 3.19 x 10$^{-5}$.

<table>
<thead>
<tr>
<th>Samples</th>
<th>$I_{Ln}^a$ / counts</th>
<th>$T_{Ln}^b / 10^{-5}$</th>
<th>$N_{Ln}^c / 10^7$</th>
<th>$CV_{Ln}^d / %$</th>
</tr>
</thead>
<tbody>
<tr>
<td>V27-LaPO$_4$</td>
<td>182</td>
<td>2.14</td>
<td>0.85</td>
<td>51</td>
</tr>
<tr>
<td>V27-NdPO$_4$</td>
<td>222</td>
<td>2.58</td>
<td>0.86</td>
<td>46</td>
</tr>
<tr>
<td>V27-EuPO$_4$</td>
<td>317</td>
<td>3.48</td>
<td>0.91</td>
<td>35</td>
</tr>
<tr>
<td>V27-TbPO$_4$</td>
<td>516</td>
<td>5.49</td>
<td>0.94</td>
<td>34</td>
</tr>
<tr>
<td>V27-HoPO$_4$</td>
<td>397</td>
<td>3.78</td>
<td>1.05</td>
<td>34</td>
</tr>
<tr>
<td>V27-TmPO$_4$</td>
<td>367</td>
<td>3.19</td>
<td>1.15</td>
<td>32</td>
</tr>
</tbody>
</table>

a. $I_{Ln}$: the mean Ln intensity measured by the mass cytometry detector.
b. $T_{Ln}$: the transmission coefficient of the detector for each Ln element.

c. $N_{Ln}$: the mean number of Ln atoms per microgel calculated by $N_{Ln} = \frac{I_{Ln}}{T_{Ln}}$.

d. $CV_{Ln}$: the coefficient of variation for the number of Ln atoms per microgel.

### 4.4 Conclusions

In this Chapter, I described the synthesis of LnPO$_4$-containing poly(NIPAm/VCL/MAA) copolymer microgels. The microgels were first ion-exchanged with Ln$^{3+}$ ions. Then various sources of phosphate ions were added as a precipitant to convert the Ln$^{3+}$ ions into LnPO$_4$. The only effective protocol to confine the LnPO$_4$ within microgels was to treat the Ln$^{3+}$-containing microgels with an excess of a PBS buffer solution. The PBS solution acted both as a precipitating reagent for Ln$^{3+}$ ions, as well as a buffering reagent to neutralize newly produced protons during the formation of LnPO$_4$. All other protocols that I examined led to LnPO$_4$ structures on the exterior of the microgels. I suspect that protons released during the formation of LnPO$_4$ caused release of Ln$^{3+}$ ions, allowing them to react outside the microgels. This result is different from that found in the preparation of microgel hybrids containing LnF$_3$.

By mass cytometry, I showed that the ion exchanged microgels contain ca. $10^7$ Ln atoms per microgel, with small variations for the different Ln ions examined. I also found that precipitation of the Ln ions as LnPO$_4$ within the microgels using PBS buffer conserved the number of Ln ions per microgel. This aspect of the results is similar to that found previously for EuF$_3$-containing microgels. In the next Chapter, I will describe the stability of LnPO$_4$-containing microgels toward leaching under different experimental conditions.
Reference

Appendix to Chapter 4

Scheme 4A-1 Structure of succinic acid and citric acid.

Figure 4A-1. Elemental analysis by EDX. A linear scan of Eu containing microgels with the presence of 10 mol% of succinic acid before adding phosphorous precipitant. The microgel was first combined with 10 mol% of succinic acid before pretreated with 1 molar eq. of NaOH solution, and then 1/3 molar eq. of EuCl₃ was combined.
**Figure 4A-2.** Elemental analysis by EDX. A map scan of Eu containing microgels with the presence of 10 mol% of citric acid before adding phosphorous precipitant. The microgel was first combined with 10 mol% of citric acid before pretreated with 1 molar eq. of NaOH solution, and then 1/3 molar eq. of EuCl$_3$ was combined.
Chapter 5 Stability of Lanthanide-Containing Microgels

In this chapter, I examine the stability of the lanthanide-containing microgels in the presence of acid, base or various buffers that are commonly utilized during cell analysis. I used conventional inductively coupled plasma-mass spectrometry solution analysis to follow the leakage of Ln ions into the aqueous medium as a function of time.

5.1 Introduction

For biological assays based upon mass cytometry, polymer beads are labeled with metal ions. The lanthanide (Ln) elements are particularly interesting as encoding species. They have low natural abundance and thus low background signals. They have similar chemistry, and there are in principle 54 natural non-radioactive isotopes available for encoding [1]. To achieve an encoding variability comparable to or greater than that of current Luminex beads, the polymer beads have to contain $10^5 \sim 10^8$ Ln ions per bead. Another requirement is that the beads should be stable to ion release into the continuous medium, upon prolonged storage and upon exposure to reaction conditions used to attach bioaffinity agents to their surface.

In our group, the initial approach to lanthanide-encoded polymer microbeads were based upon polystyrene microspheres [2-5]. Lanthanide-encoded poly(styrene-co-methacrylic acid) (PS-PMAA) or poly(styrene-co-acrylic acid) (PS-PAA) microspheres with diameters on the order of 2 μm were synthesized by two-stage dispersion polymerization (2-DisP) [2-4, 6], in which problematic reagents, such as functional monomers and crosslinkers that commonly disrupt the particle-forming process, were added to the reaction after the nucleation stage was complete [7]. Different lanthanide ions were incorporated into these microspheres during the synthesis, by addition of LnCl$_3$ salts and excess MAA or AA to the reaction, in the second stage, after the microsphere nucleation stage was complete. With these polystyrene microspheres, we could achieve good control of Ln content per particle and maintain a low particle-to-particle variation of Ln content. Even better results were obtained by three-stage dispersion polymerization (3-DisP) [2-5].
The students who synthesized these Ln-encoded polystyrene microbeads used traditional ICP-MS to follow the loss of lanthanide ions into the aqueous medium as a function of time. For PS-MAA particles, no detectable ion loss was found in 2-(N-morpholino)ethanesulfonic acid (MES, at pH 6.5), phosphate buffered saline (PBS, at pH 7.2), and ammonium acetate (AmAc, at pH 9.0) [3]. They found that only when the chelating agents ethylenediaminetetraacetic acid (EDTA) or diethylenetriaminepentaacetic acid (DTPA) were present in the buffer was there a significant loss of Ln ions [5]. In the most severe case, only 15% of La content from PS-MAA particles prepared by 2-DisP was released into the PBS buffer in presence of EDTA after 8 weeks under stirring. For PS-AA particles, the results were somewhat different [2]. At pH 3 (50 mM sodium acetate buffer), there was no detectable leakage of Tm atoms from Tm encoded PS-AA beads prepared by 2-DisP. Under other conditions, ion loss was also small, and lower for particles prepared by 3-DisP than 2-DisP. In one example, the highest detectable loss of Tm$^{3+}$ ions was found at high pH (pH 10.6, 200 mM sodium carbonate/bicarbonate), but amounted to only 0.1% of the Tm content after 3 weeks.

The motivation for the current investigation on lanthanide-containing microgels as a platform for polymer microbead-based biological assays was as an alternative to Ln-encoded polystyrene (PS). In Chapter 3 and Chapter 4, I showed that I could synthesize functional microgels containing up to $10^7$ Ln ions / microgel in the form of LnF$_3$ and LnPO$_4$ nanoparticles. In this chapter, I describe the stability of different types of lanthanide-containing microgels toward metal loss into aqueous media as studied by traditional ICP-MS analysis. This study provided additional information about the properties of different types of lanthanide-containing microgels, as well as important guide-lines for their storage and use in bead-based bioassays by mass cytometry.

5.2 Experimental

We consider the stability of lanthanide encoded microgels in water and in buffer solutions toward loss of lanthanide ions to the continuous media. Before any measurements were carried out, the microgel solutions were purified by three centrifugation and redispersion cycles with DI water. The leaching of Ln atoms from microgels to the continuous media was examined by
traditional ICP-MS. We tested both a centrifugation method and a dialysis approach to separate the free Ln ions from Ln ions trapped inside the microgel network.

In the centrifugation method, Ln-tagged microgels were combined with buffer solutions with vigorous stirring. The buffers included 0.1 M MES buffer pH 4.7, 0.03 M Bis-tris buffer pH 6.1, 0.01 M HEPES buffer pH 7.4, 0.01 M AmAc buffer pH 9, and DI water. Typically, 0.5 g of hybrid microgel solution, containing 3 x 10^{-6} mol Ln metals in total, was combined with 9.5 g of buffer solution, so that the initial concentration of metal was adjusted to ca. 40 to 50 ppm. Then 1 mL aliquots were transferred into centrifuge tubes from time-to-time over two weeks and centrifuged at 5000 rpm for 30 min. After centrifugation, the supernatant was diluted 10^4 times with 3% v/v HNO_3 solution for ICP-MS analysis. The sedimented microgel particles were reserved for mass cytometric analysis.

To test whether the microgels would undergo continuous metal loss into fresh buffer after the first leakage experiments, we reincubated microgels with fresh buffer (MES pH 4.7 and Bis-tris pH 6.1) and analyzed the serum after centrifugation. The microgels samples were first incubated in buffer solutions for 200 min. Then a 1 mL aliquot was transferred into a centrifuge tube and sedimented at 5000 rpm for 30 min. The supernatant was removed and kept for analysis. The microgel particles were resuspended in 1 mL fresh buffer solution. After incubation for another 200 min, the solution was again centrifuged at 5000 rpm for 30 min. This cycle of incubation and centrifugation with buffer solution was repeated, and the supernatant solutions from the three cycles were analyzed by ICP-MS.

In the dialysis approach, a cellulose membrane with a molecular weight cut-off of 50,000 was used to separate the free Ln ions from the microgels. The dialysis bags were filled with 100 µL of Tb-coded microgel solution (containing 0.1 mg Tb) and immersed in 2 L of aqueous solution. The continuous phase was either dilute HCl (10^{-4} M, pH 4), NaOH solution (10^{-4} M, pH 10) or Milli-Q water (pH 7), with the ionic strength adjusted by addition of 0.02 mol (1.170 g) of NaCl. 20 µL of aliquots were removed from the continuous phase outside of the dialysis bag from time-to-time over two weeks for ICP-MS measurements. At the end of two weeks, we removed the microgel solution from the dialysis bag, and measured the metal content of the microgels by mass cytometry.
5.3 Results and Discussions

We now consider the stability of different Ln-encoded microgels in water and in buffer toward loss of lanthanide elements to the continuous media. Prior to carrying out these experiments, the microgels were purified by three successive sedimentation-redispersion cycles with DI water. We begin with a description of experiments involving the ion exchanged microgels (MG-Ln) containing Ln$^{3+}$ ions as their carboxylate salts. Then we examine the corresponding behavior of the hybrid microgels containing LnF$_3$ NPs (MG-LnF$_3$) and LnPO$_4$ NPs (MG-LnPO$_4$). Poly(NIPAm/VCL/MAA) microgels (V27) were used as a common lanthanide carrier in the following discussion.

5.3.1 Ion Leakage from MG-Ln Microgels in Buffers.

In the first set of experiments, the MG-Ln microgels were incubated with DI water or with buffer solutions over two weeks. Aliquots were removed from time-to-time, centrifuged to separate free Ln$^{3+}$ ions from Ln$^{3+}$ bound microgels and then monitored by ICP-MS to measure the extent of metal loss to the continuous media. The buffers used in this experiment included 0.1 M MES buffer pH 4.7, 0.03 M bis-tris buffer pH 6.1, 0.01 M HEPES buffer pH 7.4, 0.01 M PBS buffer pH 7.4, 0.01 M AmAc buffer pH 9.

In Figure 5-1 we plot the percent of ions released from the MG-Ln microgels over time in different buffer media. We found that in HEPES buffer at pH 7.4, PBS buffer at pH 7.4, ammonium acetate buffer at pH 9.0, and DI water there was essentially no significant loss (< 1%) of Ln ions from the microgels into the buffers over the period of two weeks. In MES buffer at pH 4.7 and bis-tris buffer at pH 6.1, there was a rapid (< 20 min) initial ion loss from the microgels, but no further loss over the rest of the two week period.
Figure 5-1. Ion release profiles of Ln$^{3+}$ ions from MG-Ln particles at 0.005 wt% concentration into different buffer media: MES buffer (0.1 M, pH 4.7), Bis-tris buffer (0.03 M, pH 6.1), HEPES buffer (0.01 M, pH 7.4), PBS buffer (0.01 M, pH 7.4), AmAc buffer (0.01 M, pH 9.0), and DI water. Samples
were centrifuged as described in the man text to sediment the microgels, and then the supernatant was analyzed by ICP-MS.

The data are replotted in Figure 5-2, where we compare the profiles of metal leaching from different metal-containing microgels under different buffering conditions after stirring for 200 min. We took this time as our reference point, where the leaching of Ln$^{3+}$ ions from microgels reached their maximum. After stirring the MG-Ln microgels in different buffer media for 200 min, there were large extents of metal loss from the serum under the acidic buffers (25-41% of metal loss from MES buffer at pH 4.7 and 6-15% of metal loss from Bis-tris buffer at pH 6.1), however, no more than 1% of metal was detected from the serum for all metal-containing microgels under neutral or basic buffer conditions (DI water, PBS buffer at pH 7.4, HEPES buffer at pH 7.4, AmAc buffer at pH 9.0). From MES buffer media, 41% of La, 33% of Nd, 29% of Eu, 29% of Tb, 26% of Ho, and 25% of Tm was detected by ICP-MS. In the case of Bis-tris buffer at pH 6.1, 15% of La and Nd, 11% of Eu and Tb, 8% of Ho and 6% of Tm. These data show a distinctive trend of metal leakage as the early lanthanides (La and Nd) are less stable toward loss of ions from the microgel frameworks compared to the middle lanthanides (Eu and Tb) and the late lanthanides (Ho and Tm).

![Figure 5-2](image-url)

**Figure 5-2.** Leaching of Ln$^{3+}$ ions from MG-Ln into different buffer solutions after stirring for 200 min, as determined by ICP-MS. Buffer media include: MES buffer (0.1 M, pH 4.7), Bis-tris buffer (0.03 M, pH 6.1), HEPES buffer (0.01 M, pH 7.4), PBS buffer (0.01 M, pH 7.4), ammonium acetate buffer (AmAc, 0.01 M, pH 9.0), and DI water.
As a check on quantitative data obtained at 200 min incubation by analysis of the continuous phase by traditional ICP-MS, we also analyzed the corresponding sedimented microgel particles for one sample (MG-Eu) by mass cytometry. Table 5-1 shows the characteristics of the Eu$^{3+}$-containing microgel particles. By ICP-MS we determined that 29% of Eu ions in the MG-Eu sample were lost to the serum of MES buffer. From mass cytometry analysis of the microgel particles themselves, we found that the average number of metals per microgel was reduced from $1.0 \times 10^7$ to $0.67 \times 10^7$ Eu ions. The data from mass cytometry shows similar trends of metal loss as compared to the ICP-MS results. After incubation in Bis-tris buffer for 200 min, 11.4% of original metal was detected in the serum, while the sedimented and resuspended microgels contained $0.86 \times 10^7$ Eu atoms per microgel. These results are in excellent agreement with one another and attest to the quantitative complementarity of the two techniques.

**Table 5-1.** Characteristics of sedimented microgel particles after ageing in buffer solutions for 200 min

<table>
<thead>
<tr>
<th>Samples</th>
<th>$N_{Eu} (10^7)^b$</th>
<th>$CV_{Eu} (%)^b$</th>
<th>Loss of Ln (%)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG-Eu-MES</td>
<td>0.67</td>
<td>51</td>
<td>29.0</td>
</tr>
<tr>
<td>MG-Eu-Bis-tris</td>
<td>0.86</td>
<td>42</td>
<td>11.4</td>
</tr>
<tr>
<td>MG-EuF$_3$-MES</td>
<td>0.86</td>
<td>39</td>
<td>13.0</td>
</tr>
<tr>
<td>MG-EuF$_3$-Bis-tris</td>
<td>0.93</td>
<td>37</td>
<td>8.5</td>
</tr>
</tbody>
</table>

a. Before exposure to buffer, the Eu content per microgel was $1.01 \times 10^7$ for MG-Eu and $1.09 \times 10^7$ for MG-EuF$_3$.
b. $N_{Eu}$, the number of Eu ions per microgel and $CV_{Eu}$ were obtained by mass cytometry.
c. The loss of Ln ions was determined by ICP-MS analysis of the supernatant and is reported as the percentage of total Ln content originally present in the microgel prior to incubation in buffer.

Since metal leaching under acidic buffer conditions occurred rapidly in the experiments described above, I decided to test whether further leaching would occur if the microgel samples were sedimented and redispersed in fresh buffer. These experiments were carried out on a MG-Tb microgel sample isolated from buffer solution after stirring with the buffer for 200 min. Table
5-2 lists the extent of Tb released into the buffer after the initial and two subsequent exposures to MES and bis-tris buffer solutions. From MES buffer at pH 4.7, 25% of the Tb was lost during the first incubation, with an additional 9% lost on the second incubation, and a further ca. 7% lost on the third incubation. For bis-tris buffer at pH 6.1, the amounts lost were smaller, but Tb ions continued to leach from the MG-Tb sample upon further exposure to buffer. The implication of these experiments is that Ln ion binding to the –COO(-) ions in the microgel is strong at neutral and basic pH, but ion leaching is prominent at acidic pH.

**Table 5-2.** Tb release from microgels into acidic buffer solutions after repeated centrifugation and redispersion with fresh buffer.

<table>
<thead>
<tr>
<th>Microgels</th>
<th>Buffers</th>
<th>MG-Tb</th>
<th>MG-TbF3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st (%)</td>
<td>2nd (%)</td>
<td>3rd (%)</td>
</tr>
<tr>
<td>MES (4.7)</td>
<td>25.3</td>
<td>9.0</td>
<td>6.8</td>
</tr>
<tr>
<td>Bis-tris (6.1)</td>
<td>10.5</td>
<td>5.8</td>
<td>1.6</td>
</tr>
</tbody>
</table>

a. The extent of metal leaching was detected from the serum by ICP-MS. The results are reported as the percentage of the total metal content originally present in the microgel solutions before incubation with buffer.

As one further test of factors affecting leaching, to see whether pH or the buffer anion is responsible for ion loss, we carried out dialysis experiments, using a large excess of unbuffered water containing 0.01 M NaCl, a NaOH solution at pH 10, and an HCl solution at pH 4. Aliquots were removed from the solution outside the dialysis bag and monitored by ICP-MS, and at the end of the experiment, the microgels within the dialysis bag were examined by mass cytometry. The details are presented in Figure 5-3. The main result is that there was negligible loss of Tb ions in DI water and at pH 10, but over 1000 min, about 35% of the Tb ions in the MG-Tb sample were lost at pH 4.
Figure 5-3. Leaching profile of Tb$^{3+}$ ions from MG-Tb microgels contained within dialysis bags under different pH conditions: pH 4: $10^{-4}$ M HCl; pH 7: DI water; pH 10: $10^{-4}$ M NaOH solution. The concentrations of Tb ions in the solutions outside the dialysis bags were measured by ICP-MS.

5.3.2 Ion Leakage from MG-LnF$_3$ Microgels in Buffers.

In this section, I describe a systematic study examining ion leakage in the presence of various buffers from hybrid microgels containing different LnF$_3$ NPs. The experiments were carried out as described above for the MG-Ln samples, and the buffers examined were 0.1 M MES at pH 4.7, 0.03 M bis-tris at pH 6.1, 0.01 M HEPES at pH 7.4, 0.01 M PBS at pH 7.4, and 0.01 M AmAc at pH 9. Individual plots of the kinetics of metal ion release are plotted in Figure 5-4. For the case of MG-LnF$_3$ samples in HEPES buffer at pH 7.4, in PBS buffer at pH 7.4, in ammonium acetate buffer at pH 9.0, and in DI water, there was no significant loss (< 1%) of Ln atoms from the microgels into the buffers over two weeks. In MES buffer at pH 4.7 and bis-tris buffer at pH 6.1, there was an immediate (< 20 min) loss of ions to the continuous medium, followed by a slow increase in ion loss over the next several hundred min.
Figure 5-4. Ion release profiles of Ln$^{3+}$ ions from MG-LnF$_3$ particles at 0.005 wt% concentration into different buffer media: MES buffer (0.1 M, pH 4.7), Bis-tris buffer (0.03 M, pH 6.1), HEPES buffer (0.01 M, pH 7.4), PBS buffer (0.01 M, pH 7.4), Ammonium acetate buffer (AmAc, 0.01 M, pH 9.0),
and DI water. Samples were centrifuged as described in the main text to sediment the microgels, and then the supernatant was analyzed by ICP-MS.

In Figure 5-5, I plot the percent of ion leaching measured after 200 min in buffer against the particular choice of buffer for the six different Ln ions. One sees first, that there is a greater extent of ion loss in MES buffer at pH 4.7 than in bis-tris buffer at pH 6.1. For these two buffers, one also sees that extent of loss of metal ions from the microgels decreased across the series, greatest for early Ln ions and smallest for late Ln ions. For MES buffer, there was a loss of 16% of La, 15% of Nd, 13% of Eu, 14% of Tb, 13% of Ho and 11% of Tm; whereas for bis-tris buffer at pH 6.1, the percent of ions released into the continuous phase was 10% of La, 9% of Nd, 8% of Eu, 6% of Tb, and 5% of Ho and Tm. Thus the leaching of Ln ions from MG-LnF$_3$ was much smaller compared to the respective MG-Ln microgels. It appears that the low solubility of the LnF$_3$ NPs helped to resist ion leakage.

![Figure 5-5. Leaching of Ln atoms from MG-LnF$_3$ into different buffers after stirring in buffer for 200 min, as determined by ICP-MS. Buffer media include: MES (0.1 M, pH 4.7), Bis-tris (0.03 M, pH 6.1), HEPES (0.01 M, pH 7.4), PBS (0.01 M, pH 7.4) Ammonium acetate (AmAc, 0.01 M, pH 9.0), and DI water.](image)

Dialysis experiments (Figure 5-6) show that there was no leaching from MG-TbF$_3$ microgels into DI water or NaOH solution at pH 10. For dialysis against aqueous HCl at pH 4, the extent of Tb leaching increased gradually from 2% at 1 hour, up to 5% at 4 hours, and reached at 17% at the end of two weeks. These results support the idea that in acidic media, there is less Ln ion leakage from LnF$_3$ nanoparticle-containing microgels into the continuous media.
than from MG-Ln microgels. Nevertheless, the LnF$_3$ NPs in the microgel are not stable to acidic pH.

![Figure 5-6](image)

**Figure 5-6.** Leaching profile of Tb$^{3+}$ ions from MG-TbF$_3$ microgels contained within dialysis bags under different pH conditions: pH 4: $10^{-4}$ M HCl; pH 7: DI water; pH 10: $10^{-4}$ M NaOH solution. The concentrations of Tb ions in the solutions outside the dialysis bags were measured by ICP-MS.

In Table 5-1, I compare the extent of ion loss for the MG-EuF$_3$ samples followed by ICP-MS analysis of the continuous phase with the remaining content of the microgels determined by mass cytometry. These values are in good agreement and emphasize the much smaller loss of Eu ions from the MG-EuF$_3$ microgels than the MG-Eu microgels. In Table 5-2, I report results of experiments on repeated exposure of MG-TbF$_3$ microgels to MES and bis-tris buffer. Here we find that the loss of Tb ions on these subsequent exposures to buffer is very small. This is an important result, as it suggests that there is a component of Ln ions in the MG-LnF$_3$ microgels that is less tightly bound than those present as LnF$_3$ NPs. If these leachable ions are removed in an acid wash, then the resultant microgels should be suitable for use in various biological assays.

**5.3.3 Ion Leakage from LnPO$_4$- Containing Microgels in Buffers.**

In this section, I describe leaching experiments carried out on hybrid microgels containing different LnPO$_4$ NPs. The stability of the MG-LnPO$_4$ samples was tested with 0.1 M MES at pH 4.7, 0.03 M bis-tris at pH 6.1, 0.01 M HEPES at pH 7.4, 0.01 M PBS at pH 7.4 and 0.01 M AmAc at pH 9. Individual plots of the kinetics of metal ion release are plotted in Figure 5-7. Over the period of two weeks, I found very small amounts of leaching ($< 2\%$) from the all the MG-LnPO$_4$ particles. For the case of MG-LnPO$_4$ samples in HEPES buffer at pH 7.4, in PBS
buffer at pH 7.4, in ammonium acetate buffer at pH 9.0, and in DI water, there was no significant loss (< 0.1%) of Ln atoms from the microgels into the buffers over two weeks. In MES buffer at pH 4.7 and bis-tris buffer at pH 6.1, unlike the MG-LnF$_3$ or MG-Ln$^{3+}$ particles, there was no immediate loss of ions to the continuous medium over the first one to two days, after that a slow increase in ion loss was found over the next several days. After one week in bis-tris buffer at pH 6.1, the loss of Ln ions from MG-LnPO$_4$ microgels was 1% of La and Nd, 0.8% Eu, 0.7% Tb, 0.4% of Ho and 0.3% of Tm. For the MG-LnPO$_4$ samples in MES buffer at pH 4.1, after one week I found 1.2% of La, 1% of Nd, 0.9% of Eu and Tb, 0.4% of Ho and 0.3% of Tm in the continuous phase.

After incubating the MG-LnPO$_4$ microgels in acidic buffers for one week, we analyzed the corresponding sedimented microgel particles by mass cytometry. In Table 5-3, I compare three series of LnPO$_4$-containing microgels (Ln = La, Tb and Ho) obtained after incubating with MES buffer, Bis-tris buffer or DI water for one week. From mass cytometry analysis of the microgel particles, I found that after treatment with acidic buffers, the MG-LnPO$_4$ samples contain similar amounts of metal per microgel as the MG-LnPO$_4$ stored in DI water. These results are in good agreement with the ICP-MS results from the serum. We also found that the MG-LnPO$_4$ microgels are more stable than MG-LnF$_3$ microgels against metal leakage under acidic condition, which is probably due to the smaller solubility of LnPO$_4$ than LnF$_3$. For LnPO$_4$, the solubility products range from $10^{-25}$ to $10^{-27}$ M$_2$ [8], while for LnF$_3$ the solubility products range from $10^{-15}$ to $10^{-19}$ M$_4$[9-10]. These results show great promise for future work.
**Figure 5-7.** Ion release profiles of Ln$^{3+}$ ions from MG-LnPO$_4$ particles at 0.005 wt% concentration into different buffer media: MES buffer (0.1 M, pH 4.7), Bis-tris buffer (0.03 M, pH 6.1), HEPES buffer (0.01 M, pH 7.4), PBS buffer (0.01 M, pH 7.4), Ammonium acetate buffer (AmAc, 0.01 M, pH
9.0), and DI water. Samples were centrifuged at 5000 rpm for 30 min to sediment the microgels, and then the supernatant was analyzed by ICP-MS.

**Table 5-3.** Characteristics of sedimented microgel particles after aging in buffer solutions for one week

<table>
<thead>
<tr>
<th></th>
<th>MES</th>
<th>Bis-tris</th>
<th>DI water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$N_{Ln}$</td>
<td>$CV_{Ln}$</td>
<td>$N_{Ln}$</td>
</tr>
<tr>
<td>MG-LaPO$_4$</td>
<td>0.84</td>
<td>51%</td>
<td>0.85</td>
</tr>
<tr>
<td>MG-TbPO$_4$</td>
<td>0.92</td>
<td>35%</td>
<td>0.94</td>
</tr>
<tr>
<td>MG-HoPO$_4$</td>
<td>1.10</td>
<td>37%</td>
<td>1.15</td>
</tr>
</tbody>
</table>

a.$N_{Ln}$ is the number of Ln ions per microgel and $CV_{Ln}$ were obtained by mass cytometry.

When we tested the stability of the MG-TbF$_3$ microgels in phosphate buffer (0.01 M, pH 7.4), we observed no leaching from the particles. What is more interesting is that after sedimentation of these microgels and resuspension in MES or bis-tris buffer, we found very little ion loss to the continuous medium. By mass cytometry, the corresponding sedimented microgels showed high levels of Tb content per microgel. Figure 5-8 shows the box-and-whisker plots of mass cytometry distributions of the numbers of Tb atoms per microgels for sedimented MG-Tb microgels from MES buffer and bis-tris buffer.

![Figure 5-8. Box-and-whisker plots of mass cytometry distribution of number of Tb atoms per microgels for sedimented MG-Tb microgels from MES buffer (4.7), bis-tris buffer (6.1) and DI water.](image)
The microgels were incubated with 0.01 M PBS buffer at pH 7.4 for 200 min and washed with DI water before being resuspended into MES buffer, Bis-tris buffer and DI water.

In a separate experiment, we incubated MG-TbF$_3$ with PBS buffer (0.01M, pH 7.4) for two hours with magnetic stirring. After sedimentation, I redispersed the microgels with DI water. From EDX analysis of TEM images of these microgels (Figure 5-9), one can see the phosphorus signals from the microgels. It appears that these conditions led to the formation of LnPO$_4$ NPs in the microgel, and that in this form, the ions are less susceptible to leaching than the MG-LnF$_3$ microgels. This process can be described by the reaction:

$$\text{TbF}_3 + 2\text{HPO}_4^{2-} \xrightleftharpoons{K} \text{TbPO}_4 + 3\text{F}^- + \text{H}_2\text{PO}_4^- \quad (5.1)$$

The thermodynamic equilibrium constant, $K$, can be calculated as:

$$K = \frac{K_{sp(\text{TbF}_3)}K_{a3(\text{H}_3\text{PO}_4)}}{K_{sp(\text{TbPO}_4)}K_{a2(\text{H}_3\text{PO}_4)}}. \quad (5.2)$$

At 25 °C, $K_{sp(\text{TbPO}_4)} \sim 10^{-27}$, $K_{sp(\text{TbF}_3)} \sim 10^{-19}$, $K_{a2(\text{H}_3\text{PO}_4)} = 10^{-7.21}$, $K_{a3(\text{H}_3\text{PO}_4)} = 10^{12.67}$ [11], the thermodynamic equilibrium constant $K$, for eq 5.2 is on the order of $10^3$. 
Figure 5-9. EDX analysis on Tb-containing microgels. The MG-TbF$_3$ microgels were incubated with PBS buffer (0.01 M, pH 7.4) with stirring for 2 hours then redispersed with DI water. Titanium signals were chosen as an internal blank for the EDX analysis.

5.4 Conclusions

To conclude, in this chapter I compared the stability of different types of Ln-containing microgels towards their loss of metals from the microgel framework into the continuous media, focusing on three aspects of this process: a) Under which condition can one find metal leakage from the microgels? b) When did the leakage start and end (or reach its equilibrium)? c) How much metal was lost from the microgels? I summarize the results in Table 5-4.
Table 5-4. Stability of lanthanide-containing microgels towards metal leaching

<table>
<thead>
<tr>
<th></th>
<th>Acidic buffers: MES (4.7), Bis-tris (6.1)</th>
<th>Neutral or basic buffers: DI water, PBS (7.4), HEPES (7.4), AmAc (9.0)</th>
</tr>
</thead>
</table>
| MG-Ln    | a) Large leaching: MES (25-41%); Bis-tris (6-15%)  
b) Starts immediately, reaches maximum immediately.  
c) Continuous leaching | No leaching                                                             |
| MG-LnF₃  | a) Small leaching: MES (11-16%); Bis-tris (5-10%)  
b) Starts immediately, reaches maximum in two hours  
c) No continuous leaching | No leaching                                                             |
| MG-LnPO₄ | a) Few leaching: MES buffer (< 1%) Bis-tris buffer (< 1%)  
b) Starts after the first day, reaches maximum in two days | No leaching                                                             |

Under neutral or basic conditions, the leakage of Ln ions into the aqueous medium was unlikely to be a source of problems in using my lanthanide-containing microgels as classifier beads for the mass cytometry analysis. In acidic buffer solutions, however, the leakage of metal from Ln³⁺ ions-containing microgels was the most prominent. On subsequent exposures to buffers, the microgels underwent continuous loss of metals. For LnF₃-containing microgels, there was much smaller extent of metal leakage and very small of continuous loss of metal upon subsequent exposures to buffers. For LnPO₄-containing microgels, over the period of one week, there was very little (< 1%) detectable leakage of metals to the acidic buffers. These results provided guidance for storage and application of different types of lanthanide microgels in future experimental assays.
**Reference**


Chapter 6 Hybrid Microgels by Ligand Exchange

6.1 Introduction

In this chapter, I describe an experiment in which I attempt to promote incorporation of preformed nanoparticles (NPs) into carboxylated microgels. The experiments were performed by stirring oleic acid stabilized nanoparticles with MAA functionalized microgels. The nanoparticles themselves were not water soluble. Following a ligand exchange process, the incorporation of surface passivated nanoparticles into the multidentate polymer ligands would allow the dispersion of the microgel/nanoparticle hybrids into aqueous media. In Chapter 1, I described two general criteria for incorporating nanoparticles into microgels. First, the nanoparticles must adhere to the microgels by interacting with functional groups that are part of the microgel structure. Second, the size of nanoparticles must be small enough to penetrate the gel [1-3]. Since polycarboxylic acids [4-5] have been used as a multidentate ligand to replace oleic acid from OA-capped NPs, one might anticipate that poly(NIPAm/VCL/MAA) microgels carrying multiple MAA functional groups would interact effectively with the OA-capped NPs if a suitable exchange medium could be found. To check the validity of these criteria, we tested the microgels with nanoparticles of different sizes and shapes. We found that, only when the size of the nanoparticles matched well with the mesh size of microgels, we could load the poly(NIPAm/VCL/MAA) microgels with significant amounts of metal atoms. Finally, the hybrid microgels were analyzed and quantified by mass cytometry.

6.2 Experimental

6.2.1 Materials

Trioctylphosphine (TOP, 90%), oleylamine (97%), oleic acid (OA, 90%), 1-octadecene (ODE, 90%), CdO (99.999%), Se powder (99.999%), HoCl₃·6H₂O (99.9%), NaOH (98%) and NH₄F (99.99%) were purchased from Aldrich and used as received. Absolute ethanol, methanol, hexane, THF and toluene were used as received. Aqueous solutions were prepared with deionized water (Elix/Gradient water purification system, Millipore).
6.2.2 Synthesis of Oleic Acid (OA)-Capped Nanoparticles (NPs)

*Synthesis of CdSe/OA QDs.* Oleic acid-capped CdSe QDs (CdSe/OA) were prepared by Yasser Hassan from the Scholes’ group according to a recipe reported by Zhang [6]. All manipulations were performed using standard air-free techniques. A saturated Se stock solution in TOP (2.1 M) was obtained by dissolving Se powder in TOP. A Cd stock solution (0.3 M) was prepared by dissolving CdO (1.1556 g, 9 mmol) in oleic acid (15.0 mL) and ODE (15.0 mL) at 250 °C to get a clear colorless solution and then stored at 60 °C. 5.0 mL of oleylamine and 0.15 mL of Se stock solution were loaded in a 50-mL three-neck round-bottom flask, and the mixture was heated to ca. 90 °C and degassed under a vacuum of 10 Pa for 20 min. The reaction vessel was then filled with argon, and its temperature was increased to 300 °C with stirring. A 1.0 mL volume of the Cd stock solution (0.3 M in oleic acid) loaded in a syringe was then injected quickly (in less than 0.2 s) into the reaction flask. The temperature was then set at 280 °C for the subsequent growth and annealing of nanocrystals. An aliquot of the sample were taken at 1 min and injected into cold toluene to terminate the growth of nanocrystals immediately for the use of recording their optical spectra. After the completion of particle growth, the reaction mixture was allowed to cool to 60 °C, and 10 mL of methanol was added thereafter. The obtained CdSe nanocrystals were precipitated by adding methanol into the toluene solution and were further isolated and purified by repeated cycles of centrifugation-sedimentation (by adding methanol to toluene each time) and decantation.

*Synthesis of NaHoF₄/OA nanoparticles.* The OA-capped NaHoF₄ nanoparticles (NaHoF₄/OA) were synthesized by Dr. Yi Hou from the Winnik group following a protocol described by Yan et al [7]. HoCl₃·6H₂O (1 mmol) was dissolved in a mixture of oleic acid (16 mL) and ODE (16 mL). The solution was heated to 150 °C under nitrogen protection to form a homogeneous solution, and then cooled to room temperature. A methanol solution (10 mL) containing NaOH (2.5 mmol) and NH₄F (4 mmol) was slowly added into the flask and stirred at 50 °C for 30 min. Subsequently, the solution was slowly heated to remove methanol, degassed at 100 °C for 10 min, and then heated to a certain temperature with an electromantle, and maintained for a certain time under nitrogen protection. The particle size was tuned by different combinations of the reaction time and temperature. NaHoF₄/OA nanoparticles with a diameter $d$
= 5.1 nm were obtained at 270 °C when reacted for 45 min. NaHoF₄/OA nanoparticles with \( d = 10.2 \) nm were obtained at 300 °C when reacted for 60 min. NaHoF₄/OA nanoparticles with \( d = 33.5 \) nm were obtained at 320 °C when reacted for 45 min. After the reaction, the solution was allowed to cool in air, and the nanocrystals were precipitated by adding ethanol into the solution. The nanoparticles were purified by repeated cycles of centrifugation-redispersion (by adding ethanol into hexane each time) and finally redispersed in hexane.

### 6.2.3 Nanoparticle-Containing Microgels Obtained by Ligand Exchange.

Nanoparticle-containing microgels were prepared by ligand exchange at room temperature between microgels and OA-capped NPs in THF as a common solvent. Poly(NIPAM/VCL/MAA) microgels containing 27 mol % MAA were used. As a typical example, aqueous solution poly(NIPAM/VCL/MAA) microgel (MG, 200 \( \mu \)L, containing 0.0445 mmol MAA/ g solution) was initially neutralized with NaOH solution (0.10 M, 90 \( \mu \)L, 1 molar equivalent based on the total MAA content of the microgels). Then the microgel solution was dried by rotary evaporator at room temperature and then resuspended into 400 \( \mu \)L THF. A homogenous solution was formed. The OA-capped nanoparticles (ca. 5 mg, according to the solids content), dispersed in hexane or toluene, were dried by rotary evaporation, and then THF (200 \( \mu \)L) was added immediately. Then the THF solutions of the microgels and the OA-capped nanoparticles were combined in a 2 mL centrifuge tube and mixed with magnetic stirring overnight. The solution was then centrifuged at 5000 rpm for 30 min to sediment the microgels. The excess nanoparticles that remained in supernatant solution were removed. The product was purified by three cycles of centrifugation (5000 rpm, 30 min) and redispersion in THF. After evaporation of the THF, the resultant microgels could readily be dispersed in water to form a robust colloidal solution that remained stable for weeks. A cartoon depicting the ligand exchange process is presented in Scheme 6-1.
6.3 Results and Discussions

6.3.1 Properties of Hybrid Microgels by Ligand Exchange

All the nanocrystals described in this Chapter were synthesized by well-established protocols of high-temperature thermolysis of nanocrystal precursors in organic media using oleic acid (OA) as stabilizers for both CdSe and NaHoF$_4$ nanocrystals. The roles of OA include passivating the nanocrystal surface and imparting colloidal stability in nonpolar solvents (e.g. hexane, toluene, and THF), but not in polar solvents such as alcohols and water. Ligand exchange of these alkane-capped nanocrystals with MAA functionalized microgels enables the dispersion of these nanocrystals into water.

6.3.1.1 CdSe-Containing Microgels (MG-CdSe).

To prepare CdSe nanoparticle-containing microgels, I mixed the CdSe/OA nanoparticles (d = 4.6 nm) with carboxylate microgels with stirring in THF as a common solvent. After ligand exchange, the microgels carrying nanoparticles were purified from excess of CdSe QDs and free OA ligands by repeated centrifugation-redispersion and transferred from THF into aqueous solution. Figure 6-1 shows dark field TEM images of CdSe-encoded microgels from THF (Figure 6-1A) and from corresponding aqueous solutions (Figure 6-1B). In both images, the
microgels are closely packed and form multiple layers on the TEM grids. The signals from the CdSe QDs are quite bright in contrast to their microgel carriers. In the packed structures, the diameters of the CdSe-containing microgels from THF solution are ca. 240 nm, and from aqueous solution are ca. 300 nm. Figure 6-1C and D show the images of individual microgels obtained when the aqueous solution was diluted from the sample shown in Figure 6-1B. In this image, we see more QDs located in the core of the microgels. The average diameter of QD-containing microgel is ca. 300 nm.

The presence of Cd and Se atoms in the CdSe-containing microgels was confirmed by energy dispersive X-ray (EDX) analysis. Figure 6-2 shows a linear scan profile across one hybrid microgel on the grid used to obtain the TEM image in Figure 6-1C. While the signal from Cd and Se are not as pronounced as that from carbon, they are much higher than that of La, chosen as a negative control, i.e., as a lanthanide element not expected to be present in the sample, except as an impurity.

Confocal microscopy images with excitation at 488 nm in Figure 6-3 show bright structures of well separated CdSe-containing microgels from samples in both aqueous (Figure 6-3A) and THF (Figure 6-3B) solutions, with emission peaks located at $\lambda_{em} = 594$ nm from THF solution and $\lambda_{em} = 602$ nm from aqueous solution. The absorption and emission spectra are presented in the Appendix. These results indicate that the microgels carry large amounts of CdSe QDs.

Figure 6-1. Dark field TEM images of CdSe-containing microgels. The CdSe NPs are with diameter of $d = 4.6$ nm. (A) CdSe-containing microgels from THF solution (B) CdSe-containing microgels from
H₂O solution, (C) CdSe-containing microgels from dilute H₂O solution. This sample is a dilution of the sample used to prepare the grid in (B), (D) a magnified image of a single CdSe-containing microgel from H₂O.

**Figure 6-2.** Elemental analysis of a CdSe-containing microgel by EDX: linear scan of CdSe-containing microgel from dilute aqueous solution.

**Figure 6-3.** Confocal images of CdSe-containing microgels, the CdSe NPs are with diameter of \( d = 4.6 \text{ nm} \) (A) in water, (B) in THF. The excitation wavelength was set at \( \lambda_{\text{ex}} = 488 \text{ nm} \). The emission peak from CdSe-containing microgels was at \( \lambda_{\text{em}} = 594 \text{ nm} \) in THF, and \( \lambda_{\text{em}} = 602 \text{ nm} \) in water.
6.3.1.2 NaHoF₄-Containing Microgels (MG-NaHoF₄).

To prepare NaHoF₄ nanoparticle-containing microgels, I mixed solutions of the NaHoF₄/OA nanoparticles and MAA-containing microgels in THF. After stirring overnight, the microgels carrying the nanoparticles were purified from excess nanoparticles and free OA ligands by repeated centrifugation-redispersion, and then transferred from THF into aqueous solution. Different NaHoF₄/OA nanoparticles were used, including cylindrical particles with a long axis of ca. 33.5 nm, spherical nanoparticles with a diameter of ca. 10.2 nm, and spherical nanoparticles with a diameter of ca. 5.1 nm.

I prepared TEM grids of the various hybrid microgel samples. In these TEM images, we observed that for nanoparticles with larger sizes (33.5 nm and 10.2 nm), the microgels only adsorb limited amounts of nanoparticles on their surface, as shown in Figure 6-4A and D. In contrast, using the small nanoparticles with \( d = 5.1 \) nm, larger amounts of nanoparticles were incorporated into the microgels, as shown in Figure 6-4G.

For poly(NIPAM/VCL/MAA) microgels, most of the –COOH functional groups were buried in the core of microgels. My hypothesis was that for large nanoparticles, which could not diffuse into the core of microgels, the strength of intermolecular association with the microgel surface would be relatively weak. Therefore only a small number of nanoparticles would adhere to the microgel surface following the microgel purification process. For small nanoparticles, which could diffuse into the interior of microgel framework and undergo ligand exchange with the -COOH functional groups inside the microgels, the interaction of the nanoparticles with the microgel would be much stronger. The critical particle size for the effective interaction should be comparable to the mesh size of microgels. In following section, I examined the validity of the above hypothesis by estimating the average mesh size of microgels based on Flory-Rehner theory of affine gel swelling in solution.
Figure 6-4. TEM images of NaHoF₄ nanoparticles and microgels, to which the NaHoF₄ nanoparticles are attached. The histograms of NaHoF₄ nanoparticle size distribution corresponding to the TEM image of the NPs to the left of the histogram. (A) Hybrid microgels obtained with 33.5 nm NaHoF₄/OA nanoparticles after transfer to water; (B) 33.5 nm NaHoF₄/OA nanoparticles from hexane; (C) histogram of 33.5 nm NaHoF₄/OA nanoparticle size distribution; (D) hybrid microgels obtained with 10.2 nm NaHoF₄/OA nanoparticles after transfer to water; (E) 10.2 nm NaHoF₄/OA nanoparticles from hexane; (F) histogram of 10.2 nm NaHoF₄/OA nanoparticle size distribution; (G) hybrid microgels obtained with 5.1 nm NaHoF₄/OA nanoparticles after transfer to water; (H) 5.1 nm NaHoF₄/OA nanoparticles from hexane; (I) histogram of 5.1 nm NaHoF₄/OA nanoparticle size distribution.
6.3.2 Estimation on Average Mesh Size of Microgels

The mesh size defines the space between macromolecular chains in a crosslinked network, and is characterized by the correlation length, $\xi$ between two adjacent crosslinks. The calculations for the mesh size can be conducted as follows using the following equations [8]:

$$\xi = \alpha (r_0^2)^{1/2}$$  \hspace{1cm} (6.1)

Here $\alpha$ is the elongation coefficient of the polymer chains in any direction, and $(r_0^2)^{1/2}$ is the root mean squared, unperturbed end to end distance of the polymer chains between two neighboring crosslinks.

For isotropically swollen hydrogels, $\alpha^3$ is the swell ratio of the polymer, the ratio of $V$, the swollen volume of polymer, to $V_0$, the dry volume of polymer.

$$\alpha^3 = \frac{V}{V_0}$$  \hspace{1cm} (6.2)

$(r_0^2)^{1/2}$ can be calculated using the following equation:

$$(r_0^2)^{1/2} = l(C_nN)^{1/2}$$  \hspace{1cm} (6.3)

where $C_n$ is the Flory characteristic ratio, $l$ is the length of the bond along the polymer backbone, and $N$ is the number of bonds between crosslinks that is calculated as:

$$N = \frac{2\bar{M}_c}{M_r} = 2N_c = 2 \times \frac{1}{2r_c} = \frac{1}{r_c}$$  \hspace{1cm} (6.4)

$\bar{M}_c$ is the average molecular mass between crosslinks in the network; $M_r$ is the molecular mass of the repeat units; $N_c$ is the number average degree of polymerization between crosslinks, which can be estimated from the reaction stoichiometry; $r_c$ is the feed ratio of the crosslinking reagent in the gel synthesis.
Combining the above equations, and rearranging them, we obtain the equation for calculation of the mesh size in a swollen hydrogel:

$$\xi = \alpha \times l \times \left( \frac{C_n}{r_c} \right)^{1/2}$$

(6.5)

If we assume the mole ratios of all monomers polymerized into microgels is equal to the mole ratio of monomers in the feed, then we can calculate the mass of a single microgel molecule as:

$$m_{\text{microgel}} = \frac{n_{\text{MAA}} \times M_{\text{MAA}}}{N_A \times r_{\text{MAA}}}$$

(6.6)

where $m_{\text{microgel}}$ is the mass of one microgel in g; $n_{\text{MAA}}$ is the number of MAA functional groups per microgel, $n_{\text{MAA}} = 3.3 \times 10^7$ [9]; $M_{\text{MAA}}$ is the molar mass of MAA (86.06 g/mol) ; $N_A$ is the avogadro’s number (6.02 x 10^{23} mol^{-1}); $r_{\text{MAA}}$ is the mol fraction of MAA in the reaction based on total monomers, $r_{\text{MAA}} = 0.212$. The mass of a single microgel can be calculated (equation 6.6) as 2.2 x 10^{-14} g. We assume that the density of the dry microgel polymer, $\rho_{\text{microgel}}$, is equal to 1 g/mL. In the swollen state, at 20 °C, the hydrodynamic radius of the hybrid microgels was $R_h = 240$ nm in THF. The polymer swell ratio $\alpha^3$ can be estimated by equation 6.2 as:

$$\alpha^3 = \frac{V}{V_0} = \frac{\frac{4}{3} \pi R_h^3}{m_{\text{microgel}} \rho_{\text{microgel}}} = \frac{\frac{4}{3} \pi \times (240 \times 10^{-7} \text{cm})^3}{2.2 \times 10^{-14} \text{g} / (1 \text{g/cm}^3)} = 2.6$$

(6.7)

from which we deduce an expansion coefficient value of $\alpha = 1.4$.

The Flory characteristic ratio for poly(NIPAm) is $C_n = 11.7$ [10], and for poly(MAA) $C_n = 14.6$ [11]. If we consider only the NIPAm and MAA components of the microgel, we can calculate a mean Flory characteristic ratio of $C_n(\text{ave}) = 12.6$. Based on the weighted composition of the microgel polymer, we take $l = 0.154$ nm as the C-C bond length and $r_c = 0.03$ as the mole fraction of crosslinker.

The average mesh size of poly(NIPAm/VCL/MAA) microgels can be calculated as:
\[
\xi = \alpha \times l \times \left( \frac{C_n}{r_c} \right)^{1/2} = 1.4 \times 0.154 \text{ nm} \times \frac{12.6}{0.03} = 4.4 \text{ nm} \tag{6.8}
\]

This estimate is in agreement with my hypothesis that nanoparticles with size on the order of or smaller than the average mesh size of microgels (CdSe QDs with \(d = 4.6\) nm and NaHoF\(_4\) NPs with \(d = 5.1\) nm) could be taken up by these microgels in high amounts; while nanoparticles with sizes larger than the mesh size of microgels (NaHoF\(_4\) NPs with \(d = 10.2\) nm and \(d = 33.5\) nm) can only adsorb in small amounts on the surface of microgels.

6.3.3 Quantification of Hybrid Microgels by Mass Cytometric Analysis.

6.3.3.1 CdSe-Containing Microgels (MG-CdSe).

In Figure 6-6A I present \(^{112}\text{Cd}-^{114}\text{Cd}\) dot-dot plots obtained from mass cytometry measurements on MG-CdSe hybrid microgels. The units on the x- and y-axes of the plot are the measured intensities for the respective isotopes. In the Figure 6-6B and Figure 6-6C, the data are replotted as histograms showing the relative abundance of microgels characterized by the isotope intensities displayed on the x-axes. The Pearson correlation between the \(^{112}\text{Cd}\) and \(^{114}\text{Cd}\) signal intensities is 0.99, which indicates these signals are highly correlated, as expected.

![Figure 6-5](image-url)

**Figure 6-5.** A) Isotopic \(^{112}\text{Cd}-^{114}\text{Cd}\) dot-dot plot of mass cytometry measurements on MG-CdSe hybrid microgels prepared from CdSe QDs with \(d = 4.6\) nm; B) Histograms of \(^{112}\text{Cd}\) signal distribution among microgels determined by mass cytometry; C) Histograms of \(^{114}\text{Cd}\) signal distribution among microgels determined by mass cytometry. The data collection was gated to exclude “cell” debris and microgel aggregates. At least 10,000 microgels were analyzed per sample.
In Figure 6-6, I show the distributions of $^{112}\text{Cd}$ and $^{114}\text{Cd}$ among the microgels by box-and-whisker plots. The small box in the middle of each plot shows the mean value of Cd signal intensity per microgel. The larger box includes the middle one half of the data, in which the ends of the box are the upper and lower quartiles. The bar near the middle of the box is the median. The two lines outside the box are from the upper and lower 5th % of data. The two ×'s show the upper and lower 99th percentile of data. The upper outlier is the maximum and the lower outlier is the minimum value recorded.

**Figure 6-6.** Box-and-whisker plots displaying the distribution of $^{112}\text{Cd}$ and $^{114}\text{Cd}$ signal intensity for hybrid microgels determined by mass cytometry.

The mean number of Cd atoms per microgel can be calculated from the expression:

$$N_{\text{Cd/microgel}} = \frac{I_{^{112}\text{Cd}}}{T_{^{112}\text{Cd}} \times RA_{^{112}\text{Cd}}} \quad (6.9)$$

where the $I_{^{112}\text{Cd}}$ is the mean intensity of $^{112}\text{Cd}$ determined from mass cytometry, $I_{^{112}\text{Cd}} = 67.0$; $T_{^{112}\text{Cd}}$ is the transmission coefficient determined for $^{112}\text{Cd}$, $T_{^{112}\text{Cd}} = 8.3 \times 10^{-6}$, and $RA_{^{112}\text{Cd}}$ is the relative abundance of $^{112}\text{Cd}$ in nature, $RA_{^{112}\text{Cd}} = 24.13\%$. The calculated mean number of Cd atoms per microgel was $3.3 \times 10^7$ atoms/microgel. The coefficient of variance (CV) was 63%.

The diameter of the CdSe QDs, $d_{\text{CdSe}}$ (nm), can be calculated based on an empirical fit of the wavelength, $\lambda$ (nm), of the first excitonic absorption peak as follows [12]:

$$d_{\text{CdSe}} = (1.6122 \times 10^{-9})\lambda^4 - (2.6575 \times 10^{-6})\lambda^3 + (1.6242 \times 10^{-3})\lambda^2 - (0.4277)\lambda + (41.57) \quad (6.10)$$
where the wavelength of the first excitonic absorption peak, \( \lambda \), is at 600 nm (as shown in Figure A6-1), and the calculated diameter of the CdSe QDs, \( d_{\text{CdSe}} \), is at 4.6 nm.

Assuming the CdSe QDs are spherical, then the number of Cd atoms per QD, \( N_{\text{Cd/QD}} \), can be estimated as:

\[
N_{\text{Cd/QD}} = \frac{4}{3} \pi (r_{\text{CdSe}})^3 \rho_{\text{CdSe}} \times N_A / M_{\text{CdSe}}
\]
\[
= \frac{4}{3} \pi (2.3 \times 10^{-7} \text{cm})^3 \times 5.663 \text{g/cm}^3 \times 6.02 \times 10^{23} \text{mol}^{-1} / (191.37 \text{g/mol})
\]
\[
= 9.0 \times 10^2
\]

where \( r_{\text{CdSe}} \) is the radius of CdSe QDs, \( r_{\text{CdSe}} = 2.3 \) nm, \( \rho_{\text{CdSe}} \) is the density of CdSe solid, \( \rho_{\text{CdSe}} = 5.663 \) g/cm\(^3\) [13], and \( M_{\text{CdSe}} \) is the molar mass of CdSe, \( M_{\text{CdSe}} = 191.37 \) g/mol.

The number of QDs per microgel, \( N_{\text{QD/microgel}} \), can be estimated as:

\[
N_{\text{QD/microgel}} = \frac{N_{\text{Cd/microgel}}}{N_{\text{Cd/QD}}}
\]

where the measured number of Cd atoms per microgel, \( N_{\text{Cd/microgel}} \), was \( 3.3 \times 10^7 \) atoms per microgel. Then the mean number of QDs per microgel could be calculated as 37,000.

### 6.3.3.2 NaHoF\(_4\) Nanoparticle-Containing Microgels (MG-NaHoF\(_4\))

In Figure 6-7A, I show the histograms of \(^{165}\)Ho signal distribution of mass cytometry measurements on MG-NaHoF\(_4\) hybrid microgels prepared from NaHoF\(_4\) NPs with \( d = 5.1 \) nm, showing the relative abundance of microgels characterized by the isotope intensities displayed on the x-axes. In Figure 6-7B, I show the distributions of \(^{165}\)Ho isotopes among NaHoF\(_4\) nanoparticle encoded microgels by box-and-whisker plots.

The number of Ho atoms per microgel was calculated as:

\[
N_{\text{Ho/microgel}} = \frac{I_{^{165}\text{Ho}}}{T_{^{165}\text{Ho}} \times RA_{^{165}\text{Ho}}}
\]

where the \( I_{^{165}\text{Ho}} \) is the mean intensity of \(^{165}\)Ho determined from mass cytometry, \( I_{^{165}\text{Ho}} = 408 \), \( T_{^{165}\text{Ho}} \) is the transmission coefficient determined for \(^{165}\)Ho, \( T_{^{165}\text{Ho}} = 2.8 \times 10^{-5} \), and \( RA_{^{165}\text{Ho}} \) is the relative abundance of \(^{165}\)Ho in nature, \( RA_{^{165}\text{Ho}} = 100\% \). The calculated mean number of Ho atoms per microgel was \( 1.5 \times 10^7 \) atoms per microgel. The coefficient of variance was 67\%.
Assuming that the NaHoF₄ nanoparticles are spherical with a radius of 2.55 nm, the number of Ho atoms per particle, \( N_{\text{HoNP}} \), can be calculated as:

\[
N_{\text{HoNP}} = \frac{4}{3} \pi (r_{\text{NP}})^3 \times \rho_{\text{NaHoF}_4} \times N_{\text{Av}} / M_{\text{NaHoF}_4}
\]

\[
= \frac{4}{3} \pi (2.55 \times 10^{-7} \text{ cm})^3 \times 5.979 \text{ g/cm}^3 \times 6.02 \times 10^{23} \text{ mol}^{-1} / (263.91 \text{ g/mol})
\]

\[
= 9.5 \times 10^2
\]

where the radius of NaHoF₄ nanoparticle, \( r_{\text{NP}} = 2.55 \text{ nm} \), the density of solid NaHoF₄, \( \rho_{\text{NaHoF}_4} = 5.979 \text{ g/cm}^3 \) [14], \( N_{\text{Av}} = 6.023 \times 10^{23} \text{ mol}^{-1} \), and \( M_{\text{NaHoF}_4} = 263.91 \text{ g/mol} \).

The mean number of NaHoF₄ nanoparticles per microgel, \( N_{\text{NP/microgel}} \), was calculated as:

\[
N_{\text{NP/microgel}} = \frac{N_{\text{Ho/microgel}}}{N_{\text{HoNP}}},
\]

with \( N_{\text{Ho/microgel}} \) equal to \( 1.5 \times 10^7 \) atoms per microgel. Then the number of NPs per microgel could be estimated as 15,000 per microgel.

![Figure 6-7. A) Histograms of \(^{165}\text{Ho}\) signal distribution of mass cytometry measurement of MG-NaHoF₄ hybrid microgels prepared from NaHoF₄ NPs with \( d = 5.1 \text{ nm} \). B) Box-and-whisker plots displaying the distribution of \(^{165}\text{Ho}\) signal intensity for NaHoF₄ encoded microgels determined by mass cytometry. Data collection was gated to exclude cell debris and cell aggregates. Approximately 10,000 microgels were analyzed.](image)

**6.4 Conclusions**

The methodology described in this chapter represents a simple and straightforward way to construct hybrid microgels via ligand exchange with OA-capped colloidal nanocrystals that are
synthesized independently. The favorable interaction between the MAA functionality in the microgel and the NPs plays an essential role for organization of QDs within the microgel framework. Since most of the MAA-derived –COOH groups are in the interior of the microgels, the microgels showed selective affinity to nanoparticles small enough to penetrate into the interior of the microgels. Only NPs with a diameter comparable to the mesh size of microgels can penetrate inside of microgels and reacted effectively with the functional groups at large amounts. For hybrid microgels containing CdSe QDs (MG-CdSe, \(d_{\text{CdSe}} = 4.6 \text{ nm}\)), and NaHoF\(_4\) NPs (MG-NaHoF\(_4\), \(d_{\text{NaHoF4}} = 5.1 \text{ nm}\)), from the TEM images, I found most NPs are still located on the surface of microgels. This might be explained by the distribution of mesh size within the microgels. The mesh size distribution originated from the distribution of crosslinker within the microgels. In NIPAm-based microgels crosslinked with methylenebisacrylamide, the crosslinker distribution was predicted to be core-localized [15-16], due to its higher reactivity than NIPAm. With a higher degree of crosslinking in the core, smaller meshes were formed in the core. With a lower degree of crosslinking on the surface, larger meshes were formed on the surface. Since both CdSe QDs (\(d = 4.6 \text{ nm}\)) and NaHoF\(_4\) NPs (\(d = 5.1 \text{ nm}\)) carried a larger diameter than the average mesh size of microgels in THF (\(\xi = 4.4 \text{ nm}\)), they probably diffused into the large meshes located close to the surface of microgels, resulting in a surface-localized distribution of NPs.

The hybrid microgels prepared by ligand exchange contained significant amounts of metals (in the order of \(10^7\) metal atoms per microgel) as measured by mass cytometry. The variation in metal content from these hybrid microgels (> 60%) was higher than the variation of Ln contents in LnF\(_3\)- or LnPO\(_4\)-containing microgels reported in the previous chapters.
Reference

[9] Titration experiments show that Ln ion binding is saturated at 1 lanthanide ion per 3 –COO-groups. Mass cytometry measurements show an average of 1.1 x 10^7 Tm ions/microgel. Thus each microgel contains on average ca. 3.3 x 10^7 –CO_2H groups.
Appendix to Chapter 6

**Figure A6-1.** A normalized absorption spectrum of CdSe/OA QDs in toluene solution. The first exitonic absorption peak is at 600 nm.

**Figure A6-2.** A normalized emission spectrum of CdSe encoded microgels in THF and aqueous solutions. The excitation wavelength of 488 nm was used. The emission peak was at 594 nm in THF and 602 nm in water.

This Chapter addresses the question of whether one can use lanthanide nanoparticles (e.g., NaHoF₄) to detect and quantify surface biomarkers expressed at low levels by mass cytometry. To avoid many of the complications of experiments on live or fixed cells, I carried out proof-of-concept experiments using aqueous microgels with a diameter on the order of 700 nm as “model cells”. These surrogate cells were used to test whether nanoparticle (NP) reagents would allow the detection as few as 100 proteins per “cell” in cell-by-cell assays. Streptavidin (SAv), which served as the model biomarker, was attached to the microgel in two different ways. Covalent coupling to surface carboxyls of the microgel led to large numbers (>10⁴) of proteins per microgel, whereas biotinylation of the microgel followed by exposure to SAv led to much smaller numbers of SAv per microgel. Using mass cytometry, I compared two biotin-containing reagents, which recognized and bound to the SAvs on the microgel. One was a metal chelating polymer (MCP), a biotin end-capped polyaspartamide containing 50 Tb³⁺ ions per probe. The other was a biotinylated NaHoF₄ NP containing 15,000 Ho atoms per probe. Non-specific binding was determined with microgels with bovine serum albumin (BSA) conjugated microgels. The MCP was effective at detecting and quantifying SAvs on the microgel with covalently bound SAv (20,000 SAvs per microgel), but was unable to give a meaningful signal above that of the BSA-coated microgel for the samples with low levels of SAv. Here the NP reagent gave a signal two orders of magnitude stronger than that of the MCP and allowed determination of SAv numbers ranging from 100 to 500 per microgel. Sensitivity was limited by the level of non-specific adsorption. This proof of concept experiment demonstrates the enhanced sensitivity possible with NP reagents in cell-by-cell assays by mass cytometry. Unless otherwise stated, all the bioconjugation experiments, synthesis and characterization presented in this chapter were done by me. The biotinylated metal chelating polymer (Bi-PAsp(DTPA)₅₀) was synthesized and characterized by Dr. Yijie Lu. The biotinylated NaHoF₄ nanoparticle sample (Bi-NaHoF₄) was synthesized and characterized by Dr. Yi Hou.
7.1 Introduction

One of the goals of modern bioanalytical chemistry is the simultaneous (multiplexed) detection of multiple biomarkers in individual cells. Biomarkers are defined as characteristic proteins, genes, or small molecules that can be measured and evaluated as indicators of normal biological or pathogenic processes [1]. In flow cytometry, bioaffinity agents are labeled with fluorescent dyes or quantum dots (QDs) to allow selectively excited and individually detected, allowing rapid cell-by-cell analysis of multiple biomarkers. One of the limitations of flow cytometry is the breadth of the emission bands of the luminescent species used as antibody (Ab) labels. The spillover of overlapping emissions requires compensation and restricts the number of species that can be detected simultaneously for each cell. The Roederer group has shown that 18-color flow cytometry is possible [2], but this level of multiplexing is not routine.

Mass cytometry is a new technique designed to address the challenges of polychromatic flow cytometry by replacing fluorophores with stable heavy metal isotopes as Ab tags. [3] In this technique, cells are introduced individually but stochastically into the plasma torch of an inductively coupled plasma mass spectrometer (ICP-MS) equipped with time-of-flight detection. Each Ab is labeled with a specific metal isotope, and the multiplexing capability comes from instrument’s ability to resolve metal ions that differ in mass by a single atomic mass unit. To achieve a signal strong enough for detection by ICP-MS, Abs have to be labeled with multiple copies of a metal isotope. This has been accomplished with metal-chelating polymers (MCPs) with 30 to 80 pendant chelating groups and appropriate end group functionality [4-6]. Abs labeled with these polymers typically carry 150 to 250 metal atoms per Ab [6]. Ln ions are attractive for mass cytometry because of their low natural abundance, similar chemistry, and the availability of isotopically enriched samples.

The strength of mass cytometry is the multiplexing capability [7]. For example, the Nolan group examined regulatory cell signaling behavior across hematopoietic cells using two 34-parameter panels that included 31 antibody targets, a DNA intercalator, and measures of viability and cell size [8]. Newell et al. [9] reported a 37-parameter study of virus-specific T cell function and phenotype. Sample throughput can be further enhances with mass-tag cellular barcoding, analogous to fluorescent cell barcoding [10]. On the other hand, it lacks sensitivity compared to
fluorescence detection. Cellular protein expression levels range from a few copies to \(10^7\) copies per cell [11], and many important proteins, such as cytokine receptors, are expressed at levels too low to detect easily, even by fluorescence [12]. With current instrumentation and MCP reagents, mass cytometry can detect and quantify target biomarkers with abundances in the range of \(10^4\) to \(10^7\) per cell [6]. The goal of this work is to demonstrate that by using NaLnF\(_4\) nanoparticle reagents in the place of MCPs, it will be possible to detect small numbers of a particular biomarker per cell, surpassing the sensitivity available by standard fluorescent flow cytometry.

Nanoparticle (NP) reagents are a promising approach to increasing the sensitivity of mass cytometry [7]. Signal strength for ICP-MS increases linearly with the number of metal ions of a particular isotope. Thus if one could attach 10,000 metal atoms per antibody, one might be able to increase the sensitivity by a factor of 500 and detect as few as 100 copies of a particular protein per cell. Many types of NPs with a 10 nm diameter (the dimensions of an IgG antibody) contain on the order of 8000 to 10000 metal atoms [13]. Cd, Se, and Te have isotopes in the range that can be detected by mass cytometry, but QDs are designed to be much smaller than 10 nm small to take advantage of quantum confinement effects in their luminescence properties [14]. The most attractive candidates for mass cytometry are NPs related to Ln doped NaYF\(_4\), which are being developed for optical up-conversion [15] and NaGdF\(_4\) [16], which are being developed as magnetic resonance imaging contrast agents.

While the function of the NPs is determined by the composition of its core, the coating plays an essential role in bioanalytical applications. The coating must provide colloidal stability in aqueous media, prevent aggregation, provide functional groups for bioconjugation and suppress non-specific adsorption. Satisfying all of these criteria is a daunting task. Many different types of nanocrystals (e.g., iron oxide, quantum dots, and lanthanide nanoparticles) are synthesized at high temperature in organic media and bear surface ligands (e.g., oleic acid) that make the NPs soluble in non-polar organic solvents and insoluble in water. Finding proper conditions for ligand exchange or coating these particles to make them colloidally stable in aqueous media is just one of the challenges associated with targeted reagent development. Not only is the in vitro and in vivo performance of nanoparticles dependent on the size, charge, hydrophilicity, and flexibility of the coating molecules, but the number, density and type of
reactive functionality on the NP surface regulate the interactions between nanoparticles and their targets [17].

There are three types of problems one faces when attempting to use NPs as Ab labels. The first is that they can be difficult to conjugate reliably. The second is the difficulty of separating Ab-NP conjugates from excess NPs used in the conjugation reaction [18]. Finally, there is the ubiquitous problem of non-specific interactions. For a reagent to be useful in a targeted assay, it not only has to recognize the target biomarker on a cell, but the corresponding signal from the reagent on cells lacking the biomarker has to be sufficiently small. QDs represent the most widely studied nanocrystals for target applications, particularly for polychromatic flow cytometry. They provide a dramatic increase in the number of parameters that can be measured simultaneously. In their 2006 Nature Medicine paper describing development of the 17-color flow cytometry assay, Chattopadhyay et al. [19] discuss the complications of non-specific binding with the QD reagents that they use to extend the color range of their assay. They found that nonspecific binding was largely overcome by coating the quantum dot shells with polyethylene glycol (PEG), allowing them to overcome a crucial hurdle in the development of these reagents for immunophenotyping use. Although one imagines that that these commercial samples have been optimized to minimize non-specific adsorption, reports continue to appear in the literature in which non-specific adsorption of QDs is a problem [20-21].

A recent paper on the use of a solvent exchange protocol to coat hydrophobic NPs with a PEG-containing phospholipid provides an interesting perspective on these problems. The authors used 1,2-distearoylphosphatidylethanolamine-methyl-poly(ethylene glycol) with a PEG chain of M = 2000 (DSPE-mPEG) to coat two sizes (d = 6.5 nm, 17 nm) of iron oxide NPs (IONPs) as well as two samples of CdSe/ZnS QDs. The IONPs were synthesized with a surface coating of oleic acid, and the CdSe/ZnS QDs had a surface coating of trioctylphosphine oxide (TOPO). By mixing the DSPE-mPEG with corresponding PEG analogues containing a terminal amino group, a terminal –COOH group, or a terminal maleimide, the authors were able to control the surface coverage of the IONPs with PEG ligands and the density of reactive functionality per NP. In addition, the authors were able to conjugate the 17 nm IONPs with anti-mouse IgGs through thiol maleimide chemistry and also to attach a goat anti-human folate receptor-1 Ab to these NPs. ELISA experiments with the former and in vitro experiments with the latter demonstrated high
target recognition efficiency and low non-specific binding. While these results are impressive and hold promise for the future of this coating protocol, the authors comment in the Supporting Information, that when they applied this approach to QDs, the coating efficiency varied significantly among different batches of QDs that they purchased. In the current state of the art, it is clear that I have much to learn about how to optimize the surface coatings for different types of NPs, and that the approach to finding the optimal surface coating may vary with the nature of the core of the nanoparticle.

While there have been important advances in the past several years in the synthesis of lanthanide nanoparticles [22-24], control over surface chemistry is less well advanced than for QDs or iron oxide NPs. Several strategies have been examined with good success to provide colloidal stability in aqueous media in the presence of phosphate buffer or serum proteins. These include flash nanoprecipitation [25-26], ligand exchange with PEG bidentate [27] or tetradentate phosphonates [28], and encapsulation with amphiphilic polymers [29]. These technologies have not yet advanced to the point where meaningful mass cytometry experiments have been carried out with lanthanide NPs labeled with Abs.

My goal in this Chapter is to assess the enhancement in sensitivity that nanoparticle reagents can provide for detection of biomarkers by mass cytometry. I want to ask the question, can one detect as few as 100 protein molecules per cell. In order to sidestep many of the problems of reagents that have to function in the complex environment of cell suspensions, I have chosen to create “model cells” consisting of microgels of uniform size. These cross-linked carboxylated microgels consist of a copolymer of N-isopropyl acrylamide (NIPAm), N-vinyl caprolactam (VCL), and 27 mol % methacrylic acid (MAA) (denoted as V27 in Chapter 2). I employ streptavidin (SAv) as a model biomarker. The choice of SAv as a model biomarker enables us to use biotinylated reagents to detect the SAv entities on the model “cell” surface. In this way, I can compare the mass cytometry signal of metal-chelating polymers with a biotin end group and ca. 50 metal ions per polymer as a mass cytometry reagent with biotinylated NaHoF₄ NPs with a core diameter $d = 13$ nm, containing ca. 15,000 Ho atoms per NP.

In mass cytometry experiments with cell suspensions, the cells are fixed, permeabilized, and then stained with an iridium intercalator [30]. Each cell entering the plasma creates an ion cloud generating signals for $^{191}\text{Ir}$ and $^{193}\text{Ir}$, which the instrument recognizes as the signature of a cell
event. For my model cells, the microgels are loaded with ca. $10^7$ TmF$_3$/MG. In analogy with experiments on cell suspensions, the mass cytometer recognizes a strong $^{169}$Tm signal as the signature of a “cell” event.

These experiments are enabled by a lucky happenstance. While it is straight forward to conjugate proteins such as SAv to the surface of microgels using typical peptide coupling agents, this approach leads to covalent attachment of ca. $10^4$ SAv/MG. It is difficult to attach only small numbers of proteins, on the order of 100 to 500 SAv to the microgels. When I reacted the microgels with biotin, to be used in a sandwich assay, the reaction could be carried out to only low conversion. At higher conversion, the microgels precipitated. In this way, I obtained three samples of microgels containing small but different numbers of biotins per microgels. These are the samples that permit us to examine whether I can detect as few as 100 SAvs per microgel.

### 7.2 Experimental Procedures

#### 7.2.1 Materials

Streptavidin from Streptomyces avidinii (SAv, salt free lyophilized powder, Sigma-Aldrich), Albumin from bovine serum (BSA, lyophilized powder, 96+%, Sigma-Aldrich), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, 99+%, Acros Organics), 6-aminocaproic acid (6-ACA, 99+% , Sigma-Aldrich) were used without purification. N-Biotinyl-3,6-dioxaoctane-1,8-diamine (Bi-NH$_2$) was synthesized in our lab as described in the Appendix, which is also commercially available from Pierce Biotechnology. Biotinlated NaHoF$_4$ nanoparticles (Bi-NaHoF$_4$) were synthesized by Dr. Yi Hou. Biotinylated metal chelating polymer (Bi-PAsp(DTPA)$_{50}$) were synthesized by Dr. Yijie Lu. Details of the synthesis were reported in the Appendix.

#### 7.2.2 Synthesis

##### 7.2.2.1 Synthesis of Tm-Encoded Poly(NIPAm/VCL/MAA) Microgel (MG(Tm)).

The poly(NIPAm/VCL/MAA) copolymer microgel sample employed here is sample V27 described in Chapter 3. It was prepared at approximately 0.85 wt % solids from a monomer mixture containing 56 mol% N-isopropylacrylamide (NIPAm), 14 mol% N-vinylcaprolactam
(VCL), 27 mol% methacrylic acid (MAA) and 3 mol% N,N′-methylenebis(acrylamide) (BIS) as a crosslinker. The purified sample was characterized by acid-base titration to contain 2.66 mmole/(g dry weight) -COOH groups. The sample employed here was repurified by sedimentation-redispersion in deionized water and its concentration in terms of total acid content was determined by titration.

The microgels were then loaded with Tm$^{3+}$ ions, followed by addition of NaF to form TmF$_3$ nanoparticles within the microgels, following the procedure described in Chapter 3 for Eu$^{3+}$ ions. A microgel solution (4.0 g, 2 wt%, containing 44.5 µmol MAA/mL) was neutralized with 1.0 eq. NaOH solution (0.10 M, 1.8 mL). After stirring for 10 min, a TmCl$_3$ solution (20 mM, 3.0 mL) was added, and the solution was stirred overnight to promote ion exchange between the Tm$^{3+}$ and Na$^+$ ions. Then NaF (0.030 M, 6.0 mL) was added slowly (1 drop per second), followed by stirring overnight. These microgels, denoted MG(Tm), were purified by centrifugation and redispersion in deionized water. I used acid-base titration data for the microgels prior to ion exchange in conjunction with a value of 3.3 x 10$^7$ -CO$_2$H groups/microgel (see below) to calculate microgel concentrations in units of number of microgels per mL of solution in a sample.

### 7.2.2.2 Synthesis of Streptavidin-Coated Microgels via Covalent Attachment (SAv-MG(Tm)).

A sample of MG(Tm) (65 µL, ca. 2.5 x 10$^{10}$ microgels) was diluted with phosphate buffer (pH 8.5, 335 µL, 200 mM). Then the microgels were treated with DMTMM (0.29 µmol in 20 µL DI water) to activate -COOH groups. The amount of DMTMM added corresponded to 20 mol% of the total carboxylic acid group content of the microgels. After 5 min, the activated microgel solution was mixed with streptavidin (0.5 nmol in 100 µL PBS buffer, pH 7.4, 0.01 M) and stirred for 30 min at room temperature. To block unreacted, activated acid groups, the mixture was subsequently treated with an excess of 6-aminocaproic acid (6-ACA, 0.3 µmol in 6 µL DI water) for 30 min. Then the streptavidin-coated microgels (SAv-MG(Tm)) were purified by three cycles of centrifugation (5000 rpm, 40 min, 23 ºC) and redispersion in DI water and finally redispersed in PBS buffer (1.0 mL) and stored at 4 ºC prior to use.
7.2.2.3 Synthesis of BSA-Coated Microgels via Covalent Attachment (BSA-MG(Tm)).

A sample of MG(Tm) (65 μL, ca. 2.5 x 10^{10} microgels) was diluted in phosphate buffer and then activated with DMTMM as described above. It was then treated with a solution of BSA in PBS buffer (1 wt % BSA, 100 μL) and stirred magnetically for 30 min. After the remaining activated carboxyl groups were quenched with an excess of 6-aminocaproic acid, the BSA-coated microgels (BSA-MG(Tm)) were purified by three cycles of sedimentation and redispersion in DI water and finally redispersed in PBS buffer (1.0 mL) and stored at 4 °C.

7.2.2.4 Synthesis of Biotinylated Microgels (Bi-MG(Tm)).

Stock solutions were prepared of Bi-NH$_2$ (7.0 mg, 19 μmol) in DI water (0.973 g, 19 μmol/g), and DMTMM (4.0 mg, 15 μmol) in DI water (0.980 g, 15 μmol/g). Three microgel samples were prepared by diluting aliquots of MG(Tm) (65 μL, containing ca. 2.5 x 10^{10} microgels in water) with phosphate buffer (pH 8.5, 335 μL, 200 mM) to obtain 0.3 wt % solutions. To each microgel sample different amounts of DMTMM (0.15-0.29 μmol) were added (see Table 7-1 for details), and the solutions were stirred for 5 min for activation of the carboxylic acid groups. Then sufficient Bi-NH$_2$ solution was added to each to obtain a 1:1 molar ratio of Bi-NH$_2$ to DMTMM. Each reaction was allowed to proceed for 30 min at room temperature under gentle stirring. Then the biotinylated microgels were purified by three cycles of sedimentation (5000 rpm, 40 min, 23 °C) followed by redispersion in DI water to remove buffer salts, reaction byproducts and unreacted substances. After the final sedimentation, the biotinylated microgels were redispersed in PBS buffer (100 μL) and stored at 4 °C prior to use. To determine the conversion of Bi-NH$_2$ in each reaction, the supernatant solutions were collected and the concentration of unreacted Bi-NH$_2$ was determined by UV-Vis spectrometry using a Pierce® biotin quantification kit. The detailed procedure is described in the Appendix.
Table 7-1. Synthesis of biotinylated Tm-encoded microgels

<table>
<thead>
<tr>
<th>sample</th>
<th>DMTMM μmol</th>
<th>Bi-NH$_2$ μmol</th>
<th>% biotin incorporation b</th>
<th>$10^7 \times$ Bi per microgel c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bi-MG(Tm)-1</td>
<td>0.15</td>
<td>0.13</td>
<td>5.0</td>
<td>1.7</td>
</tr>
<tr>
<td>Bi-MG(Tm)-2</td>
<td>0.22</td>
<td>0.20</td>
<td>7.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Bi-MG(Tm)-3</td>
<td>0.29</td>
<td>0.26</td>
<td>9.5</td>
<td>6.2</td>
</tr>
</tbody>
</table>

a. Each MG sample contained 1.32 μmol MAA based on titration of the microgel prior to incorporation of TmF$_3$ NPs.

b. Calculated from the amount of unreacted Bi-NH$_2$ detected at the end of the reaction.

c. Calculated assuming $3.3 \times 10^7$ -COOH groups per microgel.

7.2.2.5 Synthesis of Streptavidin-Coated Microgels via Formation of a Biotin-SAv Complex: SAv-Bi-MG(Tm).

Individual solutions of the three different Bi-MG(Tm) samples (see Table 7-1, 100 μL, containing ca. $2.5 \times 10^{10}$ microgels) were each mixed with an excess of BSA (100 μL, 1 wt % in PBS buffer) and stirred magnetically at room temperature for 30 min to allow passive adsorption of BSA to the microgels. I refer to the BSA-passivated samples as BSA/Bi-MG(Tm). Then the microgels were incubated with a streptavidin solution (500 nmol/L, 100 μL, 0.05 nmol) for 30 min. After reaction, the corresponding streptavidin-coated microgels (SAv-Bi-MG(Tm)-1, SAv-Bi-MG(Tm)-2, SAv-Bi-MG(Tm)-3) were purified to remove excess protein by three cycles of sedimentation-redispersion in DI water and finally redispersed in PBS buffer (1 mL). They were stored at 4 °C prior to use.

7.2.2.6 Synthesis of BSA-Coated Microgels via Passive Absorption (BSA/MG(Tm)).

A sample of MG(Tm) (65 μL, containing ca. $2.5 \times 10^{10}$ microgels) was diluted with PBS buffer (335 μL), then sedimented at 5000 rpm for 40 min followed by redispersion in PBS buffer (400 μL). An aliquot (100 μL) of this microgel solution was treated with an excess of BSA (100 μL, 1 wt % in PBS buffer) and stirred magnetically at room temperature for 30 min. These BSA-passivated microgels (BSA/MG(Tm)) were purified to remove excess protein by three cycles of
sedimentation-redispersion in DI water and finally redispersed in PBS buffer (1 mL) and stored at 4 °C prior to use.

### 7.2.2.7 Synthesis of Biotinylated NaHoF₄ Nanoparticle Probes (Bi-NaHoF₄).

Oleic-acid-capped NaHoF₄ nanoparticles (NaHoF₄-OA) were synthesized as described by Zhang [31-34]. Note that these particles, synthesized by Dr. Yi Hou, are analogous to those described in Chapter 6, but this is a different sample. The particles were subjected to ozonolysis in hexane at -78 °C to introduce surface -COOH groups and transfer the nanoparticles to water following a protocol described by Yan [35]. Details are provided in the Appendix. A NaHoF₄-COOH nanoparticle sample (containing 0.125 mmol COOH groups by titration) was dissolved in DI water (5 mL). The solution was adjusted to pH 8.5 by adding NaOH (5 M) monitored by a pH meter. DMTMM (0.07 g, 0.25 mmol) was dissolved in DI water (2 mL) and added into the NaHoF₄-COOH solution dropwise with continuous stirring. This solution was given 5 min to pre-react before Bi-NH₂ (20 mg, 0.05 mmol) in DI water (3 mL) was added. After stirring the mixture for 10 min, the biotinylated NaHoF₄ nanoparticles (Bi-NaHoF₄) were precipitated through addition of acetone into the solution. The NPs were collected by centrifugation at 14,000 rpm for 30 min at room temperature, washed with acetone several times, and finally redispersed in DI water.

### 7.2.2.8 Synthesis of a Biotinylated Metal Chelating Polymer Probe (Bi-PAsp(Tb)₅₀).

A biotin-end-capped polyaspartamide with diethylenetriaminepentaacetic acid (DTPA) chelators on each of the 50 repeat units [Bi-PAsp(DTPA)₅₀] was synthesized by Dr. Yijie Lu as previously reported by our group [36]. Details for this polymer and its characterization are provided in the Appendix to this chapter. To incorporate Tb³⁺ ions, a solution of Bi-PAsp(DTPA)₅₀ (5 mg, 0.12 µmol) in ammonium acetate buffer (2.4 mL, 20 mM, pH 6.0) was mixed with a solution of TbCl₃.6H₂O (4.675 mg, 12.5 µmol, 1.4 mol eq./DTPA) in the same buffer (0.25 mL). After incubation at 37 °C for 30 minutes, the solution was transferred to a 4 mL 3kDa MWCO Millipore Amicon spin filter and washed three times with Tris buffer (Tris 25 mM, NaCl 150 mM, KCl 2 mM, pH 7.4) and then DI water. Then the product was filtered with a 200 nm syringe filter. The final concentration of solution was adjusted with DI water (to 3.3 µmol/L and 0.33 µmol/L) and stored at 4 °C prior to use.
7.2.3 Mass Cytometric Bioassays

7.2.3.1 Streptavidin-Biotin Coupling Assays Employing Bi-PAsp(Tb)\textsubscript{50}

**Samples.** Four aliquots of SAv-MG(Tm) solution (100 µL, containing ca. 2.5 x 10\textsuperscript{9} microgels) were incubated with different amounts of a Bi-PAsp(Tb)\textsubscript{50} solution (3.3 µmol/L: S1, 20 µL; S2, 40 µL; S3, 60 µL; S4, 80 µL) for 30 min at room temperature. Then the solutions were purified from excessive polymer by three cycles of centrifugation at 5000 rpm for 40 min at RT followed by redispersion in DI water (1 mL). The concentration of the microgel solution was adjusted to ca. 10\textsuperscript{6} microgels per mL by DI water for analysis by mass cytometry.

**Negative control.** Four aliquots of BSA-MG(Tm) solution (100 µL, containing ca. 2.5 x 10\textsuperscript{9} microgels) were incubated with different amounts of a Bi-PAsp(Tb)\textsubscript{50} solution (3.3 µmol/L: N1, 20 µL; N2, 40 µL; N3, 60 µL; N4, 80 µL) and stirred at room temperature for 30 min. Then the solutions were purified as described above by three cycles of centrifugation-redispersion with DI water (1 mL), and then diluted to 10\textsuperscript{6} microgels per mL by DI water for analysis by mass cytometry.

**Blank control.** A solution of streptavidin-coated microgel (SAv-MG(Tm), 100 µL, containing ca. 2.5 x 10\textsuperscript{9} microgels) was directly diluted to 10\textsuperscript{6} microgels per mL with DI water for analysis by mass cytometry.

7.2.3.2 Biotin-Streptavidin-Biotin Sandwich Assays.

**Sample + Bi-PAsp(Tb)\textsubscript{50}.** Four aliquots of a solution of SAv-Bi-MG(Tm)-3 (100 µL, containing ca. 2.5 x 10\textsuperscript{9} microgels) were stirred for 30 min at room temperature with 6-aminocaproic acid (6-ACA, 0.03 µmol in 3 µL), then incubated with different amounts of a Bi-PAsp(Tb)\textsubscript{50} solution (0.33 µmol/L: S1, 10 µL; S2, 20 µL; S3, 40 µL; S4, 80 µL) and stirred at room temperature for 30 min. Then the solutions were purified as described above by three cycles of centrifugation-redispersion in DI water (1 mL), and then diluted to 10\textsuperscript{6} microgels per mL with DI water for analysis by mass cytometry.
Sample + Bi-NaHoF$_4$. A solution of each of the three SAv-Bi-MG(Tm) microgels (100 μL, containing ca. 2.5 x 10$^9$ microgels) was stirred with Bi-NaHoF$_4$ NPs solution in DI water (5 mg/mL, 10 μL, 0.015 μmol) for 30 min at room temperature. The solutions were purified as described above by three cycles of centrifugation-redispersion in 1 mL of DI water, and then diluted to 10$^6$ microgels per mL by DI water for analysis by mass cytometry.

Negative control + Bi-PAsp(Tb)$_50$. Four aliquots of a solution of microgels with a surface passivated with adsorbed BSA (BSA/MG(Tm), 100 μL, containing ca. 2.5 x 10$^9$ microgels) were stirred with 6-aminocaproic acid (6-ACA, 0.03 μmol in 3 μL) for 30 min at room temperature, and then incubated with SAv solution (500 nmol/L, 10 μL, 0.005 nmol) for 30 min. After that, different amounts of a Bi-PAsp(Tb)$_50$ solution (0.33 μmol/L: S1, 10 μL; S2, 20 μL; S3, 40 μL; S4, 80 μL) were added followed by with stirring for 30 min at room temperature. Then the solutions were purified as described above by three cycles of centrifugation-redispersion in DI water (1 mL), and then diluted to 10$^6$ microgels per mL by DI water for analysis by mass cytometry.

Negative control + Bi-NaHoF$_4$. A solution of SAv (500 nmol/L, 10 μL, 0.005 nmol) was added to a solution of microgels with a surface passivated with adsorbed BSA (BSA/MG(Tm), 100 μL, containing ca. 2.5 x 10$^9$ microgels), and stirred for 30 min. Then a solution of Bi-NaHoF$_4$ in DI water (5 mg/mL, 10 μL, 0.015 μmol) was added, and stirring was continued for 30 min at room temperature. The solution was purified by three sedimentation/redispersion cycles as described above to remove excess NaHoF$_4$ nanoparticles, which did not sediment under these conditions. In the final step, the microgels were redispersed in DI water (1 mL). Before analysis, the microgel solution was diluted with DI water to ca. 10$^6$ microgels per mL.

Blank control. A solution of SAv-Bi-MG(Tm) (100 μL, containing ca. 2.5 x 10$^9$ microgels) was diluted to ca. 10$^6$ microgels per mL by DI water before characterization.

7.3 Results and Discussions

I begin this section with a description of the synthesis of the metal-encoded microgels, followed by a discussion of the attachment of streptavidin (SAv) as a model biomarker to the
microgel surface. The starting microgels are the sample V27 described in Chapter 3. They were synthesized by the precipitation copolymerization of NIPAM, VCL, MAA, and the cross-linker methylene-bis-acrylamide (mole ratio 56:14:27:3). These microgels had a narrow size distribution and a mean hydrodynamic diameter \(d_h = 700\ \text{nm}\) (in PBS buffer at pH 7.4) as characterized by multiangle dynamic light scattering (DLS). Based on the work of Hoare and Pelton [37-38], one expects, because of the reactivity ratio mismatch in the polymerization reaction, that most of the –COOH groups will be located in the core of the microgel. Some of the –COOH groups are located at or near the microgel surface. These are the groups to which proteins can be attached. The –COOH content of the entire sample was determined by acid-based titration, which showed that the carboxylic acid groups of the microgel were characterized by a distribution of \(pK_a\) values ranging from 4.5 to 7.5 [39].

Ion exchange experiments with \(\text{Tm}^{3+}\) ions began with neutralization of an aqueous microgel solution with 1 eq. NaOH, followed by 1/3 eq. TmCl\(_3\). After three centrifugation-resuspension cycles to purify the ion exchanged microgels, the suspension was treated with 1 eq. NaF to precipitate TmF\(_3\) nanoparticles in the microgel interior. I refer to these particles as MG(Tm). They were characterized for particle size by DLS \((d_h = 700\ \text{nm}, \ \text{in PBS buffer at pH 7.4})\) and transmission electron microscopy \((d_{\text{TEM}} = 350 \pm 20\ \text{nm}, \ \text{Figure 7-1A})\), and by mass cytometry to determine a mean Tm content \((1.1 \times 10^7\ \text{Tm atoms per microgel})\). In Chapter 3, I described similar ion-exchange experiments with EuCl\(_3\) that demonstrated that the lanthanide ion \((\text{Ln}^{3+})\) content of the microgels saturated at one \(\text{Ln}^{3+}\) for every three -COOH groups and did not change after conversion of the Ln ions to LnF\(_3\) nanoparticles. From these results, I infer that my sample contains an average of \(3.3 \times 10^7\) -COOH groups per microgel. I used this value to calculate microgel concentrations in units of microgels/mL.

I used two approaches to attach SAv to the microgels. In the first approach, I used -COOH activation chemistry to attach SAv covalently to the microgels, presumably by coupling to lysine amino groups. This led to a high SAv content per microgel. I refer to these samples as SAv-MG(Tm). In a second approach, I attached biotin covalently to the microgel and then exposed the biotin-modified microgels (Bi-MG(Tm)) to a solution of SAv. This led to microgels containing low copy numbers of SAv per microgel. I refer to these microgels as SAv-Bi-
MG(Tm). These reactions and the characterization of the products are described in the following sections.

**Scheme 7-1.** Covalent attachment of Bi-NH₂ to microgels via DMTMM coupling

### 7.3.1 Covalent Attachment of Streptavidin and BSA to Microgels: SAv-MG(Tm) and BSA-MG(Tm).

To prepare SAv-MG(Tm) samples, I used 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) to covalently attach SAv to the surface of the microgels. As a control, I used similar chemistry to attach BSA to these microgels. We note that for SAv, there are 100 exposed amines, consisting of 80 arginine residues and 20 lysine residues [40], while in each BSA protein, there are 60 lysine residues and 23 arginine residues [41]. MG(Tm) microgel samples were first activated with DMTMM (0.2 eq./COOH) at pH 8.5, and then a solution of streptavidin or BSA was added. These reactions were allowed to proceed for 30 min, after which excess 6-aminocaproic acid (6-ACA) was added to block unreacted activated carboxylic acid groups.

These modified microgels were characterized by both TEM and DLS. TEM images (Figure 7-1) show the morphology in the dry state. The MG(Tm) microgels in Figure 7-1A show a dense core associated with the presence of TmF₃ NPs surrounded by a diffuse corona. The BSA-MG(Tm) microgels (Figure 7-1B) and SAv-MG(Tm) microgels (Figure 7-1C) show more compact structures, which are rather different in appearance, with the SAv-MG(Tm) structures
appear to be more dense and more compact than either the BSA-MG(Tm) or MG(Tm) microgels. DLS measurements indicated a small contraction in the hydrodynamic diameters of the modified microgels compared to MG(Tm) \([d_{h}(\text{BSA-MG(Tm)})] = 640 \text{ nm}; d_{h}(\text{SAv-MG(Tm)}) = 640 \text{ nm}; d_{h}(\text{MG(Tm)}) = 700 \text{ nm}] \). These values imply a more significant contraction of the microgel host, since attachment of protein molecules to the surface should add to the diameter of the overall objects. In the cartoons accompanying the TEM images in Figure 7-1, I attempt to depict the shape of the protein molecules, recognizing that the drawing is not to scale. The BSA protein is a prolate ellipsoid, 14 x 4 nm [42]. SAv is approximately spherical \((d \approx 7 \text{ nm})\). I depict it as a square to emphasize that it can bind 4 biotin moieties.

Figure 7-1. TEM images for the functional microgels described in the text. A) MG(Tm); B) BSA-MG(Tm) C) SAv-MG(Tm). The microgels are characterized by: \(d_{\text{MG(Tm)}} = 350 \pm 20 \text{ nm}, d_{\text{BSA-MG(Tm)}} = 330 \pm 20 \text{ nm}, d_{\text{SAv-MG(Tm)}} = 230 \pm 20 \text{ nm}\). Scale bars are 500 nm.
7.3.2 Streptavidin-Coated Microgels via Formation of a Biotin-SAv Complex: SAv-Bi-MG(Tm).

To prepare SAv-Bi-MG(Tm) samples, I first synthesized biotinylated microgels. In this reaction, carboxyl groups in the MG(Tm) microgels were first activated at pH 8.5 with DMTMM followed by reaction with N-biotinyl-3,6-dioxaooctane-1,8-diamine (Bi-NH₂) as shown in Scheme 7-1. Three samples were prepared (Table 7-1) employing mole ratios of DMTMM/COOH ranging from 0.1 to 0.2 in the pre-activation step followed by addition of 1 eq. Bi-NH₂ per DMTMM. The extent of Bi-NH₂ attachment was determined by separating the microgels by centrifugation followed by analysis of the supernatant for unreacted Bi-NH₂ using a Pierce® biotin quantification kit. The attachment yields were low, with only 5 - 10 % of the added Bi-NH₂ was consumed in the reaction. Attempts to attach larger amounts of biotin led to precipitation of the microgels. I could estimate the number of biotins per microgel by assuming each microgel contains 3.3 x 10⁷ COOH functional groups. The estimated number of biotins ranges from 1.7 to 6.2 x 10⁵ biotins per microgel for these samples.

Biotinylated microgels were examined by DLS to determine their hydrodynamic diameters. See Appendix for details. These samples showed somewhat contracted dimensions (d = 630 to 660 nm) compared to the starting MG(Tm) sample (700 nm) in PBS buffer at pH 7.4. This small decrease in the hydrodynamic diameter of the microgels is likely driven by the hydrophobic nature of the biotin moieties.

To prepare SAv-Bi-MG(Tm) samples, the Bi-MG(Tm) samples (100 µL) described above were first treated with a solution of bovine serum albumin (BSA, 100 µL, 1%) to saturate sites of non-specific protein adsorption. In the Appendix to this chapter, I provide evidence that BSA adsorbs to both biotinylated and nonbiotinylated MG samples. SAv solutions were then added to the BSA-treated microgel [BSA/Bi-MG(Tm)] samples with the idea that the strong biotin-SAv interaction would lead to binding of SAv groups to the microgel. In these SAv-Bi-MG(TM) samples, I anticipate that the SAv molecules are confined to the MG surface. While I estimate that the microgels contain on average ca. 10⁵ biotin moieties, many of these may be buried in the interior of the microgel. Recall that from the design of the MG synthesis, most of the –COOH groups are localized in the core of the MG. I think that it is unlikely that SAv, with a \( d_h \approx 7 \text{ nm} \)
[43], would be able to penetrate into the interior of these microgels, for which the cross-link density imposes an average mesh size on the order of ca. 5 nm (see calculation in the Appendix). In this way I immobilize SAv as a model biomarker on the surface of my model “cells”. As we will find in a later section of this Chapter, SAv-Bi-MG(Tm) microgels contain low copy numbers of accessible SAv model biomarkers.

### 7.3.3 Reagents for Biotin-Streptavidin Coupling Bioassays.

In current mass cytometry immunoassays of cell marker populations, a suspension of cells is treated with a cocktail of antibodies, each covalently labeled with metal chelating polymers (MCPs). Each type of antibody directed against a specific epitope is labeled with a different metal isotope. In my model system, SAv serves as the model biomarker; thus I need an MCP with biotin end-functionality as the recognition element. I employ a biotin-end-capped MCP based on a polyaspartamide backbone with diethylenetriaminepentaacetic acid (DTPA) groups attached as a monoamide to each pendant group, synthesized as described in ref [36]. Its structure is shown in Scheme 7-2. It has a number average degree of polymerization $D_{\text{Pn}} = 50$ as determined by $^1$H NMR. Based on previous experiments with this type of polymer [44-45], in which each DTPA binds a lanthanide ion, I assume that the Tb$^{3+}$ labeled polymer carries ca. 50 Tb ions per polymer. I refer to this MCP as Bi-PAsp(Tb)$_{50}$. The characterization of the MCP is reported in the Appendix.

I begin with a number of simple tests of binding. For Bi-PAsp(Tb)$_{50}$, a sample of SAv-MG(Tm) (ca. 2.5 x 10$^9$ microgels in PBS buffer, 100 µL, pH 7.4) was treated with 264 pmol MCP and subsequently analyzed by TEM. An image of this sample is presented in Figure 7-2A. This image shows compact microgel structures with dense features at the perimeter that I attribute to the present of Tb ions. Analysis of the grid by EDX (Figure 7-2B) shows the presence of both Tm from the microgel and Tb from the biotinylated MCP confirming the formation of MCP-biotin-SAv-MG(Tm) complex. Additional support for the formation of the complex comes from electrophoretic mobility measurements (see Appendix). The SAv-MG(Tm) sample had a value of $\mu = -1.4 \times 10^{-8}$ m$^2$/Vs, and for BSA-MG(TM) $\mu = -1.06 \times 10^{-8}$ m$^2$/Vs. When the SAv-MG(Tm) sample described above was treated with 198 pmol Bi-PAsp(Tb)$_{50}$, the
value of $\mu$ decreased to $1.7 \times 10^{-8}$ m$^2$/Vs, whereas treatment of BSA-MG(Tm) with this polymer left the value of $\mu$ unchanged.

Figure 7-2. A) TEM images of streptavidin-coated microgels (SAv-MG(Tm)) after treatment with a biotinylated metal chelating polymer (Bi-PAsp(Tb)$_{50}$). $d_{\text{Bi-PAsp(Tb)}-\text{SAv-MG(Tm)}} = 180 \pm 10$ nm. B) EDX linear scan through assembly of streptavidin-coated microgels (SAv-MG(Tm)) with a biotinylated metal chelating polymer (Bi-PAsp(Tb)$_{50}$). The strong Tb signal indicates the success of biotin-SAv coupling. C) A cartoon scheme shows the assembly between SAv-MG(Tm) with Bi-PAsp(Tb)$_{50}$. D) Histograms of Tm, Tb and La distributions among assemblies between SAv-MG(Tm) with Bi-PAsp(Tb)$_{50}$, where the La signal is an internal blank as compared to the signals from Tm and Tb.

As a higher sensitivity model reagent, I introduce biotinylated NaHoF$_4$ nanoparticles (NPs). The NPs themselves, with oleic acid as surface ligands, were synthesized as previously described [44]. A dispersion of these NPs in hexane was subjected to ozonolysis at -78°C followed by oxidation with H$_2$O$_2$ as described by Yan and coworkers [35,46] to obtain an aqueous dispersion of NPs. These were then biotinylated with Bi-NH$_2$ using DMTMM as a coupling agent. Details are provided in the Appendix, and a dark field TEM image of these biotinylated NPs (Bi-NaHoF$_4$)
is presented in Figure 7-3A. By TEM, these NPs have a mean diameter of 12.9 nm and contain ca. 15,000 Ho atoms per NP.

**Figure 7-3.** TEM images: A) bright field image of biotinylated NaHoF$_4$ nanoparticles (Bi-NaHoF$_4$); B) dark field TEM image of microgels with BSA adsorbed non-covalently (BSA/MG(Tm)); C) dark field TEM image of biotinylated microgels treated successively with BSA, then SAv, and finally with Bi-NaHoF$_4$.

I began my tests with one sample of biotinylated microgel employing mole ratios of DMTMM/COOH of 0.29 in the pre-activation step followed by addition of 1 eq. Bi-NH$_2$/DMTMM (Bi-MG(Tm)-3). The microgels with adsorbed BSA (BSA/MG(Tm)) are characterized by a dark field TEM diameter of ca. 340 nm (Figure 7-3B). The biotinylated microgels treated successively with BSA, then SAv, and finally with Bi-NaHoF$_4$ (i.e. SAv-Bi-MG(Tm)-Bi-NaHoF$_4$), show a rough surface morphology with an average TEM diameter of 350 nm (Figure 7-3C). EDX analysis further confirms the formation of the biotin-SAv-biotin assembly from coexistence of Tm from the microgel and Ho from the Bi-NaHoF$_4$ NP probe (Figure 7-4).
**Figure 7-4.** EDX Linear scans through biotinylated microgel, streptavidin, biotinylated NaHoF$_4$ NP (biotin-SAv-biotin) sandwich assembly samples.

### 7.3.4 Biotin-Streptavidin Coupling Bioassays.

The remainder of the paper examines the sensitivity of the reagents described above for detecting and quantifying the number of SAv, as a model biomarker, per microgel, as a model cell. In immunoassays of cells by mass cytometry, the cells themselves are fixed and then the double-stranded nuclear DNA is labeled with an Ir intercalator. As each cell passes through the inductively coupled plasma source of the instrument, it is atomized and ionized. An ion cloud is generated, and the signature of a cell event is the detection of appropriately strong signals of $^{191}$Ir and $^{193}$Ir ions. In my model system, the “nucleus” of the microgel is stained with TmF$_3$ NPs. Thus the signature of a “cell” event is the detection of $^{169}$Tm ions. SAv molecules at the surface serve as model biomarkers.

The design of biotin-streptavidin coupling bioassays is shown in Scheme 7-2. I first incubated the microgels with covalently bound streptavidin (SAv-MG(Tm)) with different amounts of biotinylated metal chelating polymer (Bi-PAsp(Tb)$_{50}$). The strong interaction between biotin and streptavidin immobilized the biotinylated polymer on the microgels. After 30 min, any excess biotinylated polymer was removed from the biotin-streptavidin assembly by
centrifugation and resuspension of the microgel. As a negative control, I incubated samples of the BSA-coated microgel (BSA-MG(Tm)) with same amounts of Bi-PAsp(Tb)\(_{50}\). Since there is no specific interaction between BSA and biotin, any Tb signal detected from this sample would be due to non-specific interaction between the Bi-PAsp(Tb)\(_{50}\) and the microgel. As a background blank control, I examined the SAv-MG(Tm) without treatment with the biotinylated MCP.

Samples of SAv-MG(Tm) in PBS buffer (100 \(\mu\)L, equivalent to a total of ca. 2.5 x 10\(^9\) microgels) were treated with 66, 132, 198, 264 pmol of Bi-PAsp(Tb)\(_{50}\). The top part of Figure 7-5 shows the mass cytometry data obtained for the titration of SAv-MG(Tm) with different amounts of Bi-PAsp(Tb)\(_{50}\). The top row displays isotopic Tb-Tm dot-dot plots for the distribution of \(^{159}\)Tb and \(^{169}\)Tm signals. The units on the x- and y-axes of these plots are the measured intensities for the respective isotopes. In the second and third rows, the data are replotted as histograms showing the relative abundance of microgels characterized by the isotope intensities displayed on the x-axes. For these SAv-MG(Tm)-Bi-PAsp(Tb)\(_{50}\) assemblies, the signals for both \(^{159}\)Tb and \(^{169}\)Tm are strong. The Pearson correlation \((r)\) between the Tb and Tm signal intensities for these signals is in the range of 0.70-0.90, which indicates a strong positive linear correlation.
For comparison, I incubated samples of BSA-MG(Tm) (100 μL, equivalent to a total of ca. 2.5 x 10^9 microgels) with same amounts of Bi-PAsp(Tb)_{50}. Corresponding data for the treatment of BSA-MG(Tm) microgels with Bi-PAsp(Tb)_{50} are shown in the lower half of Figure 7-5. This system represents my negative control, since no specific interaction is expected between the polymer and the microgel or the BSA molecules bound to the surface. In the dot-dot plots, and in the histograms presented in the bottom row, the ^{169}\text{Tm} signals are strong, indicating that the BSA-MG(Tm) microgels are easily detected. The corresponding Tb signals are very weak suggesting rather weak non-specific binding. In these data the Pearson correlation coefficient between Tb and Tm signal intensities was in the range of \( r = 0.15-0.20 \), indicating the Tb signals I detect are not strongly associated with the Tm signals of the microgels.

As a blank, I examined the SAv-MG(Tm) sample itself in the absence of any intentionally added source of Tb. These data are shown in the three plots on the right-hand side of the lower portion of Figure 7-5 (i.e. Figure 7-5C). While the Tm signal is strong, the Tb signal is weak, with only a very small correlation (\( r < 0.02 \)) between them. The Tb signal in the blank is noticeably weaker in intensity than in the negative control, and the difference between them serves as a measure of non-specific binding of the polymer to the protein-coated microgel.

The histogram representation of the frequency distribution of the integrated ion intensity over the transient signal for individual microgel particles gives quantitative information about the metal ion content of the microgel population. For example, in Figure 7-6A, the population distribution is presented for the ^{169}\text{Tm} and ^{159}\text{Tb} ion signal collected for 3 min (ca. 1.1 x 10^5 microspheres) for SAv-MG(Tm) complexed with Bi-PAsp(Tb)_{50}. The x-axis of each histogram is the intensity analog output of the TOF detector and is considered here as a relative number.

The calculated number of metal ions per microgel is plotted in Figure 7-6. After treating the SAv-MG(Tm) solutions (containing ca. 2.5 x 10^9 microgels) with 66, 132, 198, 264 pmol of Bi-PAsp(Tb)_{50}, I obtained (1.9 ± 0.8) x 10^6, (2.8 ± 1.3) x 10^6, (4.1 ± 1.5) x 10^6, (3.6 ± 1.4) x 10^6 Tb atoms per microgel, respectively. For comparison, I incubated samples of BSA-MG(Tm) (containing ca. 2.5 x 10^9 microgels) with same amounts of Bi-PAsp(Tb)_{50}. Here I found Tb signals in mass cytometry corresponding to (1.1 ± 0.4) x 10^5, (1.7 ± 0.8) x 10^5, (1.7 ± 1.0) x 10^5, (2.2 ± 1.5) x 10^5 Tb atoms per microgel (also plotted in Figure 7-6A). These signal levels correspond to a non-specific binding signal of 4-6%. For comparison, the SAv-MG(Tm)
microgels that serve as a blank show a background signal corresponding to \((3.0 \pm 1.3) \times 10^4\) Tb atoms per microgel (i.e. ca. 1% of background contamination). Figure 7-6B (lower panel) shows that all of the samples exhibited a similar Tm intensity, corresponding to \(1.1 \times 10^7\) Tm atoms per cell, with a typical CV of ca. 30%. 
**Figure 7-5.** Isotopic Tb-Tm dot-dot plots (upper panel), histograms of Tb content distribution (middle panel) and histograms of Tm content distribution (lower panel) from biotin-SAv coupling assays. Part A: SAv-MG(Tm) (100 µL, containing ca. 2.5 x 10⁹ microgels in total) were incubated with different amounts of Bi-PAsp(Tb)₅₀ solution (3.3 µmol/L: S1, 20 µL; S2, 40 µL; S3, 60 µL; S4, 80 µL). Part B:
BSA-MG(Tm) (100 μL, containing ca. 2.5 x 10⁹ microgels in total) were incubated with different amounts of Bi-PAsp(Tb)₅₀ solution (3.3 μmol/L: S1, 20 μL; S2, 40 μL; S3, 60 μL; S4, 80 μL). Part C: SAv-MG(Tm) microgel solution. Data collection was gated to exclude cell debris and cell aggregates. At least 10,000 microgels were analyzed per sample.

Figure 7-6. Tb and Tm content per microgel determined by mass cytometry from biotin-SAv coupling assays. Numbers of Tb and Tm atoms per cell were calculated using the mass cytometry transmission coefficient for Tb ions of 9.88 x 10⁻⁵ and for Tm ions of 7.30 x 10⁻⁵. Microgel samples contained 100 μL solution in DI water containing 0.132 μmol total MAA functional groups within microgel network. For SAv-MG(Tm) and BSA-MG(Tm) samples, the amounts of Bi-PAsp(Tb)₅₀ employed are indicated on the x-axis. The SAv-MG(Tm) samples not treated with metal chelating polymer are indicated by the cross-hatched bars in the histograms. A. Tb content per microgel. The redline indicates a saturation level for SAv-MG(Tm) + Bi-PAsp(Tb)₅₀ of 4.0 x 10⁶ Tb atoms per cell microgel. B. Tm content per microgel. The red line indicates a mean value of 1.1 x 10⁷ Tm atoms per
microgel for all samples, with a cell-to-cell CV of 30%. The error bars indicate the cell-by-cell coefficient of variation of lanthanide ion content (CV$_{Ln}$) determined from gated mass cytometry data.

To estimate the number of SAv biomarkers per cell, I have to make some assumptions about the interaction between the SAvs on the microgel surface and the biotinylated MCP. Each Bi-PAsp(Tb)$_{50}$ carries an average of 50 Tb ions. Streptavidin has four binding sites. If I assume that each SAv binds to four biotin-end-capped polymers, then each SAv would carry 200 Tb ions at saturation. Since at saturation, the microgels contain ca. $4.0 \times 10^6$ Tb atoms per microgel, then on average, each microgel carries 20,000 SAv biomarkers. This is a reasonable number. Microgels with the same dimensions of streptavidin-coated microgels ($R_h = 320$ nm from DLS), can be fully packed by $3.0 \times 10^4$ streptavidin molecules with diameter of 7 nm. To the extent that the assumptions made above are correct, my streptavidin-coated microgels have a surface packing density of 67%.

7.3.5 Biotin-SAv-Biotin Sandwich Bioassays.

A second set of assays employed biotinylated microgels. As described above, I was limited in the amount of biotin that I could attach covalently to the microgels. Over-reaction led to precipitation of the microgels. In addition, I anticipate from the chemical structure of the microgels that not all of the covalently attached biotin groups will be on the surface and accessible to external reagents. Thus I anticipate, after exposure of these microgel samples in water to excess SAv, that the number of SAv biomarkers will be much smaller than in the examples described above.
Scheme 7-3. Biotin-streptavidin-biotin sandwich assays.

A global view of the assay strategy is presented in Scheme 7-3. The three biotinylated microgel samples (Table 7-1) were obtained by coupling of carboxylic acid from microgels to the amine group from Bi-NH$_2$ via DMTMM. Aliquots of each of these samples (each corresponding to ca. 2.5 x 10$^{10}$ microgels) were first treated with BSA/PBS solution to saturate the sites of non-specific protein adsorption, and then with SAv to promote SAv binding to accessible biotins on the microgel surface. I refer to this type of SAv-coated microgels as SAv-Bi-MG(Tm). With the SAv-Bi-MG(Tm) microgels, I carried out binding assays with two types of biotin containing reagents: Bi-PAsp(Tb)$_{50}$ and Bi-NaHoF$_4$.

The first set of binding experiments were carried out with Bi-PAsp(Tb)$_{50}$, where I treated aliquots of the SAv-coated microgels (100 µL, ca. 2.5 x 10$^{9}$ microgels, 6.2 x 10$^{5}$ biotin/microgel),
with different amounts of Bi-PAsp(Tb)$_{50}$ solution (0.33 µmol/L: S1, 10 µL; S2, 20 µL; S3, 40 µL; S4, 80 µL). The corresponding data for the treatment of BSA/MG(Tm) microgels with different amounts of Bi-PAsp(Tb)$_{50}$ solution were used as negative controls account for non-specific binding. As a blank, I examined the SAv-Bi-MG(Tm) itself in the absence of Bi-PAsp(Tb)$_{50}$. From the mass cytometry data, I obtained the strong signals from Tm, but the Tb signals were weak for all the microgel samples. The detected Tb signal intensity was in the range of 1-10 counts from the mass cytometer. With increasing amounts of added Bi-PAsp(Tb)$_{50}$, I obtained increasing amounts of Tb signals from both SAv-Bi-MG(Tm) and BSA/MG(Tm) microgels. However, the difference in Tb intensity from the Bi-Asp(Tb)$_{50}$ treated SAv-Bi-MG(Tm) samples and BSA/MG(Tm) samples was very small. The signal-to-noise ratio of the largest peak was ca. 1.2, too low to see a signal or measure it. I conclude that the number of SAv on the surface of the biotinylated microgels is too small to be detected by mass cytometry with current generation instrument using a metal chelating polymer as a reagent. The dot-dot plots and histograms for these samples are presented in the Appendix to this chapter.

In the following experiments, I incubated each of the three SAv-Bi-MG(Tm) samples (100 µL, each containing ca. 2.5 x 10$^9$ microgels in total) with excess Bi-NaHoF$_4$ nanoparticles (0.05 mg, 0.015 µmol, 3000 molar equivalent to added SAv) to build biotin-streptavidin-biotin sandwich. Excess nanocrystals were removed from the sandwich assembly via three cycles of centrifugation and resuspension of the microgels. The Ho-Tm dot-dot plots and histograms are shown in Figure 7-7A. The top row displays isotopic Ho-Tm dot-dot plots for the distribution of $^{165}$Ho and $^{169}$Tm signals. The units on the x- and y-axes of these plots are the measured intensities for the respective isotopes. In the second and the third rows, the data are replotted as histograms showing the relative abundance of microgels characterized by the isotope intensities displayed on the x-axes. For these SAv-Bi-MG(Tm)-Bi-NaHoF$_4$ assemblies, the signals for both $^{165}$Ho and $^{169}$Tm are strong. The Pearson correlation between Tm and Ho signal intensities measured by mass cytometry was ca. 0.60 from three batches of samples. This value indicates that the Ho signals I detect are associated with the Tm signals of the microgels.

In parallel, I treated BSA-passivated biotin-free microgels (BSA/MG(Tm)) with Bi-NaHoF$_4$ nanoparticles. The corresponding mass cytometry data are shown in the Figure 7-7B. The Tm signal is strong; however, the Ho signal is much weaker. The correlation coefficient between Tm
and Ho signal intensities was $r = 0.20$ for this negative control sample, which indicates a weak linear correlation between the two ions. As a blank, I examine the SAv-Bi-MG(Tm) itself in the absence of any intentionally added source of Ho. These data are shown in the three plots in Figure 7-7C. While the Tm signal was strong, the Ho signal was weak, with a very little correlation ($r < 0.01$) found between Tm and Ho signals.

The numbers of Tm and Ho atoms per microgel were calculated using the mass cytometry transmission coefficient of $T = 1.11 \times 10^{-4}$ for Tm ions and $1.23 \times 10^{-4}$ for Ho ions. The calculated numbers of metal ions per microgel are plotted in Figure 7-8. For the SAv-Bi-MG(Tm)-Bi-NaHoF$_4$ complexes, I obtained $(1.6 \pm 0.6) \times 10^6$ Ho atoms per microgel for SAv-Bi-MG(Tm)-1, $(3.1 \pm 0.7) \times 10^6$ Ho atoms per microgel from SAv-Bi-MG(Tm)-2 and $(6.0 \pm 0.9) \times 10^6$ Ho atoms per microgel from SAv-Bi-MG(Tm)-3. In contrast, for the negative control (BSA/MG(Tm), I obtained $(2.9 \pm 1.2) \times 10^5$ Ho atoms per cell. This signal level corresponds to a non-specific binding signal of 5-18%. The SAv-Bi-MG(Tm) microgels that serve as a blank and show a background signal corresponding to $(2.4 \pm 4.9) \times 10^4$ Ho atoms per microgel (ca. 1% background contamination). Figure 7-8B (lower panel) shows that all the microgel hybrids exhibited a similar Tm intensity, $1.1 \times 10^7$ Tm atoms per microgel, with a typical CV of ca. 30%.

Dividing the Ho number per cell by the Ho number per nanoparticle (15,000) yields the average number of nanoparticles per cell. From the three different SAv-Bi-MG(Tm)-Bi-NaHoF$_4$ complexes, I obtained $107 \pm 40$, $205 \pm 47$, $400 \pm 60$ NaHoF$_4$ nanoparticles per cell. For the negative control (BSA/MG(Tm), I found $(19 \pm 8)$ nanoparticles bound non-specifically per cell. From the blank, I detected a background contamination level at $(2 \pm 3)$ nanoparticles per microgel.
Figure 7-7. Isotopic Ho-Tm dot-dot plots (upper panel), histograms of Ho content distribution (middle panel) and histograms of Tm content distribution (lower panel) from biotin-SAv-biotin sandwich assays. Part A: SAv-Bi-MG(Tm) microgel solution in PBS buffer (100 µL, containing 2.5 x 10⁹ microgels) were incubated with excess of Bi-NaHoF₄ NPs (0.015 µmol in 10 µL DI water). Part B: BSA/MG(Tm) solution in PBS buffer (100 µL, containing ca. 2.5 x 10⁹ microgels) were incubated with SAv solution (500 nmol/L, 10 µL, 0.005 nmol), then with excess of Bi-NaHoF₄ NPs (0.015 µmol in 10 µL DI water). Part C: SAv-Bi-MG(Tm) microgels. Data collection was gated to exclude cell debris and cell aggregates. At least 10,000 cells were analysed per sample.
Figure 7-8. Ho and Tm intensities for cells from biotin-SAv-biotin sandwich assays. Numbers of Tm and Ho atoms per cell were calculated using the mass cytometry transmission coefficient for Tm ions of 1.11 x 10^{-4} and for Ho ions of 1.23 x 10^{-4}. A) Ho content per microgel. B) Tm content per microgel. The red line indicates a mean value of 1.1 x 10^7 Tm atoms per microgel for all samples, with a cell-to-cell CV of 30%. The error bars indicate the cell-by-cell coefficient of variation of lanthanide ion content (CV_{ln}) determined from gated mass cytometry data.

With at least one of the SAv binding sites already attached to the cell surface, a maximum of three biotin binding sites are available for additional binding with nanoparticles. To estimate whether more than one NP can bind to each SAv, I carried out a geometric analysis (see Appendix) of the space occupied by the first bound NP and its influence on access to the other binding sites of the same SAv. This analysis shows that for NPs with a radius r_{NP} ≤ 10.1 nm, 3 NPs can bind to each SAv. For NPs with radii in the range 10.1 nm < r_{NP} ≤ 13.0 nm, the
maximum packing number is 2 per SAv. For larger NPs, with $r_{NP} > 13.0$ nm, the maximum packing number is 1 per SAv. By DLS, the measured hydrodynamic radius of the biotinylated NaHoF$_4$ nanoparticles was ca. 14 nm. Thus we assume that only one NP is bound to each SAv, and the average number of biomarkers can be calculated as $107 \pm 40$ biomarkers per SAv-Bi-MG(Tm)-1, $207 \pm 47$ biomarkers per SAv-Bi-MG(Tm)-2, $400 \pm 60$ biomarkers per SAv-Bi-MG(Tm)-3.

### 7.4 Summary and Conclusions

In this Chapter, I examined the sensitivity of different reagents for detecting and quantifying by mass cytometry the number of SAv, as a model biomarker, per microgel, as a model cell. In my model system, the microgel was labeled with TmF3, so that the detection of 169Tm ions served as the signature of a “cell” event. The streptavidin biomarkers were then detected with biotinylated probes, from which I was able to quantify the number of biomarkers per model cell.

I used two approaches to attach SAv biomarkers to the microgel. Carboxyl activation chemistry to attach SAv covalently to the microgels led to a high SAv content. In contrast, covalent attachment of biotin, passivation with BSA, and subsequent treatment with SAv led to low SAv contents per microgel. Two types of biotinylated probes were used: a biotinylated MCP (Bi-PAsp(Tb)50, containing on average 50 Tb$^{3+}$ ions atoms per probe) and biotinylated NaHoF$_4$ NPs (Bi-NaHoF$_4$, containing ca. 15,000 Ho atoms per probe). For microgels carrying high abundance of SAv biomarkers, the interaction of Bi-PAsp(Tb)50 and the SAv-coated microgels were much stronger than the interaction with BSA-coated microgels, which was used as a negative control to account for non-specific absorption. From this approach a biomarker level at ~104 per cell was detected by mass cytometry.

For microgels carrying low copy number of streptavidin biomarkers, the Bi-PAsp(Tb)50 reagent gave a very low signal, not significantly different from that with the BSA-coated microgel sample. With current instrumentation, this type of MCP reagent does not generate sufficient signal to measure these low levels of biomarkers per cell.

In contrast, the biotinylated NaHoF$_4$ NPs (with 15,000 Ho atoms per NP) gave a mass cytometry signal about two orders of magnitude stronger, while maintaining a relatively low signal level from non-specific absorption. In the sandwich assay described here, for the three SAv-Bi-MG(Tm) samples examined, I determined biomarker levels of $400 \pm 60$, $207 \pm 47$, and
107 ± 40 biomarkers per microgel. While the background signal was very low, the sensitivity of the measurements was limited by non-specific interaction of the NPs with the BSA-coated microgels, determined to be ca. 19 NPs per microgel. This proof of concept experiment demonstrates the enhanced sensitivity possible with NP reagents in cell-by-cell assays by mass cytometry.

Applying this knowledge to biological samples requires significant improvement in the surface coating of lanthanide nanoparticles, to optimize the type and number of surface functional groups for attachment to antibodies, to optimize purification of the NP-Ab conjugates, and to minimize non-specific interaction with cells. This is an ongoing task in our laboratory and elsewhere.
Reference


[13] Gold nanoparticles (with 31,000 atoms in a 10 nm diameter particle) are not suitable because Au, with a high ionization potential (9.7 eV) is not fully ionized in the plasma and tends to bleed into the detector.

[22] Li, Z. Q.; Zhang, Y. Nanotechnology **2008**, 19, 345606.
[34] Liu, Q.; Sun, Y.; Yang, T.; Feng, W.; Li, C.; Li, F. J. Am. Chem. Soc. 2011, 133, 17122-17125.
Appendix to Chapter 7

A7.1 Experimental
A7.1.1 Materials
A7.1.2 Instrumentation
A7.1.3 Synthesis of NaHoF₄ nanoparticles by Dr. Yi Hou
  A7.1.3.1 Synthesis of Oleic acid-capped NaHoF₄ nanoparticles (NaHoF₄-OA)
  A7.1.3.2 Synthesis of Hydrophilic NaHoF₄ nanoparticles (NaHoF₄-COOH) by ozonolysis
A7.1.4 Synthesis of Methyl Biotinate
A7.1.5 Synthesis of N-Biotinyl-3,6-dioxaoctane-1,8-diamine(Bi-NH₂)
A7.1.6 Synthesis of Bi-PAsp(DTPA)₅₀ polymer by Dr. Yijie Lu
  A7.1.6.1 Synthesis of β-benzyl-L-aspartate-N-carboyanhydride (BLA-NCA)
  A7.1.6.2 Synthesis of Bi-poly(γ-benzyl-L-aspartate) (Bi-PBLA)
  A7.1.6.3 Synthesis of Bi-PAsp(DET)₅₀
  A7.1.6.4 Synthesis of Bi-PAsp(DTPA)₅₀
A7.2 Estimation on number of Ho atoms per NaHoF₄ nanoparticle
A7.3 pH and conductometric titrations of Bi-NaHoF₄ nanoparticles and NaHoF₄-COOH nanoparticles
A7.4 Determination of the microgel concentration by titration
A7.5 Estimation of the maximum number of Bi-NaHoF₄ NPs that is accessible to the streptavidin binding sites
A7.6 Mass cytometry screen captures from SAv-Bi-MG(Tm)/Bi-NaHoF₄ NPs assemblies
A7.7 Quantifying conversion of biotinylation reaction on MG(Tm) by UV-Vis spectrometry
A7.8 $R_h$ of microgel samples determined by dynamic light scattering
A7.9 Characterization of MG(Tm) microgels by static light scattering
A7.10 Estimation of the average mesh size of the microgels
A7.11 Apparent zeta potential and electrophoretic mobility of microgels
A7.12 Mass cytometry results for Bi-SAv-Bi sandwich assays with Bi-PAsp(Tb)₅₀

Reference
A7.1 Experimental

A7.1.1 Materials

HoCl$_3$·6H$_2$O (99.9%), oleic acid (OA, 99%), 1-octadecene (ODE, 97%), acetic acid (CH$_3$COOH, 99.7%), hydrogen peroxide solution (H$_2$O$_2$, 30%, w/w), diethylenetriamine-pentaacetic acid (DTPA, 98%), tetrahydrofuran (THF, anhydrous), $\beta$-benzyl-$L$-aspartate (BLA), diethylenetriamine (DET), phosgene solution (~ 20% in toluene) were purchased from Sigma-Aldrich and used without purification. 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, 99+%) was purchased from Fisher Scientific Canada. N-Biotinyl-3,6-dioxaoctane-1,8-diamine (Bi-NH$_2$) was synthesized in our lab, but is also commercially available from Pierce Biotechnology. All other chemical reagents of analytical grade were used directly without further purification. Water was purified through a MilliQ water purification system (18 MΩcm).

A7.1.2 Instrumentation

Particle sizes and shapes in the dry state were examined by bright field TEM (Hitachi H-7000) operated at 100 kV, and the particle sizes were analyzed using the software program ImageJ. The size and distribution of nanocrystals in solution were characterized by dynamic light scattering (DLS). A commercial spectrometer (ALV/DLS/SLS-5022F) equipped with multi-τ digital time correlation (ALV5000) and a cylindrical 22 mW UNIPHASE He-Ne laser ($\lambda_0 = 632$ nm) as the light source was used. The DLS measurements were carried out at scattering angles over the range from 30° to 90° for microgel samples, and only at 90° for nanoparticles.

$^1$H NMR (400 MHz) spectra were recorded on a Varian Mercury 400 spectrometer. All spectra were collected as 64 transients with a delay time of 10 seconds.

A7.1.3 Synthesis of NaHoF$_4$ nanoparticles by Dr. Yi Hou.

A7.1.3.1 Synthesis of Oleic acid - capped NaHoF$_4$ nanoparticles (NaHoF$_4$-OA).

HoCl$_3$·6H$_2$O (0.379 g, 1 mmol) was mixed with oleic acid (OA, 16 mL) and octadecene (ODE, 16 mL) in a 100 mL round bottom flask. The resulting mixture was heated to 150 °C under a nitrogen purge with constant stirring to remove water and oxygen. After 30 min, the
solution was cooled to room temperature. A methanol solution (10 mL) containing NaOH (0.100 g, 2.5 mmol) and NH$_4$F (0.148 g, 4 mmol) was slowly added into the flask and stirred for 30 min. Subsequently, the solution was slowly heated and degassed at 100 °C for 10 min to remove methanol. Then the solution was heated to 300 °C by an electromantle, and the temperature was maintained for 1 hour under a nitrogen atmosphere. When the reaction was completed, the solution was cooled naturally. An excess amount of ethanol was poured into the solution, and the NaHoF$_4$ nanocrystals precipitated out from the solution. The nanocrystals were centrifugally separated and washed with ethanol (three cycles) and dried in a vacuum oven overnight. The as-prepared NaHoF$_4$ nanocrystals could be easily redispersed in non-polar organic solvents such as hexane, cyclohexane and toluene [1-4]. The as prepared NaHoF$_4$-OA nanoparticles were hexagonal-shaped with diameter of ca. 12.0 nm (PDI =1.01) (as shown in TEM image in Figure A7-1A and particle size distribution in Figure A7-2A). By dynamic light scattering at 90°, the hydrodynamic radius of the NaHoF$_4$-OA nanoparticles was 8.1 nm, PDI = 0.12 (Figure A7-3A).

**A7.1.3.2 Synthesis of hydrophilic NaHoF$_4$ nanoparticles (NaHoF$_4$-COOH) by ozonolysis.**

The ozonolysis reaction was carried out according to a procedure reported by C. H. Yan [5]. The as-prepared NaHoF$_4$ nanocrystals (130 mg) were dissolved in hexane (50 mL) and stirred at -78 °C (dry ice & ethanol bath). Then, the resulting solution was reacted with a continuous steam of ozone/oxygen (0.4 L / min) for 30 min. The color of the nanoparticle solution changed from colorless to blue. Then, an oxygen steam was substituted for the ozone/oxygen steam until the color of the solution changed back to colorless, in order to remove the excess ozone dissolved in the solution. Subsequently, to modify the nanocrystals with -COOH groups, a mixture of glacial acetic acid and H$_2$O$_2$ (30%) (2:8 v/v) with a total volume of 2 mL was added to the solution and stirred for 30 min. The resulting mixture was concentrated under vacuum. The nanocrystals (NaHoF$_4$-COOH) were washed twice with acetone before being redispersed in deionized water or ethanol. The as prepared NaHoF$_4$-COOH nanocrystals have a mean diameter of ca. 12.3 nm (PDI = 1.01) by TEM image (see image in Figure A7-1B and particle size distribution in Figure A7-2B). By dynamic light scattering the hydrodynamic radius ($R_h$) of NaHoF$_4$-COOH nanoparticles was 13.2 nm, PDI = 0.20 (Figure A7-3B). Note that $R_h$ increased from 8.1 nm to 13.2 nm and the PDI increased from 0.12 to 0.20 following ozonolysis.
Figure A7-1. The TEM image of NaHoF$_4$ nanoparticles: A) Oleic acid capped NaHoF$_4$ nanoparticles (NaHoF$_4$-OA) from a solution in hexane, B) NaHoF$_4$-COOH nanoparticles obtained by ozonolysis followed by H$_2$O$_2$ oxidation from a solution in DI water.
Figure A7-2. Particle size distribution for NaHoF$_4$ nanocrystals determined by TEM: A) NaHoF$_4$-OA NPs before ozonolysis; B) NaHoF$_4$-COOH NPs obtained after the ozonolysis reaction; C) Bi-NaHoF$_4$ NPs.
Figure A7.3. Hydrodynamic radius of NaHoF₄ nanoparticles determined by DLS. A) NaHoF₄-OA nanoparticle dispersed in cyclohexane; B) NaHoF₄-COOH nanoparticles dispersed in DI water; C) Bi-NaHoF₄ nanoparticles dispersed in DI water. PDI values are shown in parentheses in the plots.

A7.1.4 Synthesis of methyl biotinate

To a solution of biotin (2 g, 8.2 mmol) in methanol (200 mL), Amberlite IR-120 resin (1.0 g) was added, and the solution was stirred at ambient temperature for 48 h. After filtration the solvent was removed under reduced pressure to give methyl biotinate as a white solid. The yield was 98 %. ¹H NMR (400 MHz, CDCl₃, 298 K, Figure A7-4): δ = 4.51-4.47 (1H, NHCH₂CH), 4.32-4.29 (1H, NHCH₂CH₂), 3.66 (3H, CH₃), 3.23-3.18 (1H, SCH), 2.95-2.69 (2H, CH₂S), 2.35 (2H, CH₂COO), 1.79-1.54 (4H, CH₂CH₂CH₂COO), 1.49-1.43 (2H, SCHCH₂).
**Figure A7-4.** $^1$H NMR spectrum (CDCl$_3$) of methyl biotinate.

### A7.1.5 Synthesis of N-Biotinyl-3,6-dioxaoctane-1,8-diamine (Bi-NH$_2$)

Methyl biotinate (1.5 g, 5.81 mmol) was dissolved in methanol (50 mL), an excess of 2,2’-(ethylenedioxy) bis-(ethylamine) (45 mL) was added. The solution was kept at 60 °C for 48 h, and methanol was removed under reduced pressure. The product N-Biotinyl-3,6-dioxaoctane-1,8-diamine (Bi-NH$_2$, as shown in scheme 7-1) was precipitated with diethyl ether, and washed with diethyl ether 5 times to remove the excess amine. The yield was 85%. This material was sufficiently pure to be used for initiating polymerization reactions. $^1$H NMR (400 MHz, D$_2$O, 298 K, Figure A7-5): $\delta = 4.51$-$4.57$ (1H, NHCH$_2$CH), 4.32-$4.29$ (1H, NHCH$_2$CH$_2$), 3.6-$3.4$ (8H, CH$_2$CH$_3$OCH$_2$CH$_2$OCH$_2$CH$_2$), 3.3 (2H, CH$_2$CH$_2$OCH$_2$CH$_2$OCH$_2$CH$_2$), 3.2 (1H, SCH), 2.9-$2.6$ (4H, CH$_2$S and OCH$_2$CH$_2$NH$_2$), 2.1 (2H, CH$_2$CH$_2$CH$_2$CONH), 1.65-$1.37$ (4H, CH$_3$CH$_2$CH$_2$CONH), 1.25-$1.35$ (2H, CH$_2$CH$_2$CH$_2$CONH).
A7.1.6 Synthesis of Bi-PAsp(DTPA)$_{50}$ polymer by Dr. Yijie Lu.

A7.1.6.1 Synthesis of $\beta$-benzyl-L-aspartate-N-carboxyanhydride (BLA-NCA)

$\beta$-Benzyl-L-aspartate (BLA, 12.2 g, 54.7 mM) and THF (100 mL anhydrous) was placed in a 500 mL round bottom three-neck flask with a reflux condenser under argon flow. Then a phosgene solution (35 mL, ~20% in toluene, ~66 mM) was added into the flask. The flask was transferred to a preheated oil bath (60 °C) and stirred for 90 min. The suspension gradually turned clear, which indicated the completion of the reaction. After 90 min, the oil bath was removed and the solution was exposed for 15 min to a vigorous argon flow to remove the excess phosgene. The gas stream was passed through aqueous NaOH to destroy residual phosgene.
Then, 300 mL dry hexane was added to the solution and purged with nitrogen to remove any remaining phosgene. Afterwards, the flask was placed in a freezer and \(\beta\)-benzyl-\(L\)-aspartate-\(N\)-carboxyanhydride (BLA-NCA) crystals formed at -20 °C. After removing the solvent and drying the product under vacuum, the product BLG-NCA was obtained (yield 13.0 g, ~89 %) in sufficient purity to be used directly for the polymerization reactions. The product was stored at -20 °C under an argon atmosphere. \(^1\)H NMR (400 MHz, acetone-\(d_6\), 25 °C ): \(\delta = 7.8\) - 7.6 (b, 1 H; NH), 7.21 - 7.41 (m, 5 H; \(C_6H_5\)), 5.08 (s, 2H, CH\(_2\)), 4.80 (t, 1H, CH), 3.1 (t, 2H, CH\(_2\)). Anal. Calcd for \(C_{12}H_{11}NO_5\): C, 57.83; H, 4.45; N, 5.62. Found: C, 57.71; H, 4.48; N, 5.62.

**A 7.1.6.2 Synthesis of Bi-poly(\(\gamma\)-benzyl-\(L\)-aspartate) (Bi-PBLA)**

Bi-PBLA was synthesized by the ring-opening polymerization of BLA-NCA. The polymerization was carried out using N-Biotinyln-3, 6-dioaoctane-1,8-diamine (60 mg, 161 \(\mu\)mol in 1 mL DMF) as the initiator and BLA-NCA (1.8 g, 7.2 mmol [M]/[I] = 45) in a DMF/DCM mixed solvent (2 mL DMF, 17 mL DCM), 24 h at 0 °C under an argon atmosphere. Here [M] refers to the concentration of BLA-NCA. The resulting solution was poured into diethyl ether to precipitate PBLA. The crude polymer was dissolved in DCM and reprecipitated in diethyl ether three times. After removal of the diethyl ether under vacuum, the PBLA solid was collected (1.1 g, 74 %).

\(^1\)H NMR (10% TFA in CDCl\(_3\)-d TFA mixed solvent): \(\delta\) (ppm, \(C_6H_5 = 5\)H as reference) 8.5-8.0 (broad, 1H per monomer, backbone amide –NHCO–, integration = 0.951), 7.4-7.0 (broad 5H, \(C_6H_5–\) integration = 5.00), 5.25-4.95 (broad, 2H per monomer, \(C_6H_5-CH_2–\), integration = 1.88), 4.95-4.75 (broad, 1H per monomer, backbone methine, integration = 0.91), 3.8-3.4 (broad, 12H per polymer, integration = 0.226, -CONH-\(CH_2CH_2OCH_2CH_2CH_2CH_2NHCO-\)), 3.2-2.8 (board, 2H per monomer, –CH\(_2\)-C=O, integration = 2.015), 2.4-2.2 (broad, 2H per polymer, -\(CH_2CH_2CH_2CH_2CONH–\), integration = 0.037), 1.8-1.6 (broad, 4H per polymer, -\(CH_2CH_2CH_2CH_2CONH–\), integration = 0.078), 1.6-1.4 (broad, 2H per polymer, -\(CH_2CH_2CH_2CH_2CONH–\), integration = 0.041) \(DP_n = 50\), calculated by comparing the integration of the \(^1\)H NMR signals at 1.8-1.6 ppm (the biotin end group) to that at 7.4-7.1 ppm (backbone –\(C_6H_5\)); GPC (NMP, RI) \(M_n = 11.2\) kDa, PDI = 1.32 (polystyrene standards).
A7.1.6.3 Synthesis of Bi-PAsp(DET)\textsubscript{50}

To obtain the Bi-PAsp(DET)\textsubscript{50}, diethylenetriamine (DET 8.0 mL, 73 mmol) was added into an \textit{N}-Methyl-2-pyrrolidone (15 mL) solution containing PBLA (300 mg, 1.46 mmol for the repeat units) and stirred at 0 °C for 2 h. The reaction mixture was slowly poured into a 20% acidic acid aqueous solution (40 mL) and transferred to a Spectra/Pro dialysis bag (MWCO 1k DA). The polymer was dialyzed against aqueous solution of HCl (0.01 M, 3L) for two days and then distilled water (3L) for three days at 4 °C. The HCl solution and distilled water were changed twice a day. Afterwards the solution was lyophilized to obtain Bi-PAsp(DET)\textsubscript{50} as the chloride salt (ca. 212 mg, 53 % yield).

\textsuperscript{1}H NMR (D\textsubscript{2}O, 60 °C): \(\delta\) (ppm, integrated peak areas are reported are based on methine proton = 1H as the reference), 4.8-4.6 (broad, 1H per monomer, backbone methine, integration = 1.00), 3.6-2.5 (board, 10H per monomer, two protons on the backbone \(-\text{CH}_2\text{-C} = \text{O}\) and \(\text{-NH-CH}_2\text{NHCH}_2\text{CH}_2\text{-NH}_2\), integration = 10.67). The fractional functionality of diethylenetriamine groups was calculated by comparing the integration of the \textsuperscript{1}H NMR signals at 4.8-4.6 ppm (backbone methine) to that at 3.6-2.5 ppm (10H two protons on the backbone \(-\text{CH}_2\text{-C} = \text{O}\) and \(\text{-NH-CH}_2\text{CH}_2\text{-NHCH}_2\text{CH}_2\text{-NH}_2\), where the DET functionality was ca. 100%.

To analyze the Bi-PAsp(DET)\textsubscript{50} polymers by SEC, they were first exhaustively succinylated. A sample of polymer (2 mg) was dissolved in 1 mL sodium carbonate/bicarbonate buffer (200 mM, pH 9.4). Succinic anhydride (20 mg) was added to the solution and then 5 N NaOH aqueous solution was added to the mixture to keep the pH at 8 to 9 until the solid of succinic anhydride was totally dissolved. Then the solution of the succinylated polymer was injected into the aqueous SEC column to characterize the nominal molecular weight (poly(methacrylic acid) standards) and polydispersity. \(M_n = 9.2 \text{ kDa}, \text{ PDI} = 1.39.\)

A7.1.6.4 Synthesis of Bi-PAsp(DTPA)\textsubscript{50}

An aqueous solution containing a large excess of DTPA was prepared by adding DTPA (3.0 g, 80 eqv per polymeric primary amine group) and 4 mL of H\textsubscript{2}O to a 100 mL round-bottom flask. Next, 5 M aq. NaOH was added to dissolve the DTPA and bring the solution pH to 8.5 (monitored by a pH meter). DMTMM (200 mg, 7 eqv per polymeric primary amine group) was
dissolved in 4 mL water with sonication and added quickly dropwise with stirring to the first solution. This solution was given 5 minutes to pre-react. Then a solution of Bi-PAsp(DET)$_{50}$ (20 mg in 4 mL water) was added quickly but dropwise with stirring. After another 10 minutes, the reaction solution was then transferred to a 15 mL 3 kDa MWCO Millipore Amicon spin filter and washed with water (9 × 15 mL). Finally, the aqueous solution was freeze-dried to yield the sodium salt of polyaspartate with the DET pendent groups fully conjugated to DTPA (Bi-PAsp(DTPA)$_{50}$). Yield: 35 mg (69 %). GPC: $M_n = 11$ kDa, PDI = 1.19 (the GPC eluent was an aqueous solution containing 0.2 M KNO$_3$, 200 ppm NaN$_3$ and 25 mM phosphate buffer at pH 8.5, and the system was calibrated with poly(methacrylic acid) standards).

$^1$H NMR (D$_2$O, 60 °C): δ(ppm, integrated peak areas reported are based on methine protons of the aspartamide repeat unit as a reference): 4.8-4.6 ppm (broad, 1H/Asp, integration = 1.00), 3.9-2.6 (broad, 2H/Asp (-CH$_2$-C=O) plus 8H per DET (−NH-CH$_2$CH$_2$-NHCH$_2$CH$_2$-NH−) plus 18H (9 CH$_2$) groups per DTPA, integration = 27.59), where the conversion of Bi-PAsp(DTPA)$_{50}$ is calculated to be ca. 98%.
**Scheme A7-1.** Synthesis of Bi-PAsp(DTPA)$_{50}$ polymer.

### A7.2 Estimation on number of Ho atoms per NaHoF$_4$ nanoparticle

The number of Ho atoms per NaHoF$_4$ nanoparticle was calculated as:

\[
N_{\text{Ho}} = \frac{4}{3} \pi r_{\text{NP}}^3 \times \rho_{\text{NaHoF}_4} \times N_A \times \frac{N_{\text{Av}}}{M_{\text{NaHoF}_4}}
\]

where the radius of nanoparticle is 6.5 nm (determined from TEM results in Figure 7-3A), the density of nanoparticle $\rho_{\text{NaHoF}_4} = 5.994$ g/cm$^3$, taken to be the density of the hexagonal NaHoF$_4$ crystal, $N_{\text{Av}}$ is Avogadro’s number, $M_{\text{NaHoF}_4}$ is the molar mass of one NaHoF$_4$ molecule (263.91 g/mol). The number of Ho atoms per NP thus obtained was 15,000.
A7.3 pH and conductometric titrations of Bi-NaHoF$_4$ nanoparticles and NaHoF$_4$-COOH nanoparticles

In the titration, a solution of nanoparticles (containing 10 mg nanoparticles by dry weight) was adjusted to ca. pH 10.5 with 0.1 M NaOH and then titrated with HCl (0.025 M) to determine the amount of –CO$_2$H functional groups per g of nanoparticle. The limits of the titration are denoted $V_{HCl,1}$ and $V_{HCl,2}$ in Figure A7-6.

![Figure A7-6](image)

**Figure A7-6.** The pH and conductometric titrations of the biotinylated NaHoF$_4$ (Bi-NaHoF$_4$) nanoparticles and the carboxylated nanoparticles obtained from the ozonolysis reaction (NaHoF$_4$-COOH). Each titration involved 10 mg (dry weight) nanoparticles, which were neutralized with a small excess of NaOH. The concentration of the HCl titrant solution was 0.025 mol/L.

From the titration results, I estimated the average mole concentration of –CO$_2$H groups per g of nanoparticle as follows:

$$C_{CO_2H\text{in NP}} = \frac{N_{CO_2H\text{in NP}}}{m_{NP}} = \frac{[HCl] \times (V_{HCl,2} - V_{HCl,1})}{m_{NP}}$$  \(\text{(A7.2)}\)

where $b_{CO_2H\text{in NP}}$ is the molar concentration of –CO$_2$H groups, in mol/ g dry nanoparticle, $N_{CO_2H\text{in NP}}$ is the total amount of –CO$_2$H groups in the NPs as determined from the titration, $c_{HCl}$ is the molar concentration of the titrant, $[HCl] = 0.025$ mol/L, $V_{HCl,1}$ and $V_{HCl,2}$ are the titration endpoints corresponding to the start and end of the acid group titration, $m_{NP}$ is the mass of the
dry NPs in g. For NaHoF₄-COOH NPs, $V_{HCl,1} = 1.22$ mL, $V_{HCl,2} = 1.45$ mL, and $m_{NP} = 10$ mg; thus $C_{CO2H in NP} = 5.8 \times 10^{-4}$ mol/g for dry NaHoF₄-COOH nanoparticles according to equation A7.2. For Bi-NaHoF₄ NPs, $V_{HCl,1} = 1.16$ mL, $V_{HCl,2} = 1.27$ mL, and $m_{NP} = 10$ mg; thus $b_{CO2H in NP} = 2.8 \times 10^{-4}$ mol/g for dry Bi-NaHoF₄ nanoparticles.

If we assume the particles are spherical and uniform, we can calculate the number of –COOH groups per NP ($n_{COOH/NP}$) and the number of –COOH groups per nm² surface area ($n_{COOH/nm²}$) as:

$$n_{COOH/NP} = C_{COOH in NP} \times N_A \times \rho_{NP} \times \frac{4}{3} \pi r_{NP}^3$$  \hspace{1cm} (A7.3)

$$n_{COOH/nm²} = n_{COOH/NP} / 4 \pi r_{NP}^2$$  \hspace{1cm} (A7.4)

where $r_{NP}$ is the radius of NaHoF₄ nanoparticle, according to TEM results $r_{NP} = 6.2$ nm for NaHoF₄-COOH NPs (Figure A7-2B), $r_{NP} = 6.5$ nm for Bi-NaHoF₄ NPs (Figure A7-2C), $\rho_{NaHoF4}$ is the density of solid NaHoF₄, $\rho_{NaHoF4} = 5.979$ g / cm³ [6], $N_A = 6.023 \times 10^{23}$ mol⁻¹. Thus for NaHoF₄-COOH NPs, there are 2000 –COOH groups per NP, or 4.3 –COOH groups per nm² surface area. For Bi-NaHoF₄ NPs, there are 1000 –COOH groups per NP, or 2.2 –COOH groups per nm² surface area.

**A7.4 Determination of the microgel concentration by titration.**

In the titration, $m_{MG} = 2.435$ g of microgel solution was neutralized with excess NaOH and back-titrated with 0.1 M HCl, from which we determine an acid group contribution from the microgels $C_{MAA in MG}$ in mmol/g.

$$C_{MAA in MG} = \frac{[HCl] \times (V_{HCl2} - V_{HCl1})}{m_{MG}} = \frac{0.100 \times (1.266 mL - 0.136 mL)}{2.435 g} = 0.0464 \text{ mmol / g}$$  \hspace{1cm} (A7.5)

Upon neutralization and ion-exchange with TmCl₃, 4.0 g of MG solution was diluted to 8.8 g ($m_{MG(Tm)}$), so that the concentration of carboxyl groups per g of Tm containing microgel
solution, $C_{\text{MAA in MG(Tm)}}$ became $(0.0464 \times 4.0 / 8.8 = 0.0211 \text{ mmol/g})$. Previous studies have shown that Ln ion exchange under these conditions saturates at one Ln ion for each 3-COOH groups in the microgel, and that precipitation of the Ln ions as LnF$_3$ conserves the number of Tm ions per microgel. Here, by mass cytometry, we determined an average of $1.1 \times 10^7$ Tm atoms per MG(Tm) microgel, from which we infer an average of $N_{\text{MAA/MG}} = 3.3 \times 10^7$ MAA groups per microgel. In eq. (A7.6), where $N_{\text{AV}}$ is Avogadro’s number, we calculate that this solution contains $C_{\text{MG}} = 3.8 \times 10^{11}$ microgels/g. We further assume that a negligible fraction of the microgels were lost upon sedimentation-redispersion cycles, so that $C_{\text{MG}}$ in successive samples can be estimated by the extent of sample dilution.

$$C_{\text{MG}} = \frac{C_{\text{MAA in MG(Tm)}} \times N_{\text{Av}}}{N_{\text{MAA/MG}}} = \frac{0.0211 \text{ mmol/g}}{3.3 \times 10^7} \times 6.02 \times 10^{23} \text{ mol}^{-1} = 3.8 \times 10^{11} / \text{ g}$$  \hspace{1cm} (A7.6)

**Figure A7-7.** pH and conductometric titration curves of poly(NIPAm/VCL/MAA) microgels. 2.435 g of a purified microgel solution was adjusted to ca. pH 10.5 with 0.1 M NaOH and then titrated with HCl (0.100 M) to determine the amount of MAA functional groups per g of microgel solution. The limits of the titration are denoted $V_{\text{HCl,1}}$ and $V_{\text{HCl,2}}$.
A7.5 Estimation of the maximum number of Bi-NaHoF$_4$ NPs that is accessible to the streptavidin binding sites

A geometric representation of a nanoparticle interacting with SAv is presented in Figure A7-8A. With at least one of streptavidin’s binding sites already attached to the “cell” surface, a maximum of three biotin binding sites are available for additional binding with nanoparticles. The Bi-NaHoF$_4$ nanoparticles can be considered as spheres with radius of $r_{NP}$. I simplify the four biotin binding sites (O, A, B, C) within streptavidin structure into a regular tetrahedron with an edge length, $a$, of 2.4 nm [7], as shown in Figure A7-8A, where site O is occupied by one of the biotin moieties on the microgel surface. All the streptavidin residues locate within a co-centered sphere with the radius, $r_{SAV}$, of 3.4 nm [8]. The distance, $c$, from each binding site to the streptavidin surface can be calculated as:

$$c = r_{SAV} - \frac{\sqrt{3}}{8}a = 1.9 \text{ nm} \quad (A7.7)$$

The local microgel surface can be considered as a plane (with curvature approaching zero). In order to bind the maximum number of NPs to streptavidin, the plane of remaining three binding sites (plane ABC) should be oriented parallel to the local microgel surface. I assume that the microgel surface is impermeable, and that limiting position of a NP attached to SAv is one in which it the NP is touching the microgel surface. Under these conditions, the distance from nanoparticle mass center to the microgel surface is equal to $r_{NP}$, and the distance, $h_1$, between base plane of tetrahedron of SAv binding sites and the local microgel surface can be calculated as:

$$h_1 = \frac{\sqrt{6}}{3}a + c = 3.9 \text{ nm} \quad (A7.8)$$

Then the distance, $h$, from the center of the (NaHoF$_4$) nanoparticle to the plane ABC is:

$$h = r_{NP} - h_1 \quad (A7.9)$$

If I attach three nanoparticles to an individual streptavidin (Figure A7-8B), the maximum distance between mass centers of the two nanoparticles can be found as:
\[ D_{\text{max}} = \sqrt{3}r + a \]  

(A7.10)

where \( r \) is the distance between the projection of nanoparticle center on the ABC plane and the corresponding binding site on the microgel where the SAv is attached; \( r \) can be calculated as:

\[ r = \sqrt{(r_{NP} + c)^2 - h^2} \]  

(A7.11)

The critical condition for binding three nanoparticles to one streptavidin is

\[ D_{\text{max}} \geq 2r_{NP} \]  

(A7.12)

From this expression, we see that three nanoparticles can bind to one SAv at the surface of the microgel only if the nanoparticle radius \( r_{NP} \leq 10.1 \) nm.

Similarly, I can generate a packing model for two nanoparticles by projecting the NP centers onto the ABC plane, as shown in Figure A7-8C. Here the maximum distance between two NP centers on the SAv is given by:

\[ D_{\text{max}} = 2r + a \]  

(A7.13)

For the critical condition \( D_{\text{max}} \geq 2r_{NP} \), I calculate that two nanoparticles can bind to one SAv at the surface of the microgel only if the nanoparticle radius \( r_{NP} \leq 13 \) nm.

The measured hydrodynamic radius of the Bi-NaHoF\(_4\) NPs is 14 nm. This is above borderline in terms of the constraints of the geometric model, therefore we consider it unlikely for two of these NPs to bind to the same SAv on a SAv-Bi-MG(Tm) microgel. Assuming only one nanoparticle bound to each SAv, the average number of biomarkers can be calculated as 107 ± 40, 207 ± 47, 400 ± 60 biomarkers per cell.
Figure A7-8. A) A schematic description of nanoparticle attachment to streptavidin surface. Point O, A, B, C represent four bioin binding sites within streptavidin. Point N is the mass center of streptavidin molecule. Point M is the mass center of nanoparticle. OA = OB = OC = AB = AC = BC = 2.4 nm, $r_{SAV} = 3.4$ nm, $c = 1.9$ nm, $h_1 = 3.9$ nm, $h = r_{NP} - h_1$, $R = c + r_{NP}$, $r = (R^2 - h^2)^{1/2}$. B) Maximum distance between mass centers ($M_1$, $M_2$, $M_3$) in three nanoparticle packing model. $r = (R^2 - h^2)^{1/2}$, $D_{max} = 1.73r + a$. C) Maximum distance between mass centers ($M_1$, $M_2$) in two nanoparticle packing model $D_{max} = 2r + a$. 

$$D_{max} = 1.73r + a$$

$$D_{max} = 2r + a$$
A7.6 Mass cytometry screen captures from SAv-Bi-MG(Tm)/Bi-NaHoF₄ NPs assemblies

![Mass Cytometry Screen Capture](image)

**Figure A7-9.** Mass cytometry screen capture. A) SAv-Bi-MG(Tm)-3 (100 µL, equivalent to a total of ca. 2.5 x 10⁹ microgels) was incubated with Bi-NaHoF₄ solution (10 µL, containing 0.015 µmol NPs). B) BSA/ MG(Tm) (100 µL, equivalent to a total of ca. 2.5 x 10⁹ microgels) was incubated with 6-ACA (0.03 µmol in 3 µL DI water solution), followed by SAv solution (0.005 nmol in 10 µL PBS buffer solution), and then with Bi-NaHoF₄ solution (10 µL, containing 0.015 µmol NPs). C) SAv-Bi-MG(Tm)-3 solution (100 µL, containing a total of ca. 2.5 x 10⁹ microgels).

A7.7 Quantifying conversion of biotinylation reaction on MG(Tm) by UV-Vis spectrometry.

Here I describe an indirect approach to determine the extent of conversion in the microgel biotinylation reaction from the amounts of unreacted biotin-NH₂ detected after the reaction with
the Pierce Biotin Quantitation Kit (Thermo Scientific). The kit contains a premix of HABA/Avidin and a biotinylated horseradish peroxidase (HRP) positive control [HABA = 4'-hydroxyazobenzene-2-carboxylic acid]. When a solution containing biotin is added to a mixture of HABA/Avidin, because of its higher affinity for binding, biotin displaces the HABA and an absorbance at 500 nm (from HABA/Avidin) decreases proportionately. The change in absorbance relates to the amounts of biotin in solution by the extinction coefficient of the HABA/Avidin complex at 500 nm ($\varepsilon_{HABA}^{500nm} = 34,000 \text{ m}^{-1}\text{cm}^{-1}$).

Following biotinylation reaction, the biotinylated microgel solution was centrifuged at 5000 rpm for 40 min. The microgels sedimented to the bottom of the centrifuge tube and unreacted biotin-NH$_2$ remained in the supernatant. I removed 100 $\mu$L of the supernatant and diluted the solution with 400 $\mu$L of ultrapure water. The HABA/Avidin premix was equilibrated at room temperature in a microtube before dilution with 100 $\mu$L of ultrapure water. PB buffer (800 $\mu$L, containing 100 mM sodium phosphate, 150 mM NaCl, pH 7.2) was pipetted into a 1 mL cuvette, and used to zero the UV-Vis spectrometer. Then the 100 $\mu$L HABA/Avidin premix solution was pipetted into the cuvette, and the absorbance of the solution at 500 nm was recorded as $A_{500}(\text{HABA/Avidin})$. Then I added 100 $\mu$L of the diluted supernatant solution to the cuvette containing HABA/Avidin and mixed the solution by inversion. The absorbance of the solution at 500 nm was recorded as $A_{500}(\text{HABA/Avidin/biotin})$.

The conversion of biotinylation reaction was evaluated using the following relations. The change in absorbance at 500 nm is calculated as:

$$\Delta A_{500} = 0.9 \times A_{500}(\text{HABA/Avidin}) - A_{500}(\text{HABA/Avidin/biotin})$$  \hspace{1cm} (A7.14)

where the 0.9 correction factor is used to adjust the dilution for HABA/Avidin mixture.

The concentration of biotin in supernatant solution is calculated as:

$$C_{\text{supernatant}} = \frac{5\Delta A_{500}}{34,000 \text{ M}^{-1}\text{cm}^{-1} \times 1 \text{ cm} \times 0.1 \text{ mL}}$$  \hspace{1cm} (A7.15)
where 1 cm is the light path length of the solution in UV-Vis spectrometer, and the factor 5 corrects for the dilution of the supernatant solution. Then the conversion of biotinylation reaction is defined as:

$$\text{conversion} = 1 - \frac{C_{\text{supernatant}}}{C_{\text{total}}}$$  \hfill (A7.16)

where $C_{\text{total}}$ is the total biotin concentration at the beginning of the biotinylation reaction. The data thus obtained is presented in Table A7-1.

**Table A7-1.** UV-Vis absorbance of HABA/Avidin and HABA/Avidin/Biotin at 500 nm and conversion of biotinylation reaction on microgels.

<table>
<thead>
<tr>
<th></th>
<th>$A_{500}$ (HABA/Avidin)</th>
<th>$A_{500}$ (HABA/Avidin/Biotin)</th>
<th>$C_{\text{supernatant}}$ $10^{-4}$ mol/L</th>
<th>$C_{\text{total}}$ $10^{-4}$ mol/L</th>
<th>conversion$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bi-MG(Tm)-1</td>
<td>1.046</td>
<td>0.573</td>
<td>2.96</td>
<td>3.12</td>
<td>5.1% ± 0.3%</td>
</tr>
<tr>
<td>Bi-MG(Tm)-2</td>
<td>1.041</td>
<td>0.639</td>
<td>4.39</td>
<td>4.71</td>
<td>7.0% ± 0.3%</td>
</tr>
<tr>
<td>Bi-MG(Tm)-3</td>
<td>1.030</td>
<td>0.726</td>
<td>5.42</td>
<td>5.99</td>
<td>9.5% ± 0.4%</td>
</tr>
</tbody>
</table>

$^a$ The error bars are the standard deviations.

**A7.8 $R_h$ of microgel samples determined by dynamic light scattering**

From light scattering, the diffusion coefficient can be calculated as:

$$D = \Gamma / q^2$$  \hfill (A7.17)

where $\Gamma (\tau = 1/f)$ is the decay rate of microgel solution. The $q$ is the scattering vector defined as:

$$q = \frac{4\pi n}{\lambda} \sin \frac{\theta}{2}$$  \hfill (A7.18)

where $n$ is the reflective index of solution, $\lambda$ is the wavelength of the incident light and $\theta$ is the scattering angle. The hydrodynamic radius $R_h$ can be determined by the Stokes-Einstein equation:

$$R_h = \frac{k_BT}{6\pi\eta D}$$  \hfill (A7.19)
Here $k_B$ is Boltzmann’s constant, $T$ is the absolute temperature in Kelvin, $\eta$ is the solvent viscosity.

For small spherical objects (eg nanoparticles with $d_h = 2R_h < 100$ nm), values of $R_h$ can be determined reliably by scattering measurements at small angles (e.g. $90^\circ$ or $173^\circ$). Larger spherical objects with $d_h \sim 1$ μm have a form factor that exhibits structure in the normally accessible range of angles at which DLS measurements are carried out. To ensure that one is in fact measuring translational diffusion, one should carry out measurements over a range of angles and show that the plot of $I$ vs $q^2$ is linear and passes through the origin.

Figure A7-10 shows the angular dependence of $R_h$ of microgels in PBS buffer solution (0.01M, pH 7.4), where the concentration of microgels solution is 0.018 mg/mL, and the temperature is at 296 K.
Figure A7-10. CONTIN plots showing the angular dependence of $R_h$ of microgels at 296K in PBS buffer (pH 7.4, 0.01 M). MG(Tm) stands for TmF$_3$-containing poly(NIPAm/VCL/MAA) microgels.

To interpret the angular dependence of microgel light scattering spectrum, I plotted the mean decay rate of microgel solutions as a function of scattering form factor squared in Figure A7-11. The mean diffusion coefficient of microgels can be obtained from the slope of the linear regression lines and the hydrodynamic radius can be calculated according to Stokes-Einstein equation as listed in Table A7-2.

![CONTIN plots for different microgels](image)

Figure A7-11. Plots of decay rates (ms$^{-1}$) vs scattering form factor squared (nm$^{-2}$), where the straight line in each plot represents the linear fitting through scattering data from 30°, 45° and 60° toward the
origin. The average diffusion coefficient can be obtained from the slopes of the linear regression lines. MG(Tm) stands for TmF₃-containing poly(NIPAm/VCL/MAA) microgels.

Table A7-2. Average diffusion coefficient and hydrodynamic radii values of microgels obtained from multi-angular dynamic light scattering.

<table>
<thead>
<tr>
<th></th>
<th>$D_{ave}$ (ms⁻¹·nm²)</th>
<th>$R_{h}$ (nm)</th>
<th>PDI²</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG(Tm)</td>
<td>659</td>
<td>351</td>
<td>0.04</td>
</tr>
<tr>
<td>SAv-MG(Tm)</td>
<td>728</td>
<td>318</td>
<td>0.10</td>
</tr>
<tr>
<td>BSA-MG(Tm)</td>
<td>721</td>
<td>321</td>
<td>0.06</td>
</tr>
<tr>
<td>Bi-MG(Tm)-1</td>
<td>702</td>
<td>329</td>
<td>0.01</td>
</tr>
<tr>
<td>Bi-MG(Tm)-2</td>
<td>732</td>
<td>316</td>
<td>0.01</td>
</tr>
<tr>
<td>Bi-MG(Tm)-3</td>
<td>733</td>
<td>315</td>
<td>0.02</td>
</tr>
</tbody>
</table>

² PDI values were obtained from the second cumulant according to the measurement at 30°.

A7.9 Characterization of MG(Tm) microgel by static light scattering

![Figure A7-12](image)

Figure A7-12. Experimental static light scattering data (squares) from dilute solution of MG(Tm) (0.018 mg/mL) in PBS buffer (0.01 M, pH 7.4), form factor as a function of the scattering vector and fits with a monodisperse homogeneous sphere model (blue line) and a polydisperse inhomogeneous...
sphere model (black line). The minimum of \( P(q) \) corresponds to a scattering angle at 98°, \( R = 228 \) nm and \( R_{q_{\text{min}}} = 4.493 \). MG(Tm) stands for TmF₃-containing poly(NIPAm/VCL/MAA) microgels.

The particle form factor \( P(q) \) describes angular dependence of the scattering intensity. According to Chapter 2, in a homogeneous sphere model the form factor is given as [9]:

\[
P_{\text{hom}}(q) = \left( \frac{3[\sin(qR) - qR \cos(qR)]}{(qR)^3} \right)^2.
\] (A7.20)

Microgel particles have an inhomogenous structure, normally with a higher density in the core and a lower density in the surface. To describe an inhomogeneous system, the form factor is rewritten as [9]:

\[
P_{\text{inhom}}(q) = \left[ \frac{3[\sin(qR) - qR \cos(qR)]}{(qR)^3} \exp\left( -\frac{(\sigma_{\text{surf}} q)^2}{2} \right) \right]^2,
\] (A7.21)

where \( \sigma_{\text{surf}} \) represents the width of the smeared particle surface and \( R \) denotes the radius of the dense core.

To consider the size polydispersity of the particles, the number distribution \( f \) with respect to the particle radius \( R \) can be described as a Gaussian function [9]:

\[
f(R, \langle R \rangle, \sigma_{\text{poly}}) = \frac{1}{\sqrt{2\pi\sigma_{\text{poly}}^2 \langle R \rangle^2}} \exp\left( -\frac{(R - \langle R \rangle)^2}{2\sigma_{\text{poly}}^2 \langle R \rangle^2} \right),
\] (A7.22)

where \( \langle R \rangle \) denotes the average particle radius, and \( \sigma_{\text{poly}} \) describes the relative particle size polydispersity. The form factor for polydisperse inhomogeneous spheres is rewritten as:

\[
P_{\text{poly,inhom}}(q) = \int_0^\infty P_{\text{inhom}}(q, R, \sigma_{\text{surf}}, \sigma_{\text{poly}}) f(R, \langle R \rangle, \sigma_{\text{poly}}) dR
\] (A7.23)

Figure A7-12 shows the form factor (squares) obtained from a dilute solution of MG(Tm) (0.018 mg/mL) in PBS buffer (0.01 M, pH 7.4) as a function of scattering vector \( q \) together with curves from fitting the form factor of monodisperse homogeneous \( P_{\text{hom}}(q, R) \) and polydisperse inhomogeneous spheres \( P_{\text{poly,inhom}}(q, \sigma_{\text{surf}}, \sigma_{\text{poly}}, \langle R \rangle) \) to the experimental data. Our fitting shows
that: the average radius of the dense core \(<R> = 228 \text{ nm}\), the relative particle size polydispersity \(\sigma_{\text{poly}} = 5\%\). The fuzziness of the density decay in the outer regions of the microgel particles is characterized by the parameter \(\sigma_{\text{surf}} = 37 \text{ nm}\). The overall size of particle obtained by static light scattering is \(R_{\text{SLS}} = R + 2\sigma_{\text{surf}} = 302 \text{ nm}\).

Due to the low scatter intensity at large angle, the signal could be affected by back scattering from the reflection of the scatter beam at low angle. Therefore, the results discussed above may be affected.

**A7.10 Estimation of the average mesh size of the microgels**

In a swollen hydrogel, the mesh size \(\xi\) can be calculated as described in Chapter 6, as follows:

\[
\xi = \alpha \times l \times \left( \frac{C_n}{r_c} \right)^{1/2}
\]

where \(\alpha\) is the elongation ratio of the polymer chains in any direction (i.e. \(\alpha^3\) is the swell ratio of the polymer), \(l\) is the length of the bond along the polymer backbone, \(C_n\) is the Flory characteristic ratio, and \(r_c\) is the feeding ratio of crosslinking reagents.

If I assume the mole ratios of all monomers polymerized into microgels are equal to the mole ratio of monomers in the feed, then I can calculate the mass of a single microgel molecule as:

\[
m_{\text{microgel}} = \frac{n_{\text{MAA}} \times M_{\text{MAA}}}{N_A \times r_{\text{MAA}}} \]

where \(m_{\text{microgel}}\) is the mass of one microgel in g; \(n_{\text{MAA}}\) is the number of MAA functional groups per microgel, \(n_{\text{MAA}} = 3.3 \times 10^7\) [10]; \(M_{\text{MAA}}\) is the molar mass of MAA, \(M_{\text{MAA}} = 86.06\) g/mol; \(N_A\) is the Avogadro’s number, \(N_A = 6.02 \times 10^{23}\) mol\(^{-1}\); \(r_{\text{MAA}}\) is the feed ratio of MAA to total monomers in wt\%, \(r_{\text{MAA}} = 21.2\) wt\%. The mass of a single microgel can be calculated
For Tm containing microgels (MG(Tm)), the mass of a single MG(Tm) can be calculated as:

\[ m_{\text{MG(Tm)}} = m_{\text{MG}} + m_{\text{TmF}} = m_{\text{MG}} + 1.1 \times 10^7 \times M_{\text{TmF}} / N_{\text{Av}} \]  
(A7.26)

where \( M_{\text{TmF}} \) is the molecular weight of one TmF molecule, \( M_{\text{TmF}} = 225.93 \text{ g/mol} \), the mass of a single Tm containing microgel can be calculated (A7.29) as \( 2.6 \times 10^{-14} \text{ g} \).

I assume the density of microgel, \( \rho_{\text{microgel}} \), is equal to 1 g/mL. In the swollen state, the hydrodynamic radius of the microgels was at \( R_h = 351 \text{ nm} \) for MG(Tm) microgels. The polymer volume fraction \( \alpha^3 \) can be estimated by equation 6.2 as:

\[ \alpha^3 = \frac{V}{V_0} = \frac{4}{3} \pi R_h^3 \frac{m_{\text{microgel}}}{\rho_{\text{microgel}}} = \frac{4}{3} \pi \times (351 \times 10^{-7} \text{ cm})^3 \frac{2.6 \times 10^{-14} \text{ g}}{(1 \text{ g/cm}^3)} = 6.96 \]  
(A7.27)

leading to \( \alpha = 1.91 \).

The Flory characteristic ratio for poly(NIPAm) is \( C_n = 11.7 \) [11], for poly(MAA) there is \( C_n = 14.6 \) [12]. I need two assumptions to proceed. First I only consider the NIPAm and MAA components in microgel. Second I assume that I can use an average \( C_n \) value based on the composition of the microgel polymer. I can obtain an average Flory characteristic ratio of \( C_n(\text{ave}) = 12.6 \). \( l \) is the C-C bond length of 1.54 Å, the feeding ratio of crosslinking reagents in mol\%, \( r_c = 3 \% \).

Combining the above calculations, I obtained the average mesh size of poly(NIPAm/VCL/MAA) microgels as:

\[ \xi = \alpha \times l \times \left( \frac{C_n}{r_c} \right)^{1/2} = 1.91 \times 0.154 \text{nm} \times \sqrt{12.6 / 3\%} = 6.0 \text{ nm} \]  
(A7.28)

Similarly, the mesh size of hybrid microgels can be estimated according to equation A7.28, as listed in the Table A7-3.
Table A7-3. Estimated polymer volume fraction ($\alpha^3$) and average mesh size ($\xi$) within hybrid microgels.

<table>
<thead>
<tr>
<th></th>
<th>$R_h$ (nm)</th>
<th>$\alpha^3$</th>
<th>$\xi$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG(Tm)</td>
<td>351</td>
<td>6.96</td>
<td>6.0</td>
</tr>
<tr>
<td>SAv-MG(Tm)</td>
<td>321</td>
<td>5.33</td>
<td>5.5</td>
</tr>
<tr>
<td>BSA-MG(Tm)</td>
<td>318</td>
<td>5.18</td>
<td>5.5</td>
</tr>
<tr>
<td>Bi-MG(Tm)-1</td>
<td>329</td>
<td>5.74</td>
<td>5.6</td>
</tr>
<tr>
<td>Bi-MG(Tm)-2</td>
<td>316</td>
<td>5.08</td>
<td>5.4</td>
</tr>
<tr>
<td>Bi-MG(Tm)-3</td>
<td>315</td>
<td>5.03</td>
<td>5.4</td>
</tr>
</tbody>
</table>

A7.11 Apparent zeta potential and electrophoretic mobility of microgels

I carried out the apparent zeta potential [13] and electrophoretic mobility measurements for different microgel samples under constant buffer condition (pH 7.0, PB, 0.01M, containing 0.0027 M KCl, 0.137 M NaCl). Table A7-4 provides the measured values of the electrophoretic mobilities of different samples under pH 7.0 in 0.01 M PB buffer, as well as the calculated zeta potentials. Tm-encoded microgels, MG(Tm), are characterized by a negative ZP = -17.4 mV. Both SAv and BSA are characterized by near neutral isoelectric points (PI), thus show small ZP values. For SAv, the zeta potential is -14.0 mV, and for BSA, the zeta potential is -8.2 mV at pH 7.0. The zeta potential for streptavidin-coated microgels, SAv-MG(Tm), is -15.0 mV. After incubation with the biotinylated polymers (Bi-PAsp(Tb)$_{50}$), the ZP of the microgel conjugate decreased to -15.5 to -21.5 mV depending on the amounts of MCP added. The lowest ZP value was found in the complex with 198 pmol of MCP added into 100 µL of SAv-MG(Tm) microgels. The BSA covalently coated microgel, BSA-MG(Tm) had a zeta potential of -13.4 mV. After incubation with Bi-PAsp(Tb)$_{50}$, there was no significant change in the ZP of the microgels.
Table A7-4. Apparent zeta potentials and electrophoretic mobilities for proteins and microgel composites

<table>
<thead>
<tr>
<th>Particles</th>
<th>ZP&lt;sub&gt;app&lt;/sub&gt; (mV)</th>
<th>μ&lt;sub&gt;e&lt;/sub&gt; (10&lt;sup&gt;-8&lt;/sup&gt; m&lt;sup&gt;2&lt;/sup&gt;/Vs)</th>
<th>Particles</th>
<th>ZP&lt;sub&gt;app&lt;/sub&gt; (mV)</th>
<th>μ&lt;sub&gt;e&lt;/sub&gt; (10&lt;sup&gt;-8&lt;/sup&gt; m&lt;sup&gt;2&lt;/sup&gt;/Vs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-15.5 (1.2)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-1.22 (0.09)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NC1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-13.6 (0.7)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-1.07 (0.06)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>S2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-17.6 (0.5)</td>
<td>-1.39 (0.04)</td>
<td>NC2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-13.8 (1.4)</td>
<td>-1.09 (0.11)</td>
</tr>
<tr>
<td>S3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-21.5 (1.3)</td>
<td>-1.69 (0.10)</td>
<td>NC3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-13.7 (1.3)</td>
<td>-1.08 (0.10)</td>
</tr>
<tr>
<td>S4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-19.2 (1.5)</td>
<td>-1.51 (0.12)</td>
<td>NC4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-13.7 (1.6)</td>
<td>-1.08 (0.13)</td>
</tr>
<tr>
<td>SAv-MG(Tm)</td>
<td>-15.0 (1.6)</td>
<td>-1.18 (0.13)</td>
<td>BSA-MG(Tm)</td>
<td>-13.4 (1.4)</td>
<td>-1.06 (0.11)</td>
</tr>
<tr>
<td>SAv</td>
<td>-14.0 (1.1)</td>
<td>-1.10 (0.09)</td>
<td>BSA</td>
<td>-8.2 (0.4)</td>
<td>-0.63 (0.03)</td>
</tr>
</tbody>
</table>

a. S1, S2, S3, S4: SAv covalently attached: SAv-MG(Tm) (100 µL, containing ca. 2.4 x 10<sup>9</sup> microgels in total) were treated with different amounts of Bi-PAsp(Tb)_{50} solution (3.3 µmol/L: S1, 20 µL; S2, 40 µL; S3, 60 µL; S4, 80 µL).

b. NC1, NC2, NC3, NC4: BSA covalently attached: BSA-MG(Tm) (100 µL, containing ca. 2.4 x 10<sup>9</sup> microgels in total) were incubated with different amounts of Bi-PAsp(Tb)_{50} solution (3.3 µmol/L: S1, 20 µL; S2, 40 µL; S3, 60 µL; S4, 80 µL).

c. Error bars: standard deviation from three replicate measurements.
A7.12 Mass cytometry results for Bi-SAv-Bi sandwich assays with Bi-PAsp(Tb)$_{50}$
**Figure A7-13.** Isotopic Tb-Tm dot-dot plots (upper panel), histograms of Tm content distribution (middle panel) and histograms of Tb content distribution (lower panel) from biotin-SAvidin-biotin sandwich assays using Bi-PAsp(Tb)$_{50}$ as a detection reagent. Part A: SAvidin-Bi-MG(Tm) solution (100 µL, containing 2.5 x 10$^9$ microgels in total, each microgels carrying a total of 5.6 x 10$^5$ biotin molecules) were incubated with different amounts of Bi-PAsp(Tb)$_{50}$ solution (0.33 µmol/L: S1, 10 µL; S2, 20 µL; S3, 40 µL; S4, 80 µL). Part B: BSA/MG(Tm) solution (100 µL, containing 2.5 x 10$^9$ microgels in total) were first treated with 6-ACA (0.03 µmol in 3 µL DI water), then with SAvidin solution (0.005 nmol in 10 µL PBS buffer), and finally combined with different amounts of Bi-PAsp(Tb)$_{50}$ solution (0.33 µmol/L: NC1, 10 µL; NC2, 20 µL; NC3, 40 µL; NC4, 80 µL). Part C: SAvidin-Bi-MG(Tm) solution (100 µL, containing 2.5 x 10$^9$ microgels in total, each microgels carrying a total of 5.6 x 10$^5$ biotin molecules). Data collection was gated to exclude cell debris and cell aggregations. At least 10,000 microgels were analyzed per sample. The mass cytometry transmission coefficient for Tb ions was 3.60 x 10$^{-5}$, and for Tm ions was 3.02 x 10$^{-5}$.
Figure A7-14. Tb and Tm content per microgel determined by mass cytometry from biotin-SAv-biotin sandwich assays using Bi-PAsp(Tb)50 as the detecting reagent. Numbers of Tb and Tm atoms per cell were calculated using the mass cytometry transmission coefficient for Tb ions of $3.60 \times 10^{-5}$ and for Tm ions of $3.02 \times 10^{-5}$. Microgel samples contained 100 µL solution in DI water containing $2.5 \times 10^9$ microgels in total. The SAv-Bi-MG(Tm) sample was prepared by coupling with biotinylated microgels (Bi-MG(Tm)-3, from which each microgel contained $5.6 \times 10^5$ biotin moieties). For SAv-Bi-MG(Tm) and BSA/MG(Tm) samples, the amounts of Bi-PAsp(Tb)50 employed are indicated on the x-axis. The Tb levels (background) detected for SAv-Bi-MG(Tm) samples not treated with metal chelating polymer are indicated by the cross-hatched bars in the histograms. A. Tb atom content per microgel. B. Tm atom content per microgel. The red line indicates a mean value of $1.1 \times 10^7$ Tm atoms per microgel for all samples, with a cell-to-cell CV of 30%. The error bars indicate the cell-by-cell variance.
cell coefficient of variation of lanthanide ion content ($CV_{Ln}$) determined from gated mass cytometry data.
Reference


[7] PDB:3ry1


[10] Titration experiments show that Ln ion binding is saturated at 1 lanthanide ion per 3 –COO-groups. Mass cytometry measurements show an average of 1.1 x 10^7 Tm ions/microgel. Thus each microgel contains on average ca. 3.3 x 10^7 –CO_2H groups.


[13] Zeta potentials are properly defined only for objects with a well-defined surface. Thus it is not correct to describe diffuse structures such as microgels in terms of zeta potentials. One should refer only to their electrophoretic mobility. For microgels with an adsorbed or covalent layer of protein at the surface, the concept of a zeta potential is more reasonable, but we report these values as “apparent” zeta potentials to draw attention to the unknown characteristics of the protein microgel surface.
Chapter 8 Summary and Future Outlook

8.1 Summary

The overall goal of the work in this dissertation was to study the design, properties and applications of lanthanide-containing polyelectrolyte microgels for bead-based bioassays based on mass cytometry. In particular, the design of microgel carriers that fulfill the requirements of bead-based mass cytometry analysis, strategies for loading lanthanide metals into the microgel carriers, the properties and stability of different types of lanthanide-containing microgels, and applications of lanthanide-containing microgels as model cells in bioassays was explored.

The synthesis and characterization of two types of functional polyelectrolyte copolymer microgels as carrier vehicles for Ln(III) ions and LnF_3 nanoparticles were described in Chapter 3. One type of microgel was based upon copolymers of N-isopropylacrylamide (NIPAm) with methacrylic acid (MAA) and N-vinylcaprolactom (VCL). The other type involved copolymers of NIPAm, MAA and poly(ethylene glycol)methacrylate (PEGMA). These highly carboxylated microgels had diameters in solution on the order of 1 μm. They were then used as templates for incorporating Ln^{3+} ions. The experiments show that by adjusting the Eu^{3+}/-COOH group ratio, the Eu content of the microgels could be controlled within the range of 10^6 to 10^7 Eu ions per microgel. The Eu-containing microgels prepared by exchanging Na^+ ions for Eu^{3+} had the same Eu content after the microgels were subsequently treated with F^- to convert the Eu^{3+} ions to EuF_3 nanoparticles. Following this general protocols, a series of Ln^{3+} ion- and LnF_3-containing microgels was prepared. The lanthanide content per microgel and their distributions were determined by mass cytometry. It is shown that there was a small variation in Ln ion uptake during the ion exchange, favoring late Ln ions (Ho, Tm) over early Ln elements (La, Nd). Conversion of the Ln carboxylates to the corresponding LnF_3 NPs conserved the number and distribution of Ln ions per microgel. We also examined a two-stage in-situ precipitation process, in which MG-EuF_3 microgels were treated with additional EuCl_3 followed, after washing, by addition of NaF. This led to a 50% increase in the Eu content to ca. 1.5 x 10^7 Eu ions per microgel.
In Chapter 4, I described the synthesis and characterization of LnPO₄-containing poly(NIPAm/VCL/MAA) microgels with microgels containing 27 mol% of MAA. These microgels were ion exchanged for Ln³⁺ ions. Then phosphate ions as a precipitant were added to convert the Ln³⁺ ions into LnPO₄. The experiments showed that to confine the LnPO₄ within the microgels, one had to treat the Ln³⁺ containing microgels with an excess of a PBS buffer solution. The PBS solution acted both as a precipitating reagent for Ln³⁺ ions, as well as a buffering agent to neutralize newly produced protons during the formation of LnPO₄. All other protocols led to LnPO₄ structures on the exterior of the microgels. We suspect that protons released during the formation of LnPO₄ lead to release of Ln³⁺ ions, allowing them to react outside the microgels. This result is different from that found in the preparation of microgel hybrids containing LnF₃ nanoparticles. From mass cytometry, the LnPO₄-containing microgels obtained from the protocol with PBS buffer conserved ~10⁷ Ln atoms per microgel, essentially at the same level as the Ln³⁺ ions and LnF₃ containing microgels.

In Chapter 5, I examined the stability of the lanthanide-containing microgels (i.e. Ln³⁺-, LnF₃⁻, and LnPO₄-containing microgels) in the presence of acid, base or various buffers that are commonly utilized during cell analysis. I used conventional inductively coupled plasma-mass spectrometry solution analysis to follow the leakage of Ln ions into the aqueous medium as a function of time. Under neutral or basic conditions, the leakage of Ln ions into the aqueous medium was not significant for all three types of lanthanide-containing microgels. In acidic buffer solutions, however, the leakage of metal from Ln³⁺ ions-containing microgels was prominent. On subsequent exposures to buffer, the microgels underwent continuous loss of metal ions. For LnF₃-containing microgels, there was a much smaller extent of metal leakage and a very small of continuous loss of metal upon subsequent exposure to buffers. For LnPO₄-containing microgels, over the period of one week, there was very little (<1%) detectable leakage of metal ions to acidic buffers. These results provided guidance for storage and application of different types of lanthanide microgels in future experimental assays.

In Chapter 6, I described an experiment in which I attempt to promote incorporation of preformed nanoparticles (NPs) into carboxylated microgels. The experiments were performed by stirring oleic acid stabilized (CdSe and NaHoF₄) nanoparticles with MAA functionalized microgels in THF. The nanoparticles themselves were not water soluble. Following a ligand
exchange process, the incorporation of surface passivated nanoparticles into the multidentate polymer ligands would allow the transfer and dispersion of the microgel/nanoparticle hybrids into aqueous media. In Chapter 1, I described two general criteria for incorporating nanoparticles into microgels. First, the nanoparticles must adhere to the microgels by interacting with functional groups that are part of the microgel structure. Second, the size of nanoparticles must be small enough to penetrate the gel. Since polycarboxylic acids have been used as a multidentate ligand to replace oleic acid from OA-capped NPs, one might anticipate that poly(NIPam/VCL/MAA) microgels carrying multiple MAA functional groups would interact effectively with the OA-capped NPs if a suitable exchange medium could be found. To check the validity of these criteria, I tested the microgels with nanoparticles of different sizes and shapes (including CdSe QDs with \(d = 4.6\) nm, NaHoF\(_4\) NPs with \(d = 5.1\) nm, 10.2 nm and 33.5 nm). I found that, only NPs with a diameter (e.g. \(d = 4.6\) nm and 5.1 nm) comparable to the mesh size of microgels (\(\xi = 4.4\) nm in THF) can penetrate inside of microgels and reacted effectively with the functional groups at large amounts. The hybrid microgels prepared by ligand exchange contained significant amounts of metal (on the order of \(10^7\) metal atoms per microgel) as measured by mass cytometry. The variation in metal content from these hybrid microgels (> 60%) was higher than the variation of Ln content of LnF\(_3\)- or LnPO\(_4\)-containing microgels.

In Chapter 7, I described the design and application of lanthanide (Ln)-encoded poly(NIPam/VCL/MAA) microgel (MG(Tm)), as model cells, coated with streptavidin (SAv), as model biomarkers, in quantitative mass cytometric bioassays. I examined the sensitivity of different reagents for detecting and quantifying the number of SAv, as a model biomarker, per microgel, as a model cell, by mass cytometry. In my model system, the microgel was labelled with TmF\(_3\) NPs. Thus the signature of a “cell” event was the detection of \(^{169}\)Tm ions. The streptavidin biomarkers were then detected using biotinylated metal containing probes. I was able to quantify the number of biomarkers per model cell based on mass cytometric analysis.

I used two approaches to attach SAv biomarkers to the microgel surface. In the first approach, I used -COOH activation chemistry to attach SAv covalently to the microgels. This led to a high SAv content per microgel. In the second approach, I attached biotin covalently to the microgel. After passivating the microgel surface with BSA, I then exposed the biotin-modified
microgels to a solution of SA\textit{v}. This led to a low content of accessible SA\textit{v}s per microgel. Two types of biotinylated probes were used: a biotinylated metal chelating polymer (Bi-PA\textit{sp}(Tb)\textsubscript{50}, containing on average 50 Tb\textsuperscript{3+} ions per probe) and a biotinylated NaHoF\textsubscript{4} nanoparticle (Bi-NaHoF\textsubscript{4}, containing ca. 15,000 Ho atoms per probe).

For microgels carrying high abundance of SA\textit{v} biomarkers, the interaction of Bi-PA\textit{sp}(Tb)\textsubscript{50} with the SA\textit{v}-coated microgels was much stronger than the interaction with BSA-coated microgels, which was used as a negative control to account for non-specific absorption. Therefore a measurable mass cytometric signal was generated. I used this signal to determine the amount of specific binding via a biotin-SA\textit{v} coupling assay. From this approach a biomarker level at \(\sim 10^4\) per cell was detected by mass cytometry.

For microgels carrying low abundance of SA\textit{v} biomarkers, the Tb intensity used to signal the interaction between Bi-PA\textit{sp}(Tb)\textsubscript{50} and SA\textit{v}-coated microgels was only slightly higher than its intensity in nonspecific interaction between Bi-PA\textit{sp}(Tb)\textsubscript{50} and BSA-coated microgels. Noting that the detection limit was determined by nonspecific adsorption, therefore I could not quantify the number of biomarkers by the metal chelating polymer reporters. The biotinylated NaHoF\textsubscript{4} nanoparticle, as a more robust probe, enhanced the signal to noise ratio, by increasing the signal intensity to two orders of magnitude higher while maintaining the noise level from non-specific absorption, thus provided an alternative approach to quantify biomarkers level as low as \(\sim 10^2\) per cell via a biotin-SA\textit{v}-biotin sandwich assay from mass cytometry. From these experiments, I demonstrated that by using lanthanide nanoparticle reporters, the detection limit by mass cytometry for number of biomarkers is on the order of 100 per cell.

### 8.2 Future Outlook

Although this thesis was effective in completing the desired research goals, given the current results there are a number of suggested directions that warrant future investigation. These proposals are provided in detail as follows.

In Chapter 3, I explored a two-stage \textit{in-situ} precipitation method in order to increase the metal content per microgel. I found when the metal content increased, the CV of metal-content-
per-microgel increased as well. This opens the question on how to increase the metal content while maintaining or narrowing the CV of metal-content-per-microgel. One solution may be to prepare monodisperse microgels of larger size and higher content of functional groups per microgel. This could be achieved by new synthetic approaches such as microfluidic synthesis.

In Chapter 5, I compared the stability of different types of Ln-containing microgels towards leakage of metal ions into buffer media, but I have yet to study the structure of LnF$_3$ or LnPO$_4$ nanoparticles inside the microgels. Small angle X-ray scattering (SAXS) will provide information on the size of small nanoparticles inside the microgels. We may learn more on the nature of NPs prepared from in-situ precipitation. One open question is: when we increase the feed ratio of Ln$^{3+}$/COOH, do we increase the amounts or the sizes of NPs in the microgels?

In Chapter 7, I used the microgels ca. 700 nm in diameter as a model cell, which is only comparable to the size of bacteria. Further experiments should aim at: a) larger size microgels, b) with more cell surface properties (such as phospholipid bilayers and glycoproteins), c) using more specific antigens as biomarkers and antibodies as detecting probes. If these can be achieved that the detection of biomarkers on the surface of microgels can be directly compared to the real cells to test how well microgels act as model cells.