Preparation, Surface Modification and Application of Lanthanide Nanoparticles as Elemental Tags in Mass Cytometric Bioassays

by

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This thesis describes the preparation, surface modification and application of lanthanide nanoparticles (Ln NPs) as elemental tags in bioassays by mass cytometry. The most common elemental tags in mass cytometry are lanthanide metals. Previous studies established polymer-based lanthanide tags for mass cytometric bioassays. My studies focused on developing a new generation of elemental tags based on Ln NPs for bioassays by mass cytometry.

The first part of my thesis describes a ligand exchange strategy for making Ln NPs that are colloidally stable in aqueous buffer media. This strategy yielded NaLnF$_4$ NPs capped with PEG-PAMAM surface ligands. These PEG-PAMAM-capped NPs were colloidally stable in water but lacked long-term colloidal stability in PBS. This made the PEG-PAMAM-capped NPs incompatible with cell assays by mass cytometry.

The second part describes the preparation of small LnF$_3$ NPs as internal labels for Ln-containing polymer microspheres. This work was done by collaboration with Dr. Jianbo Tan. He prepared PMMA microspheres labeled with those small LnF$_3$ NPs. We then developed a streptavidin-biotin binding assay using those LnF$_3$-labeled microspheres as a proof-of-concept bead-based assay by mass cytometry.
The third part describes a sandwich-type bead-based immunoassay by mass cytometry. I prepared NaYF$_4$@SiO$_2$ NPs as reporter tags for this bead-based immunoassay. By using the LnF$_3$-labeled PMMA microspheres as bead supports, I established a bead-based immunoassay that specifically targets human IgG and tested the specificity of this assay by TEM.

The fourth part discusses making a NP-based elemental tag that can specifically target cell surface markers with minimal non-specific cell binding. I synthesized PEGylated NaHoF$_4$@SiO$_2$ NPs and attached antibodies onto the PEG corona of the NPs. These NP-antibody conjugates showed excellent targeting ability for CD20 antigens on Ramos cells along with a low background level of non-specific binding. Future experiments are needed to demonstrate their usage in detecting low-abundance cellular biomarkers by mass cytometry.
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<th>Full Form</th>
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<tbody>
<tr>
<td>AA</td>
<td>acrylic acid</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>AcOH</td>
<td>acetic acid</td>
</tr>
<tr>
<td>ACVA</td>
<td>4,4’-azobis(4-cyanovaleric acid)</td>
</tr>
<tr>
<td>AEP</td>
<td>2-aminoethyl dihydrogen phosphate</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
</tr>
<tr>
<td>APTES</td>
<td>(3-aminopropyl)triethoxysilane</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CTAB</td>
<td>cetyl trimethylammonium bromide</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
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<tr>
<td>DDMAT</td>
<td>(S)-1-dodecyl-(S')-((\alpha),(\alpha')-dimethyl-(\alpha')-acetic acid)trithiocarbonate</td>
</tr>
<tr>
<td>DELFIA</td>
<td>dissociation-enhanced lanthanide fluorescent immunoassay</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>DOTA</td>
<td>1,4,7,10-tetraaza-cyclododecane-1,4,7,10-tetraacetic acid</td>
</tr>
<tr>
<td>DTAB</td>
<td>dodecyltrimethylammonium bromide</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5-dithio-bis-(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTPA</td>
<td>diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>DTTA</td>
<td>(N^1)-((p)-isothiocyanatobenzyl)-diethylenetriamine-(N^1), (N^2), (N^3), (N^3)-tetraacetate</td>
</tr>
<tr>
<td>EDC</td>
<td>(N)-(3-dimethylaminopropyl)-(N')-ethylcarbodiimide</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGMAP</td>
<td>ethylene glycol methacrylate phosphate</td>
</tr>
<tr>
<td>FCS</td>
<td>flow cytometry standard</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>GaH</td>
<td>goat anti-human</td>
</tr>
<tr>
<td>GaM</td>
<td>goat anti-mouse</td>
</tr>
<tr>
<td>GaM Fc Ab</td>
<td>goat anti-mouse IgG Fc antibody</td>
</tr>
<tr>
<td>HABA</td>
<td>4'-hydroxyazobenzene-2-carboxylic acid</td>
</tr>
<tr>
<td>HER3</td>
<td>human epidermal growth factor receptor 3</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>Ln</td>
<td>lanthanide</td>
</tr>
<tr>
<td>MAL</td>
<td>maleimide</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>MFI</td>
<td>mean fluorescence intensities</td>
</tr>
<tr>
<td>MMA</td>
<td>methyl methacrylate</td>
</tr>
<tr>
<td>MPTMS</td>
<td>(3-mercaptopropyl)trimethoxysilane</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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</tr>
<tr>
<td>NA\textsubscript{v}</td>
<td>NeutrAvidin</td>
</tr>
<tr>
<td>NHS</td>
<td>\textit{N}-hydroxysuccinimidy\textsubscript{e} ester</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NP</td>
<td>nanoparticle</td>
</tr>
<tr>
<td>OA</td>
<td>oleate</td>
</tr>
<tr>
<td>OAH</td>
<td>oleic acid</td>
</tr>
<tr>
<td>ODE</td>
<td>1-octadecene</td>
</tr>
<tr>
<td>OEGA</td>
<td>oligo(ethylene glycol) methyl ether acrylate</td>
</tr>
<tr>
<td>PAA</td>
<td>poly(acrylic acid)</td>
</tr>
<tr>
<td>PAMAM</td>
<td>poly(amidoamine)</td>
</tr>
<tr>
<td>PB</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PDA</td>
<td>polydopamine</td>
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<td>PDI</td>
<td>polydispersity index</td>
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<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PGMA</td>
<td>poly(glycidyl methacrylate)</td>
</tr>
<tr>
<td>PMAO</td>
<td>poly(maleic anhydride-\textit{alt}-1-octadecene)</td>
</tr>
<tr>
<td>PMMA</td>
<td>poly(methyl methacrylate)</td>
</tr>
<tr>
<td>PPG</td>
<td>polypropylene glycol</td>
</tr>
<tr>
<td>PS</td>
<td>polystyrene</td>
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<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
</tr>
<tr>
<td>RAFT</td>
<td>reversible addition-fragmentation chain-transfer</td>
</tr>
<tr>
<td>RB</td>
<td>rhodamine B</td>
</tr>
<tr>
<td>SA\textsubscript{v}</td>
<td>streptavidin</td>
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<tr>
<td>SEC</td>
<td>size-exclusion chromatography</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TEOS</td>
<td>tetraethyl orthosilicate</td>
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<tr>
<td>TGA</td>
<td>thermogravimetric analysis</td>
</tr>
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<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TMR</td>
<td>tetramethylrhodamine</td>
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<tr>
<td>TMSBr</td>
<td>trimethylsilyl bromide</td>
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<tr>
<td>TOF</td>
<td>time-of-flight</td>
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<tr>
<td>UCNP</td>
<td>upconversion nanoparticle</td>
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<td>UV-vis</td>
<td>ultraviolet-visible</td>
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<td>XRD</td>
<td>X-ray diffraction</td>
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1 Chapter 1: Introduction

1.1 Overview

In modern bioanalytical chemistry, there is an emerging demand for techniques that can extract a high density of bioinformation from limited amounts of samples. This requires multiplexed bioassays that are highly sensitive and specific. Researchers have developed multiplexed bioassays based on various platforms, including multiwell microtiter plates, chip-based microarrays, microfluidic systems and flow cytometers. Among those techniques, cytometric bioassays are most commonly used for functional analysis and immunophenotyping of cells in heterogeneous populations.

Cytometric bioassays have been reported for various bioanalytical applications, including cell cycle analysis, cell signaling pathways, cellular medical responses, suspension bead arrays, etc. In conventional flow cytometric bioassays, the analytes of interest are typically detected with antibodies tagged with fluorescent dyes. This technique can simultaneously measure 3 – 6 analytes. Quantitative analysis of any measurement beyond 6 channels is very challenging due to the spectral overlap of fluorophores, although the most advanced flow cytometer can accommodate 18 color channels.

Mass cytometry, a novel technique that adapts inductively coupled plasma mass spectrometry (ICP-MS) for single cell analysis, breaks the barrier of multiplexity. Instead of using fluorescent tags that interfere with each other, this technique employs stable elemental isotopes as reporter tags. Current mass cytometry instrument covers a mass range between 89 and 220, corresponding to elements from Y to Bi. Thanks to the single mass resolution of atomic mass spectrometry, mass cytometry can theoretically perform simultaneous cell assays with 70 – 100 parameters (Figure 1-1). Now more than 30 different isotopes have been commercialized, most of which are lanthanide (Ln) metals. Commercial labeling reagents for mass cytometry are antibodies tagged with metal-chelating polymers (MCP), which carry 100 – 250 metal ions per antibody.

Although mass cytometry allows bioassays with much higher multiplexity than conventional flow cytometry, the sensitivity of metal-isotope-tagged antibodies is lower than antibodies tagged with most popular fluorophores. To improve the sensitivity of bioassays based on mass cytometry, one needs to develop new elemental tags that carry more isotopes per antibody. Lanthanide nanoparticles (Ln NPs) are great candidates to fulfill this requirement. For instance, one antibody conjugated with one spherical NaLnF$_4$ nanoparticle with a diameter of 10 nm carries about 8000 lanthanide atoms per antibody, which is almost 2 orders of magnitude higher than commercial MCP-labeled antibodies.

My research focused on surface modification of Ln NPs as elemental tags for bioassays based on mass cytometry. Numerous research groups have reported surface modification methods of Ln NPs for various biological applications. Our group’s effort is the first to examine their application in mass cytometry. To provide a background of this multidisciplinary project, this introductory chapter contains a literature review of related topics (basic principles of mass cytometry, applications of mass cytometry, development of elemental tags for mass cytometry, surface modification methods of Ln NPs), a discussion of my research goals and the plan of my thesis.
1.2 Literature Review

1.2.1 Basic Principles of Mass Cytometry

Mass cytometry was first reported by Bandura et al. in 2009.\textsuperscript{12} The sample introduction of mass cytometry is similar to that of conventional flow cytometry (Figure 1-2).\textsuperscript{5} For analysis by mass cytometry, cells are stained with target-specific antibodies tagged with heavy metal isotopes instead of fluorophores. Since mass cytometers lack the light scattering measurement of cell events in flow cytometry, the cells are also stained with Rh- or Ir-labeled DNA intercalators to provide a baseline for identifying cell events.

The stained cells are introduced into the instrument in the form of an aqueous suspension and delivered to a nebulizer via a capillary tube. In the nebulizer the stained cells are converted to a fine spray of water droplets containing single cells, then the single-cell droplets are released to a heated spray chamber. The spray chamber is connected with a supply of argon make-up gas flow, which dries the water droplets and carries the remaining cells into an inductively coupled plasma (ICP) torch.

The argon plasma inside the torch is maintained at a temperature of ca. 7,000 K, which ionizes approximately 0.1% of the argon atoms.\textsuperscript{10} The cells are delivered concentrically to the plasma core, where the cells are completely vaporized, atomized and ionized by the argon plasma. Thus each cell is converted to an ion cloud. The ion cloud then passes through a radio-frequency quadrupole mass filter. The low mass cutoff of the quadrupole is set to $m/z = 80$ so that most abundant low mass ions generated from either the cell or argon plasma are filtered out, leaving only heavy metal ions of interest.

The filtered ion clouds are finally analyzed by a time-of-flight (TOF) mass analyzer. The TOF analyzer in a mass cytometer is a single-stage reflectron,\textsuperscript{17} which measures a mass spectrum every 10 μs. A typical cell event lasts 200 – 300 μs, thus consists of 20 – 30 mass spectra. The number of mass spectra in each cell event is recorded as ‘cell length’. Then the signal intensity of each $m/z$ channel is integrated for each cell event within its cell length. As mentioned above, since mass cytometry lacks the light scattering measurement of cell events, the presence of a real cell event is identified by comparing integrated Rh or Ir signals to a preset threshold value. Data corresponding to each identified event, including the length and the integral of every $m/z$ of
interest, is saved in a table. Events with cell length larger than a certain value are considered to be generated by either doublets or cell aggregates, which are saved separately during the data processing. The data is by default saved in FCS 3.0 format, compatible with conventional flow cytometry data processing software e.g. FlowJo.

Briefly, mass cytometry can be considered as an adaptation of ICP-MS to single cell analysis. Each cell is distinguished by a ‘fingerprint’ of signal intensities from each mass channel, which contains information about type and number of biomarkers on the cell. Since ICP-MS is a quantitative technique, the signal intensity of metal ions in mass cytometry can be directly converted to the number of metal-tagged antibodies bound to every single cell without the need for an external calibration standard. This is an advantage over conventional flow cytometry. Also, since different isotope peaks do not overlap in mass spectra, compensation is not required in mass cytometry. This greatly facilitates quantitative analysis in highly multiplexed bioassays. State-of-the-art mass cytometry is suitable for measuring isotopes with \( m/z \) ranging from 80 to 220. Among all elements in this range, lanthanide isotopes are most commonly used because of their low natural abundance, similar properties of coordination chemistry between each other as well as commercial availability of enriched isotopes. For multiplexed bioassays in mass cytometry, it is essential to develop high-quality lanthanide-based elemental tags for antibody labeling of cellular or serum biomarkers.
Figure 1-2. Workflow of mass cytometry. In mass cytometry, the cells are labeled with antibodies tagged with elemental isotopes. The labeled cells are injected into the instrument, pass through a nebulizer and delivered to the ICP torch as single-cell droplets. In the ICP torch (ca. 7,000 K), each cell droplet is pyrolyzed and converted to an ion cloud. The ion clouds are filtered by a quadrupole mass filter to remove low mass ions and finally analyzed by a time-of-flight mass analyzer. The signal intensity of each element represents the type and number of antigens that are present on the cell surface. Reprinted with permission from ref. 5.

1.2.2 Development of Lanthanide Elemental Tags for Mass Cytometry

1.2.2.1 Small-Molecule Lanthanide Complexes as Elemental Tags

Before the invention of mass cytometry, various research groups have used conventional ICP-MS to quantitatively measure biomolecules of interest. The earliest ICP-MS-based bioassay using lanthanide (Ln) isotopes as elemental tags was reported by Zhang et al. in 2001. They used antibodies tagged with Eu-DTTA complex (DTTA = N\textsubscript{1}-(p-isothiocyanatobenzyl)-diethylenetriamine-N\textsubscript{1}, N\textsubscript{2}, N\textsubscript{3}, N\textsubscript{3} -tetraacetate) for quantitative determination of thyroid-stimulating hormone in human serum and obtained results consistent with traditional radioimmunoassay and chemiluminescent immunoassays. In the following year, Baranov et al. reported another example of an ICP-MS-based immunoassay. In their study, the targeting antibodies were tagged with commercial DELFIA (dissociation-enhanced lanthanide fluorescent immunoassay, PerkinElmer) Ln complexes and were thought to contain 6 – 10 Ln ions per antibody. Since DELFIA-Ln complexes are luminescent, the antibodies tagged with DELFIA-Ln
can also be used for fluorescent immunoassays according to the procedure given by the manufacturer. The authors found that although the fluorescent and ICP-MS immunoassays provided comparable accuracy and precision, the detection limit of fluorescent immunoassays was roughly one order of magnitude better than ICP-MS-based assays. To increase sensitivity and lower detection limit of ICP-MS-based assays, elemental tags that carry more Ln ions per antibody are required.

1.2.2.2 Lanthanide Metal-Chelating Polymers as Elemental Tags

In 2007, Lou et al. published the first example of polymer-based elemental tags for ICP-MS bioassays. They synthesized metal-chelating polymers (MCPs) with ca. 30 DOTA (DOTA = 1,4,7,10-tetraaza-cyclododecane-1,4,7,10-tetraacetic acid) groups as chelators for Ln metals on each polymer chain. When mouse anti-CD33 antibodies tagged with those MCP-Eu complexes were used to label microtitre plates coated with goat anti-mouse antibodies, the signals in ICP-MS were 80 times higher than same antibodies tagged with DELFIA-Eu complexes. To further increase the number of Ln ions per tag, Majonis et al. synthesized MCPs with a higher degree of polymerization that carried DTPA (DTPA = diethylenetriaminepentaacetic acid) as metal chelators instead of DOTA. Isothermal titration calorimetry (ITC) analysis showed that each polymer chain bound 68 ± 7 Ln ions and ICP-MS analysis showed that a CD45 antibody tagged with a Tb-conjugate of this newer MCP had 161 ± 4 Tb per antibody. A mixture of 11 antibodies tagged with different Ln complexes of this MCP were used to stain whole umbilical cord blood for single cell analysis by mass cytometry. Illy et al. prepared another type of MCP that has two DTPA groups on each repeat unit of the polymer. This polymer can carry 200 – 300 Ln ions per antibody, which was used to quantitatively determine antibody binding capacity of cells in a 10-plex assay yielding values similar to literature results determined by conventional flow cytometry.

1.2.2.3 Lanthanide-Containing Nanoparticles as Elemental Tags

To date, MCPs are the most commonly used elemental tags in mass cytometry studies. A number of Ln-MCP complexes and Ln-MCP-tagged antibodies have been commercialized by DVS Sciences (now part of Fluidigm Inc.) as reagent kits for mass cytometry. Nevertheless, the sensitivity of Ln-MCP-tagged antibodies in mass cytometry is still lower than antibodies tagged with the most popular fluorescent dyes in conventional flow cytometry. Due to the limited degree of polymerization of MCPs, it is necessary to seek other elemental tags that can carry more Ln ions.
ions for ultrasensitive detection of biomarkers. Lanthanide-containing nanoparticles (Ln NPs) are natural candidates for this purpose. A spherical $d = 10$ nm NaLnF$_4$ NP contains $\sim$8,000 Ln ions, which is two orders of magnitude higher than one MCP chain (Figure 1-3). However, preparation of monodisperse Ln NPs that cover the whole Ln series from La to Lu for mass cytometry is very difficult. Since the nucleation and growth mechanism and kinetics of Ln NPs vary from one Ln element to another, reaction conditions suitable for synthesizing some of the Ln NPs may not apply to other Ln elements.\textsuperscript{22,23} Also, due to the high affinity of phosphate ions to Ln metals, it is challenging to prepare Ln NPs that are colloidally stable in physiological buffer media, since many of those media contain phosphate ions. These ions can competitively replace the original surface ligands of the Ln NPs and reduce their colloidal stability. Among all different types of Ln NPs, the preparation of NaLnF$_4$ NPs is most well studied due to their outstanding properties for biomedical imaging. For instance, NaYF$_4$:Yb,Er NPs have been widely used for upconversion luminescence imaging and NaGdF$_4$ NPs have been widely used for magnetic resonance imaging (MRI).\textsuperscript{24} The most popular synthetic method of NaLnF$_4$ NPs was developed by Zhang and co-workers.\textsuperscript{25} In this method, LnCl$_3$ precursors first react with NaOH and NH$_4$F at a relatively low temperature (room temperature or 50 °C) to form amorphous lanthanide fluoride nuclei, then the reaction mixture was eventually heated to 300 °C, during which the nuclei crystallize and then grow to form monodisperse NaLnF$_4$ NPs. Since the reaction takes place in a solvent mixture of oleic acid (OAH) and 1-octadecene (ODE), NaLnF$_4$ NPs prepared by this method are capped with a layer of oleates (OA) as surface ligands.
1.2.3 Surface Modification Methods of NaLnF₄ NPs

As mentioned above, the surface of as-prepared NaLnF₄ NPs is capped with hydrophobic OA ligands, thus the NPs are only dispersible in nonpolar organic solvents such as hexane, toluene and chloroform. To render NaLnF₄ NPs water-dispersible and colloidally stable in aqueous buffers, researchers have developed many different surface modification strategies, including surface ligand exchange, encapsulation with amphiphilic molecules, silica coating, etc.²⁶

1.2.3.1 Surface Ligand Exchange

Ligand exchange on NaLnF₄ NPs means replacing the original hydrophobic ligands on the surface of NPs by polymeric or small-molecule hydrophilic ligands, to make the NPs dispersible in aqueous media (Figure 1-4). To develop an efficient ligand exchange method one needs to consider various aspects. First, the new ligand has to bind more tightly to NaLnF₄ NPs than the original OA ligand. The binding affinity of ligands to NaLnF₄ NPs is determined by the number and type of anchoring groups on the ligand. Multidentate ligands bind more tightly than monodentate ligands, and the binding strength of anchoring groups vary in the order phosphate/phosphonate > carboxylate > amine. Also, the solvent of ligand exchange has to be both suitable for dispersing the OA-capped NPs and dissolving the new ligand. Last but not least, a complete ligand exchange usually requires excess amount of new ligands. Sometimes the excess
ligands may cause undesirable side effects in the next steps, so they have to be properly removed after the ligand exchange reaction.

A pioneering work in this field is reported by Yin and co-workers who developed a ligand exchange method to transfer OA-capped inorganic NPs to water by exchanging the OA ligands with poly(acrylic acid) (PAA) in diethylene glycol at 240 °C.\textsuperscript{27} To prepare PAA-capped NaLnF\textsubscript{4} NPs under milder conditions, Lin and co-workers developed a two-step ligand exchange strategy.\textsuperscript{28} They first coated the surface of OA-capped NaYF\textsubscript{4}:Yb,Er nanoparticles (NPs) with cetyl trimethylammonium bromide (CTAB) in a chloroform-water mixture. Then they evaporated the chloroform to obtain a colloidal solution of CTAB-OA-capped NPs in water and exchanged CTAB-OA with PAA in water at room temperature. Nevertheless, the authors did not provide any information about the colloidal stability of their PAA capped NPs in aqueous buffer media.

Polyethylene glycol (PEG) is a flexible and water-soluble polymer with low toxicity. Thus it is widely used to coat inorganic NPs for biomedical applications. Liu and co-workers showed that PEG-capped NaYF\textsubscript{4}:Yb,Tm NPs exhibited prolonged blood circulation half-lives compared to the same NPs capped with PAA.\textsuperscript{29} In their experiment, either PEG- or PAA-capped NPs were intravenously injected into Balb/c mice via the tail vein. The blood circulation curves of both NPs was fitted to a two-compartmental model. For PEG-capped NPs, the first and second phase blood circulation half-lives were 5.1 ± 2.5 min and 13.1 ± 6.2 min, respectively, whereas PAA-capped NPs showed much shorter first and second phase half-lives of 0.13 ± 0.11 min and 3.5 ± 0.4 min.

In other literature, Gorris and co-workers used PEG dicarboxylic acid as a surface ligand to prepare water-dispersible NaYF\textsubscript{4}:Yb,Er NPs.\textsuperscript{30} Van Veggel and co-workers synthesized PEG-monophosphoric acid as an alternative ligand that can also stabilize NaYF\textsubscript{4}:Yb,Er NPs in aqueous media.\textsuperscript{31} Nevertheless, because of the high affinity between phosphate ions and Ln ions, neither PEG-dicarboxylic acid nor PEG-monophosphoric acid can provide sufficient colloidal stability for Ln NPs in phosphate-containing buffer media. To further enhance the binding between Ln NPs and PEG-based surface ligands, Cao et al. synthesized multidentate PEG compounds that bear two or four phosphonate anchoring groups on each PEG molecule.\textsuperscript{32} They found that NaHoF\textsubscript{4} NPs capped with PEG-tetraphosphonate could maintain good colloidal stability in phosphate buffered saline (PBS), whereas same NPs capped with PEG-monophosphate rapidly flocculated upon transfer from water to PBS.
Small molecules containing carboxylate or phosphate anchoring groups are also used to render Ln NPs hydrophilic via ligand exchange. Li and co-workers used citrate to exchange the OA ligands on UCNPs in diethylene glycol at 220 °C, a similar reaction condition for ligand exchange with PAA. The citrate-capped UCNPs are water-dispersible. In a later report by Li’s group, the authors incubated the citrate-capped UCNPs in a water solution of radioisotope $^{18}$F$^-$ to exchange some of the F$^-$ on the NP surface and prepare $^{18}$F-labeled UCNPs for both luminescence and positron emission tomography (PET) imaging. This exchange of surface ions on the NPs is more difficult for NPs capped with polymeric ligands since the kinetics of free ions in the solution passing through the coating layer of polymeric ligands is much slower. Acidic molecules with long alkyl chains, e.g. hexanedioic acid, 1,10-decanedicarboxylic acid and 11-mercaptododecanoic acid have also been used for ligand exchange on NaLnF$_4$ NPs since surface ligands with alkyl chains can provide colloidally stability for the NPs via steric repulsion between each other.

Figure 1-4. Common ligand exchange strategies to render NaLnF$_4$ NPs hydrophilic. Hydrophilic NaLnF$_4$ NPs can be prepared by replacing the original oleate (OA) ligands with hydrophilic ligands, including poly(acrylic acid) (PAA), poly(ethylene glycol) (PEG) phosphate or phosphonate, or small molecules e.g. citrates.
1.2.3.2 Surface Silica Coating

Hydrophilic NaLnF$_4$ NPs can also be obtained by coating the NPs with a silica shell. The silica shell not only provides colloidal stability for NaLnF$_4$ NPs, but also reduces the toxicity of NaLnF$_4$ NPs by preventing leakage of heavy metal Ln ions from the NPs. This modification makes the NPs more compatible with biochemical applications. There are two strategies to coat OA-capped NaLnF$_4$ NPs with a silica shell. One strategy is to first make the NPs hydrophilic by either ligand exchange or ligand encapsulation, then coat the NP surface with silica using a Stöber process. Van Veggel and co-workers used polyvinylpyrrolidone (PVP) to exchange the OA ligands on the surface of NaYF$_4$:Yb,Er NPs with an average diameter of 21.0 nm, then dispersed the PVP-capped NPs in ethanol and coated the NPs with a 9-nm-thick silica shell via a Stöber process.$^{37}$ Zhang and co-workers used CTAB to coat OA-capped UC NPs and transferred the NPs to water followed by a sol-gel reaction of NaOH-catalyzed hydrolysis of tetraethyl orthosilicate (TEOS). After the reaction CTAB was removed by ethanol washing, leaving a mesoporous silica shell on the NP surface. In this case CTAB not only renders the NPs hydrophilic, but also plays the role of a porogen for making the silica shell mesoporous.$^{38}$

The other strategy, which is more commonly used to coat OA-capped NaLnF$_4$ NPs with a silica shell, is a reverse microemulsion method. In this method the OA-capped NPs are encapsulated with reverse micelles formed by non-ionic detergents e.g. Igepal CO-520, Triton X-100, etc. in a nonpolar organic solvent, typically cyclohexane.$^{39}$ On the surface of each NP a small aqueous compartment is enclosed, where ammonia-catalyzed hydrolysis of TEOS occurs leading to formation of a silica shell. The ratio between the concentration of detergent, NPs and aqueous ammonia needs to be well adjusted to obtain uniform coating of single NPs.$^{40}$ In addition, the thickness of the silica shell can be adjusted by varying the amount of TEOS added to the reverse microemulsion.

Silica coated NaLnF$_4$ (NaLnF$_4$@SiO$_2$) NPs can be further functionalized to introduce reactive groups for bioconjugation. The functionalization can be done by either adding functional silane reagents during the silica coating reaction or reacting purified NaLnF$_4$@SiO$_2$ NPs with functional silane reagents. For instance, amine-functionalized NaLnF$_4$@SiO$_2$ (NaLnF$_4$@SiO$_2$-NH$_2$) NPs can be prepared by condensing (3-aminopropyl)trimethoxysilane (APTMS) or (3-aminopropyl)triethoxysilane (APTES) onto the surface of NaLnF$_4$@SiO$_2$ NPs.$^{41,42}$ Carboxylate-
functionalized NaLnF₄@SiO₂ (NaLnF₄@SiO₂-COOH) NPs can be obtained by condensing carboxyethylsilanetriol onto the surface of silica shell. For bioconjugation reactions at physiological pH, NaLnF₄@SiO₂-COOH NPs are more favourable than NaLnF₄@SiO₂-NH₂ NPs since the positively charged amines and negatively charged ions in the buffer media electrostatically attract each other and may cause aggregation of the NPs. Other functional groups, such as thiols, epoxides, etc. can also be introduced onto the surface of NaLnF₄@SiO₂ NPs by using corresponding functional silanes.

The surface of NaLnF₄@SiO₂ NPs can be further coated with a layer of PEG to suppress non-specific protein adsorption and cell uptake. Wang and co-workers attached PEG-amine onto NaYF₄:Yb,Er@SiO₂-COOH NPs by using a EDC/NHS mediated coupling reaction. Xing and co-workers used N-hydroxysuccinimidyl-ester(NHS)-terminated PEG to directly react with NaYF₄:Yb,Tm@SiO₂-NH₂ NPs in PBS and obtained PEGylated NaYF₄:Yb,Tm@SiO₂ NPs. Shi and co-workers reported one interesting example for indirectly PEGylating NaLnF₄@SiO₂ NPs. They first attached small (d = 1 – 2 nm) Au NPs onto the surface of upconverting NaLnF₄@SiO₂-NH₂ NPs and then added PEG-thiols that bound to Au NPs, thus PEGylating the surface of NaLnF₄@SiO₂ NPs. This composite nanomaterial, which consists of both upconverting luminescent and CT contrast agents, was applied for multimodal biomedical imaging in vivo.
1.2.3.3 Other Surface Modification Strategies

1.2.3.3.1 Ligand Oxidation

OA ligands on the surface of NaLnF₄ NPs contain an unsaturated double bond between C9 and C10, which can be cleaved by oxidizing reagents to yield azelaic acid remaining on the NP surface, making the NPs hydrophilic. Li and co-workers used the Lemieux-von Rudloff reagent to perform this oxidation reaction.⁵⁰ Lemieux-von Rudloff reagent is a mixture of a catalytic amount of MnO₄⁻ and a stoichiometric amount of IO₄⁻. In the reaction, the C=C double bond is first oxidized by MnO₄⁻, then the reduced manganese reagent is reoxidized by IO₄⁻. Huang and co-workers showed that this oxidation reaction does not affect the size, shape and crystal phase of NaLnF₄ NPs.⁵¹ Moreover, the newly formed carboxylic acid group can be used to conjugate biomolecules such as streptavidin and peptides.⁵⁰,⁵¹

The ligand oxidation on NaLnF₄ NPs can also be achieved by ozonolysis of the OA surface ligand. By using different reaction conditions, the oxidation of double bonds can lead to formation of either aldehydes or carboxylic acids on the NP surface.⁵² If the intermediate of the ozonolysis
reaction was oxidized by a mixture of hydrogen peroxide and acetic acid, then the resulting surface ligand was azelaic acid. If the intermediate was reduced by using dimethyl sulfide, then the NP surface ligand became azelaic aldehyde instead. The aldehyde functionalized NaLnF₄ NPs could directly react with amine groups on biomolecules to form an imine linker.

1.2.3.3.2 Ligand Removal

As an alternative to ligand exchange reactions, Capobianco and co-workers showed that the OA ligands on the surface of NaLnF₄ NPs can be removed by treatment with mild aqueous acid.⁵³ At pH 4, the OA ligands can be protonated and detach from the NP surface. This yields water-dispersible ligand-free NaLnF₄ NPs. The ligand-free NPs appeared to be stabilized only by their surface charge. At pH < 5 the NPs bear a net positive surface charge since Ln ions on the NP surface are coordinated to H₂O in the form of [Ln-OH₂⁺]···Cl⁻. When pH was increased, the coordinated water was deprotonated to form Ln-OH and Ln-O⁻, and the NPs displayed a negative zeta potential. Those ligand-free NaLnF₄ NPs can be further functionalized with biomolecules e.g. heparin and β-carboxyphthalocyanine zinc, for diagnostic imaging and therapeutic treatment of biological objects.⁵⁴,⁵⁵

1.2.3.3.3 Encapsulation with Amphiphilic Molecules

The long alkyl chain of OA or OM ligands on the surface of NaLnF₄ NPs allows attachment of amphiphilic molecules via hydrophobic interaction. The interaction between OA or OM ligands and the hydrophobic part of amphiphilic molecules is driven by van der Waals forces in an aqueous environment. The amphiphilic molecules can be detergents, lipids or modified polymers. Schuck and co-workers attached octylamine to a PAA backbone and used this partially alkylated PAA to encapsulate OA-capped NaYF₄:Yb,Er NPs.⁵⁶ The authors stated that the ratio of alkylated to non-alkylated free carboxylic acid groups has to be well adjusted to provide strong enough interaction between the polymer and the OA ligands while maintaining enough free acid groups to make the encapsulated NPs hydrophilic. Alternatively, Liu and co-workers attached PEG to poly(maleic anhydride-alt-1-octadecene) (PMAO) by reacting amine-terminated PEG with the anhydride groups on PMAO backbone.⁵⁷ They used this PMAO-PEG amphiphilic polymer to encapsulate OA-capped UCNPs via hydrophobic interaction between OA and octadecene chains on the PMAO-PEG polymer and demonstrated that the encapsulated NPs are well dispersed in water by
DLS. Nevertheless, neither of the researcher groups studied the colloidal stability of their encapsulated NaLnF$_4$ NPs at high salt concentrations or in buffer media.

Amphiphilic polymers bearing hydrophobic parts other than long alkyl chains have also been used to encapsulate OA-capped NaLnF$_4$ NPs. Pluronic F127, a triblock copolymer consisting of one central hydrophobic block of polypropylene glycol (PPG) linked with hydrophilic blocks of PEG on both ends, was used to coat OA-capped NaYF$_4$:Yb,Er(or Tm) NPs and render the NPs hydrophilic.$^{58}$ In this report the authors mixed a colloidal solution of OA-capped NPs (average diameter 38 nm) in chloroform and a solution of Pluronic F127 in water, stirred the mixture vigorously to make a chloroform in water emulsion, then evaporated the chloroform to produce water-dispersible Pluronic F127-encapsulated NPs. They used DLS to confirm that the NPs encapsulated with Pluronic F127 are colloidally stable in both water and common cell incubation media for at least one week. They also showed that the Pluronic F127-encapsulated NPs have low cytotoxicity, which makes the NPs compatible with biomedical imaging. In another study, Prud’homme and co-workers used a block copolymer of PEG and poly(lactic acid) (PEG-$b$-PLA) to coat large NaYF$_4$:Yb,Er NPs with diameter about 100 nm.$^{59}$ Those NPs are colloidally stable in water, PBS and cell culture medium at 4 °C for three months. At elevated temperature (37 °C) that simulates physiological conditions, no sign of aggregation was observed over a period of 25 hours, which is sufficient for most biological applications.

Non-polymeric surfactants, such as sodium dodecyl sulfate (SDS), dodecyltrimethylammonium bromide (DTAB) and PEG-$t$-octylphenyl ether, have been reported to render NaYF$_4$:Yb,Er NPs hydrophilic via ligand encapsulation as well.$^{60}$ One interesting observation in this paper was that when a high concentration of DTAB was used to encapsulate the NPs, well-separated individual NPs could be obtained. With the decrease of DTAB concentration, the authors observed aggregated NPs forming spherical structures composed of several to several dozens of NPs. They explained this phenomenon by claiming that at very low DTAB concentrations, DTAB formed large-size microemulsion droplets that encapsulated multiple NPs to form large aggregates of NPs. As the surfactant concentration increased, the size of emulsion droplets became smaller thus contained fewer NPs. When the surfactant concentration was high enough, the size of the micelles became so small that only one individual NP could be encapsulated. According to the authors, this resulted in well-separated single NPs coated by a layer of DTAB.
1.2.4 Applications of Mass Cytometry in Bead-Based Bioassays

Cytometric bead-based bioassays have been widely used in genomics and proteomics as a high-throughput screening platform for cellular and serum biomarkers. Multiplexed bead-based assays require beads encoded with at least two different markers as ‘classifiers’. For bead-based bioassays by mass cytometry, the ‘classifier’ markers are Ln isotopes (Ln1 and Ln2, Figure 1-6). The beads have to be loaded with a high enough Ln content to generate detectable signals in mass cytometry. Also, the beads need to have a narrow size distribution and an average diameter preferably between 1 and 5 μm for complete pyrolysis in the ICP torch. Furthermore, the surface of the beads should be functional for conjugating bioaffinity agents with minimal non-specific binding.

Figure 1-6. A brief scheme of a 3-plex bead-based bioassay by mass cytometry.

Our research group has spent many years developing methods to prepare polymer beads that satisfy the above requirements. In 2009, Thickett et al. reported synthesis of Ln-encoded polystyrene (PS) beads via a seeded emulsion polymerization. The PS beads carried carboxyl groups on their surface introduced by the initiator 4,4’-azobis(4-cyanovaleric acid) (ACVA).
Those beads had average diameters between 600 and 900 nm and \( \sim 10^6 \) Ln ions per bead.\(^{61}\) Abdelrahman et al. developed an alternative approach to prepare carboxyl-functionalized PS beads using a two-stage dispersion polymerization. In this method, the carboxyl groups were introduced by acrylic acid (AA) or methacrylic acid (MAA) that copolymerizes with styrene during the synthesis.\(^{62,63}\) This method yielded PS beads with diameters on the order of 2 \( \mu \)m with a very narrow size distribution and a narrower bead-by-bead distribution of Ln content than the beads prepared by Thickett’s method. Nevertheless, the researchers were only able to attach a limited number of NeutrAvidin, as a model protein, to the surface of the beads. This problem was attributed to the presence of a polyvinylpyrrolidone (PVP) corona on the bead surface. The PVP corona was introduced during the bead synthesis as a surface-stabilizer. However, this corona impeded biomolecules from approaching the bead surface, thus caused a low protein binding capacity of the beads. To circumvent this problem, Abdelrahman et al. used their Ln-encoded PS beads as seeds for seeded emulsion polymerization of glycidyl methacrylate (GMA) to grow a poly(glycidyl methacrylate) (PGMA) shell on the bead surface.\(^ {64}\) The glycidyl groups, which are reactive towards amino groups on proteins, provided anchoring points for protein conjugation. This approach increased the amount of NeutrAvidin conjugated to the beads by a factor of 10 or more compared to the PVP-coated PS beads. This approach improved the potential of the beads for mass cytometric bioassays.

As an alternative approach, Lin et al. synthesized polymer microgels loaded with precipitated LnF\(_3\) as bead classifiers. They synthesized polymer microgels via a copolymerization of \( N \)-isopropylacrylamide (NIPAM), \( N \)-vinylcaprolactam (VCL) and MAA. The microgels were first loaded with LnCl\(_3\) in aqueous media, then NaF or NH\(_4\)F was added to precipitate LnF\(_3\) inside the microgels.\(^ {65}\) In a later report, they attached streptavidin to the surface of microgels and developed a streptavidin-biotin binding assay between those microgels and biotin-functionalized NaHoF\(_4\) NPs. The NaHoF\(_4\) NPs showed a much higher sensitivity in detecting low copy numbers of streptavidin on the surface of microgels than a biotinylated metal-chelating polymer.\(^ {66}\) In general, mass cytometric bead-based assays are still in a proof-of-concept stage compared to the numerous bead-based assays that have been developed and commercialized for flow cytometry.
1.2.5 Applications of Mass Cytometry in Cell Biology

In mass cytometry, most applications reported in the literature addressed questions in cell biology. For example, mass cytometric cell analysis can yield high-dimensional proteomics data, which allows deep immunophenotyping of cells and reveals rare cell subsets that cannot be identified by any other analytical techniques.\(^{67}\) It helps people better understand heterogeneity of patients by providing information on the pathogenesis of disease as well as guidance of therapeutic regimens, leading to development of personalized medicine.\(^{68}\) Also, the quantitative nature of mass cytometry allows unsupervised data analysis by computational tools, which circumvents user-dependent biased gating.\(^{69}\) A few selected applications of mass cytometry in cell biology are presented below.

1.2.5.1 Quantification of Cell Surface Receptors

Quantification of cell surface receptors, namely antigens or other ligand binding sites, is essential for various aspects of cell biology, including theoretical modeling of cellular networks, predicting saturation level of therapeutic compounds as well as kinetics of drug internalization into the cells.\(^{70}\) Quantitative assays of cell surface antigens based on flow cytometry have been realized for almost 20 years.\(^{71}\) Those assays require both calibration beads and antibodies conjugated with phycoerythrin (PE) with a 1:1 PE-antibody ratio. This increases the cost of analysis since preparation of monovalent PE-antibody conjugate usually results in low yield and a loss of the antibody during purification. Because of the quantitative nature of ICP-MS, mass cytometry is a more advantageous tool for quantifying cellular biomarkers than flow cytometry. Quantitative measurement of cell surface receptors can be achieved by directly staining cells with mass-tagged antibodies excluding the usage of calibration beads or antibodies conjugated with tags at a precise ratio. Wang and co-workers compared methods based on either conventional flow cytometry or mass cytometry for quantifying CD4 expression of peripheral blood mononuclear cells (PBMCs) either freshly prepared or cryopreserved.\(^{72}\) They used \(^{142}\)Nd-tagged anti-CD4 antibodies to stain the cells for mass cytometry analysis and obtained results that were consistent with flow cytometric assays using PE-conjugated anti-CD4 antibodies to stain the cells. Moreover, the authors demonstrated the potential of mass cytometry for simultaneous quantification of multiple cell surface receptors in one single assay, which is very challenging for flow cytometry due to the spectral overlap of different fluorophores.
1.2.5.2 Cell Barcoding

In conventional flow cytometry, cell barcoding is a technique that labels individual cells of interest with a mixture of fluorescent dyes of different intensities, leaving each cell sample tagged with a ‘barcode’ of fluorophores as an identifier. It allows multiple cell samples to be mixed and analyzed in a single tube and greatly increases the throughput of multiplexed cell assays. In mass cytometry, thiol- or amine-reactive mass-tagged reagents are used instead of fluorescent dyes for cell barcoding. Nolan and co-workers used maleimido-DOTA chelator with seven different Ln isotopes to generate $2^7 = 128$ combinations, which is enough to barcode each sample in a 96-well plate. Since antibody labeling of cell surface biomarkers also require Ln isotopes, the same research group later developed a functional Pd-EDTA complex for cell barcoding. The isotopes of Pd do not interfere with Ln-based cell labeling reagents, thus avoid reduction of available antibody measurement channels. One drawback of this technology is that the barcoding staining requires cell fixation by paraformaldehyde (PFA) and at least partial permeabilization of cell membrane by methanol treatment, which makes it incompatible with PFA- or methanol-sensitive cell surface epitopes. To circumvent this problem, Mei and co-workers used anti-CD45 antibodies tagged with six different metal isotopes (Pd or In) for barcoding of live cells without fixation and cell membrane permeabilization. For cells that express low levels of CD45 e.g. non-leukocytes, this approach can be modified by using antibodies against other biomarkers that are particularly suitable to the cells of interest.

1.2.5.3 Cell Cycle Analysis

Cell cycle analysis distinguishes cells in different phases of the cell cycle. It is a useful tool for measuring cell activation, malignancy and apoptosis. In conventional flow cytometry, cell cycle assays use DNA-intercalating fluorescent dyes, typically propidium iodide, to differentiate cells in the three major stages of the cell cycle (G1 vs S vs G2/M). The extent of dye-staining is correlated with the amount of DNA they contain. In 2012, Nolan and co-workers adapted mass cytometry for cell cycle analysis. They developed a multiplexed assay that measures both stage-specific cell cycle protein biomarkers (cyclin B1, phosphorylated histone H3 and retinoblastoma protein) and DNA content as well as DNA replication of each cell. They used idoxuridine, a nucleobase-like molecule that can be incorporated into DNA replication, to specifically label cells in the S stage. Since idoxuridine contains $^{127}$I, an isotope inside the detectable mass range of mass cytometry, the incorporation of idoxuridine could be directly measured, bypassing the need for an
antibody or DNA intercalation. The researchers compared the results of their mass cytometric cell cycle assay with an analogous assay based on flow cytometry and found that the two assays yielded results in agreement with each other.\textsuperscript{77}

1.2.5.4 Studies of Cell Signaling States

Antigen- or cytokine-triggered cell signaling pathways commonly involve phosphorylation of signaling proteins. This step generates a binding site for a different protein and induces protein-protein interactions for signal transduction. Measurement of phosphorylation states of signaling proteins can reveal information about antigen-induced cell activation, kinetics of signaling and downstream biomarkers involved in the signaling cascade.\textsuperscript{7} Nolan and co-workers reported a phospho-protein assay based on mass cytometry that measured 14 phosphorylation sites of 14 types of human peripheral blood mononuclear cells under 96 conditions, resulting in 18,816 quantified phosphorylation levels from each sample.\textsuperscript{74} Benoist and co-workers used mass cytometry to study T-cell receptor signaling kinetics in non-obese diabetic (NOD) mice. They uncovered a mode of signal propagation in T cells in which small initial differences could lead to large defects in activation of downstream signaling proteins. They used this mode to explain the relatively low ERK (extracellular signal-regulated kinase) activation in NOD mice.\textsuperscript{78}

1.3 Research Objectives

My doctoral research had three main objectives:

1. To synthesize lanthanide nanoparticles with diameters between 10 and 30 nm and with a narrow size distribution.

2. To develop surface modification strategies that make the lanthanide nanoparticles colloidally stable in phosphate buffers. Thus the nanoparticles are suitable for applications in mass cytometry.

3. To synthesize surface-functionalizable polymer microspheres encoded with lanthanide nanoparticles and to develop a proof-of-concept test of their use in bead-based bioassays by mass cytometry.

In the following sections, I will provide more details about how I planned to achieve these objectives.
1.3.1 Preparation of Functional NaLnF₄ NPs as Elemental Tags for Bioassays in Mass Cytometry

As I mentioned in Section 1.2.2.3, to improve the sensitivity of bioassays based on mass cytometry, scientists require high-quality NaLnF₄ NPs as next-generation elemental tags. For mass cytometry applications, one has to prepare NaLnF₄ NPs with a narrow size distribution and an average diameter preferably between 10 nm and 30 nm, since each NaLnF₄ NP in this size range can carry enough Ln ions to generate detectable mass signal without causing incomplete pyrolysis in the ICP torch. The size of NPs in this range is also close to the dimension of an antibody (Figure 1-7). Moreover, the surface of NaLnF₄ NPs has to be modified to satisfy the multiple requirements described as follows.

![Figure 1-7. Structure of a whole IgG antibody with approximate dimensions. Reprinted with permission from ref. 79.](image)

First of all, since all the bioassays take place in aqueous media, the Ln NPs must be water-dispersible. Syntheses of NaLnF₄ NPs are typically performed at high temperatures (between 250 and 330 °C) in high-boiling-point solvents such as OAH, OM and ODE. The surface of these as-prepared NPs is coated with hydrophobic ligands (OA or OM). For biological applications, the surface of Ln NPs has to be modified to render the NPs hydrophilic while maintaining their colloidal stability and physiochemical properties.

Secondly, the surface of the Ln NPs needs to be modified to prevent non-specific binding to cells. Non-specific adherence of Ln NPs to cell surface reduces their targeting specificity and leads
to false positive signals in CyTOF measurements. The most common strategy to reduce non-specific cell binding of NPs is to coat the surface of NPs with a layer of poly(ethylene glycol) (PEG) molecules. The effectiveness of PEG coating is affected by both grafting density and the length of the PEG.\footnote{80–82}

Thirdly, the surface of Ln NPs should have functional groups, such as –COOH, –NH$_2$, –SH, etc. to conjugate with targeting ligands. In the context of bioassays based on mass cytometry, the ligands could either be antibodies that directly target cell surface biomarkers or avidin/streptavidin/neutravidin that target biotinylated secondary antibodies. Optional methods of conjugating those ligands to the surface of Ln NPs include EDC/NHS mediated coupling between –NH$_2$ and –COOH groups or Michael addition reaction between –SH and maleimide groups.

In my research, I first synthesized monodisperse NaLnF$_4$ NPs with average diameters between 10 and 20 nm, then I developed two surface modification strategies to prepare NaLnF$_4$ NPs that satisfy the requirements above. One method was to replace the original oleate ligands on the surface of NaLnF$_4$ NPs with functional PEG-tetraphosphonate ligands. The tetraphosphonate anchoring groups bind to the surface of NaLnF$_4$ NPs and the hydrophilic PEG chains provide colloidal stability for the NPs in aqueous media. The PEG coating layer also suppresses non-specific binding of NPs to proteins or cell surfaces. For conjugation with targeting biomolecules e.g. antibodies or neutravidins, functional groups can be introduced to the surface of NaLnF$_4$ NPs by using heterobifunctional PEG-tetraphosphonate ligands.

The other method is to coat the surface of NaLnF$_4$ NPs with a silica shell. The silica coating was realized by a reverse microemulsion method, then the surface of silica-coated NaLnF$_4$ (NaLnF$_4$@SiO$_2$) NPs is modified by different silane reagents to introduce functional groups such as –NH$_2$, –COOH or –SH. The functional groups can either be used to directly conjugate bioaffinity agents or attach PEG molecules to create a second PEG coating layer that reduces non-specific cell binding of NaLnF$_4$ NPs.

1.3.2 Preparation of Poly(methyl methacrylate) (PMMA) Microspheres Encoded with Small LnF$_3$ NPs for Bead-Based Assays in Mass Cytometry

As I stated in Section 1.2.4, the PVP corona on the surface of PS microbeads impeded conjugation of bioaffinity agents, thus limited their application in bead-based bioassays. To
circumvent this problem, I decided to seek an alternative strategy to prepare Ln-encoded polymer microspheres with accessible functional groups on their surface corona. For this purpose, I collaborated with Dr. Jianbo Tan, a visiting student from Sun Yat-sen University, to establish a separate project for synthesizing PMMA microspheres by photo-initiated RAFT dispersion polymerization, a synthetic method invented by Dr. Tan. The RAFT agent used in the synthesis was a copolymer of oligo(ethylene glycol) methyl ether acrylate (OEGA) and acrylic acid (AA) with a trithiocarbonate terminal group. Both the OEGA chains and carboxyl groups on AA provide colloidal stability for the PMMA microspheres in aqueous media. The carboxyl groups also provide anchoring points for bioaffinity agents. The PMMA microspheres were internally labeled with small LnF₃ NPs for mass cytometry analysis. Those LnF₃ NPs carried polymerizable double bonds on their surface, which made it possible for the NPs to be incorporated into the PMMA microspheres via copolymerization during the polymer synthesis. In this part of the project, I was in charge of the preparation of the polymerizable small LnF₃ NPs and Dr. Tan was in charge of the synthesis of PMMA microspheres encoded with those small LnF₃ NPs.

The applicability of the PMMA microspheres as hosts for attaching bioaffinity agents was first tested by a biotin binding assay. In this assay, FITC-labeled streptavidin (FITC-SAv) was covalently attached to the surface of COOH-functionalized PMMA microspheres, then the biotin-binding capacity of this SAv-coated PMMA microspheres was quantified by three independent methods: fluorescence titration, ICP-MS and mass cytometry.

After the surface functionality of PMMA microspheres was tested, I moved forward to develop a sandwich-type bead-based immunoassay. In this assay, I covalently attached capture antibodies onto the PMMA microspheres. The capture antibodies bind to a certain type of analyte, which is then labeled by biotinylated detection antibodies. I prepared NeutrAvidin-conjugated NaYF₄@SiO₂ NPs as reporter tags that bind to biotinylated antibodies. The result of this bead-based immunoassay was characterized by TEM.

1.4 Thesis Organization

This thesis contains seven chapters, including this one. Chapter 2 covers general experimental methods. Chapter 3 describes synthesis of a poly(amidoamine)-tetraphosphonate (PAMAM-4P) ligand, ligand exchange of PAMAM-4P with OA-capped NaLnF₄ (Ln = Gd or Tb) NPs following by PEGylation and colloidal stability tests of PEG-PAMAM-4P capped NaLnF₄
NPs. Chapter 4 describes preparation of PMMA microspheres encoded with small LnF\textsubscript{3} NPs, characterization of Ln-encoded PMMA microspheres with TEM and CyTOF, conjugation of FITC-SAv onto PMMA microspheres and subsequent biotin-binding assays. Chapter 5 first describes preparation, surface modification and bioconjugation of NaYF\textsubscript{4}@SiO\textsubscript{2} NPs, then reports a sandwich bead-based immunoassays using Ln-encoded PMMA microspheres as support and NaYF\textsubscript{4}@SiO\textsubscript{2} NPs as reporter tags. Chapter 6 describes an alternative way to functionalize the surface of NaHoF\textsubscript{4}@SiO\textsubscript{2} NPs following by PEGylation and antibody attachment, then displays the performance of NaHoF\textsubscript{4}@SiO\textsubscript{2}-PEG NPs and their immunoconjugates as reporter tags in cell assays based on mass cytometry. The final chapter, Chapter 7, discusses possible future extensions of my project.
Chapter 2: General Experimental Methods

2.1 Overview

This chapter contains general experimental methods applied in subsequent chapters.

2.2 Instrumentation and Characterization

2.2.1 Transmission and Scanning Electron Microscopy (TEM and SEM)

For TEM measurements, hydrophobic materials, mostly OA capped NaLnF₄ NPs, were first dispersed in hexane or cyclohexane and then directly drop-cast onto hydrophobic Formvar-carbon-coated copper TEM grids. Hydrophilic materials e.g. PAMAM-4P or PEG-PAMAM-4P capped NaLnF₄ NPs, NaLnF₄@SiO₂ NPs and PMMA microspheres, were dispersed in DI-water. Then a drop of the aqueous particle solution was drop-cast onto the uncoated side of TEM grids and the grids were left still until water completely evaporated. All the TEM images were obtained were obtained with a Hitachi H-7000 TEM instrument at an accelerating voltage of 100 kV.

For SEM measurements, the PMMA microspheres were dispersed in DI-water and the particle solution was drop-cast onto mica. All the SEM images were obtained on a Hitachi S-5200 field-emission SEM instrument at an accelerating voltage of 1 kV.

2.2.2 Confocal Microscopy

Confocal microscopy measurements were done on a Leica TCS SP2 microscope. The fluorescein fluorophore of FITC-SA or FITC-NA was excited at 488 nm and examined with the green fluorescence channel (490–540 nm) of the instrument.

2.2.3 Nuclear Magnetic Resonance (NMR) Spectroscopy

¹H-, ¹³C- and ³¹P-NMR spectra of organic compounds were acquired on either a Bruker Avance III 400 MHz or a Varian VNMRS 400 MHz NMR spectrometer at ambient temperature. Small molecules were analyzed with 64 transients and a delay time of 1 second. Polymers were analyzed with 512 transients and a delay time of 10 seconds.
2.2.4 Mass Spectrometry

The accurate masses of all organic compounds were measured on an Agilent 6538 Q-TOF system.

ICP-MS measurements were performed on a PerkinElmer ELAN 9000 ICP-MS spectrometer under normal conditions (1400 W forward plasma power, 17 L/min Ar plasma gas flow, 1.2 L/min auxiliary Ar flow, and 0.95 L/min nebulizer Ar flow). A cross-flow double pass spray chamber (PerkinElmer SCIEX) was employed in all instances. All the experiments were conducted using an autosampler (PerkinElmer AS 93) modified for operation with Eppendorf 1.5 mL tubes. Sample volumes were fixed at 1.0 mL. The sample uptake rate was adjusted depending on the particular experiment, typically 100 μL/min. A standard solution was prepared from 1000 μg/mL PerkinElmer pure Single-Element Standard solution (PerkinElmer, Shelton, CT) by sequential dilution with high-purity HNO₃. Diluted high-purity HNO₃ (3%) was measured as a blank signal, which was subtracted from the sample signal.

NPs for ICP-MS measurements were digested in concentrated HNO₃ (ICP grade, Seastar Chemical Inc.) by treating an aliquot (0.5 mL) of NP dispersion in water with concentrated HNO₃ (4.5 mL) in a capped glass vial and stirring at room temperature overnight.

2.2.5 Dynamic Light Scattering (DLS) and Zeta Potential Measurement

Both DLS and zeta potential measurements were performed on a Malvern Zetasizer Nano ZS instrument at a backscattering angle of 173°. For zeta potential measurements, the solutions were placed in DTS 1060 folded capillary cells (Malvern, Worcestershire, UK). All the NP solutions were passed through a 0.2 μm cellulose syringe filter before any DLS or zeta potential measurement.

2.2.6 X-Ray Diffraction (XRD)

XRD measurements were performed on a Philips XRD system, which is composed of a PW 1830 HT generator, a PW 1050 goniometer and PW3710 control electronics. All the materials for XRD measurements were lyophilized overnight and ground to obtain a fine powder.
2.2.7 Thermogravimetric Analysis (TGA)

TGA measurements were performed on a TA SDT Q600 thermogravimetric analyzer. All of the TGA measurements on NPs were performed in an air atmosphere. The NP samples were heated from room temperature to 100 °C at a ramping rate of 10 °C/min, equilibrated at 100 °C for 30 min to remove any absorbed water, then heated to 800 °C at the same rate of 10 °C/min.

2.2.8 Fourier Transform Infrared (FT-IR) Spectroscopy

FT-IR spectra were recorded on a PerkinElmer Spectrum 1000 infrared spectrometer using the KBr pellet method. All the materials for FT-IR measurements were lyophilized overnight and ground to obtain a fine powder.

2.2.9 Ultraviolet-visible (UV-vis) and Fluorescence Spectroscopy

UV-vis absorption measurements were performed on a PerkinElmer Lambda 35 UV/vis spectrometer. Fluorescence measurements were carried out with a HORIBA Jobin Yvon SPEX Fluorolog-3 spectrometer.

2.2.10 Size-Exclusion Chromatography (SEC)

The molecular weight and the polydispersity of polymers were measured with a Viscotek size-exclusion chromatography (SEC) instrument equipped with a Viscotek VE 3580 refractive index detector and Viscotek ViscoGEL G4000PWXL and G2500PWXL columns (kept at 30 °C). The flow rate was maintained at 1.0 mL/min using a Viscotek VE 1122 solvent delivery system and VE7510 degasser. An eluent of 0.2 mol/L KNO₃, 200 ppm of NaN₃, and 25 mmol/L pH 8.5 phosphate buffer was used. The system was calibrated with poly(methacrylic acid) standards.

2.2.11 Mass Cytometry

Labeled cells or Ln-containing PMMA microspheres (beads) for bioassays were analyzed by mass cytometry using a CyTOF® 2 instrument (Fluidigm Canada, formerly DVS Sciences, Markham, ON, Canada). In general, cells or beads were suspended in DI-water, individually introduced and pyrolyzed in an ICP flame, thus generated a cloud of ions. The resultant ion cloud was analyzed by a TOF mass analyzer. Data were collected in FCS 3.0 format and processed by FlowJo vX software.
For bead-based assays, the Ln-containing polymer beads were diluted in DI-water to a final concentration of ~$10^6$ beads/mL, then the bead suspension was directly injected to the instrument.

For cell assays, a solution of 0.1X beads was prepared by diluting 1 part of EQ™ Four Element Calibration Beads (Fluidigm Canada, Markham, ON, Canada) with 9 parts of DI-water, then the washed cell pellet was resuspended in the 0.1X bead solution and filtered through a 35 to 45-micron mesh filter right before injection to the instrument. The data were normalized against the Calibration Beads according to the user guide provided by Fluidigm.
Chapter 3: Preparation and Characterization of Functional PEG-PAMAM-4P Capped NaLnF$_4$ Nanoparticles

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3.1 Introduction

Researchers have reported various ligand exchange strategies to render the surface of NaLnF$_4$ NPs hydrophilic. Although those studies often use photographic images or DLS measurements to indicate that their NPs are dispersible in water, most of them lack proper tests of colloidal stability of their NPs in buffer-containing media. This is crucial since people have shown that particle aggregation can dramatically affect their biological activities.$^{83}$ In addition, many biological buffers contain phosphate ions, which can competitively replace the original ligands on the surface of NaLnF$_4$ NPs because of the high affinity between phosphate and Ln ions. For instance, Boyer et al. reported surface modification of upconverting NaYF$_4$ NPs with PEG-monophosphate ligands and used the NPs for cell imaging experiments. They did not, however, report the colloidal stability of NPs in the buffer media they used.$^{31}$ I followed their strategy to prepare NaLnF$_4$ NPs capped with the same PEG-monophosphate ligands and noticed that the NPs rapidly aggregate and precipitate in phosphate-containing buffers. Our group previously reported a PEG-tetraphosphonate ligand that can stabilize NaLnF$_4$ NPs in phosphate buffered saline (PBS).$^{32}$ Nevertheless, the synthesis was relatively tedious. Inspired by our previous studies, I developed an alternative method to prepare surface functional NaLnF$_4$ NPs that are colloidally stable in phosphate-containing buffer media.

In this chapter, I first report the synthesis of a tetraphosphonate ligand based on a poly(amidoamine) (PAMAM) skeleton. This ligand is abbreviated as PAMAM-4P. Then I describe a two-step ligand exchange protocol to replace the oleates on the as-synthesized NaLnF$_4$ NPs with PAMAM-4P ligands to make the NPs dispersible in aqueous media. PEGs were subsequently attached onto the surface of PAMAM-4P capped NaLnF$_4$ NPs, and the colloidal
stability of NaLnF4 NPs capped with different ligands were tested against water and phosphate-containing buffer media by DLS.

To better interpret the binding nature of ligands to the surface of NaLnF4 NPs, I performed an experiment to show that the presence of excess ligands in water may lead to surface etching of NaLnF4 NPs. As a result, it is essential to remove excess ligands right after ligand exchange. After removal of excess ligands, I quantified the surface ligand density of NaLnF4 NPs by TGA. To introduce surface functionality for the NaLnF4 NPs, I used a mixture of methoxy-PEG and biotin-PEG to PEGylate the surface of PAMAM-4P capped NaLnF4 NPs. The surface ligand density of biotinylated NaLnF4 NPs was quantified independently by a HABA(4’-hydroxyazobenzene-2-carboxylic acid)/Avidin assay, and the result agreed well with ligand density values given by TGA analysis.

In addition, I compared the colloidal stability of my PEG-PAMAM-4P capped NPs with same NPs capped with a different ligand, PEG-lysine-4P. This ligand was synthesized by my colleague Dr. Pengpeng Cao. PEG-lysine-4P also has four phosphonate anchoring groups that bind to the surface of NaLnF4 NPs but structurally differs from PEG-PAMAM-4P. DLS studies disclosed considerable difference between PEG-PAMAM-4P and PEG-lysine-4P capped NaLnF4 NPs in terms of their long-term colloidal stability in phosphate-containing buffer, which implied that factors other than the number of anchoring groups, e.g. spacing of the anchoring groups, also greatly impact the ability of ligands stabilizing NPs.

### 3.2 Experimental Details

#### 3.2.1 Materials

LnCl3·6H2O (99.9% trace metals basis), oleic acid (90%), 1-octadecene (90%), NH4F (98%), N-boc-ethylenediamine (98%), methyl acrylate (99%), ethylenediamine (99%), diethyl vinylphosphonate (97%), PEG monomethyl ether (Mn 750 or 2,000), Arsenazo III and most solvents were purchased from Sigma-Aldrich and used without further purification. NaOH (reagent grade pellets) was obtained from Caledon (Georgetown, ON, Canada). All other PEG derivatives were purchased from JenKem Technology (Plano, TX, USA). Dry THF was obtained from an Innovative Technology Solvent Purification System (Amesbury, MA, USA). Ultrapure water (R = 18.2 megaohm) was obtained from a Milli-Q water purification system (EMD
Millipore, Billerica, MA, USA). The Pierce Biotin Quantitation Kit and Slide-A-Lyzer G2 Dialysis Cassettes were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All the dialyses employed 10k MWCO, 3 mL capacity Slide-A-Lyzer G2 Dialysis Cassettes unless otherwise noted.

3.2.2 Synthesis of OA Capped NaLnF₄ NPs (Ln = Gd or Tb)

NaLnF₄ NPs were synthesized according to a literature procedure with some modifications. First, LnCl₃·6H₂O (1 mmol), oleic acid (OAH) (16 mL) and 1-octadecene (ODE) (16 mL) were heated to 120 °C in a three-necked flask under vacuum for 1 hour to form a clear solution. Then the solution was cooled to 50 °C. A CH₃OH solution (10 mL) of NH₄F (0.15 g, 4.0 mmol) and NaOH (0.10 g, 2.5 mmol) was added dropwise via a syringe and the resulting cloudy mixture was stirred for 30 min after the addition was completed. The solution was slowly heated to 100 °C under gentle vacuum to evaporate CH₃OH. When all the CH₃OH was evaporated the solution was heated to 300 °C at a rate of 13 °C/min under N₂ atmosphere and maintained at 300 °C for 1 h. Then the solution was cooled to room temperature and the as-prepared NaLnF₄ NPs were stored in the reaction medium.

3.2.3 Synthesis of Organic Compounds

3.2.3.1 Synthesis of Poly(ethylene glycol) Monomethyl Ether Phosphoric Acid (mPEG-OPO₃H₂)

The monophosphoric acid ligands, mPEG750- and mPEG2000-OPO₃H₂, were synthesized according to a literature procedure. In a typical synthesis, 18.5 mmol of mPEG750 (13.9 g) or mPEG2000 (37 g) was dried at 70 °C in a vacuum oven overnight. The dried mPEG was then dissolved in 60 mL of dry THF. To the resulting solution, a solution of POCl₃ (1.7 mL, 18.5 mmol) in 10 mL of dry THF was added dropwise. Then the mixed solution was heated to 55 °C under a nitrogen flow to remove HCl formed in the reaction. The reaction mixture was maintained at 55 °C for 6 hours and then cooled to room temperature. A portion of cold DI-water (5 mL) was added dropwise to the solution and the mixture was allowed to react for 1 hour. Then the THF was gently removed in vacuo on a rotary evaporator, and the resulting product was dried in a vacuum oven at 70 °C overnight to remove any possible water residue. The crude product was recrystallized from isopropanol to obtain a white solid.
### 3.2.3.2 Synthesis of PAMAM Dendrimer, N-Boc-Ethylenediamine Core, Generation 0.0 (Compound 2)

This synthesis followed the strategy described by Cao et al.\textsuperscript{32} N-Boc-ethylenediamine (1.94 mL, 12.5 mmol) was dissolved in CH\textsubscript{3}OH (7 mL), then a CH\textsubscript{3}OH solution (8 mL) of methyl acrylate (4.53 mL, 50.0 mmol) was added. The solution was stirred at room temperature for 2 days, then the CH\textsubscript{3}OH and excess methyl acrylate were evaporated in vacuo to afford compound 1 as a colorless liquid. Yield: 4.07 g (98%). \textsuperscript{1}H-NMR (400 MHz, CDCl\textsubscript{3}): \(\delta\) 5.12 (1H, br s, BocNH\textsubscript{2}), 3.69 (6H, s, -OCH\textsubscript{3}), 3.18 (2H, d, BocNHCH\textsubscript{2}), 2.75 (4H, t, -CH\textsubscript{2}N(CH\textsubscript{2})\textsubscript{2}), 2.52 (2H, t, -CH\textsubscript{2}N(CH\textsubscript{2})\textsubscript{2}), 2.43 (4H, t, -CH\textsubscript{2}CO\textsubscript{2}), 1.45 (9H, s, -C(CH\textsubscript{3})\textsubscript{3}).

Compound 1 (3.78 g, 11.3 mmol) was dissolved in CH\textsubscript{3}OH (20 mL), then a CH\textsubscript{3}OH solution (30 mL) of ethylenediamine (44 mL, 660 mmol) was added. The solution was stirred at room temperature for 5 days, then the CH\textsubscript{3}OH and excess ethylenediamine were evaporated in vacuo to afford compound 2 as a light yellow liquid. Yield: 4.33 g (99%). \textsuperscript{1}H-NMR (400 MHz, CDCl\textsubscript{3}): \(\delta\) 7.41 (2H, br, -CONH\textsubscript{2}), 6.49 (1H, br, BocNH\textsubscript{2}), 3.29 (4H, m, -CH\textsubscript{2}N(CH\textsubscript{2})\textsubscript{2}), 3.18 (2H, d, BocNHCH\textsubscript{2}), 2.85 (4H, t, -CH\textsubscript{2}CH\textsubscript{2}N\textsubscript{2}), 2.73 (4H, t, -CH\textsubscript{2}N(CH\textsubscript{2})\textsubscript{2}), 2.53 (2H, br, -CH\textsubscript{2}N(CH\textsubscript{2})\textsubscript{2}), 2.34 (4H, t, -CH\textsubscript{2}CONH\textsubscript{2}), 1.44 (9H, s, -C(CH\textsubscript{3})\textsubscript{3}). The NMR results of 1 and 2 were in accord with literature values.\textsuperscript{84}

### 3.2.3.3 Synthesis of PAMAM-4P Ligand (Compound 4)

Diethyl vinylphosphonate (3.60 mL, 23.8 mmol) was added to a solution of 2 (1.99 g, 5.1 mmol) in DI-water (10 mL) and stirred at room temperature for 5 days. Then water and unreacted diethyl vinylphosphonate were removed on a rotary evaporator. The crude product was purified by silica column chromatography (eluent: 40:3 CHCl\textsubscript{3}−CH\textsubscript{3}OH) to afford compound 3 as a light-yellow liquid. Yield: 1.59 g (30%). \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}): \(\delta\) 7.89 (2H, br, -CONH\textsubscript{2}), 5.62 (1H, br, BocNH\textsubscript{2}), 4.09 (16H, m, -OCH\textsubscript{2}CH\textsubscript{3}), 3.30 (4H, m, -CONHCH\textsubscript{2}CH\textsubscript{2}), 3.17 (2H, d, BocNHCH\textsubscript{2}), 2.77−2.82 (12H, m, -CONHCH\textsubscript{2}CH\textsubscript{2} and -CH\textsubscript{2}CH\textsubscript{2}PO(OCH\textsubscript{2}CH\textsubscript{3})\textsubscript{2}), 2.56 (6H, m, BocNHCH\textsubscript{2}CH\textsubscript{2} and BocNHCH\textsubscript{2}CH\textsubscript{2}N(CH\textsubscript{2})\textsubscript{2}), 2.35 (4H, t, -CH\textsubscript{2}CH\textsubscript{2}CONH\textsubscript{2}), 1.92 (8H, m, -CH\textsubscript{2}PO(OCH\textsubscript{2}CH\textsubscript{3})\textsubscript{2}), 1.43 (9H, s, -C(CH\textsubscript{3})\textsubscript{3}), 1.33 (24H, t, -OCH\textsubscript{2}CH\textsubscript{3}). \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}): \(\delta\) 172.6, 156.1, 78.9, 61.7, 61.6, 52.5, 51.4, 49.9, 46.8, 46.2, 38.4, 36.9, 33.6, 28.5, 23.6, 22.2, 16.5, 16.4. \textsuperscript{31}P NMR (160 MHz, CDCl\textsubscript{3}): \(\delta\) 30.6 (s, -PO(OCH\textsubscript{2}CH\textsubscript{3})\textsubscript{2}). HRMS \(m/z\) for C\textsubscript{41}H\textsubscript{89}N\textsubscript{6}O\textsubscript{16}P\textsubscript{4} ([M + H]) calculated 1045.5280, found 1045.5270.
Bromotrimethylsilane (TMSBr) (2.0 mL, 14.5 mmol) was added dropwise to a solution of 3 (0.77 g, 0.73 mmol) in anhydrous CH$_2$Cl$_2$ (10 mL). The solution was sealed and stirred at room temperature for 24 h, and then CH$_2$Cl$_2$ and excess TMSBr were removed on a rotary evaporator. The residue was then dissolved in water (5 mL) and stirred for 10 min to hydrolyze the TMS esters. To the resulting turbid solution, was added 1 M NaOH aq solution dropwise until pH ≈ 10, during which the solution turned clear. Water was removed by vacuum evaporation, and the residue was dissolved in CH$_3$OH (5 mL). The CH$_3$OH solution was filtered to remove any insoluble inorganic salts. Then the filtrate was dried again, and the residue was lyophilized overnight to remove any water residue and generate the product 4 as a light-yellow powder. Yield: 0.52 g (79%). $^1$H NMR (400 MHz, 0.1 M NaOD in D$_2$O): δ 3.24 (4H, t, −CONHC$_2$H$_2$−), 2.71 (4H, t, NH$_2$CH$_2$CH$_2$N(CH$_2$)$_2$−), 2.58–2.62 (12H, m, NH$_2$CH$_2$H$_2$− and −CH$_2$CH$_2$PO(OCH$_2$CH$_3$)$_2$−), 2.51 (4H, t, −CONHCH$_2$CH$_2$−), 2.46 (2H, t, NH$_2$CH$_2$H$_2$−), 2.33 (4H, t, −CH$_2$CH$_2$CONH−), 1.49 (8H, m, −CH$_2$PO$_3$Na$_2$). $^{13}$C NMR (100 MHz, 0.1 M NaOD in D$_2$O): δ 174.9, 54.8, 50.6, 49.2, 48.7, 37.8, 36.7, 32.7, 26.2, 24.9. $^{31}$P NMR (160 MHz, 0.1 M NaOD in D$_2$O): δ 19.7 (s, −PO$_3$Na$_2$). HRMS $m/z$ for C$_{20}$H$_{47}$N$_6$O$_{14}$P$_4$ ([M − H]−) calculated 719.2106, found 719.2109.

3.2.4 Ligand Exchange

A sample of OA capped NaLnF$_4$ NPs stored in the OAH and ODE mixture (4.5 mL) was precipitated by adding EtOH (22 mL) followed by centrifugation (2700 × g, 30 min). After decanting the supernatant, the precipitated NPs were redispersed in THF (3 mL) by sonication and precipitated with fresh EtOH (15 mL), then sedimented again by centrifugation. This washing process was repeated three times. Often the NPs purified in this way were stored as a colloidal solution in cyclohexane.

To estimate the concentration of NPs in the OAH-ODE reaction mixture, we took 1.0 mL of the reaction mixture, purified the NPs as described and dried the precipitate. Typically, the weight of the dry powder was between 6 and 7 mg.

3.2.4.1 Preparation of mPEG750- or mPEG2000-OPO$_3$ Capped NaLnF$_4$ NPs

For ligand exchange experiments, freshly purified NaLnF$_4$ NPs were precipitated with ethanol. The ethanol was decanted to obtain a moist precipitate. A still-moist sample of NaLnF$_4$
NPs (∼30 mg) was redispersed in THF (3 mL) with sonication. A THF solution (1.5 mL) of mPEG750-OPO\textsubscript{3}H\textsubscript{2} (75 mg) or mPEG2000-OPO\textsubscript{3}H\textsubscript{2} (200 mg) or was added. The solution was stirred at room temperature for 24 h, then the solution was mixed with 3 mL of DI water. The mixed solution was extracted with hexanes (1 mL) to remove the displaced OA, and the aqueous phase was gently warmed on a water bath in vacuo to evaporate THF. The resulting aqueous solution was dialyzed in a 10k MWCO dialysis cassette against 3 L of DI-water for 24 h to remove the excess mPEG-OPO\textsubscript{3}H\textsubscript{2}.

3.2.4.2 Preparation of PAMAM-4P Capped NaLnF\textsubscript{4} NPs

To a dialyzed aqueous solution containing mPEG750-OPO\textsubscript{3} capped NaLnF\textsubscript{4} NPs, an aqueous solution (1 mL) of PAMAM-4P (25 mg) was added. The solution was stirred at room temperature for 24 h, then dialyzed in a 10k MWCO dialysis cassette against 3 L of DI-water for 24 h to obtain PAMAM-4P capped NaLnF\textsubscript{4} NPs without excess free ligands.

3.2.5 Particle Etching Experiments

A sample of mPEG750- or mPEG2000-OPO\textsubscript{3} capped NaGdF\textsubscript{4} NPs (∼30 mg) was prepared according to the procedure described in section 3.2.4.1, except that excess ligands were not removed by dialysis. The NP solution in water, along with excess ligands, were diluted to a total volume of 15 mL by adding DI-water. Then the solution was divided into three 5-mL portions. Each was added to a D-Tube Dialyzer Mega (10 mL, 6-8k MWCO, EMD Millipore). The D-Tube Dialyzer was then placed in a 250 mL beaker containing 150 mL DI-water or two different concentrations (1.0 mM, 2.5 mM) of mPEG-OPO\textsubscript{3}H\textsubscript{2} in water. The dialysis was continued for a period of one week. Aliquots (1 mL) of solution outside the D-Tube Dialyzer were taken periodically to analyze for Gd\textsuperscript{3+} ion concentration.

The concentration of Gd\textsuperscript{3+} ions in the dialysis solution was quantified by a colorimetric assay based on the chromogenic reaction between Gd\textsuperscript{3+} ion and Arsenazo III dye. At pH 7.4, Arsenazo III has an absorption peak at 530 nm. The addition of Gd\textsuperscript{3+} to Arsenazo III gives rise to two new peaks at 655 nm and 610 nm, respectively. The absorbance at 655 nm A(655), which quantitatively corresponds to the Gd-Arsenazo III complex, was used to determine the concentration of Gd\textsuperscript{3+}.
To obtain a standard curve of $A_{(655)}$ versus [Gd$^{3+}$], in each UV-vis cuvette we pipetted 2500 μL of 2.5 mM 1:1 Tris-AcOH buffer, 150 μL of 5.0 mM mPEG-OPO$_3$H$_2$ aqueous solution, 40 μL of 1.0 mM Arsenazo III aqueous solution, 10 μL of DI-water and 300 μL of 0, 10, 20, 40, 60, 80 or 100 μM freshly prepared GdCl$_3$ aqueous solution, which led to a total volume of 3,000 μL. The absorption spectra were measured on a PerkinElmer Lambda 35 UV-vis spectrometer. The standard curve of $A_{(655)}$ versus [Gd$^{3+}$] is shown in Figure 3-18.

To quantify the concentration of Gd$^{3+}$ in the external dialysis solution, we added 300 μL of the dialysis solution taken at different times to UV-vis cuvettes to which had been added 1:1 (v/v) Tris-AcOH buffer (2500 μL, 2.5 mM), aqueous mPEG-OPO$_3$H$_2$ (150 μL, 5.0 mM), Arsenazo III dye solution (40 μL of 1.0 mM in water), and DI-water (10 μL, to make the total volume 3.00 mL). The absorbance at 655 nm was converted to [Gd$^{3+}$] using the $A_{(655)}$ vs [Gd$^{3+}$] standard curve shown in Figure 3-18.

3.2.6 PEGylation and Biotin Functionalization of PAMAM-4P Capped NaLnF$_4$ NPs

To an aqueous solution (3 mL) containing PAMAM-4P capped NaLnF$_4$ NPs (∼15 mg) was added 1 mL of 0.5 M NaHCO$_3$ buffer (pH 8.5), followed by addition of an aqueous solution (1 mL) of mPEG2000-NHS (20 mg). The solution was stirred at room temperature for 4 hours and then dialyzed against 3 L of DI-water for 48 hours to obtain mPEG2000-PAMAM-4P capped NaLnF$_4$ NPs. To prepare functional biotin−PEG2000−PAMAM-4P capped NaLnF$_4$ NPs, a mixture of mPEG2000-NHS (18 mg) and biotin−PEG2000-NHS (2 mg) was used instead of pure mPEG2000-NHS.

We performed a HABA colorimetric assay by using a Pierce Biotin Quantitation Kit to determine the average number of biotin groups on each biotin-PEG2000-PAMAM-4P capped NaLnF$_4$ NP. The kit contains a premix of the HABA/Avidin complex. When a solution containing biotin is added to a solution of HABA/Avidin complex; biotin, which has a higher affinity for binding, 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.
First, 100 μL of Milli-Q ultrapure water (R = 18.2 megaohm) was added to one microtube containing the HABA/Avidin Premix and mixed with a pipette tip. To a 1 mL disposable UV cuvette, 800 μL of TBS buffer (25 mM Tris, 150 mM NaCl, pH 7.2) was added and the cuvette with TBS was used to zero the UV-Vis spectrometer. Then the 100 μL HABA/Avidin Premix solution was added to the cuvette and mixed by inverting the cuvette. The absorbance of the mixed solution at 500 nm was measured and recorded as $A_{500}(H-Av)$.

To quantify the biotin groups on the NaLnF$_4$ NP surface, we used NaTbF$_4$ NPs capped with 90% mPEG2000-PAMAM-4P and 10% biotin-PEG2000-PAMAM-4P ligands, which were prepared according to the procedure above, as the analyte. We also used NaTbF$_4$ NPs capped with 100% mPEG2000-PAMAM-4P ligands as a control to account for non-specific interactions between the mPEG2000-PAMAM-4P ligands and the HABA/Avidin complex that may contribute to the decrease of $A_{500}$. The concentration of NaTbF$_4$ NPs in the solution was measured beforehand by ICP-MS. We pipetted 100 μL of either mPEG2000-PAMAM-4P capped or biotin-PEG2000-PAMAM-4P capped NaTbF$_4$ NP solution to a cuvette containing the diluted HABA/Avidin Premix, mixed the solution by inverting the cuvette and waited for 1 minute to allow the mixture to reach equilibrium. Then the absorbance of the solution in the cuvette at 500 nm was recorded as $A_{500}(H-Av-NP)$. The calculations of the biotin concentration and the average number of biotin groups per NP are described in section 3.3.4.

3.2.7 Comparing Colloidal Stability of NaTbF$_4$ NPs Capped with Either mPEG-PAMAM-4P or mPEG-lysine-4P

Solutions of mPEG2000-PAMAM-4P capped and mPEG2000-lysine-4P capped NaTbF$_4$ NPs were prepared in water, and the particle concentrations were determined by ICP-MS. These samples were transferred to various aqueous media, at a particle concentration of 1.0 mg NaTbF$_4$ NPs/mL. The aqueous media included water, 10 mM sodium phosphate buffer (PB), and 1× phosphate buffered saline (PBS containing 10 mM Na$_2$HPO$_4$, 2.0 mM KH$_2$PO$_4$, 137 mM NaCl, 2.0 mM KCl), and 150 mM PB. All the buffer solutions were adjusted to pH 7.4. The colloidal stability of the two different NaTbF$_4$ NPs in various aqueous media was analyzed by DLS at different time after mixing the NP stock solution and the buffer media.
3.3 Results and Discussion

3.3.1 Synthesis of OA Capped NaLnF$_4$ NPs

I synthesized OA capped NaGdF$_4$ and NaTbF$_4$ NPs by using a protocol developed by Zhang et al.$^{25}$ The NPs could be stored in the original reaction media of OAH and ODE for more than one year while maintaining their initial size and morphology. Both NPs have a nearly spherical shape. The average diameter, given by statistical analysis of TEM images for more than 500 individual NPs, is 11.3 ± 0.6 nm for NaGdF$_4$ and 11.1 ± 0.6 nm for NaTbF$_4$ NPs (Figure 3-1). The size analysis of NPs was performed by Fovea Pro, an image processing plugin of Adobe Photoshop.

![TEM images of OA capped NaGdF$_4$ and NaTbF$_4$ NPs.](image)

For surface modification experiments, the NPs were purified by adding excess EtOH to precipitate the NPs from their storage media. The NPs were collected by centrifugation and then washed with a 1:5 v/v THF-EtOH mixture for three cycles to remove excess OAH and ODE. The freshly purified NPs were dispersible in various organic solvents including cyclohexane, THF, chloroform or toluene to yield clear colloidal solutions. It is not recommended to store the purified NPs as a dry solid since the dried NPs sometimes could not be redispersed in any solvent.

DLS CONTIN plots of OA capped NaLnF$_4$ NPs in cyclohexane are shown in Figure 3-2. Mean hydrodynamic diameter was 14.8 nm (PDI = 0.03) for NaGdF$_4$ and 14.6 nm (PDI = 0.02) for NaTbF$_4$ NPs. The distributions are symmetric and narrow.
3.3.2 Synthesis of the PAMAM-4P Ligand

The tetradeutate ligand PAMAM-4P (compound 4) was synthesized via a four-step strategy in which all the reactions were performed at room temperature (Scheme 3-1). The first two steps followed the general synthetic method for PAMAM dendrimers. Both reactions produced the product in almost quantitative yield without chromatographic purification. In the third step, the generation 0.0 PAMAM dendrimer reacted with 4 equivalents of diethyl vinylphosphonate to give the phosphonate-ethyl-ester terminated PAMAM dendrimer (compound 3). The relatively low yield (30%) can be possibly attributed to the affinity of the polar phosphonate groups to silica gel, which causes a portion of the product to be retained on the stationary phase during the column chromatography. A side reaction between water and diethyl vinylphosphonate may also take place and lower the yield of desired product.

In the last step, both the Boc protecting group and the eight ethyl groups were cleaved by treatment with TMSBr. Then the residue was hydrolyzed with water to yield free phosphonic acid and amine groups. Since the compound itself contains both acidic and basic functional groups, it can potentially form a zwitterion. The isolated product was oily and difficult to weigh on a balance. To solve this problem, a stoichiometric amount of NaOH was added to convert the PAMAM-4P
to its octasodium salt. After removal of water and lyophilization, the PAMAM-4P-Na$_8$ salt was obtained as a pale-yellow solid. This sodium salt is hygroscopic and needs to be stored at 4 °C in a desiccator.

![Scheme 3-1. Synthesis of the PAMAM-4P Ligand 4 as its octasodium salt.](image)

3.3.3 Ligand Exchange and Particle Etching by Excess Ligands

3.3.3.1 Two-Step Ligand Exchange to Prepare PAMAM-4P Capped NaLnF$_4$ NPs

As I stated in section 3.3.1, the OA capped NaLnF$_4$ NPs are only dispersible in nonpolar and semipolar organic solvents. In contrast, the PAMAM-4P ligand is only soluble in water, methanol and ethanol. I was unable to find a solvent that can both disperse the NPs and dissolve the ligand. I tried one-step ligand exchange in mixed solvents such as THF–CH$_3$OH or CHCl$_3$–CH$_3$OH but only obtained aggregated NPs that were not dispersible in water.

To overcome this problem, I develop a two-step method to prepare PAMAM-4P capped NPs (Scheme 3-2). In the first step, a colloidal solution of OA capped NaLnF$_4$ NPs in THF was treated with mPEG750-OPO$_3$H$_2$. After stirring at room temperature overnight, the THF solution
was diluted with same volume of water. Upon mixing the solution remained transparent, which suggests that the NPs were still colloidally stable. The free oleic acid replaced from the surface of NaLnF₄ NPs was removed by extraction with hexane, then the aqueous phase was separated and gently heated in vacuo to evaporate THF. The remaining aqueous solution was dialyzed against DI-water to remove excess mPEG750-OPO₃H₂. In this way, the original OA surface ligands on the NaLnF₄ NPs were replaced by mPEG750-OPO₃.

In the second step, the monodentate mPEG750-OPO₃ ligand on the NP surface is replaced by the tetradeutate ligand PAMAM-4P since the latter one can bind more tightly to the NP surface. The reaction was carried out in water in the presence of excess PAMAM-4P sodium salt. After 20 hours of reaction, the displaced mPEG750-OPO₃ ligands and excess PAMAM-4P were both removed by dialysis. Taking NaGdF₄ NPs as an example, TEM images of both mPEG750-OPO₃ and PAMAM-4P capped NPs (Figure 3-3) show that the NPs maintained their initial size and morphology, and remained well separated, after each step of the two-step ligand exchange experiment.

Scheme 3-2. Two-step ligand exchange strategy for preparing PAMAM-4P capped NaLnF₄ NPs.
Figure 3-3. TEM images of NaGdF₄ NPs capped with (A) mPEG750-OPO₃ (B) PAMAM-4P.

The surface ligands on the NPs before and after each ligand exchange reaction were characterized by FT-IR spectroscopy (Figure 3-4). In the IR spectra of OA capped NaGdF₄ NPs, the absorption bands at 2920 and 2850 cm⁻¹ (attributed to the stretching vibration of C=C−H bonds) as well as 1560 and 1460 cm⁻¹ (attributed to the stretching vibration of carboxylate C=O bonds) indicated the presence of OA on the NP surface. After the first ligand exchange step, in the IR spectra of mPEG750-OPO₃ capped NaGdF₄ NPs, all the above bands belonging to OA disappeared, accompanied by the appearance of bands of a C−H stretching vibration at 2880 cm⁻¹ and a C−O stretching vibration at 1110 cm⁻¹, associated with the backbone of the PEG chains. After the second ligand exchange step (PAMAM-4P capped NaGdF₄ NPs), neither band associated with the PEG backbone bond appeared in the IR spectra. The presence of PAMAM on the NP surface was detected by the characteristic amide I and II vibrational bands at 1650 and 1540 cm⁻¹.
3.3.3.2 Particle Etching by Excess Ligands

When I investigated reaction conditions for ligand exchange, I noticed that in the exchange process, prolonged exposure of the NPs in water to excess ligands led to particle etching. In Figure 3-5, the TEM image of a freshly prepared sample of NaGdF$_4$ NPs capped with mPEG2000-OPO$_3$H$_2$ shows a spherical morphology and an average diameter of $11.1 \pm 0.7$ nm. At the moment excess mPEG2000-OPO$_3$H$_2$ ligands were not removed. After 24 hours of dialysis against DI-water, the NPs maintained their original morphology and size ($\bar{d} = 11.3 \pm 0.5$ nm). On the contrary, after 24 hours of dialysis against excess mPEG2000-OPO$_3$H$_2$ ligands (2.5 mM in water), the particle size became smaller and polydisperse ($\bar{d} = 10.1 \pm 1.4$ nm). Besides, many of NPs appeared to be angular and less spherical. This morphological change suggests that the NaGdF$_4$ NPs underwent surface etching in the presence of excess mPEG2000-OPO$_3$H$_2$ ligand.

Figure 3-4. FT-IR spectra of NaGdF$_4$ NPs capped with different surface ligands (a) OA (b) mPEG750-OPO$_3$ (c) PAMAM-4P (d) mPEG2000–PAMAM-4P.
Figure 3-5. TEM images of mPEG2000-OPO$_3$ capped NaGdF$_4$ NPs (A) immediately after ligand exchange and transfer to water (B) after 24 h of dialysis against water (C) after 24 h of dialysis against 2.5 mM mPEG2000-OPO$_3$H$_2$ solution.

To better interpret the surface etching process, I carried out dialysis experiments of NaGdF$_4$ NPs capped with mPEG2000-OPO$_3$H$_2$ against different concentrations of excess mPEG2000-OPO$_3$H$_2$ ligand and monitored the kinetics of ligand etching by quantifying the concentration of free Gd$^{3+}$ ions released to the solution using an Arsenazo colorimetric assay. In this experiment, the NP solutions were placed in a 6 – 8k MWCO D-Tube Dialyzer Mega (10 mL capacity, EMD Millipore). The dialysis membrane prevents passage of NPs into the external solution and allows only Gd$^{3+}$ ions or Gd$^{3+}$ ions coordinated by excess mPEG2000-OPO$_3$H$_2$ ligand to pass through the membrane and be detected. At different time intervals, an aliquot of the external solution was analyzed by the Arsenazo assay to quantify [Gd$^{3+}$]. As shown in Figure 3-6(A), when the NPs were dialyzed against water there was no detectable Gd$^{3+}$ in the external solution. When the NPs were dialyzed against excess ligands increasing amounts of Gd$^{3+}$ were detected over a period of 150+ hours, and the rate of metal ion loss from the particles increased with increasing PEG-phosphoric acid in the solution. After 1 week (∼170 h), the concentration of Gd$^{3+}$ appeared to be leveling off at 40 μM in the presence of 1.0 mM mPEG2000-OPO$_3$H$_2$ and at 60 μM in the presence of 2.5 mM mPEG2000-OPO$_3$H$_2$.

Figure 3-6(B) shows a corresponding set of experiments for mPEG750-OPO$_3$ capped NaGdF$_4$ NPs prepared by ligand exchange in THF with mPEG750-OPO$_3$H$_2$ and then subjected to dialysis. Again there was no detectable loss of metal ions in the presence of DI water, and growing loss of metal ions in the presence of excess mPEG750-OPO$_3$H$_2$. Surprising to us was the finding that the amount of Gd$^{3+}$ ions lost to the aqueous medium over 1 week in the presence of excess
PEG-phosphate was less for mPEG750-OPO\textsubscript{3} capped NPs exposed to mPEG750-OPO\textsubscript{3}H\textsubscript{2}, than for mPEG2000-OPO\textsubscript{3} capped NPs exposed to mPEG2000-OPO\textsubscript{3}H\textsubscript{2}.

Figure 3-6. Kinetics of Gd\textsuperscript{3+} release as NaGdF\textsubscript{4} NPs were dialyzed against DI water and excess mPEG-phosphate. (A) Particles capped with mPEG2000-OPO\textsubscript{3}H\textsubscript{2} and then dialyzed against DI water or mPEG2000-OPO\textsubscript{3}H\textsubscript{2}. (B) Particles capped with mPEG750-OPO\textsubscript{3}H\textsubscript{2} and then dialyzed against DI water or mPEG750-OPO\textsubscript{3}H\textsubscript{2}. In each part: dialysis against DI water (bottom trace), against 1.0 mM mPEG-phosphate (middle trace), 2.5 mM mPEG-phosphate (upper trace).

The ligand etching experiments revealed a key point about the storage condition of NaLnF\textsubscript{4} NPs after ligand exchange. The NPs are stable to metal leaching over at least a period of days if stored in DI water, but subject to etching if stored in the presence of excess ligands. In separate experiments monitored by TEM, we found that NaGdF\textsubscript{4} NPs and NaTbF\textsubscript{4} NPs, capped with either mPEG2000-OPO\textsubscript{3} or mPEG750-OPO\textsubscript{3}, were stable over a period of weeks if the excess ligands were immediately removed by dialysis after ligand exchange. It is known that NaLnF\textsubscript{4} NPs capped with PEG-monophosphate are colloidal unstable in phosphate-containing buffers, but it has been reported colloidal stability can be preserved in the presence of excess ligands.\textsuperscript{31} My experiments suggest that storing NPs with excess ligands may be inappropriate since particle etching can occur.
Beyond that, the differences seen in the susceptibility to etching in the presence of the two PEG-phosphate ligands that differ only in the length of the PEG chains indicate that there are other factors involved in the ligand exchange process that remain poorly understood.

3.3.4 PEGylation and Biotin Functionalization of PAMAM-4P Capped NaLnF$_4$ NPs

PEGylation is a common strategy to reduce non-specific protein binding and cell adhesion of NPs. In my experiment, the PEG molecules were attached to the NP surface by reacting the –NH$_2$ group on the PAMAM-4P capped NPs and methoxy PEG-succinimidy carboxymethyl ester (mPEG2000-NHS). This step was performed in a pH 8.5 sodium bicarbonate buffer solution, an optimal pH for reactions between amines and NHS esters. After the reaction, we removed unreacted free PEG molecules and all excess salts by dialysis against water for 48 hours in a 10k MWCO dialysis cassette. A TEM image of the product mPEG2000-PAMAM-4P capped NaGdF$_4$ NPs is shown in Figure 3-7. When the PAMAM-4P capped NaGdF$_4$ NPs were conjugated with mPEG2000-NHS, the PEG backbone vibrational bands at 2880 and 1110 cm$^{-1}$ appeared again in the IR spectra of the product, mPEG2000–PAMAM-4P capped NaGdF$_4$ NPs (Figure 3-4(d)).

![Figure 3-7. TEM image of mPEG2000-PAMAM-4P capped NaGdF$_4$ NPs.](image)

To introduce surface functionality for conjugating bioaffinity agents, a mixture of mPEG2000-NHS and biotin-PEG2000-NHS with a molar ratio of 9:1 was used to PEGylate the surface of PAMAM-4P capped NaTbF$_4$ NPs. The product was named as Bi-PEG-NP. The biotin groups on the NP surface were quantified by a HABA/Avidin colorimetric assay. I also prepared a sample of mPEG2000–PAMAM-4P capped NaTbF$_4$ NPs, named mPEG-NP, as a control in the
HABA/Avidin assay since mPEG-NP has no biotin group on the NP surface (Scheme 3-3). According to the vendor’s instruction, the apparent biotin concentration in the analyte solution was calculated based on $\Delta A_{500}$, which is the difference in the absorbance of HABA/Avidin complex at 500 nm before and after the addition of NP solution. Raw absorbance data are presented in Table 3-1 and a sample calculation is given in equation (1). The same experiment was performed with mPEG-NP as a control. The difference between the apparent molar biotin concentrations of Bi-PEG-NP and mPEG-NP is taken to be the true biotin concentration in the sample of Bi-PEG-NP.

Scheme 3-3. Preparation of mPEG-NP and Bi-PEG-NP.
Table 3-1. Raw data from the HABA/Avidin assay.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bi-PEG-NP</th>
<th>mPEG-NP</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_{500}(H$-Av)$^a$</td>
<td>1.003</td>
<td>1.029</td>
</tr>
<tr>
<td>$A_{500}(H$-Av-NP)$^b$</td>
<td>0.559</td>
<td>0.900</td>
</tr>
<tr>
<td>$\Delta A_{500} = 0.9 \times A_{500}(H$-Av$) - A_{500}(H$-Av-NP)$^c$</td>
<td>0.344</td>
<td>0.026</td>
</tr>
<tr>
<td>Apparent [biotin]</td>
<td>$1.01 \times 10^{-4}$ M</td>
<td>$7.6 \times 10^{-6}$ M</td>
</tr>
</tbody>
</table>

a. Absorbance of the HABA/Avidin complex at 500 nm before the addition of NaTbF$_4$ NP solution.

b. Absorbance of the HABA/Avidin complex at 500 nm after the addition of NaTbF$_4$ NP solution.

c. Change in absorbance, taking account of sample dilution.

$$[\text{biotin}]_{\text{Bi-PEG-NP}} = \frac{\Delta A_{500,\text{Bi-PEG-NP}}}{\varepsilon l} \times 10 = \frac{0.344 \times 10}{34000 \, \text{M}^{-1}\text{cm}^{-1} \times 1 \, \text{cm}} = 1.01 \times 10^{-4} \, \text{M}$$ (1)

The NP concentration $c$(NaTbF$_4$ NP) (mg/mL) used in each assay was determined by ICP-MS on NP samples digested overnight with concentrated HNO$_3$ (70 vol. %). Nanoparticle number concentrations in NPs/ml were calculated from these results using equation (2). The calculation employed values of $\bar{d}$(NP) = 11.1 nm given by TEM (assuming that the NPs are spherical) and the bulk density of NaTbF$_4$, $\rho$(NaTbF$_4$) = 5.74 g/cm$^3$.

$$\text{NaTbF}_4 \, \text{(NPs/mL)} = c \, (\text{NaTbF}_4 \, \text{NPs}) / \left( \frac{1}{6} \pi d^3 (\text{NP})^{3} \times \rho (\text{NaTbF}_4) \times 10^{21} \, \text{cm}^3 \, \text{nm}^{-3} \right)$$ (2)

For the Bi-PEG-NP sample, we determined an apparent biotin concentration of 101 $\mu$M for a NP sample containing 2.1 mg/mL of NaTbF$_4$ NPs. As a control, we used the results for mPEG-NP (signal corresponding to 7.6 $\mu$M biotins for a sample containing 2.7 mg/mL of NaTbF$_4$ NPs) to estimate the contribution of non-specific binding between the mPEG2000-PAMAM-4P ligand and the HABA/Avidin complex. Combining the apparent concentrations of biotin and the number concentration of NaTbF$_4$ NPs, we calculated the apparent average numbers of biotins per NP which were 72 for Bi-PEG-NP and 4 for mPEG-NP. The difference between those two values gives us an average number of 68 biotins per Bi-PEG-NP. These results and the estimated errors associated with the calculated values are collected in Table 3-2.

The number of ligands per NP and the mean area per ligand can be calculated if we assume no significant difference in the NHS-group reactivities of mPEG2000-NHS and biotin-PEG2000-
NHS with PAMAM-4P capped NPs. For a 9:1 mixture of mPEG2000-NHS and biotin-PEG2000-NHS, 68 biotins/NP correspond to 680 ligands per NP. A spherical NaTbF₄ NP with \( \bar{d} = 11.1 \text{ nm} \) has a surface area of 380 nm², thus yields an average number of 1.8 ligands per nm². For PEGs in water, their radius of gyration values can be estimated from the expression \( R_g = 0.181N^{0.58} \) (nm), where \( N \) is the number of repeating units. This equation provides \( R_g = 1.6 \) nm for PEG2k (\( N = 44 \)). A literature study by Kataoka and co-workers assigned the conformation of surface-tethered PEGs to four different regimes based on their reduced tethering density \( \sigma \pi R_g^2 \): isolated mushrooms for \( \sigma \pi R_g^2 < 1 \), overlapping mushrooms for \( 1 < \sigma \pi R_g^2 < 3 \), squeezed chains for \( 3 < \sigma \pi R_g^2 < 6 \) and scalable brushes for \( \sigma \pi R_g^2 > 6 \). In this case, the grafting density \( \sigma = 1.8 \text{ nm}^{-2} \), so the reduced tethering density \( \sigma \pi R_g^2 = 1.8 \text{ nm}^{-2} \times \pi \times (1.6 \text{ nm})^2 = 15 \), which indicates that the PEG2k chains on the NP surface adopted a scalable brush conformation.

### Table 3-2. ICP-MS and HABA assay results for Bi-PEG-NP and mPEG-NP.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bi-PEG-NP</th>
<th>mPEG-NP</th>
</tr>
</thead>
<tbody>
<tr>
<td>( c(\text{Tb}) ) by ICP-MS (^a) (mg/mL)</td>
<td>2.1 ± 0.1</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>( c(\text{NaTbF}_4 \text{NPs}) )(^a,b) (mg/mL)</td>
<td>3.4 ± 0.1</td>
<td>4.4 ± 0.1</td>
</tr>
<tr>
<td>( \text{NaTbF}_4 ) (NPs/mL)</td>
<td>( (8.3 \pm 0.2) \times 10^{14} )</td>
<td>( (1.1 \pm 0.03) \times 10^{15} )</td>
</tr>
<tr>
<td>Apparent [biotin] (( \mu \text{M} )) (^c)</td>
<td>101 ± 5</td>
<td>7.6 ± 0.4</td>
</tr>
<tr>
<td>Apparent [biotin] / ( c(\text{NaTbF}_4 \text{NPs}) ) mmol/mg NP</td>
<td>( (3.0 \pm 0.2) \times 10^{-5} )</td>
<td>( (1.7 \pm 0.1) \times 10^{-6} )</td>
</tr>
<tr>
<td>Apparent biotins per NP</td>
<td>72 ± 4</td>
<td>4.2 ± 0.2</td>
</tr>
</tbody>
</table>

\(^a\) \( c(\text{Tb}) \) and \( c(\text{NaTbF}_4 \text{NPs}) \) refer to the mass concentrations in mg/mL as determined by ICP-MS. The error bars were calculated assuming an error of 3% in measured values.

\(^b\) The number concentrations of NaTbF₄ NPs were calculated from the mass concentration using equation (2).

\(^c\) From Table 3-1. An error of 5% in absorbance measurements was assumed in calculating the values obtained by the HABA assay.

#### 3.3.5 Quantification of Surface Ligand Density by TGA

TGA is a common technique for quantifying surface ligand density of NPs. The NaLnF₄ NPs, after removal of excess ligands by either washing or dialysis, were heated in air and the mass...
loss was attributed to oxidative degradation of the ligands. Thus the surface ligand density (molecules/nm$^2$) can be calculated using the equation below.

$$\text{NP surface ligand density} = \frac{\text{total number of ligands}}{\text{surface area of all the NPs}}$$

$$= \frac{\Delta w\% / M(\text{ligand}) \times N_A}{(1 - \Delta w\%) / \left[ \frac{4}{3} \pi r^3 \times \rho(\text{NP}) \right] \times 4\pi r^2}$$ (3)

The TGA trace for OA capped NaGdF$_4$ NPs (Figure 3-8) showed a mass loss of 15.0%. Assuming spherical NPs ($\bar{d} = 11.3$ nm) and bulk density ($\rho = 5.647$ g/cm$^3$) for the inorganic core, the ligand density was calculated to be 4.4 molecules/nm$^2$. This value corresponds to an average parking area of 0.23 nm$^2$/OA, consistent with a literature value for OA capped NaYF$_4$:Yb,Er NPs, which is on the order of 0.25 nm$^2$/OA. In another paper published by my colleague Dr. Lemuel Tong, he obtained a higher surface ligand density of 8.2 molecules/nm$^2$ for OA capped NaYF$_4$ NPs ($\bar{d} = 17$ nm) by TGA measurement.$^{87}$ This difference may be due to several reasons, including the difference between size and composition of the NPs, the synthetic conditions of the NPs as well as the washing protocol for removing free oleic acids.

For mPEG750-OPO$_3$ capped NaGdF$_4$ NPs, the mass loss corresponds to 5.5 molecules/nm$^2$ (i.e., an average area of 0.18 nm$^2$ per molecule). The mass loss for mPEG2000-OPO$_3$ capped NaGdF$_4$ NPs implied an even higher ligand density (6.4 molecules/nm$^2$), corresponding to 0.16 nm$^2$ per PEG-phosphate molecule. When the capping ligand was mPEG2000–PAMAM-4P, the mass loss was 42.5%. Since the molar mass of PAMAM-4P (excluding the sodium ions) is 712.46, we used a molecular weight 2710 for mPEG2000–PAMAM-4P to convert the mass loss into surface density. In this way, we obtained a value of 1.7 molecules/nm$^2$. This number is in excellent agreement with the result given by the HABA/Avidin assay (1.8 PEG molecules/nm$^2$).
If we assume that the four phosphonates of the mPEG2000–PAMAM-4P ligand all bind to the NP surface, the average area occupied by each phosphonate group would be 0.15 nm\(^2\). These average parking area values for the phosphate and phosphonate anchoring groups determined for different PEG-based ligands are similar to one another. This result implies that the average area occupied by the ligand on the NaLnF\(_4\) NP surface is determined primarily by the interaction of phosphate and phosphonate groups with Ln ions in the particle surface, rather than by the dimensions of the PEG chains. For mPEG2000-PAMAM-4P, the value of 1.7 molecules/nm\(^2\) corresponds to an average PEG-to-PEG distance $\bar{d}$ of 0.8 nm. The Flory radius $R_F$ of a PEG2000 random coil in a good solvent is estimated to be about 3.4 nm.\(^88,89\) Thus, for mPEG2000–PAMAM-4P capped NaGdF\(_4\) NPs, the distance between anchoring points of the PEG chains on the particle surface is significantly smaller than $2R_F$.\(^90\) Under these conditions, the PEG chains on the NP surface become extended and are in the so-called “brush” regime.\(^91\) For PEG-monophosphate...
ligands, the mean distance between PEG chains is even smaller, and the brush should be more extended. Previous studies on PEGylated Au NPs indicate that this high PEG surface density is effective at resisting protein absorption. Moreover, the grafting density \( \sigma \) of different PEG ligands can be converted to reduced tethering density \( \sigma \pi R_g^2 \), which considers the effect of both PEG grafting density and the PEG chain length to the conformation of surface-grafted PEG chains. The calculated results are summarized in Table 3-3. The reduced tethering density of all the three PEG ligands was much higher than 6, indicating that the conformations of all three PEG ligands grafted on the NP surface were in the scalable brush regime.

Table 3-3. TGA percentage mass loss, average surface ligand density, ligand parking area and reduced tethering density of ligands of NaGdF\(_4\) NPs capped with OA, mPEG750-OPO\(_3\), mPEG2000-OPO\(_3\) or mPEG2000-PAMAM-4P.

<table>
<thead>
<tr>
<th>NaGdF(_4) NPs capped with</th>
<th>OA</th>
<th>mPEG750-OPO(_3)</th>
<th>mPEG2000-OPO(_3)</th>
<th>mPEG2000-PAMAM-4P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGA mass loss</td>
<td>15.0%</td>
<td>39.0%</td>
<td>66.7%</td>
<td>42.5%</td>
</tr>
<tr>
<td>Ligand molecules/nm(^2)</td>
<td>4.4</td>
<td>5.5</td>
<td>6.4</td>
<td>1.7</td>
</tr>
<tr>
<td>Mean area per ligand (nm(^2))</td>
<td>0.23</td>
<td>0.18</td>
<td>0.16</td>
<td>0.59</td>
</tr>
<tr>
<td>Reduced tethering density</td>
<td>–</td>
<td>15</td>
<td>53</td>
<td>14</td>
</tr>
</tbody>
</table>

3.3.6 Colloidal Size and Stability Measurements

3.3.6.1 Colloidal Properties of NaGdF\(_4\) NPs Capped with Different Ligands

The hydrodynamic radii of NaGdF\(_4\) NPs capped with different ligands were measured by DLS to assess their colloidal stability in different media. The original OA capped NaGdF\(_4\) NPs in cyclohexane showed a z-average hydrodynamic radius \( R_z \) of 7.4 nm (Figure 3-2). After the first ligand exchange with mPEG750-OPO\(_3\)H\(_2\), the NPs became dispersible in water and the aqueous solution of mPEG750-OPO\(_3\) capped NaGdF\(_4\) NPs showed a monomodal size distribution on DLS CONTIN plot without any sign of aggregation. The \( R_z \) value of this sample was 12 nm. Upon transfer to 10 mM pH 7.4 phosphate buffer (PB), the sample aggregated immediately with an increase of \( R_z \) to 54 nm (Figure 3-9(A)). Within 30 minutes, the NPs precipitated.

When the surface ligand of NaGdF\(_4\) NPs was exchanged from mPEG750-OPO\(_3\) to PAMAM-4P, the solution of PAMAM-4P capped NPs in water remained clear. The DLS CONTIN
plot was bimodal, which showed a main peak at 16 nm and a weaker peak at 90 nm. In 10 mM PB, the intensity of the latter peak increased with a shift in magnitude to lower hydrodynamic radius (~60 nm) (Figure 3-9(B)). After storage in 10 mM PB for 1 week, the PAMAM-4P capped NPs precipitated.

Corresponding CONTIN plot of mPEG2000-PAMAM-4P capped NaGdF₄ NPs became monomodal again. When those NPs were dispersed in either 10 mM PB or PBS at pH 7.4, they retained their monomodal size distribution yet the peak values shifted to larger size accompanied by a small increase in polydispersity. The \( R_z \) values increased from 12.0 nm (in water) to 20.6 nm (in 10 mM PB) and to 21.2 nm (in PBS). The above results indicate that the PAMAM-4P ligand, whether PEGylated or not, can efficiently stabilize NaGdF₄ NPs in phosphate-containing buffer where same NPs capped with PEG-monophosphate ligands rapidly aggregate and precipitate. Nevertheless, in 150 mM PB where the concentration of competitive phosphate ions was much higher, even mPEG2000-PAMAM-4P was no longer able to stabilize the NPs (Figure 3-9(C)).
Figure 3-9. CONTIN plots of the hydrodynamic radius distribution of 1.0 mg NP/mL solutions of NaGdF₄ NPs capped with (A) mPEG750-OPO₃; (B) PAMAM-4P in water and in 10 mM phosphate buffer (PB, pH 7.4); (C) mPEG2000–PAMAM-4P in different aqueous media, all adjusted to pH 7.4. The mPEG750-OPO₃-capped NPs in PB aggregated immediately to give the plot shown, but then precipitated over the subsequent 30 min. For the mPEG2000–PAMAM-4P-capped NPs in (C), all of the measurements were performed immediately after mixing the NP stock solution in DI water with the concentrated buffer solution to obtain the final buffer concentrations shown in the Figure. During the measurement, the sample in 150 mM PB buffer became slightly turbid, and this turbidity increased upon standing afterward.

3.3.6.2 Comparing Colloidal Stability of NaTbF₄ NPs Capped with Either mPEG-Lysine-4P or mPEG-PAMAM-4P

An alternative tetradeutate ligand, mPEG-lysine-4P, was reported by Cao et al. in an earlier publication. The pairs of phosphonate anchoring groups in this ligand are spaced differently (1 methylene in between) compared to the anchoring groups in mPEG-PAMAM-4P (2 methylenes in between). The structure of both ligands is shown in Figure 3-10(A). To investigate whether this structural difference affects the ability of the ligands to stabilize NaLnF₄ NPs in phosphate-containing buffer media, I used DLS to study the kinetic evolution of hydrodynamic radii of NaTbF₄ NPs capped by either ligand.
One set of experiment was carried out in 10 mM pH 7.4 PB. In the initial solution \((t = 0)\) both samples showed monomodal CONTIN plots with \(R_z = 19.3\) nm for the mPEG2000-lysine-4P capped NPs and 18.6 nm for the mPEG2000–PAMAM-4P capped NPs. For the both samples, there was essentially no change in size over 20 hours and no formation of aggregates detected by DLS (Figure 3-10(B) and Figure 3-11).

In PBS containing 12 mM phosphate, 2.7 mM KCl and 137 mM NaCl, mPEG2000-lysine-4P capped NPs could still maintain their colloidal stability over a time span of 20 hours. However, striking difference was observed for mPEG2000-PAMAM-4P capped NPs, in which a shift in the peak maximum could be seen after only 2 hours with substantial peak broadening in the CONTIN plot after 4 hours. When the NPs were stored in PBS for 20 hours, an aggregate peak centered around 300 nm dominated the CONTIN plot along with a residual peak of the initial sample hydrodynamic size (Figure 3-12). The \(R_z\) value was calculated to be 100 nm, which was an obvious sign of the NPs becoming colloidally unstable (Figure 3-10(C)). All the \(R_z\) values and corresponding PDI values are summarized in Table 3-4.
Figure 3-10. (A) Structure of mPEG2000–PAMAM-4P and mPEG2000-lysine-4P ligands. The phosphonates on the dendrimer are shown as ionized as a reminder that ligand exchange was carried out in water with the octasodium salt of the ligand. The phosphonates on the lysine-based ligand are shown as protonated as a reminder that ligand exchange was carried out in THF solution with the fully protonated form of the ligand. (B and C): Comparison of the temporal evolution of $z$-average hydrodynamic radii of 1 mg NP/mL solutions of NaTbF$_4$ NPs capped with the two different PEG-tetraphosphonate ligands in either (B) 10 mM PB or (C) PBS, both pH 7.4.
Figure 3-11. DLS CONTIN plots of NaTbF$_4$ NPs capped with either mPEG2000-PAMAM-4P or mPEG2000-lysine-4P ligands in 10 mM PB (pH 7.4) at different time.
Figure 3-12. DLS CONTIN Plots of NaTbF$_4$ NPs Capped with Either mPEG2000-PAMAM-4P or mPEG2000-lysine-4P Ligands in 1× PBS (pH 7.4) at Different Times.
Table 3-4. Calculated DLS z-average hydrodynamic radii\(^a\) (PDI values in parentheses) of NaTbF\(_4\) NPs capped with PEG-tetraphosphonate ligands in 10 mM PB or 1× PBS solutions (both at pH 7.4) for different storage times.

<table>
<thead>
<tr>
<th>Dispersion Medium</th>
<th>10 mM PB</th>
<th>1× PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mPEG2000-PAMAM-4P</td>
<td>mPEG2000-lysine-4P</td>
</tr>
<tr>
<td>Capping Ligand</td>
<td>Aging Time</td>
<td>Aging Time</td>
</tr>
<tr>
<td></td>
<td>0 h</td>
<td>18.6 nm (0.16)</td>
</tr>
<tr>
<td></td>
<td>2 h</td>
<td>18.1 nm (0.14)</td>
</tr>
<tr>
<td></td>
<td>4 h</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>20 h</td>
<td>18.3 nm (0.14)</td>
</tr>
</tbody>
</table>

\(\text{a. Calculated by the software of the Malvern Zetasizer.}\)

The difference in colloidal stability for the NPs capped with the two tetradeutate ligands was unexpected, which raises questions that go beyond their structural difference. It is worth mentioning that the ligand exchange protocols for the two ligands also differ. mPEG-lysine-4P capped NaLnF\(_4\) NPs were prepared by a one-step ligand exchange in THF between OA capped NPs and mPEG-lysine-4P ligands, which involves the fully protonated form of phosphonate anchoring groups. This also applies for the mPEG-monophosphate ligands. We imagine that each phosphate or phosphonate group, when protonated, may replace one oleate, which carries away one proton to form oleic acid. Thus, each phosphate or phosphonate group carries (on average) one negative charge, with which to form a P−O−Ln bond. In contrast, when mPEG-monophosphate is displaced from the NPs with PAMAM-4P, this ligand exchange involves the fully deprotonated dendrimer as its octasodium salt. These differences may affect how the phosphonate groups of the two different tetradeutate ligands are bound to the particle surface and consequently lead to the difference between the NP colloidal stabilities.
3.4 Summary

I prepared OA capped NaLnF$_4$ (Ln = Gd or Tb) NPs with mean diameters about 11 nm. To render the NPs hydrophilic, I synthesized a tetradeutate PAMAM-4P ligand and developed a two-step ligand exchange process to attach the PAMAM-4P ligand onto the surface of NPs. The first step replaced the OA on the NP surface with mPEG750-OPO$_3$H$_2$ in THF, which allows the NPs to be dispersed in water. The second step replaced mPEG750-OPO$_3$ with PAMAM-4P in water, which yielded PAMAM-4P capped NaLnF$_4$ NPs. For long-term storage of the NPs, the excess ligands employed in the ligand exchange reaction need to be removed immediately after the reaction. A particle etching experiment showed that storing with excess mPEG-OPO$_3$H$_2$ ligands can cause the NaGdF$_4$ NPs to eventually degrade and free Gd$^{3+}$ ions to be released to solution.

The primary amine group on the PAMAM-4P ligand allows attachment of amine-reactive species to further functionalize the surface of NPs. In my studies I used mPEG-NHS to react with PAMAM-4P capped NaLnF$_4$ NPs and obtained mPEG-PAMAM-4P capped NPs. I also used a mixture of mPEG-NHS and biotin-PEG-NHS to prepare biotin-PEG-PAMAM-4P capped NPs. The average number of biotin per NP was $68 \pm 4$, which was quantified by a HABA/Avidin assay. From this value I calculated a surface ligand density of 1.8 ligands/nm$^2$.

A more generic method I employed to quantify the surface ligand density of NaLnF$_4$ NPs was TGA. By analyzing NaGdF$_4$ NPs capped with four different ligands (OA, mPEG750-OPO$_3$, mPEG2000-OPO$_3$ and mPEG2000-PAMAM-4P) I noticed that the ligands bearing phosphate or phosphonate anchoring groups have similar values in terms of parking area of each anchoring group (0.15 – 0.18 nm$^2$ / phosphate or phosphonate). This number is somewhat smaller than the parking area of OA (0.23 nm$^2$ / oleate). This is surprising since I expected that the interchain repulsion of PEG ligands would reduce the surface density of PEG ligands on the NP surface. One possible explanation is that although most of the free PEG ligands were removed by dialysis, there might still be ligands loosely associated with the NP corona yet not directly bound to NP surface, which increased the values of mass loss in TGA and decreased the values of parking area per anchoring group.

The most surprising result in this chapter was obtained by a head-to-head comparison between mPEG2000-PAMAM-4P and mPEG2000-lysine-4P, an alternative ligand reported by Cao et al.$^{32}$ Given that both ligands have four phosphonate groups, I initially supposed they would
show similar properties on stabilizing NaLnF₄ NPs in phosphate-containing buffer media. However, while both ligands provide long-term colloidal stability for NaTbF₄ NPs in 10 mM PB, the colloidal stability of mPEG2000-lysine-4P capped NPs was much better than mPEG2000-PAMAM-4P capped NPs after 20 hours of storage in PBS (10 mM Na₂HPO₄, 2.0 mM KH₂PO₄, 137 mM NaCl and 2.0 mM KCl). This difference may result from either the structural difference of the two ligands (e.g. the spacing between the phosphonate anchoring groups) or differences in the ligand exchange protocol.

3.5 Appendix

3.5.1 NMR Spectra of New Organic Compounds (Compound 3 and 4).

Figure 3-13. ¹H NMR spectrum of compound 3 in CDCl₃.
Figure 3-14. $^{13}$C and $^{31}$P NMR spectra of compound 3 in CDCl$_3$.

Figure 3-15. $^1$H and $^{31}$P NMR spectra of the PAMAM-4P octasodium salt 4 in 0.1 M NaOD/D$_2$O.
Figure 3-16. 2D-COSY NMR spectrum of the PAMAM-4P octasodium salt 4 in 0.1 M NaOD/D$_2$O.

Figure 3-17. $^{13}$C NMR spectrum of compound 4 in 0.1 M NaOD/D$_2$O.
3.5.2 Calibration Curve of the Arsenazo Assay

Figure 3-18. Arsenazo colorimetric assay: calibration curve of $A(655)$ vs $[\text{Gd}^{3+}]$. 
Chapter 4: Preparation of PMMA Microspheres Encoded with Small LnF₃ NPs

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4.1 Introduction

Bead-based bioassays play an important role in quantitative bioanalysis. Researchers have developed bead-based assays that target diverse analytes such as cytokines, kinases, gene expression markers, etc. In the field of mass cytometry, bead-based bioassays are still in an early stage. The foundation of bead-based assays for mass cytometry is to prepare lanthanide-encoded polymer microspheres (beads). The microspheres should not only have a narrow size distribution, but also have sufficient lanthanide content in each bead as well as a low bead-to-bead variation of lanthanide contents. Besides, the microspheres have to be colloidally stable in aqueous buffer and bear surface functional groups for bioconjugation. In our group, people have spent several years to develop and optimize the preparation of lanthanide-encoded polystyrene (PS) microspheres. The PS microspheres were synthesized by two- or three-stage dispersion polymerization using acrylic acid (AA) to incorporate lanthanide ions into the microspheres via copolymerization and poly(vinylpyrrolidone) (PVP) as surface stabilizer for the microspheres. The method can yield highly monodisperse PS microspheres with sufficient lanthanide content, yet the conjugation of bioaffinity agents to the surface of the beads is impeded by the PVP stabilizer. Although the bioconjugation efficiency was somehow improved by replacing the PVP corona with a poly(glycidyl methacrylate) (PGMA) shell for the PS microspheres, the synthesis was relatively long and tedious.

To overcome this problem, I planned to use a molecule that can provide both colloidal stability and surface functionality for the polymer microspheres. My colleague Dr. Jianbo Tan, during his visit from Sun Yat-sen University, proposed a polymeric macro-RAFT agent for this application. This macro-RAFT agent is a copolymer of oligoethylene glycol monomethyl ether
acrylate (OEGA, $M_n = 475$ g/mol) and acrylic acid (AA). The OEGA component can provide colloidal stability as well as repellency for non-specific protein adherence for the polymer microspheres. The AA component can provide surface functional groups for covalent attachment of bioaffinity agents.

In this chapter, I describe the synthesis of PMMA microspheres via a photoinitiated RAFT dispersion polymerization using the above macro-RAFT agent as the surface stabilizer. Dr. Tan and I collaborated to develop the synthetic methodology. We first tried the conventional technique using AA and LnCl$_3$ salts to incorporate Ln ions into the PMMA microspheres. This attempt was unsuccessful since Ln$^{3+}$ ions were barely incorporated into the microspheres. Then we developed a second method inspired by Lin et al. who used a similar strategy to prepare LnF$_3$ NP-encoded microgels.$^{65}$ This was achieved by adding LnF$_3$ NPs, which bear polymerizable double bonds on their surface, to the reaction mixture and the NPs were incorporated in the microspheres via copolymerization. I synthesized the LnF$_3$ NPs and Dr. Tan synthesized the PMMA microspheres encoded with the LnF$_3$ NPs. The bead-by-bead distribution of lanthanide contents in the PMMA microspheres was analyzed by mass cytometry. To test the surface functionality of the PMMA microspheres, we covalently attached FITC-streptavidin (FITC-SAv) onto the surface of the microspheres and used three methods to quantify the biotin binding capacity of the SAv labeled microspheres.

4.2 Experimental Details

4.2.1 Materials

Ethylene glycol methacrylate phosphate (EGMAP) was kindly donated by Esstech, Inc. (Essinton, PA, USA), and used without further purification. S-1-dodecyl-S’-(α,α’-dimethyl-α”-acetic acid)trithiocarbonate (DDMAT, 98%), 4,4’-azobis(4-cyanovaleric acid) (ACVA, 98), N-(3-(dimethylamino)propyl)-N-ethylcarbodiimide hydrochloride (EDC, BioXtra), N-hydroxysuccinimide (NHS, 98%), oligo(ethylene glycol) methyl ether acrylate (OEGA, $M_n = 475$ g/mol), acrylic acid (AA, 99%), 2-aminoethyl dihydrogen phosphate (AEP), sodium fluoride (NaF, 99%), and 2,2-dimethyl-2-phenylacetophenone (Darocur 1173, 97%) and lanthanide salts were purchased from Sigma-Aldrich and used without purification. Methyl methacrylate (MMA, 99%, Aldrich) was passed through a column of basic alumina and stored at 4 °C. Fluorescein-conjugated bovine serum albumin (FITC-BSA), FITC-SAv and 5-(and 6)-tetramethylrhodamine biocytin
(biotin-TMR) were purchased from Life Technologies Inc. (now acquired by Thermo Fisher Scientific). DOTA-biotin-sarcosine (C-100) (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) was purchased from Macrocyclics Inc. (Dallas, TX, USA). The synthetic procedures of macro-RAFT agent poly(OEGA-co-AA) and Lu-DOTA-biotin are described in the appendix of this chapter.

4.2.2 Synthesis of Small LnF$_3$ NPs

To synthesize LnF$_3$ NPs capped with both AEP and EGMAP, a solution of the ligands AEP (0.102 g, 0.746 mmol) and EGMAP (0.076 g, 0.36 mmol) (molar ratio of AEP/EGMAP is 3:1) in water (10 mL) was adjusted to pH 6.5 by adding 1.5 M aqueous NH$_3$. Then a lanthanide nitrate salt Ln(NO$_3$)$_3$·6H$_2$O (Ln = La, Ce, Pr or Tb) (1.33 mmol) or a mixture of lanthanide nitrates ($n_{La}$:$n_{Ce}$:$n_{Tb}$ = 40:45:15, total amount = 1.33 mmol) in water (10 mL) was added to the solution to form the Ln-AEP/EGMAP complex at room temperature with stirring at 1,000 rpm for 10 min. Then NaF (0.13 g, 3.00 mmol) in water (13 mL) was added dropwise at 0.2 mL/min via a syringe pump. After 16 hours of stirring at 1,000 rpm, the NPs in the clear solution were purified by dialysis at room temperature for 2 – 3 days using a 1 kDa MWCO Spectra/Pro 7 dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA, USA). The concentrations of these NP solutions, determined by ICP-MS, were 3.7 mg/mL for LaF$_3$ NPs, 5.3 mg/mL for TbF$_3$ NPs, 5.2 mg/mL for CeF$_3$ NPs, 4.3 mg/mL for PrF$_3$ NPs, and 5.2 mg/mL for LaF$_3$:Ce,Tb NPs, respectively. The synthesis of unreactive LaF$_3$ NPs without double bonds on the surface was carried out in the same way but without the addition of EGMAP. The concentration of LaF$_3$ NPs capped with AEP was 6.1 mg/mL.

4.2.3 Preparation of PMMA Microspheres by Three Different Methods

4.2.3.1 Two-Stage Photoinitiated RAFT Dispersion Polymerization of MMA in the Presence of LnCl$_3$·6H$_2$O and AA

A mixture of the monomer MMA (2.0 g, 10 wt % of the whole mixture), the macro-RAFT agent poly(OEGA-co-AA) (0.3 g, 15 wt % relative to MMA), the RAFT agent DDMAT (5 mg, 0.25 wt % relative to MMA), and the photoinitiator Darocur 1173 (60 mg, 3 wt % relative to MMA) was dissolved in an ethanol/water mixture (7.2 g/10.8 g) to form a homogeneous solution. The mixture was gently purged with nitrogen for 15 min and then sealed. A LED UV lamp ($\lambda$ = 365 nm, light intensity 16 mW/cm$^2$) was employed to irradiate the reaction mixture from the top
of the reaction cell. The reaction mixture turned turbid after 30 s of UV irradiation. After 15 min of irradiation, a degassed solution containing 1.0 g of MMA, 40 mg of AA, 30 mg of Darocur 1173, 20 mg of TbCl₃·6H₂O, and 3.6 g/5.4 g of ethanol–water was added into the reaction. The reaction was further irradiated for another 45 min. The product PMMA microspheres were precipitated by centrifugation, washed three times with ethanol/water (40/60, w/w) mixtures and finally dispersed in DI-water.

4.2.3.2 One-Stage (Batch) Dispersion Polymerization of MMA in the Presence of LnF₃ NPs

A mixture of the monomer MMA (2.0 g, 10 wt % of the whole mixture), the macro-RAFT agent poly(OEGA-co-AA) (0.3 g, 15 wt % relative to MMA), the RAFT agent DDMAT (5 mg, 0.25 wt % relative to MMA), and the photoinitiator Darocur 1173 (60 mg, 3 wt % relative to MMA) was dissolved in an ethanol/water mixture (7.2 g/9.8 g) to form a homogeneous solution. Then 1.0 g of LaF₃ NP solution (3.7 mg/mL LaF₃ in water) was added. The mixture was gently purged with nitrogen for 15 min and then sealed. After 1 hour of UV irradiation a stable dispersion was formed without any observable coagulum. The product was precipitated by centrifugation, washed three times with ethanol/water (40/60, w/w) mixtures and finally dispersed in DI-water.

4.2.3.3 Two-Stage Dispersion Polymerization of MMA in the Presence of LnF₃ NPs.

A mixture of the monomer MMA (2.0 g, 10 wt % of the whole mixture), the macro-RAFT agent poly(OEGA-co-AA) (0.3 g, 15 wt % relative to MMA), the RAFT agent DDMAT (5 mg, 0.25 wt % relative to MMA), and the photoinitiator Darocur 1173 (60 mg, 3 wt % relative to MMA) was dissolved in an ethanol/water mixture (7.2 g/10.8 g) to form a homogeneous solution. The mixture was gently purged with nitrogen for 15 min and then sealed. After 45 min of UV irradiation, a degassed solution containing 1.5 g of MMA, 60 mg of Darocur 1173, 1.0 g of LnF₃ NP solution and 5.4 g/7.1 g of ethanol–water was added into the reaction. The reaction mixture was irradiated for another 45 min. The product was precipitated by centrifugation, washed three times with ethanol/water (40/60, w/w) mixtures and finally dispersed in DI-water.
4.2.4 Titration of –COOH Groups on the Surface of PMMA Microspheres

The number of –COOH groups on the surface of PMMA microspheres was quantified by acid-base titration. Titrations were carried out at room temperature with simultaneous potentiometric and conductometric monitoring using a Traceable™ conductivity meter (Fisher Scientific, Pittsburg, PA, USA) and an Ecomet P25 pH meter (Istek Inc., Seoul, Korea). Samples of microspheres suspended in water (5 g solution, solid content 10%) were diluted in 40 mL distilled water. A standardized 0.1 M NaOH solution was added by micropipette to the solution of microspheres with continuous magnetic stirring. The solution was then back-titrated using 0.025 M HCl added in volume increments of 20 μL. A blank titration to correct for CO₂ uptake was carried out by the same way.

4.2.5 Ion Release Test of the PMMA Microspheres

For testing ion release upon storage in different media, each microsphere sample was washed by three cycles of sedimentation-redispersion in ethanol/water (40/60, w/w) followed by washing twice with distilled water. Then the samples were redispersed at 0.5 wt % solids content in different media, namely MES buffer (0.1 M, pH 5.5), phosphate buffer (0.1 M, pH 7.4) and PBS (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl, pH 7.4). The solutions were stirred continuously during the test. Aliquots (100 μL) were taken at different time intervals over a two-week period. Each sample was mixed with deionized water (900 μL) and spun down by centrifugation (12,000 × g, 30 min) immediately to separate the microspheres. The supernatant was collected, diluted 10-fold with 3% aqueous HNO₃ (ICP grade) and analyzed by ICP-MS.

4.2.6 Preparation of FITC-SAv Labeled PMMA Microspheres

An aliquot of PMMA microsphere dispersion in water (160 μL, solids content 10 wt %, prepared by the two-stage method with LaF₃ NPs as described above) was washed twice with 500 μL of MES buffer (pH 5.5, 100 mM) in a 2.0 mL Eppendorf tube with a slanted bottom, and the microspheres were redispersed in 400 μL of MES buffer. A solution of EDC and NHS in MES buffer (400 μL, containing 32 mg of EDC and 88 mg of NHS) was added to the vial with gentle vortexing for 25 min, and then the microspheres were sedimented by centrifugation at 8,000 × g and washed twice with 500 μL of PBS. The activated microspheres were redispersed in 100 μL of PBS buffer, which contained 160 μL of FITC-SAv (2 mg/mL in PBS). The samples were incubated overnight at room temperature with gentle vortexing, then sedimented at 8,000 × g, and
resuspended in PBS. This washing process was repeated twice more, and the supernatant was discarded. An aliquot of the FITC-SAv labeled microspheres was examined by confocal microscopy.

As a negative control, we also attached FITC-BSA to the microspheres in the same way to prepare FITC-BSA labeled PMMA microspheres.

4.2.7 Quantification of Biotin Binding Capacity of FITC-SAv Labeled PMMA Microspheres

4.2.7.1 Quantification by Fluorescence Titration

For fluorescence titration experiments, a freshly prepared sample of FITC-SAv labeled microspheres (~ 16 mg) was sedimented and redispersed in 1800 μL of PBS and equally split into nine centrifuge tubes (200 μL solution in each). Then different amounts of biotin-TMR were added to the tubes and incubated for 90 min under gentle vortexing. The samples were sedimented by centrifugation at 8,000 × g. The supernatants were collected, and the intensity of the TMR fluorescence was measured (excitation at 530 nm, emission measured at 580 nm).

The same experiment was performed on a sample of FITC-BSA labelled microspheres as a negative control.

4.2.7.2 Quantification by ICP-MS and Mass Cytometry

A freshly prepared sample of FITC-SAv labeled microspheres (6 mg) was sedimented and redispersed in 300 μL of PBS, then treated with an solution of Lu-DOTA-biotin (2 μL, 2.4 mM in water, concentration determined by ICP-MS). After 90 min of incubation under gentle vortexing, the sample was sedimented by centrifugation at 8,000 × g. The supernatant was collected and analyzed by ICP-MS after 50 × dilution with 3% aqueous HNO₃ (ICP grade). The precipitated microspheres were washed with PBS (500 μL × 2) and dispersed in 500 μL of PBS. For mass cytometry analysis, the microspheres were precipitated and redispersed in DI-water, diluted and analyzed by a CyTOF 2 mass cytometer.

The same experiment was performed on a sample of FITC-BSA labeled microspheres as a negative control.
4.3 Results and Discussion

4.3.1 Preparation of Ln-Containing PMMA Microspheres by Using LnCl$_3$·6H$_2$O and AA

The key component of preparing colloidally stable PMMA microspheres by photoinitiated dispersion polymerization is the macro-RAFT agent, poly(OEGA-co-AA). This macro-RAFT agent is synthesized by a RAFT copolymerization of OEGA and AA, which is shown in Scheme 4-1. The OEGA component provides a barrier to suppress nonspecific interaction between proteins and the surface of microspheres, whereas the –COOH groups on AA provide functionality for covalent attachment of bioaffinity agents to the microspheres.

Scheme 4-1. Synthesis of the macro-RAFT agent poly(OEGA-co-AA) used for photoinitiated dispersion polymerization.

A typical photoinitiated RAFT dispersion polymerization for preparing monodisperse PMMA microspheres requires the monomer MMA, the photoinitiator Darocur™ 1173 (2-hydroxy-2-methyl-1-phenyl-propan-1-one) that generates free radical under UV irradiation, the macro RAFT agent poly(OEGA-co-AA) and a small-molecule RAFT agent DDMAT. Without the addition of DDMAT the resulting PMMA microspheres will not be monodisperse.$^{93}$ Besides, an appropriate Ln source is needed to introduce Ln ions into the microspheres. When Dr. Tan and I started preparing Ln-containing PMMA microspheres, we first attempted to adopt the strategy developed by Abdelrahman et al., which used AA and LnCl$_3$·6H$_2$O to incorporate Ln$^{3+}$ ions into polystyrene (PS) microspheres.$^{62}$ The synthesis was achieved by a two-stage dispersion polymerization, in which AA and LnCl$_3$·6H$_2$O were added to the reaction in the second stage. When we did our reaction, we also started the photoinitiated polymerization without addition of AA and LnCl$_3$·6H$_2$O (here Ln = Tb). After 15 min of reaction, we temporarily removed the light source, added a solution of AA and LnCl$_3$·6H$_2$O in an ethanol-water mixture and then restarted
the irradiation. The whole reaction took 1 hour, which was much shorter than the conventional preparation of PS microspheres.

An SEM image of the PMMA microspheres showed that the microspheres have a narrow size distribution ($CV_d = 2.9\%$) with a mean diameter of 0.98 μm (Figure 4-1). However, when the microspheres were characterized by mass cytometry almost no Tb signal was observed. The result indicated that Tb$^{3+}$ ions were not actually incorporated in the microspheres.

![Figure 4-1. SEM image and diameter distribution histogram of PMMA microspheres prepared by two-stage photoinitiated RAFT dispersion polymerization with poly(OEGA-co-AA) as stabilizer in the presence of 2 wt % AA and 1 wt % TbCl$_3$·6H$_2$O (relative to MMA).](image)

The failure could be attributed to multiple factors. First, the two-stage synthesis of Ln-containing PS microspheres took 24 hours in total, whereas the synthesis of PMMA microspheres by photoinitiated polymerization took only 1 hour to finish. The kinetics might be too rapid for the AA monomer to be copolymerized with MMA. Secondly, the PS microspheres were synthesized in pure ethanol whereas the PMMA microspheres were synthesized in an ethanol-water mixture. The LnCl$_3$·6H$_2$O salts are more soluble in water and less likely to be incorporated in the polymer phase. Thirdly, the difference in reaction temperature may also play a role. The synthesis of PS microspheres was performed at 70 °C whereas the synthesis of PMMA microspheres was performed at room temperature.
4.3.2 Preparation of Ln-Containing PMMA Microspheres by Using Small LnF₃ NPs

4.3.2.1 Synthesis of Small LnF₃ NPs

To solve the problem, we turned to an alternative strategy inspired by Lin et al. They prepared small LnF₃ NPs by a coprecipitation method. The NPs bear polymerizable double bonds on their surface and were incorporated into microgels by copolymerization. We first reproduced their synthesis of LaF₃ NPs by reacting La(NO₃)₃·6H₂O and NaF in aqueous media in the presence of a mixture of AEP and EGMAP. The reaction yielded relatively uniform NPs with diameters ranging from 4 to 6 nm (Figure 4-2). We then extended the synthesis to prepare CeF₃, PrF₃ and TbF₃ NPs as well as doped LaF₃:Ce,Tb NPs by using a mixture of La(NO₃)₃·6H₂O, Ce(NO₃)₃·6H₂O and Tb(NO₃)₃·5H₂O (molar ratio 40:45:15). The chemical structures of LnF₃ NPs were characterized by powder XRD (Figure 4-3). We also prepared a sample of LaF₃ NPs with only AEP as the ligand so that the NPs have no polymerizable groups on their surface. These NPs are referred as ‘unreactive’ LaF₃ NPs.

Figure 4-2.TEM images of LnF₃ NPs. (A) LaF₃ NPs capped with EGMAP and AEP (B) LaF₃ NPs capped with AEP only (C) CeF₃ NPs (D) PrF₃ NPs (E) TbF₃ NPs (F) LaF₃:Ce,Tb NPs. Scale bar: 100 nm.
4.3.2.2 Preparation of Ln-Containing PMMA Microspheres by One-Stage Polymerization

We first tried the preparation of PMMA microspheres in the presence of small LnF$_3$ NPs by mixing all the reactants together at the beginning of the reaction. The microspheres thus prepared showed a broad size distribution. Also, many microspheres were non-spherical (Figure 4-4(a)). This may be due to the fact that the addition of small NPs interferes with the nucleation stage of the polymerization reaction. The nucleation stage is essential for make uniform polymer microspheres.

Figure 4-3. XRD spectra of the various LnF$_3$ NPs prepared in the presence of AEP and EGMAP. The sharp vertical lines at the bottom of each spectrum correspond to lines for a corresponding powder X-ray reference from the data base of the International Centre for Diffraction Data as indicated by the JCPDS number.
4.3.2.3 Preparation of Ln-Containing PMMA Microspheres by Two-Stage Polymerization

We then tried a two-stage protocol to prepare the PMMA microspheres by adding the LnF$_3$ NPs in the second stage. A previous paper by Tan et al. showed that under this condition, the nucleation stage lasted for 45 min. So we added LaF$_3$ NPs 45 min after the reaction was started as well as additional MMA monomers and photoinitiators, then we allowed the polymerization to proceed for another 45 min. Using this strategy we obtained PMMA microspheres with a narrow size distribution ($CV_d = 2.9\%$) with a mean diameter of 1.02 μm (Figure 4-4(b) and (e)). On a magnified TEM image of LaF$_3$-containing microspheres, the LaF$_3$ NPs can observed at the edge of the microspheres (Figure 4-4(d)).
Figure 4-4. (a) SEM image of PMMA microspheres prepared by batch (one-stage) photoinitiated RAFT dispersion polymerization in the presence of 1.0 g LaF$_3$ NP solution (3.7 mg/mL); (b) SEM image of PMMA microspheres prepared by two-stage photoinitiated RAFT dispersion polymerization in which the LaF$_3$ NP solution plus additional monomer and photoinitiator were added in the second stage; (c), (d) TEM image of PMMA microspheres prepared by two-stage photoinitiated RAFT dispersion polymerization with the LaF$_3$ NP solution added in the second stage; (e) diameter histogram of the PMMA microspheres prepared in the two-stage dispersion polymerization; (f) Analysis by mass cytometry of the La content distribution of the PMMA microspheres prepared by two-stage photoinitiated RAFT dispersion polymerization.

The lanthanide content of the microspheres was quantified by mass cytometry. When LaF$_3$ NPs were employed as the Ln source, the average content was $1.4 \times 10^6$ La atoms per microsphere with a coefficient of variation $CV_{La} = 42\%$ (Figure 4-4(f)). The PS microspheres previously prepared by Abdelrahman et al. have a mean diameter of 1.9 $\mu$m and average Ln content of $10^7$ Ln atoms per microsphere (bead). Considering the size difference between the two microspheres, the loading of Ln into the PMMA microspheres is almost as efficient. For PMMA microspheres incorporated with TbF$_3$, CeF$_3$ and PrF$_3$ NPs respectively, we obtained values of $2.7 \times 10^5$ Tb per bead, $1.4 \times 10^6$ Ce per bead and $1.5 \times 10^6$ Pr per bead. The SEM images of the microspheres and their corresponding mass cytometry histograms are shown in Figure 4-5.
Figure 4-5. SEM images, diameter distribution histogram and lanthanide content distributions of PMMA microspheres prepared by two-stage photoinitiated RAFT dispersion polymerization in the presence of 15 wt% poly(OEGA-co-AA) with the addition of (a) 1.0 TbF$_3$ NP solution (5.3 mg/mL), $d = 1.06 \mu$m, $CV_d = 3.1\%$, $CV_{\text{Tb}} = 29\%$; (b) 1.0 g CeF$_3$ NP solution (5.2 mg/mL), $d = 1.03 \mu$m, $CV_d = 2.9\%$, $CV_{\text{Ce}} = 25\%$; (c) 1.0 g PrF$_3$ NP solution (4.3 mg/mL), $d = 1.08 \mu$m, $CV_d = 4.5\%$, $CV_{\text{Pr}} = 27\%$.

To assess whether the EGMAP ligands on the NP surface actually copolymerize with MMA monomers to incorporate the LnF$_3$ NPs into the PMMA microspheres, a control experiment was carried out to prepare PMMA microspheres with ‘unreactive’ LaF$_3$ NPs that have only AEP as their surface ligand. On the TEM images of PMMA microspheres thus prepared, hardly any NPs could be observed at the edge of the microspheres (Figure 4-6(b)). Mass cytometry analysis of those microspheres also showed no La signal above background. Those results provide clear evidence that the EGMAP ligands on the surface of LnF$_3$ NPs play a key role to incorporate the NPs into the PMMA microspheres (Figure 4-6(d)).
Figure 4-6. (a) SEM image, (b) TEM image and (c) diameter distribution histogram of PMMA microspheres prepared by two-stage photoinitiated RAFT dispersion polymerization with the addition of a solution of ‘unreactive’ LaF₃ NPs (1.0 g solution, 6.1 mg/mL LaF₃) plus additional monomer and photoinitiator in the second stage; (d) Mass cytometry measurement of the La content of the PMMA microspheres.

Since the microspheres are to be used for bioconjugation, we did an acid-base titration experiment to quantify the functional –COOH groups on the surface of the microspheres. We chose LaF₃-containing PMMA microspheres as an example. The mean value of –COOH groups on each microsphere was $6.8 \times 10^6$.

We also tested ion leakage of LaF₃-containing PMMA microspheres upon storage in different buffer media. Over 2 weeks of storage in PBS, no loss of La ions was detected by ICP-MS. In 0.1 M pH 7.4 phosphate buffer, the loss was about 1% but did not increase upon storage. In MES buffer at pH 5.5, the microspheres lost about 3% of its La content over 2 weeks (Figure 4-7). These values indicate that ion leakage is unlikely to be a problem for the microspheres to be applied in bioassays based on mass cytometry.
Figure 4-7. La ion release from colloidal suspensions of La-containing PMMA microspheres in three different buffer solutions. The La-containing PMMA microspheres were prepared by photoinitiated RAFT dispersion polymerization with poly(OEGA-co-AA) as stabilizer in the presence of 1.0 g ‘reactive’ LaF$_3$ NPs solution (3.7 mg/mL). The buffer solutions are 0.1M MES buffer (pH 5.5), 0.1M PB (pH 7.4) and PBS (pH 7.4).

4.3.3 Preparation of Ln-Encoded PMMA Microspheres by Using Small LnF$_3$ NPs with Two or More Ln Elements

For multiplexed bead-based assays on mass cytometry, the beads need to contain at least two Ln elements as ‘classifiers’ on a bivariate data plot. We explored two methods to prepare Ln-encoded PMMA microspheres containing two or more Ln elements. One is to use doped LnF$_3$ NPs. We prepared LaF$_3$ NPs doped with Ce and Tb (LaF$_3$:Ce,Tb NPs) and used this type of NP to prepare Ln-encoded PMMA microspheres. SEM analysis of the product showed a mean diameter of 0.92 μm with a relatively narrow size distribution ($CV_d = 2.8\%$) (Figure 4-8(a) and (b)). Mass cytometry analysis for both Ce and Tb in this sample is presented as an isotopic ‘dot-dot’ diagram in Figure 4-8(c) as well as a histogram for La in Figure 4-8(d). The average Ln content in each microsphere was $4.5 \times 10^5$ Ce, $7.3 \times 10^4$ Tb and $4.7 \times 10^5$ La. This method, despite its effectiveness, has a major disadvantage. When we change the ratio of Ln elements in the microspheres we will have to resynthesize LnF$_3$ NPs with a different doping ratio, which is time consuming.
To circumvent this problem, we developed a second method to prepare Ln-encoded PMMA microspheres by using a mixture of two LnF$_3$ NPs. Here we chose CeF$_3$ and TbF$_3$ NPs as the Ln source. The microspheres prepared by this method with different NP ratios all showed average diameters around 1 μm and relatively narrow size distributions ($CV_d < 4\%$). Figure 4-9 display mass cytometry data of three individual samples prepared by adding different ratios of CeF$_3$/TbF$_3$ NPs in the second stage of the polymerization reaction. To better illustrate this method does lead to distinguishable signals from different microspheres on mass cytometry data plot, we mixed the three samples and analyzed the mixture by mass cytometry. As shown in Figure 4-9(d), three well-separated populations of beads were observed on the bivariate isotopic diagram. Thus we
established that Ln-encoded PMMA microspheres can be obtained by adding LnF₃ NPs with different ratios during the synthesis.

![Figure 4-9. Bivariate dot-dot plots of mass cytometry results for PMMA microspheres prepared by two-stage photoinitiated RAFT dispersion polymerization with the addition of (a) 1.0 g TbF₃ NP solution and 0.2 g CeF₃ NP solution; (b) 1.0 g TbF₃ NP solution and 1.0 g CeF₃ NP solution; (c) 0.2 g TbF₃ NP solution and 1.8 g CeF₃ NP solution; (d) mass cytometry data of a mixed sample with microspheres (a), (b) and (c) showing three separate populations.]

4.3.4 Conjugation of FITC-SAβ onto the PMMA Microspheres

We have shown that the surface of PMMA microspheres bears a considerable number of –COOH groups, and we need to examine their capability for conjugating bioaffinity agents. As a proof-of-concept experiment we used FITC-SAβ as the bioaffinity agent, which can be easily detected by the fluorescence of FITC.

We first tested the noncovalent adsorption of FITC-SAβ onto the PMMA microspheres. In this experiment, we took an aliquot of LaF₃-containing PMMA microsphere suspension in water (20 μL, solids content 10%), diluted with PBS and then added a solution of FITC-SAβ (100 μL,
0.1 mg/mL in PBS). After 1 hour of incubation, the sample was washed by five cycles of sedimentation-redispersion with 1 mL of PBS in each cycle.

Figure 4-10(a) and (b) show an optical and a confocal fluorescence microscopic image of the microspheres after the noncovalent protein binding test. No fluorescence was observed from the microspheres upon excitation at 488 nm, which suggested that noncovalent adsorption of FITC-SAv onto the microspheres is negligible. This is crucial for bead-based bioassays since noncovalent adsorption of proteins negatively affects the specificity of the assays. In our case, the OEGA component of the macro-RAFT agent, while providing colloidal stability for the microspheres, also impedes noncovalent adsorption of proteins onto the bead surface.

We then used the –COOH groups, which come from the AA component of the macro-RAFT agent, for covalent conjugation of proteins to the microspheres. The –COOH groups were activated with EDC and NHS at pH 5.5 in MES buffer, then the activated microspheres were treated with FITC-SAv under the same condition applied for noncovalent binding test. After 1 hour of incubation, the sample was washed with PBS to remove any free protein. Figure 4-10(c) and (d) show an optical and a confocal fluorescence microscopic image of the microspheres after the covalent protein binding experiment. Clear fluorescence signal can be observed from individual microspheres, which indicates that FITC-SAv was successfully conjugated to the surface of microspheres. Thus we confirmed the potential of Ln-containing PMMA microspheres for bioconjugation and bead-based bioassays.
4.3.5 Quantification of Biotin Binding Capacity of FITC-SAv Labeled PMMA Microspheres

After we conjugated FITC-SAv onto the PMMA microspheres, we used three independent methods to quantify the biotin binding capacity of those microspheres (Scheme 4-2). The first method is fluorescence titration, in which the FITC-SAv labeled microspheres were treated with different amount of biotin-TMR. After 90 min of incubation the microspheres were precipitated by centrifugation. The supernatants containing unbound biotin-TMR were collected and their fluorescence intensities were measured. We also prepared FITC-BSA labeled microspheres as a negative control to assess the influence of any nonspecific adsorption of biotin-TMR to the microspheres. In both experiments, the fluorescence intensity was measured at 580 nm where only TMR emits.
Scheme 4-2. Three different methods to quantify the biotin binding capacity of FITC-SAv labeled PMMA microspheres: 1) Fluorescence titration with biotin-TMR. 2) ICP-MS measurement of unbound Lu-DOTA-biotin. 3) CyTOF measurement of PMMA microspheres labeled with Lu-DOTA-biotin.

Figure 4-11 shows the plot of fluorescence intensities from unbound biotin-TMR from the supernatant versus the amount of biotin-TMR added to the titration. For FITC-BSA labeled microspheres, the intensity values increased linearly and the linear fit extrapolates to zero, which suggests little nonspecific binding of biotin-TMR to the microspheres. For FITC-SAv labeled microspheres, the fluorescence intensity of unbound biotin-TMR was close to zero when the amount of biotin-TMR added for titration was below a certain threshold. After that the intensity also increased linearly. This trend indicates at the crossover point, which corresponds to 0.25 nmol of biotin-TMR, the binding sites on the FITC-SAv labeled microspheres were saturated. From this result we calculated a binding capacity of $5.6 \times 10^4$ biotin-TMR per microsphere.
The second and the third methods for quantifying biotin binding capacity employed ICP-MS and mass cytometry, respectively. For both methods Lu-DOTA-biotin was used as the probe. In the experiment, an aliquot of LaF₃-containing microspheres labeled with FITC-SAv (6 mg of microspheres in 300 μL of PBS, ca. 9.0 × 10⁹ beads) was incubated with 4.8 nmol of Lu-DOTA-biotin. After 90 min, the microspheres were sedimented by centrifugation and the supernatant containing unbound Lu-DOTA-biotin was analyzed by ICP-MS. In the supernatant we detected 4.0 nmol of Lu-DOTA-biotin, based on which we calculated a value of 5.3 × 10⁴ Lu-DOTA-biotin per microsphere. This result is close to the one obtained by fluorescence titration. Details of the calculation procedure are presented in the appendix of this chapter.

The same batch of FITC-SAv labeled microspheres, after treatment with Lu-DOTA-biotin, were analyzed by mass cytometry. We also performed the same experiment with FITC-BSA labeled microspheres and the results of both experiments are shown in Figure 4-12. On the bivariate pseudocolor plot of FITC-SAv labeled microspheres, the ¹²⁹La signal from the microsphere and the ¹⁷⁵Lu signal from the Lu-DOTA-biotin probe showed a strong correlation. The biotin binding capacity calculated from the ¹⁷⁵Lu average intensity in mass cytometry is 6.6 × 10⁴ Lu-DOTA-biotin per microsphere, which is close to the values determined by fluorescence titration and ICP-MS analysis. In the case of FITC-BSA labeled microspheres, this was only very weak signal of ¹⁷⁵Lu above the baseline, which proved that nonspecific binding between Lu-DOTA-biotin and the microspheres is minimal.
We also include screen captures of either FITC-SAv or FITC-BSA labeled microspheres when they were analyzed by CyTOF. Figure 4-12(b) shows a nice correlation between $^{139}$La and $^{175}$Lu signals for FITC-SAv labeled microspheres, which further confirmed the binding of Lu-DOTA-biotin to those microspheres. On the contrary, the data of FITC-BSA labeled microspheres treated with Lu-DOTA-biotin (Figure 4-12(d)) show only $^{139}$La signal from the microspheres but no $^{175}$Lu signal from the probe. This result supports the conclusion that Lu-DOTA-biotin did not bind to FITC-BSA labeled microspheres.

![Figure 4-12](image)

**Figure 4-12.** (a) $^{139}$La/$^{175}$Lu bivariate plot and (b) mass cytometry screen capture of FITC-SAv labeled PMMA microspheres treated with Lu-DOTA-Biotin; (c) $^{139}$La/$^{175}$Lu bivariate plot and (d) mass cytometry screen capture of FITC-BSA labeled PMMA microspheres treated with Lu-DOTA-Biotin.

### 4.4 Summary

In this chapter, I described a photoinitiated RAFT dispersion polymerization to prepare Ln-containing PMMA microspheres using a macro RAFT-agent, poly(OEGA-co-AA). This macro RAFT-agent not only provided colloidal stability for the microspheres, but also introduced functional groups to the surface of the microspheres for bioconjugation. The polymerization was
carried out in the presence of small LnF₃ NPs (\(d < 5\) nm) bearing polymerizable methacrylate groups on their surface. The LnF₃ NPs include LaF₃, CeF₃, PrF₃ and TbF₃ NPs. This method produced Ln-containing PMMA microspheres with \(d \sim 1\) μm and a narrow size distribution. Mass cytometry analysis showed that the microspheres have an average Ln content of \(\sim 10^5\) Ln atoms per microsphere.

We also prepared PMMA microspheres encoded with two or more Ln elements by two different methods. One used doped LnF₃ NPs and the other used a mixture of two different LnF₃ NPs. By using the second method we prepared three different Ce,Tb-encoded PMMA microspheres with different Ce/Tb ratios. When the three different microspheres were mixed and analyzed by mass cytometry, they showed three well-separated populations on the Ce-Tb bivariate plot, which demonstrated the potential of those microspheres to be used in multiplexed bead-based assays.

A proof-of-concept biotin-avidin binding assay was performed to test the reactive functionality of Ln-containing PMMA microspheres for conjugating bioaffinity agents. When FITC-SA\(v\) was simply mixed with the microspheres without any crosslinking reagent, there was minimal non-covalent protein adsorption on the surface of the microspheres, a consequence of the protein-repellency feature of the macro RAFT-agent. Nevertheless, FITC-SA\(v\) could still be covalently attached to the bead surface via EDC/NHS coupling. The same protocol was employed to prepare FITC-BSA labeled microspheres. In addition, I also described how I quantified the biotin binding capacity of FITC-SA\(v\) labeled microspheres by three independent methods. The first one involved fluorescence titration with biotin-TMR. The second one used a treatment with excess Lu-DOTA-biotin followed by measuring the amount of unbound Lu-DOTA-biotin. The third method used mass cytometry analysis of beads treated with Lu-DOTA-biotin. FITC-BSA labeled microspheres were used in all the three methods as a negative control. Fluorescence titration and ICP-MS analysis presented similar results (ca. \(5 \times 10^4\) biotin per microsphere), whereas mass cytometry analysis gave a slightly higher value (\(6.6 \times 10^4\) biotin per microsphere).
4.5 Appendix

4.5.1 Synthesis of the Macro-RAFT Agent Poly(OEGA-co-AA)

DDMAT (0.22 g, 0.55 mmol), ACVA (0.031 g, 0.11 mmol), AA (4.7 g, 65 mmol), OEGA monomer (31.2 g, 65 mmol) and ethanol (25 g) were added into a 100 mL three-neck round-bottomed flask and purged under N₂ for 30 min. The flask was sealed and heated in an oil bath at 70 °C for 24 h. Then the flask was cooled to room temperature and placed in ice water to quench the reaction. The product was precipitated by adding diethyl ether (100 mL) and washed several times with diethyl ether. Then the solvent was removed by rotary evaporation. The degree of polymerization of the polymer was determined by ¹H-NMR by comparing the integration of the end group protons on the RAFT agent to the methoxy group signals as well as the backbone. In this case, the degree of polymerization of OEGA is 131 and the degree of polymerization of AA is 113. The polymer was also analyzed by SEC and the molecular weight distribution $M_w/M_n$ is 1.41. The NMR spectrum and SEC trace are shown below.
Figure 4-13. $^1$H-NMR spectrum of poly(OEGA-co-AA) in CDCl$_3$. The peak k of the end group was used for end-group analysis. The degree of polymerization of the OEGA component $x = \text{integral (peak g) / 3}$. The overall degree of polymerization of both OEGA and AA $x + y = \text{integral (peak a+b+c+d) / 3}$. For this polymer $x = 131$, $x + y = 244$, so $y = 113$. 
Figure 4-14. SEC trace of poly(OEGA-co-AA) in water at 30 °C containing 0.2 M KNO₃, 25 mM phosphate buffer (pH 8.5), and 200 ppm NaN₃. The trace was analyzed by comparison to PMAA standards.

4.5.2 Synthesis of Lu-DOTA-Biotin

DOTA-biotin-sarcosine (5.0 mg, 5.5 μmol) was added to 2 mL of 0.1 M pH 6.0 NH₄OAc buffer solution, then an aqueous solution (0.2 mL) of LuCl₃·6H₂O (4.6 mg, 12 μmol) was added. The mixed solution was sealed and stirred in an oil bath preheated to 90 °C for 1 h. The solution was cooled to room temperature, the solvent was evaporated in vacuo and the residue was redissolved in 1 mL of water. The crude product solution was loaded on a short C18 SPE cartridge. The unbound Lu ions were first eluted with pure water, then a mixture of H₂O-CH₂CN (80:20 v/v) was used to elute the product Lu-DOTA-Biotin. The ¹H-NMR spectrum of Lu-DOTA-Biotin is shown below. ¹H-NMR (400 MHz, D₂O, δ (ppm)): 1.28–1.63 (m, 6H), 2.2–3.8 (m, 33H), 3.18 (s, 3H), 4.19–4.54 (m, 4H), 7.22–7.44 (m, 4H). HRMS m/z for C₃₆H₅₁LuN₈O₁₁S ([M + H]⁺) calculated 979.2800, found 979.2873.
4.5.3 Calculation of the Biotin Binding Capacity of FITC-SAv Labeled PMMA Microspheres

4.5.3.1 For Quantification by Biotin-TMR

The mean number of biotin-TMR per microsphere (bead) is calculated based on following equations.

\[ V_{\text{bead}} = \frac{4}{3} \pi R^3 = \frac{4}{3} \times 3.14 \times (1.02/2/10000)^3 = 5.6 \times 10^{-13} \text{ cm}^3 \]

\[ m_{\text{bead}} = \rho \times V_{\text{bead}} = 1.19 \times (5.6 \times 10^{-13}) = 6.7 \times 10^{-13} \text{ g} \]

\[ m_{\text{total}} = 160 \times 10^{-3} \times 1.0 \times 10\% / 9 = 1.8 \times 10^{-3} \text{ g} \]

\[ N_{\text{bead}} = \frac{m_{\text{total}}}{m_{\text{bead}}} = (1.8 \times 10^{-3}) / (6.7 \times 10^{-13}) = 2.7 \times 10^9 \]

\[ N_{\text{biotin-TMR}} = n_{\text{biotin-TMR}} \times N_A = 0.25 \times 10^{-9} \times 6.02 \times 10^{23} = 1.5 \times 10^{14} \]
\[ N_{\text{biotin-TMR/bead}} = \frac{N_{\text{biotin-TMR}}}{N_{\text{bead}}} = \frac{(1.5 \times 10^{-3})}{(2.7 \times 10^{-13})} = 5.6 \times 10^4 \]

In the above equations, \( V_{\text{bead}} \) is the mean bead volume, \( R \) is the mean bead radius, \( \rho (= 1.19 \, \text{g/cm}^3) \) is the density of PMMA, \( m_{\text{bead}} \) is the mass of one single bead, \( m_{\text{sample}} \) is the total mass of beads used in the bioconjugation, \( N_{\text{bead}} \) is the number of PMMA beads in the sample, \( N_{\text{biotin-TMR}} \) is the number of biotin-TMR determined by the titration curve, \( N_{\text{biotin-TMR/bead}} \) is the number of biotin-TMR bound to each bead.

**4.5.3.2 For Quantification by Lu-DOTA-Biotin**

The mean number of Lu-DOTA-Biotin per bead is calculated based on following equations.

\[ V_{\text{bead}} = \frac{4}{3} \pi R^3 = \frac{4}{3} \pi \times (1.02/2/10000)^3 = 5.6 \times 10^{-13} \, \text{cm}^3 \]

\[ m_{\text{bead}} = \rho \times V_{\text{bead}} = 1.19 \times (5.6 \times 10^{-13}) = 6.7 \times 10^{-13} \, \text{g} \]

\[ m_{\text{total}} = 60 \times 10^{-3} \times 1.0 \times 10^0 = 6.0 \times 10^{-3} \, \text{g} \]

\[ N_{\text{bead}} = \frac{m_{\text{total}}}{m_{\text{bead}}} = \frac{(6.0 \times 10^{-3})}{(6.7 \times 10^{-13})} = 9.0 \times 10^9 \]

\[ N_{\text{Lu-DOTA-Biotin}} = 0.8 \times 10^{-9} \times 6.02 \times 10^{23} = 4.8 \times 10^{14} \]

\[ N_{\text{Lu-DOTA-Biotin/bead}} = \frac{N_{\text{Lu-DOTA-Biotin}}}{N_{\text{bead}}} = \frac{(4.8 \times 10^{14})}{(9.0 \times 10^9)} = 5.3 \times 10^4 \]

In these equations, \( V_{\text{bead}} \) is the mean bead volume, \( R \) is the mean bead radius, \( \rho (= 1.19 \, \text{g/cm}^3) \) is the density of PMMA, \( m_{\text{bead}} \) is the mass of one single bead, \( m_{\text{total}} \) is the total mass of beads used in the bioconjugation, \( N_{\text{bead}} \) is the number of PMMA beads in the sample, \( N_{\text{Lu-DOTA-Biotin}} \) is the total number of Lu-DOTA-Biotin bound to beads determined by ICP-MS, \( N_{\text{Lu-DOTA-Biotin/bead}} \) is the number of Lu-DOTA-Biotin bound to each bead.
Chapter 5: Preparation and Application of NaYF$_4$@SiO$_2$ NPs as Reporter Tags in Bead-Based Immunoassay

5.1 Introduction

In Chapter 3, I described preparation of functional PEG-PAMAM-4P capped NaLnF$_4$ NPs. Those NPs were colloidally stable in phosphate-containing buffer to a certain extent. They also bear functional groups (e.g. biotin) that could be used for bioconjugation reactions. Nevertheless, it is hard to sediment PEG-PAMAM-4P capped NaLnF$_4$ NPs from aqueous media by centrifugation, which makes them difficult to be separated from unbound biomolecules in bioconjugation experiments. Besides, the aqueous solution of those NPs was colorless, which makes it quite difficult to track the NPs.

To overcome these problems, I decided to design an alternative surface modification strategy, in which I coated the surface of NaLnF$_4$ NPs with a thin silica shell. This strategy has multiple advantages. The silica shell not only renders the NPs hydrophilic, but also protects the NPs from any possible surface degradation. Also, there are abundant literature methods for introducing functional groups onto the surface of silica shell. For instance, Yeh and co-workers used (3-aminopropyl)triethoxysilane to introduce –NH$_2$ groups onto the surface of silica coated NaYF$_4$::Yb,Tm NPs.$^{41}$ Wang and co-workers added carboxyethylsilanetriol together with TEOS during the silica coating to prepared COOH-functionalized NaYF$_4$:Yb,Er@SiO$_2$ NPs.$^{43}$ Cai and co-workers reacted mesoporous silica NPs with (3-mercaptopropyl)trimethoxysilane to attach –SH groups to the silica NPs.$^{94}$ Moreover, it is possible to incorporate organic dyes or quantum dots into the silica shell, which makes the silica coated NPs easily trackable by naked eyes.$^{39}$

Silica coated NaLnF$_4$ NPs have been employed by many research groups for biological applications. Zvyagin and co-workers coated NaYF$_4$:Yb,Er@NaGdF$_4$ core-shell upconversion luminescent NPs with a 6-nm-thick uniform layer of silica, then attached a recombinant protein G’ modified with a silica-binding peptide linker onto the surface of silica coated NPs. The protein G’ coated NPs were conjugated with the Fc region of cell-specific antibodies and applied for targeted imaging of cancer cells.$^{95}$ Kanaras and co-workers attached single-strand DNA onto silica coated NaYF$_4$:Yb,Er NPs and developed a DNA sensor based on luminescence energy transfer.
between the NPs and graphene oxide.\(^\text{96}\) Shi and co-workers prepared a series of silica coated Gd\(^{3+}\) doped NaYF\(_4\):Yb,Er NPs by varying the silica shell thickness from 2.6 nm to 16.9 nm, then studied the influence of silica shell thickness on the magnetic relaxivities of Gd\(^{3+}\) doped NaYF\(_4\):Yb,Er NPs.\(^\text{97}\) Up to now, silica coated NaLnF\(_4\) NPs have been used for imaging, biosensing, drug delivery and therapeutic applications.\(^\text{98}\)

In this chapter, I first describe the preparation of NaYF\(_4\)@SiO\(_2\) NPs via the reverse microemulsion method. The microemulsion consists of cyclohexane, Igepal CO-520, aqueous ammonia and TEOS. Ammonia catalyzed the hydrolysis of TEOS to form a thin layer of silica on the surface of the NPs. Rhodamine B (RB) dye was embedded into the silica shell by adding small amount of RB to the microemulsion, which made the NaYF\(_4\)@SiO\(_2\) NPs red-colored and much easier to track in next-stage experiments. Then I modified the surface of NaYF\(_4\)@SiO\(_2\) NPs to introduce functional groups, namely –NH\(_2\) and –COOH groups. NeutrAvidin (NAv) was covalently attached to the surface of NaYF\(_4\)@SiO\(_2\)-COOH NPs and the average number of NAv per NP was quantified by a micro bicinchoninic acid (BCA) assay.

To test the applicability of the NaYF\(_4\)@SiO\(_2\)-NAv NP-protein conjugate, I performed a sandwich-type bead-based immunoassay using this NP-protein conjugate as the reporter. I first covalently attached goat anti-human (GaH) IgG as the capture antibody to the surface of Ce,Tb-encoded PMMA microspheres, which selectively captures human IgG as the target analyte. Then a detection antibody, biotinylated GaH IgG, binds to the analyte human IgG that is captured by the microspheres. The presence of biotin groups on the detection antibody was revealed by either a conventional fluorescent reporter, FITC-NAv, or the NaYF\(_4\)@SiO\(_2\)-NAv NP-protein conjugate. By comparing the results we confirmed that the NAv protein maintained its biological activity when conjugated to the NaYF\(_4\)@SiO\(_2\) NPs. In this immunoassay, mouse and rabbit IgGs were selected as controls to examine the specificity of the assay.

5.2 Experimental Details

5.2.1 Materials

YCl\(_3\)-6H\(_2\)O (99.9% trace metals basis), oleic acid (90%), 1-octadecene (90%), NH\(_4\)F (98%), Igepal CO-520, TEOS (98%), rhodamine B isothiocyanate (RBITC, mixed isomers), (3-aminopropyl)triethoxysilane (APTES, 99%), cyclohexane (99%), acetic acid (AcOH, 99%),
diglycolic anhydride (90%), \(N,N\)-dimethylformamide (DMF, anhydrous, 99.8%), \(N\)-(3-(dimethylamino)propyl)-\(N\)-ethylcarbodiimide hydrochloride (EDC, BioXtra), \(N\)-hydroxysulfosuccinimide sodium salt (sulfo-NHS, 98%), MES monohydrate (99%, BioXtra), bovine serum albumin (BSA, lyophilized powder) and regular solvents were purchased from Sigma-Aldrich and used without purification. RB-APTES stock solution was prepared by mixing 7 mg of RBITC, 50 \(\mu\)L of APTES and 450 \(\mu\)L of absolute EtOH overnight. The stock solution was stored at -20 °C. NaOH (reagent grade pellet) was purchased from Caledon (Georgetown, ON). Ammonium hydroxide (28%, reagent grade) was purchased from ACP Chemicals (Saint-Léonard, QC). Micro BCA protein assay kit was purchased from Thermo Scientific. NA\(_v\), FITC-NA\(_v\) and all the antibodies were purchased from Life Technologies (now part of Thermo Fisher Scientific).

5.2.2 Synthesis of OA Capped Na\(_{2}YF\(_4\) NPs

\(YCl_3\cdot 6H_2O\) (1 mmol), oleic acid (OAH) (6 mL) and 1-octadecene (ODE) (15 mL) were heated to 120 °C in a three-necked flask under vacuum for 1 h to form a clear solution. Then the solution was cooled to room temperature with continuous stirring. A freshly prepared CH\(_3\)OH solution (10 mL) of NH\(_4\)F (0.15 g, 4.0 mmol) and NaOH (0.10 g, 2.5 mmol) was added dropwise via a syringe and the resulting cloudy mixture was stirred for 30 min after the addition was completed. The solution was slowly heated to 100 °C in open air to evaporate CH\(_3\)OH. When all the CH\(_3\)OH was evaporated the solution was heated to 298 °C at a rate about 10 °C/min under N\(_2\) atmosphere and maintained at 298 °C for 1 h. Then the solution was cooled to room temperature and the as-prepared Na\(_{2}YF\(_4\) NPs were stored in the reaction medium.

5.2.3 Silica Coating on Na\(_{2}YF\(_4\) NPs

1.5 mL of the OAH-ODE reaction mixture containing about 10 mg of OA capped Na\(_{2}YF\(_4\) NPs was transferred to a conical centrifuge tube, then 7.5 mL of EtOH was added to precipitated the NPs. The NPs were sedimented by centrifugation at 2700 \(\times\) g for 30 min. The supernatant was decanted, the pelleted NPs were redispersed in 0.5 mL of cyclohexane by sonication and precipitated again by adding EtOH (8.5 mL) and centrifugation. This washing cycle was repeated once more and the purified NPs were finally dispersed in 10 mL of cyclohexane.

To the NP colloidal solution in cyclohexane, 0.15 mL of Igepal CO-520 was added with stirring and the mixture was stirred for 20 min. Then more Igepal CO-520 (0.50 mL) and
ammonium hydroxide (0.08 mL) were added with vigorous stirring. The solution was sealed and sonicated in a Branson 1210 ultrasonic cleaner (max power output 80 W) (Branson Ultrasonics, Danbury, CT) for 40 min to form a reverse microemulsion. When the sonication finished, a mixture of TEOS (36 μL) and RB-APTES stock solution (4 μL) was injected to the resulting transparent homogeneous solution via a syringe pump at a rate of 2 μL/min. The reaction mixture was sealed again, shielded from light and stirred at room temperature for 24 hours. Then 20 μL of TEOS was injected at the same rate and the reaction mixture was stirred for another 24 hours. The product NaYF₄@SiO₂ NPs were precipitated by adding 30 mL of EtOH and centrifugation at 24,000 × g for 20 min. The NPs were washed twice with EtOH (20 mL each time, 24,000 × g, 20 min) and finally dispersed in 5 mL of DI-water.

5.2.4 Surface Functionalization of NaYF₄@SiO₂ NPs

5.2.4.1 Preparation of NaYF₄@SiO₂-NH₂ NPs

An aqueous solution (2 mL) of NaYF₄@SiO₂ NPs (~ 4 mg) was mixed with 40 μL of glacial AcOH and 20 μL of APTES. The mixture was stirred at room temperature for 4 hours, then the NPs were precipitated by centrifugation at 35,000 × g for 20 min, washed with EtOH (2 mL × 2) and finally dispersed in 2 mL of EtOH.

5.2.4.2 Preparation of NaYF₄@SiO₂-COOH NPs

NaYF₄@SiO₂-NH₂ NPs (~ 4 mg) were precipitated from their EtOH stock solution by centrifugation and redispersed in anhydrous DMF (1.5 mL), then a solution of diglycolic anhydride (0.1 g) in DMF (0.5 mL) was added. The mixture was shielded from light and stirred at room temperature for 20 hours. The NPs were precipitated by centrifugation at 35,000 × g for 20 min, washed with DI-water (2 mL × 2) and finally dispersed in 2 mL of DI-water.

5.2.5 Preparation of NaYF₄@SiO₂-NAv Conjugate

An aqueous solution (0.5 mL) of NaYF₄@SiO₂-COOH NPs (~ 1 mg) was mixed with a freshly prepared solution of EDC (4 mg) and sulfo-NHS (11 mg) in 0.1 M pH 5.5 MES buffer (0.1 mL). The mixture was gently shaken on a vortex mixer for 30 min, then the activated NPs were precipitated by centrifugation at 35,000 × g for 20 min, washed with 10 mM pH 7.4 phosphate buffer (PB) once and redispersed in 0.25 mL of the same PB. To this solution, 10 μL of NAv solution (2 mg/mL in PBS) was added and then the mixture was gently shaken for 3 hours. The
NPs were precipitated by centrifugation at 35,000 × g for 20 min, washed with 10 mM PB (0.25 mL × 2) to remove unbound NAv and finally redispersed in 10 mM PB containing 0.5% w/v BSA to block unreacted surface groups.

The average number of NAv per NP was quantified by a micro BCA assay. The assay was performed by using a Pierce micro BCA assay kit from Thermo Fisher Scientific. According to the instruction provided by the vendor, 5.0 mL of reagent A (buffer solution), 4.8 mL of reagent B (BCA solution) and 0.2 mL of reagent C (Cu²⁺ solution) was mixed to make a freshly prepared working reagent (BCA-Cu conjugate). A series of NAv standard solution (40, 20, 10, 5 and 2.5 μg/mL) were prepared by sequential dilution from a 0.2 mg/mL stock solution of NAv in 10 mM PB. In each 1.5 mL microcentrifuge tube, 0.5 mL of working reagent and 0.5 mL of NAv standard solution were mixed. The tubes were heated at 60 °C in a water bath for 1 hour, then cooled to room temperature and taken to measure absorbance at 562 nm $A_{562}$. Plotting $A_{562}$ against [NAv] gave the calibration curve of micro BCA assay for NAv, which is shown in Figure 5-7.

To quantify the average number of NAv per NP, 50 μL of the supernatant containing unbound NAv after the conjugation reaction was diluted by adding 450 μL of 10 mM PB, then mixed with 0.5 mL of working reagent and heated at 60 °C in a water bath for 1 hour. The mixture was cooled to room temperature and then measured $A_{562}$. This value was converted to [NAv] using the calibration curve in Figure 5-7. The total amount of NAv bound to NaYF₄@SiO₂-COOH NPs was calculated by subtracting the amount of unbound NAv from the amount of NAv added to the conjugation reaction. Detailed calculations are presented in Section 5.3.4.

5.2.6 Bead-Based Immunoassay

In a 1.5 mL microcentrifuge tube, 50 μL of the Ce,Tb-encoded PMMA bead solution (9.0 wt % in DI-water) was diluted by adding 250 μL of 0.1 M pH 5.5 MES buffer (activation buffer), then the beads were sedimented by centrifugation at 1,000 × g for 3 min. The beads were washed with activation buffer (300 μL × 2) and then dispersed in activation buffer (200 μL). To the bead solution, a freshly prepared solution of EDC (5 mg) and NHS (12.5 mg) in activation buffer (50 μL) was added. The tube was gently shaken on a vortex mixer for 25 min, then the beads were sedimented by centrifugation and washed with PBS (200 μL × 2).
To the tube containing activated beads, a solution of GaH IgG (40 μL, 2.5 mg/mL in PBS) was added. The tube was shaken overnight, then the beads were sedimented by centrifugation, washed with PBS (200 μL × 2) and then redispersed in 200 μL of PBS containing 1% w/v BSA to block unreacted sites. The GaH IgG labeled beads thus prepared were stored at 4 ºC for further experiments.

For immunoassays, the GaH IgG labeled beads were sedimented again and redispersed in 150 μL of PBS containing 0.1% w/v Tween-20 (reaction buffer). The solution was equally divided into three microcentrifuge tubes labeled as Tube A to C. To Tube A, 150 μL of reaction buffer containing 12.5 mg of human IgG was added. To Tube B and C, same amount of solution containing mouse IgG or rabbit IgG was added instead of human IgG. All the three tubes were shaken for 3 hours. The beads were sedimented, washed with reaction buffer (200 μL × 2 per tube) and redispersed in reaction buffer (190 μL per tube).

After that, a solution of biotinylated GaH IgG (1.5 mg/mL in PBS) was added to each of the three tubes (10 μL per tube). The tubes were shaken for 1.5 hours. The beads were sedimented, washed with reaction buffer (200 μL × 2 per tube) and stored in reaction buffer (200 μL per tube) for the next step.

5.2.6.1 Detection by FITC-NAv

From each tube (A, B or C), a 50 μL aliquot of beads solution was transferred to a new tube. The beads were sedimented and redispersed in PBS without Tween-20 (100 μL per tube). To each new tube, a solution of FITC-NAv (2.5 μL per tube, 2 mg/mL in PBS) was added. The tubes were shaken for 1 hour. The beads were sedimented, washed with PBS (200 μL × 2 per tube) and stored in PBS (200 μL per tube).

5.2.6.2 Detection by NaYF₄@SiO₂-NAv NPs

From each tube (A, B or C), a 50 μL aliquot of beads solution was transferred to a new tube. The beads were sedimented and redispersed in 10 mM pH 7.4 PB containing 0.5% w/v BSA (100 μL per tube). To each new tube, a solution of NaYF₄@SiO₂-NAv (100 μL per tube, ~ 2 mg/mL in 10 mM PB) was added. The tubes were shaken for 1 hour. The beads were sedimented by centrifugation at 1000 × g for 3 min, washed with 10 mM PB (200 μL × 2 per tube) and stored in 10 mM PB (200 μL per tube).
5.3 Results and Discussion

5.3.1 Preparation of OA Capped NaYF$_4$ NPs

OA capped NaYF$_4$ NPs were prepared by my colleague Dr. Jothir Pichaandi following a literature procedure as described above.$^{25}$ The average diameter of the NPs given by TEM analysis was 20 ± 1 nm (Figure 5-1(a)). For the silica coating reaction, it was necessary to quantify the concentration of NPs in the storage media. To determine the NP concentration, I transferred 1.0 mL of the OAH-ODE reaction mixture to a centrifuge tube and added 5 mL of EtOH to precipitate the NPs. The NPs were pelleted by centrifugation. Then the supernatant was decanted, the pelleted NPs were redispersed in 0.5 mL of cyclohexane and precipitated again by adding 5 mL of EtOH. After three cycles of washing, the NPs were dried under vacuum overnight. The weight of dry NPs was typically between 6 and 7 mg. Taking one batch as an example of which the weight was 6.4 mg, the NPs were then subjected to TGA analysis. In that case, the mass loss at 700 °C was 17.9% (Figure 5-1(b)). The molar concentration of NaYF$_4$ NPs in their original storage media was calculated as follows, which gave a value of 28 mM.

$$c(\text{NaYF}_4) = \frac{w(\text{NP}) \times (1 - \Delta w\%) \div M_w(\text{NaYF}_4)}{V(\text{reaction mixture})}$$

$$= \frac{6.4 \text{ mg} \times 0.821 \div 187.89 \text{ g/mol}}{1.0 \text{ mL}} = 28 \text{ mM}$$

(4)

The surface ligand density of OA capped NaYF$_4$ NPs can also be calculated based on the TEM and TGA analysis. Assuming the same density ($\rho = 4.21 \text{ g/cm}^3$) for NaYF$_4$ NPs with the bulk material of NaYF$_4$, the mass of one $d = 20$ nm NaYF$_4$ NP is

$$m(\text{NP}) = \rho \times \frac{1}{6} \pi d^3 = 1.8 \times 10^{-17} \text{ g}$$

and the surface area of one NP is

$$A(\text{NP}) = \pi d^2 = 1.3 \times 10^3 \text{ nm}^2$$

The mass of OA ligands on one NP is
\[ m(OA) = m(NP) \times \frac{\Delta w\%}{1 - \Delta w\%} = 1.8 \times 10^{-17} \text{ g} \times \frac{0.179}{0.821} = 3.9 \times 10^{-18} \text{ g} \]

which corresponds to a total number of

\[ N(OA) = \frac{m(OA)}{M_w(OA)} \times N_A = 8.2 \times 10^3 \text{ OA molecules} \]

thus the surface density of OA on \( d = 20 \text{ nm} \) NaYF\(_4\) NPs is

\[ \sigma(OA) = \frac{N(OA)}{A(NP)} = \frac{8.2 \times 10^3}{1.3 \times 10^3 \text{ nm}^2} = 6.3 \text{ nm}^{-2} \]

and the footprint of OA ligand is 0.16 OA/nm\(^2\).

Figure 5-1. (a) TEM image of OA capped NaYF\(_4\) NPs (prepared by Dr. J. Pichaandi) (b) TGA trace of OA capped NaYF\(_4\) NPs.

5.3.2 Silica Coating on NaYF\(_4\) NPs

Because the surface of OA capped NaYF\(_4\) NPs is hydrophobic, silica coating on those NPs cannot be achieved by the Stöber method.\(^{99}\) Here I used a reverse microemulsion method to coat a layer of silica shell on NaYF\(_4\) NPs.\(^{43}\) I first dispersed around 10 mg of OA capped NaYF\(_4\) NPs in 10 mL of cyclohexane. According to result of Equation (4), the molar concentration of NaYF\(_4\) NPs was 4.2 mM. Then a non-ionic surfactant, Igepal CO-520, was added to the NP colloidal solution and the mixture was stirred to dissolve the surfactant. The following step was to add more surfactant and aqueous NH\(_4\)OH to the mixture. Upon the addition of NH\(_4\)OH the solution
temporarily turned cloudy but became transparent again within 1 min under vigorous stirring. The resulting transparent solution was sealed and sonicated for 40 min to form the reverse microemulsion.

One major advantage of silica coating is that dyes or other functional small molecules can be readily loaded into the silica layer, which facilitates the preparation of multifunctional nanocomposites. In this case, I used a mixture of TEOS and RB-APTES to load RB dye into the silica shell. The main purpose of this step is to make the NaYF$_4$@SiO$_2$ NPs colorful and easier to track in future experiments. It is crucial to slowly inject the silicate reagents at a rate of 2 $\mu$L/min into the reaction mixture. My own experience indicated that, if the silicate reagents were added all at once, the resulting product NPs would not be well dispersible in ethanol or water.

After 48 hours of reaction, ethanol was added to break the microemulsion and to precipitate the NPs. The NaYF$_4$@SiO$_2$ NPs were collected by centrifugation and formed a red pellet at the bottom of centrifuge tubes. The NPs were washed twice with ethanol to remove free surfactant and unreacted silicate reagents. After the silica coating, the NPs became readily dispersible in methanol, ethanol and water. The NaYF$_4$@SiO$_2$ NPs were finally dispersed in 5 mL of water, which corresponded to a concentration of 8.4 mM NaYF$_4$ NPs. Both the pelleted NPs and the NP colloidal solution showed yellow fluorescence of the RB dye under 365 nm UV irradiation (Figure 5-2).
TEM analysis showed that the average diameter of NaYF₄@SiO₂ core-shell NPs was 39 nm. Given that the NaYF₄ core NP has an average diameter of 20 nm, the thickness of SiO₂ shell was around 10 nm (Figure 5-3).
5.3.3 Surface Functionalization of NaYF\textsubscript{4}@SiO\textsubscript{2} NPs

The as-prepared NaYF\textsubscript{4}@SiO\textsubscript{2} NPs only bear Si–OH groups on their surface. The NP surface needs to be modified to introduce functional groups for bioconjugation. I first treated the NaYF\textsubscript{4}@SiO\textsubscript{2} NPs with APTES to introduce –NH\textsubscript{2} groups to the NP surface. The –NH\textsubscript{2} groups were reacted with diglycolic anhydride to yield –COOH groups (Scheme 5-1).

In DI-water, the $z$-average hydrodynamic diameters ($D_z$) of NaYF\textsubscript{4}@SiO\textsubscript{2}, NaYF\textsubscript{4}@SiO\textsubscript{2}-NH\textsubscript{2} or NaYF\textsubscript{4}@SiO\textsubscript{2}-COOH NPs were similar to each other. However, when the NPs were dispersed in 10 mM pH 7.4 PB they showed very different colloidal properties. NaYF\textsubscript{4}@SiO\textsubscript{2} and NaYF\textsubscript{4}@SiO\textsubscript{2}-COOH NPs were colloidally stable in 10 mM PB, whereas the $D_z$ of NaYF\textsubscript{4}@SiO\textsubscript{2}-COOH NPs (61.4 nm) was smaller than NaYF\textsubscript{4}@SiO\textsubscript{2} NPs (71.5 nm) (Figure 5-4). On the contrary, NaYF\textsubscript{4}@SiO\textsubscript{2}-NH\textsubscript{2} NPs became colloidally unstable in 10 mM PB and rapidly precipitated. This may result from the surface charge of NaYF\textsubscript{4}@SiO\textsubscript{2}-NH\textsubscript{2} NPs being neutralized by phosphate ions, which decreased the electrostatic repulsion between the NPs and caused the NPs to aggregate.
The difference between surface properties of those NPs was also characterized by zeta potential measurement. Zeta potentials were measured at pH 6 since NaYF₄@SiO₂-NH₂ NPs were colloidally unstable at neutral pH. The starting material, NaYF₄@SiO₂ NPs, showed a zeta potential of –28 mV. The –OH groups on the NP surface are weakly acidic and partially deprotonate, explaining the negative charge. When the NPs were treated with APTES to introduce –NH₂ groups, the zeta potential increased to +13 mV. When the –NH₂ groups were converted to –COOH groups by reacting with diglycolic anhydride the zeta potential of NPs decreased to –48 mV, which was lower than the original NaYF₄@SiO₂ NPs. This result is reasonable, since carboxylic acids have a lower pKₐ and dissociate more than silicic acids. The results are summarized in Table 5-1.
Table 5-1. Z-average diameter (PDI in parentheses) and zeta potential of NaYF₄@SiO₂ NPs with different surface functional groups.

<table>
<thead>
<tr>
<th></th>
<th>NaYF₄@SiO₂</th>
<th>NaYF₄@SiO₂-NH₂</th>
<th>NaYF₄@SiO₂-COOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_z$ in DI-water</td>
<td>77.1 nm (0.180)</td>
<td>81.1 nm (0.225)</td>
<td>79.3 nm (0.219)</td>
</tr>
<tr>
<td>$D_z$ in 10 mM PB at pH 7.4</td>
<td>71.5 nm (0.142)</td>
<td>(aggregation)</td>
<td>61.4 nm (0.108)</td>
</tr>
<tr>
<td>Zeta Potential at pH 6.0</td>
<td>−28 mV</td>
<td>+13 mV</td>
<td>−48 mV</td>
</tr>
</tbody>
</table>

5.3.4 NAv Conjugation onto NaYF₄@SiO₂-COOH NPs

NAv conjugation onto NaYF₄@SiO₂-COOH NPs was achieved by an EDC/NHS mediated coupling reaction. The −COOH groups on the NP surface were first activated by EDC and sulfo-NHS at pH 5.5. Then the activated NPs were transferred to 10 mM PB at pH 7.4 and reacted with −NH₂ groups on the NAv protein (Scheme 5-2). TEM images of NaYF₄@SiO₂-COOH NPs and NaYF₄@SiO₂-NAv NP-protein conjugates showed that the protein conjugation did not affect the size or morphology of the NPs (Figure 5-5). DLS analysis indicated that both NaYF₄@SiO₂-COOH NPs and NaYF₄@SiO₂-NAv NP-protein conjugates were colloidally stable in 10 mM PB (Figure 5-6). After the NAv conjugation the $D_z$ of NPs slightly decreased from 61.4 nm to 54.7 nm and the zeta potential of NPs increased from −48 mV to −31 mV.

![Scheme 5-2. NAv conjugation onto NaYF₄@SiO₂-COOH NPs.](image-url)
The average number of NAv conjugated to each NP was quantified by a micro BCA protein assay. In this assay, Cu$^{2+}$ ions are reduced to Cu$^{+}$ by peptide bonds of proteins. The Cu$^{+}$ ions form a purple-colored complex with BCA, which has an absorption maximum at 562 nm. Compared to the Bradford assay, the micro BCA assay is more suitable for quantifying proteins at low concentrations (< 100 μg/mL). Nevertheless, this assay is incompatible with reductants and...
metal chelators. Figure 5-7 shows a calibration curve of the micro BCA assay for NAv, which has a good linearity when the NAv concentration ranged from 0 to 45 μg/mL.

![Calibration curve of micro BCA assay for NAv](image)

**Figure 5-7. Calibration curve of micro BCA assay for NAv.**

After the NAv conjugation reaction, the concentration of unbound NAv left in the supernatant (diluted to 1.0 mL) was determined to be 15.9 μg/mL. Since the total amount of NAv added to the reaction was 20 μg, the amount of NAv attached to the NPs was 4.1 μg. This value corresponds to 68 pmol or $4.1 \times 10^{13}$ molecules of NAv. On the other hand, the conjugation reaction involved 0.5 mL of 8.4 mM NaYF₄@SiO₂ NPs. The mass of NPs indicated by NaYF₄ was

$$0.5 \text{ mL} \times 8.4 \text{ mM} \times 187.89 \text{ g mol}^{-1} = 790 \text{ μg NaYF}_4$$

For $d = 20$ nm NaYF₄ NPs, the mass of each NP is

$$\frac{\pi}{6} \times (20 \text{ nm})^3 \times 4.21 \text{ g cm}^{-3} = 1.8 \times 10^{-11} \text{ μg}$$

so 790 μg NaYF₄ contains $790 / (1.8 \times 10^{-11}) = 4.5 \times 10^{13}$ NPs. Thus the average number of NAv conjugated to each NP was
\[
\frac{4.1 \times 10^{13}}{4.5 \times 10^{13}} = 0.9
\]

In summary, each NaYF₄@SiO₂ NP was conjugated with an average of one NAv.

5.3.5 Bead-Based Immunoassay

Previous studies showed that conjugation to NPs may affect the activity of proteins. To test whether the NaYF₄@SiO₂-NAv conjugates still maintain their biotin binding ability, I performed a sandwich-type bead-based immunoassay using the NaYF₄@SiO₂-NAv conjugate as a reporter (Scheme 5-3). This assay targeted human IgG as the analyte. Mouse IgG and rabbit IgG were selected as controls.

In this assay, PMMA microspheres encoded with CeF₃ and TbF₃ NPs were used as bead supports. Those microspheres were prepared according to the procedure described in Chapter 4, Section 4.2.3.3. The first step of the assay was to conjugate the capture antibody, goat anti-human IgG (GaH IgG), onto the surface of the beads via an EDC/NHS mediated coupling reaction. The antibody-conjugated beads were then incubated with a solution containing human IgG, mouse IgG or rabbit IgG. The capture antibody GaH IgG selectively captured human IgG but not mouse IgG or rabbit IgG. The third step was to incubate the beads with the detection antibody, biotinylated GaH IgG, which only bound to beads treated with human IgG.
Scheme 5-3. Design of the sandwich-type bead-based immunoassay.

The last step of the assay was to incubate the beads with a reporter compound. In conventional multiplexed bead-based immunoassays the reporter is typically a fluorescent dye-labeled SAv or NAv protein. Here I used FITC-NAv as a fluorescent reporter. Figure 5-8 presents confocal microscope images of the beads after incubation with the FITC-NAv reporter. Beads treated with mouse or rabbit IgG did not bind to the detection antibody, thus were not labeled by the reporter and barely fluoresced under 488 nm laser excitation (Figure 5-8(b) and (d)). Only beads treated with the target analyte, human IgG, had the biotinylated detection antibody bound to their surface that could be detected by the FITC-NAv reporter and showed fluorescence under 488 nm laser excitation (Figure 5-8(f)).
Figure 5-8. (a) Optical and (b) confocal fluorescent microscopic image of bead-based immunoassay incubated with mouse IgG (control analyte). (c) Optical and (d) confocal fluorescent microscopic image of bead-based immunoassay incubated with rabbit IgG (control analyte). (e) Optical and (f) confocal fluorescent microscopic image of bead-based immunoassay incubated with human IgG (the target analyte). Scale bar: 4 μm.

Now that the specificity of the assay was confirmed by the experiment above, I used the NaYF₄@SiO₂-NAv conjugates as the reporter to test the biotin binding ability of the conjugates. Figure 5-9 presents TEM images of beads after incubation with the NaYF₄@SiO₂-NAv reporter. Similar to the results given by the FITC-NAv reporter, only beads incubated with the target analyte, human IgG, had the NaYF₄@SiO₂-NAv NPs bound to their surface due to the presence of the detection antibody, biotinylated GaH IgG. Beads incubated with control analytes, namely
mouse or rabbit IgG, had hardly any NaYF@SiO2-NAv NPs bound to their surface. These results establish the applicability of NaYF@SiO2-NAv NPs as a reporter for bead-based immunoassays.

Figure 5-9. TEM images of bead-based immunoassay using NaYF@SiO2-NAv as the reporter. (a) incubated with mouse IgG (control analyte) (b) incubated with rabbit IgG (control analyte) (c) incubated with human IgG (the target analyte). Scale bar: 200 nm.

5.4 Summary

In this chapter, I described the preparation of NaYF@SiO2 NPs by using a reverse microemulsion method. This water-in-oil microemulsion was stabilized by a non-ionic surfactant Igepal CO-520. Silica coating on the NP surface was achieved by ammonia-catalyzed hydrolysis of TEOS. To make the NaYF@SiO2 NPs visible to naked eyes and easy to track in the subsequent experiments, a small amount of RB-APTES dye was embedded in the SiO2 shell. The product NaYF@SiO2 NPs were red-colored and showed yellow fluorescence under 365 nm UV light irradiation.

For conjugation with bioaffinity agents, the surface of NaYF@SiO2 NPs was first functionalized with APTES to introduce –NH2 groups. The –NH2 groups were reacted with diglycolic anhydride to yield NaYF@SiO2-COOH NPs. The change of surface functional groups on the NaYF@SiO2 NPs was characterized by zeta potential measurement. The difference of functional groups on the NP surface also affected their colloidal stability. NaYF@SiO2, NaYF@SiO2-NH2 and NaYF@SiO2-COOH NPs were all colloidally stable in water. Nevertheless, only NaYF@SiO2 and NaYF@SiO2-COOH NPs but not NaYF@SiO2-NH2 NPs were colloidally stable in 10 mM PB at pH 7.4. This may result from the –NH2 groups being neutralized by phosphate ions, thus the surface of NaYF@SiO2-NH2 NPs lack enough electrostatic repulsion to stabilize the NPs.
NAv was conjugated to the NaYF₄@SiO₂-COOH NPs by using a EDC-NHS coupling reaction. The average number of NAv bound to each NP was quantified to be 0.9 by a micro BCA protein assay. The biological activity of the NaYF₄@SiO₂-NAv NP-protein conjugate was tested by a sandwich-type bead-based immunoassay. The target analyte of the assay was human IgG. The assay employed PMMA microspheres encoded with small CeF₃ and TbF₃ NPs as the bead support. The assay began with conjugating the capture antibody, GaH IgG, onto the beads by using an EDC/NHS mediated coupling reaction. The antibody-conjugated beads were then incubated with a solution containing the target analyte human IgG or control analytes, namely mouse or rabbit IgG. Only human IgG was captured by the GaH IgG bound to the surface of the beads. Then human IgG, after being captured by the beads, was labeled by a detection antibody, biotinylated GaH IgG. The last step of the assay was to label the biotinylated detection antibody with a reporter. I first used FITC-NAv as a fluorescence reporter and characterized the beads by fluorescence confocal microscopy. Then I used the NaYF₄@SiO₂-NAv NP-protein conjugate as an alternative reporter tag and characterized the beads by TEM. In both cases, only beads incubated with the target analyte, human IgG, has either the FITC-NAv or the NaYF₄@SiO₂-NAv NP reporter bound to their surface, which confirmed both the specificity of the assay and the applicability of the NaYF₄@SiO₂-NAv NP-protein conjugate in biolabeling experiments.
6 Chapter 6: Preparation and Application of NaHoF₄@SiO₂-PEG NPs as Reporter Tags in Single Cell Analysis Based on Mass Cytometry

6.1 Introduction

Highly sensitive cytometric cell analysis is crucial for detection of rare cell population and cell events as well as identification of novel biomarkers that allows in-depth analysis of heterogeneous cell samples. The importance of effectively detecting low-abundance cells and biomarkers is demonstrated by its clinical impact. It not only provides people with detailed information about physiological and pathological mechanisms, but also helps to improve the accuracy of early diagnosis of diseases. Fluorescence-based conventional flow cytometry can detect as few as 3100 copies of HER3 per cell. Detection of low-abundance cellular biomarkers requires antibodies tagged with highly bright fluorophores, such as phycoerythrin (PE), allophycocyanin (APC) and Brilliant Violet™ dyes.

A normalized measure of brightness of different fluorophores in flow cytometry is the staining index (S.I.). S.I. is defined as $D/W$, where $D$ is the difference between the median fluorescence intensity of positive and negative cell populations and $W$ is twice the standard deviation of the negative cell population histogram (Figure 6-1).

![Figure 6-1](image.png)

Figure 6-1. A sample scheme showing histograms of negative and positive cell populations on flow cytometry to explain the concept of staining index. Red: positive cell population histogram. Blue and Green: negative cell population histogram. $D$: the difference between the median fluorescence intensity of positive and negative cell populations. $W_1$ and $W_2$: the spread of the negative cell population histogram, which is equal to twice the standard deviation. Reprinted with permission from ref. 105.
Quantifying cell surface biomarker by flow cytometry is arduous. It requires a series of polymer beads with predetermined antibody binding capacities to generate a calibration curve between arbitrary mean fluorescence intensities and absolute values of antibodies per bead or cell. Also, the accuracy of measurement is affected by various factors including autofluorescence of cells, photobleaching of dyes and photon-saturation of the detector.111

Thanks to the inherently quantitative nature of ICP-MS measurement, quantitation of cellular biomarkers by mass cytometry is much easier. For a mass cytometry analysis, the cells are labeled with antibodies tagged with lanthanide (Ln) metal chelating polymers (MCPs) instead of fluorescent reporters. The average intensity of cell population histogram obtained by mass cytometry yields the number of Ln ions per cell. By dividing the number of Ln ions per cell by the number of Ln ions per antibody, one can obtain the absolute number of antibodies per cell, also known as antibody binding capacity per cell, without any external calibration curve. Illy et al. showed that antibody binding capacity values determined by mass cytometry correspond well with literature values given by quantitative flow cytometry, which confirmed mass cytometry as a reliable method for quantifying cell surface biomarkers.16

Nevertheless, the sensitivity of MCP-tagged antibodies in mass cytometry is lower than that of the brightest fluorophores, e.g. PE and APC, in flow cytometry.5 For instance, APC-tagged anti-human CD8 antibody showed a S.I. value of 349 on human peripheral blood mononuclear cells, whereas the same antibody tagged with 152Sm-MCP showed a S.I. value of only ~150 (Figure 6-2).5,109 This limitation, which stems from the current polymer-based metal tags carrying only 100 – 200 Ln ions per antibody, sets a barrier for the detection of low-abundance biomarkers by mass cytometry. The need to get over this barrier stimulates the effort of developing surface functional lanthanide nanoparticles (Ln NPs) as the next-generation metal tags for high-sensitivity cell assays based on mass cytometry, since NP-based tags can carry many more Ln ions per antibody than MCPs. For instance, a spherical \( d = 10 \text{ nm} \) NaLnF\(_4\) NP contains \( \sim 8,000 \) Ln ions, which is two orders of magnitude higher than one MCP chain.
In this chapter, I first introduce the preparation of NaHoF\(_4@\)SiO\(_2\) NPs by the same reverse microemulsion method I described in Chapter 5. Then I describe the attachment of PEG molecules with different lengths (\(M_w\) 2,000 or 5,000) and different functional end-groups (–OMe or –COOH) onto the surface of the NaHoF\(_4@\)SiO\(_2\) NPs. The resulting NaHoF\(_4@\)SiO\(_2\)-PEG NPs displayed satisfactory colloidal stability in PBS. Mass cytometry studies showed that PEGylation of the NaHoF\(_4@\)SiO\(_2\) NPs did suppress non-specific cell binding of those NPs to KG1a cells.

To specifically target cell surface biomarkers, I performed antibody conjugation with NaHoF\(_4@\)SiO\(_2\)-PEG5k NPs, using a thiol-maleimide coupling reaction. The NP surface was functionalized by maleimide groups. The antibody, namely a goat anti-mouse IgG Fc antibody (GaM Fc Ab), was reacted with Traut’s reagent to introduce thiol groups, then the maleimide-activated NPs and the thiolated Ab were reacted overnight to form a NP-GaM-Ab conjugate. In the specific cell binding experiment, the CD20 surface antigen on Ramos cells was first labeled with a mouse anti-human CD20 primary antibody. Then the primary antibody was detected by the NP-GaM-Ab conjugate and the NP-labelled cells were analyzed by mass cytometry. As a control,
the same Ramos cells were directly incubated with the NP-GaM-Ab conjugate without adding the primary antibody, to evaluate the non-specific cell binding of the NP-GaM-Ab conjugate.

6.2 Experimental Details

6.2.1 Materials

\( \text{HoCl}_3 \cdot 6\text{H}_2\text{O} \) (99.9\% trace metals basis), oleic acid (90\%), 1-octadecene (90\%), \( \text{NH}_4\text{F} \) (98\%), Igepal CO-520, TEOS (98\%), cyclohexane (99\%), (3-mercaptopropyl)trimethoxysilane (MPTMS, 95\%), \( L \)-cysteine hydrochloride monohydrate (98\%), methoxypolyethylene glycol maleimide, \( M_n \) 2,000 or 5,000 (mPEG2k-MAL and mPEG5k-MAL, 90\%), \( N \)-(3-(dimethylamino)propyl)-\( N \)-ethylcarbodiimide hydrochloride (EDC, BioXtra), \( N \)-hydroxysulfosuccinimide sodium salt (sulfo-NHS, 98\%), \( N \)-(2-aminoethyl)maleimide trifluoroacetate salt (95\%) and regular solvents were purchased from Sigma-Aldrich and used without purification. \( \text{NaOH} \) (reagent grade pellet) was purchased from Caledon (Georgetown, ON). Ammonium hydroxide (28\%, reagent grade) was purchased from ACP Chemicals (Saint-Léonard, QC). RB-APTES solution was prepared according to the recipe in Chapter 5. Carboxyl-PEG-maleimide (COOH-PEG2k-MAL and COOH-PEG5k-MAL) were purchased from Nanocs (New York, NY, USA). Bradford reagent, Ellman’s reagent (5,5-dithio-bis-(2-nitrobenzoic acid) = DTNB), Traut’s reagent (2-iminothiolane, HCl salt) and goat anti-mouse IgG Fc secondary antibody (GaM Fc Ab) were purchased from Thermo Scientific.

6.2.2 Synthesis of OA Capped NaHoF₄ NPs

A mixture of \( \text{HoCl}_3 \cdot 6\text{H}_2\text{O} \) (2 mmol), oleic acid (OAH) (8 mL) and 1-octadecene (ODE) (34 mL) were heated to 120 °C in a three-necked flask under vacuum for 1 h to form a clear solution. Then the solution was cooled to room temperature with continuous stirring. A freshly prepared CH₃OH solution (20 mL) of \( \text{NH}_4\text{F} \) (0.30 g, 8.0 mmol) and \( \text{NaOH} \) (0.20 g, 5.0 mmol) was added dropwise via a syringe and the resulting cloudy mixture was stirred for 30 min after the addition was completed. The solution was slowly heated to 100 °C in open air to evaporate \( \text{CH}_3\text{OH} \). When all the \( \text{CH}_3\text{OH} \) was evaporated, the solution was heated to 285 °C at a rate of about 10 °C/min under a \( \text{N}_2 \) atmosphere and maintained at 285 °C for 100 min. Then the solution was cooled to room temperature, and the as-prepared NaHoF₄ NPs were stored in the reaction medium.
6.2.3 Silica Coating on NaHoF$_4$ NPs

1.5 mL of the OAH-ODE reaction mixture containing about 10 mg of OA capped NaHoF$_4$ NPs was transferred to a conical centrifuge tube. Then 7.5 mL of EtOH was added to precipitate the NPs. The NPs were sedimented by centrifugation at $2,700 \times g$ for 30 min. The supernatant was decanted. The pelleted NPs were redispersed in 0.5 mL of cyclohexane by sonication and precipitated again by adding EtOH (8.5 mL) and centrifugation. This washing cycle was repeated once more and the purified NPs were finally dispersed in 10 mL of cyclohexane.

To 10 mL of the NP colloidal solution in cyclohexane, 0.15 mL of Igepal CO-520 was added with stirring, and the mixture was stirred for 20 min. Then more Igepal CO-520 (0.50 mL) and ammonium hydroxide (0.08 mL) were added with vigorous stirring. The solution was sealed and sonicated in a Branson 1210 ultrasonic cleaner (max. output 80 W) (Branson Ultrasonics, Danbury, CT, USA) for 40 min to form a reverse microemulsion. When the sonication was finished, a mixture of TEOS (36 $\mu$L) and the RB-APTES stock solution (4 $\mu$L) was injected into the resulting transparent microemulsion solution via a syringe pump at a rate of 2 $\mu$L/min. The reaction mixture was sealed again, shielded from light and stirred at room temperature for 24 hours. Then 20 $\mu$L of TEOS was injected at the same rate, and the reaction mixture was stirred for another 24 hours. The product NaHoF$_4$@SiO$_2$ NPs were precipitated by adding 15 mL of EtOH followed by centrifugation at $2,700 \times g$ for 10 min. The NPs were washed twice with EtOH (20 mL each time, $16,000 \times g$, 20 min) and finally dispersed in 5 mL of absolute EtOH.

6.2.4 Surface Functionalization of NaHoF$_4$@SiO$_2$ NPs

6.2.4.1 Preparation of NaHoF$_4$@SiO$_2$-SH NPs

An EtOH solution (1 mL) containing NaHoF$_4$@SiO$_2$ NPs (~ 2 mg) was mixed with MPTMS (100 $\mu$L). Then the mixed solution was placed in a sealed glass vial and heated in an oil bath set at 90 °C with stirring overnight. The NaHoF$_4$@SiO$_2$-SH NPs were precipitated by centrifugation at $16,000 \times g$ for 20 minutes, washed with 1:1 EtOH-H$_2$O (1 mL \times 3) and finally redispersed in 0.8 mL of DI-water. The NaHoF$_4$@SiO$_2$-SH NPs should be used for the next step as soon as possible, since long-term storage of NaHoF$_4$@SiO$_2$-SH NPs leads to a loss of reactive thiol groups by oxidative formation of disulfide bonds. The number of thiol groups per NP was quantified by an Ellman’s assay. The detailed procedure of this Ellman’s assay is presented in the appendix of this chapter.
6.2.4.2 Preparation of NaHoF\textsubscript{4}@SiO\textsubscript{2}-PEG NPs

For a typical PEGylation reaction, an aqueous solution (400 μL) containing NaHoF\textsubscript{4}@SiO\textsubscript{2}-SH NPs (~ 1 mg) was mixed with a freshly prepared solution (100 μL) of PEG-maleimide (20 mM in 0.1 M pH 7.2 phosphate buffer). The mixed solution was gently shaken on a vortex mixer at room temperature overnight. Then the PEGylated NPs were precipitated by centrifugation at 16,000 × g for 20 minutes, washed with H\textsubscript{2}O (500 μL × 3) and finally redispersed in 400 μL of DI-water.

6.2.5 Cell Culture and Preparation

Cell culture and preparation were performed by Alexandre Bouzekri at Fluidigm Canada, Inc. The cells used for mass cytometry studies were grown in proper culture media at 37 °C, 5% CO\textsubscript{2} in a T75 flask at a density of 1 – 2 × 10\textsuperscript{6} cells/mL in 30 mL of culture media. For KG1a cells, the culture media was Iscove's Modified Dulbecco's Medium. For Ramos cells, the culture media was Advanced RPMI 1640 Medium. Both media contained 2 mM of L-glutamine and 100 U/mL penicillin-streptomycin.

To take the cells for future experiments, the cells were stained with Trypan Blue and counted by a TC20 Cell Counter, then the amount of cells needed were transferred to a 50 mL Falcon\textsuperscript{®} tube. CTL Anti-Aggregate Wash\textsuperscript{™} was added to prevent cell aggregation. The cells were then sedimented by centrifugation, resuspended in 5 mL of culture media and subjected to the next step.

6.2.6 Non-Specific Cell Binding Experiments of NaHoF\textsubscript{4}@SiO\textsubscript{2}-SH NPs and NaHoF\textsubscript{4}@SiO\textsubscript{2}-PEG NPs

Non-specific cell binding experiments of NPs were performed on KG1a cells by Dr. Jothirmayanathan Pichaandi. The cells were stained with Cell-ID\textsuperscript{™} Intercalator-Rh (Fluidigm Canada, Markham, ON) in the cell culture medium at 37 °C, 5% CO\textsubscript{2} for 15 min to identify dead cells, then washed with PBS containing 0.5 % (w/v) BSA once (1,500 × g, 3 min) and resuspended in chilled PBS/BSA buffer. Then the cells were stained with a mixture of \textsuperscript{154}Sm-labelled anti-human CD45 antibody and \textsuperscript{166}Er-labelled anti-human CD34 antibody at room temperature for 30 min, washed once and resuspended again in chilled PBS/BSA buffer at a final concentration of 2 × 10\textsuperscript{6} cells in 100 μL of PBS/BSA.
To test for non-specific cell binding of NPs, the cells were incubated with either NaHoF$_4$@SiO$_2$-SH NPs, NaHoF$_4$@SiO$_2$-PEG5k-OMe or NaHoF$_4$@SiO$_2$-PEG5k-COOH NPs with a series of NP concentrations (33, 170 and 830 NPs/cell) in PBS/BSA buffer at 4 °C for 2 hours. After that, the cells were sedimented by centrifugation and washed with chilled PBS three times to remove unbound NPs, then fixed with 1.6% paraformaldehyde/PBS solution at 4 °C overnight. The fixed cells were washed with PBS, stained with Cell-ID™ Intercalator-Ir (Fluidigm Canada, Markham, ON) and washed with PBS again. The pelleted cells were resuspended in an aqueous solution containing EQ™ Four Element Calibration Beads (Fluidigm Canada, Markham, ON) and subjected to mass cytometry analysis.

6.2.7 Conjugation of GaM Fc Ab onto NaHoF$_4$@SiO$_2$-PEG NPs

To an aqueous solution (100 μL) of NaHoF$_4$@SiO$_2$-PEG5k-COOH NPs (~ 0.25 mg), a freshly prepared solution of EDC (1.1 mg) and sulfo-NHS (2.7 mg) in 0.1 M pH 5.5 MES buffer (25 μL) was added. The mixture was gently shaken on a vortex mixer at room temperature for 30 min, then the NPs were pelleted by centrifugation at 16,000 × g for 20 min and redispersed in 100 μL of DI-water. To the sulfo-NHS-activated NP solution, a freshly prepared solution (25 μL) of N-(2-aminoethyl)maleimide trifluoroacetate salt (0.5 mg) in 0.1 M pH 8.0 phosphate buffer was added. The mixed solution was gently shaken at room temperature for 2 h. Then the NPs were pelleted by centrifugation, washed with DI-water (250 μL × 2) and redispersed in 250 μL of DI-water. This surface modified product is denoted as NaHoF$_4$@SiO$_2$-PEG5k-MAL NPs.

For conjugation with NaHoF$_4$@SiO$_2$-PEG5k-MAL NPs, the antibody GaM Fc Ab was thiolated by reaction with Traut’s reagent. 100 μL of the GaM Fc Ab solution (2.4 mg/mL in PBS) was adjusted to pH 8.0 by adding 1 μL of 0.1 M NaOH solution and 1 μL of EDTA solution (0.5 M, pH 8.0). Then 1 μL of freshly prepared solution of Traut’s reagent (6.7 mg/mL) in 0.1 M pH 8.0 phosphate buffer was added. The mixed solution was incubated at room temperature for 1 h, then 300 μL of coupling buffer (C-buffer = 0.1 M sodium phosphate, 0.15 M NaCl, 4 mM EDTA, pH 7.2) was added to the Ab solution. The Ab was purified from unreacted small molecules by a 30k MWCO centrifugal filter (12,000 × g, 5 min) and washed once with 350 μL of C-buffer. The concentrated Ab solution was adjusted to a total volume of 100 μL by adding more C-buffer, and the final concentration of thiolated Ab (Ab-SH) was determined by $A_{280}$. 
For NP-Ab conjugation by thiol-maleimide coupling, 100 μL of NaHoF₄@SiO₂-PEG5k-MAL NP solution (~1 mg/mL) in DI-water was mixed with 100 μL of Ab-SH solution in C-buffer. The mixed solution was gently shaken on a vortex mixer at room temperature for 16 h. Then the NPs were pelleted by centrifugation, washed with PBS (200 μL × 2), redispersed in 200 μL of PBS containing 0.5% (w/v) BSA and stored at 4 °C. The accurate concentration of the NP-GaM-Ab immunoconjugate was determined by ICP-MS. The amount of unbound GaM Fc Ab left in the supernatant was quantified by a Bradford assay. The detailed procedure for the Bradford assay is presented in the appendix of this chapter.

6.2.8 Non-Specific and Specific Cell Binding Experiments of the NaHoF₄@SiO₂-PEG NP-GaM-Ab Immunoconjugate

Non-specific and specific cell binding experiments of the NP-GaM-Ab immunoconjugate were performed on Ramos cells by Dr. Jothirmayanantham Pichaandi. The cells were stained with Cell-ID™ Intercalator-¹⁰³Rh in the cell culture media at 37 °C, 5% CO₂ for 15 min to identify dead cells, then washed with PBS containing 0.5 % (w/v) BSA once (1,500 × g, 3 min) and resuspended in chilled PBS/BSA buffer at a final concentration of 2 × 10⁶ cells in 100 μL of PBS/BSA.

To test non-specific cell binding of the NP-GaM-Ab conjugate, the cells were incubated with the NP-GaM-Ab conjugate with a series of NP dosages (130, 670, 3,300 and 16,700 NPs/cell) in PBS/BSA buffer at 4 °C for 2 hours. After that the cells were sedimented by centrifugation and washed with chilled PBS three times to remove unbound NPs, then fixed with 1.6% paraformaldehyde/PBS solution at 4 °C overnight. The fixed cells were washed with PBS, stained with Cell-ID™ Intercalator-Ir and washed with PBS again. The pelleted cells were resuspended in an aqueous solution containing EQ™ Four Element Calibration Beads and subjected to mass cytometry analysis.

To test specific cell binding of the NP-GaM-Ab conjugate, the cells were first incubated with mouse anti-human CD20 primary antibody (5 μg / 2 × 10⁶ cells) in PBS/BSA buffer at room temperature for 30 min. Then the cells were sedimented by centrifugation, washed with PBS/BSA and then incubated with the NP-GaM-Ab conjugate with a series of NP dosages (same as the non-specific cell binding experiments) in PBS/BSA buffer at 4 °C for 2 hours. After that the cells were sedimented by centrifugation and washed with chilled PBS three times to remove unbound NPs, then fixed and stained with the Ir-intercalator according to the procedure described above. The
cells were finally resuspended in an aqueous solution containing EQ™ Four Element Calibration Beads and subjected to mass cytometry analysis.

6.3 Results and Discussion

6.3.1 Silica Coating and Surface PEGylation of NaHoF₄ NPs

OA capped NaHoF₄ NPs were prepared by my colleague Elsa Lu by a modified high-temperature co-precipitation method.²⁵ The average diameter of the NPs given by TEM analysis was 12.0 ± 0.6 nm (Figure 6-3(A)). To render the NaHoF₄ NPs hydrophilic, the NPs were coated with a silica shell by a reverse microemulsion method. TEM analysis showed an average diameter of 21.3 ± 1.1 nm for NaHoF₄@SiO₂ NPs, which corresponds to a shell thickness of 4.7 nm (Figure 6-3(B)). For this method a uniform coating on single NP was achieved.

In order to suppress undesirable non-specific binding in cell assays, the surface of NaHoF₄@SiO₂ NPs was PEGylated via a two-step strategy.⁹⁴,¹¹² The first step was to react NaHoF₄@SiO₂ NPs with MPTMS in EtOH at an elevated temperature. The reaction took place in a sealed vial placed in an oil bath set at 85 °C. In this reaction, MPTMS hydrolyzed and condensed on the surface of NaHoF₄@SiO₂ NPs to introduce thiol groups. The NaHoF₄@SiO₂-SH NPs were readily dispersible in DI-water. Since thiol groups are susceptible to oxidation, the NaHoF₄@SiO₂-SH NPs should be used for the next step as soon as possible. In the second step, the thiol groups on NP surface were reacted with carboxyl-PEG5,000-maleimide (COOH-PEG5k-MAL) to attach PEGs onto the surface of NaHoF₄@SiO₂ NPs and yielded NaHoF₄@SiO₂-PEG5k-COOH NPs. TEM analysis showed average diameters of 21.6 ± 1.0 nm for NaHoF₄@SiO₂-SH NPs (Figure 6-3(C)) and 21.4 ± 1.2 nm for NaHoF₄@SiO₂-PEG5k-COOH NPs (Figure 6-3(D)). These results indicated that the surface modification did not affect the size and morphology of NaHoF₄@SiO₂ NPs.
Figure 6-3. TEM image of (A) NaHoF$_4$ NPs, $d = 12.0 \pm 0.6$ nm, (B) NaHoF$_4$@SiO$_2$ NPs, $d = 21.3 \pm 1.1$ nm, (C) NaHoF$_4$@SiO$_2$-SH NPs, $d = 21.6 \pm 1.0$ nm, (D) NaHoF$_4$@SiO$_2$-PEG5k-COOH NPs, $d = 21.4 \pm 1.2$ nm. All the images showed that every single NP was coated with a uniform silica shell (thickness = 4.7 nm). The surface modification did not affect the shell thickness and uniformity.

Since it is difficult to directly quantify the PEG density on NaHoF$_4$@SiO$_2$-PEG NPs, I used an Ellman’s assay to measure the number of thiol group per NP instead. In Ellman’s assay, thiol groups react with DTNB to yield a yellow-colored dianion 2-nitro-5-thiobenzoate at pH 8.0, which has an absorption maximum at 412 nm. A qualitative Ellman’s test of NaHoF$_4$@SiO$_2$, NaHoF$_4$@SiO$_2$-SH and NaHoF$_4$@SiO$_2$-PEG5k-COOH NPs showed that only NaHoF$_4$@SiO$_2$-SH NPs displayed a yellow color upon mixing with DTNB, whereas NaHoF$_4$@SiO$_2$ and NaHoF$_4$@SiO$_2$-PEG5k-COOH NPs remained colorless (Figure 6-4). This result indicated that no detectable thiol groups were present on NaHoF$_4$@SiO$_2$-PEG5k-COOH NPs, and implied that all of the thiol groups originally on the NPs reacted with COOH-PEG5k-MAL. In this way I infer that
the number of thiol groups per NaHoF₄@SiO₂-SH NP equaled the number of PEG molecules per NaHoF₄@SiO₂-PEG NP.

Figure 6-4. Qualitative Ellman’s test of (A) NaHoF₄@SiO₂ NPs (B) NaHoF₄@SiO₂-SH NPs (C) NaHoF₄@SiO₂-PEG5k-COOH NPs. In tube (A), the NaHoF₄@SiO₂ NPs had no thiol group on the surface, so the Ellman’s test did not show any color change. In tube (B), the NaHoF₄@SiO₂-SH NPs had thiol groups on the surface, so a yellow color developed after the NPs reacted with Ellman’s reagent. In tube (C), all the thiol groups originally on the NP surface reacted with MAL-PEG5k-COOH, so the Ellman’s test showed no color change again.

The number of thiol groups per NP was determined by a quantitative Ellman’s assay. The calibration curve was obtained by using cysteine as a standard for thiol groups (Figure 6-12). For a sample of NaHoF₄@SiO₂-SH NPs, whose concentration was determined by ICP-MS to be 2.1 mg/mL NaHoF₄, the Ellman’s assay showed an A₄₁₂ value of 0.739. This value was converted to 0.545 mM of thiol concentration. To quantify the number of thiol groups per NP, I first calculated the mass of each

\[ m_{\text{NP}} = \rho \times \frac{1}{6} \pi d^3 = 5.4 \times 10^{-15} \text{ mg} \]

Then the total number of NPs in 1 mL of 2.1 mg NaHoF₄/ mL NaHoF₄@SiO₂-SH NP solution was calculated to be

\[ N_{\text{total-NP}} = \frac{2.1 \text{ mg}}{5.4 \times 10^{-15} \text{ mg}} = 3.9 \times 10^{14} \]
and the total number of thiol groups in 1 mL of this solution was

\[ N_{\text{total-SH}} = 0.545 \text{ mM} \times 1 \text{ mL} \times N_{Av} = 3.28 \times 10^{17} \]

so the number of thiol per NP is

\[ N_{\text{SH/NP}} = \frac{N_{\text{total-SH}}}{N_{\text{total-NP}}} = \frac{3.28 \times 10^{17}}{3.9 \times 10^{14}} = 840 \]

Furthermore, the calculated surface area of one NaHoF\textsubscript{4}@SiO\textsubscript{2}-SH NP was (for \( d = 21.6 \text{ nm} \))

\[ A_{\text{NP}} = \pi d^2 = 1.46 \times 10^3 \text{ nm}^2 \]

so the average surface density of thiol groups on NaHoF\textsubscript{4}@SiO\textsubscript{2}-SH NPs was

\[ \sigma_{\text{SH/NP}} = \frac{N_{\text{SH/NP}}}{A_{\text{NP}}} = \frac{840}{1.46 \times 10^3 \text{ nm}^2} = 0.58 \text{ nm}^{-2} \]

which I assume is also the average surface density of PEG molecules on NaHoF\textsubscript{4}@SiO\textsubscript{2}-PEG5k-COOH NPs. The calculated results are summarized in Table 6-1.

**Table 6-1. Number and surface density of thiol groups on each NaHoF\textsubscript{4}@SiO\textsubscript{2}-SH NP.**

<table>
<thead>
<tr>
<th>Concentration of NaHoF\textsubscript{4}@SiO\textsubscript{2}-SH NPs (by ICP-MS)</th>
<th>Number of NPs in 1 mL</th>
<th>Number of –SH Groups in 1 mL (by Ellman’s Assay)</th>
<th>Number of –SH Groups per NP</th>
<th>Density of –SH Groups on the Surface of NP\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 mg/mL NaHoF\textsubscript{4}</td>
<td>3.9 \times 10^{14}</td>
<td>3.28 \times 10^{17}</td>
<td>840</td>
<td>0.58 nm\textsuperscript{-2}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} i.e. a docking area of 1.7 nm\textsuperscript{2} per –SH group.

6.3.2 Colloidal Stability of NaHoF\textsubscript{4}@SiO\textsubscript{2}-PEG NPs with Different PEG Length and Terminal Groups

To study the influence of PEG length and terminal groups on colloidal stability of PEGylated NaHoF\textsubscript{4}@SiO\textsubscript{2} NPs, I used four different PEG-maleimides: mPEG2k-MAL, mPEG5k-MAL, COOH-PEG2k-MAL and COOH-PEG5k-MAL to react with NaHoF\textsubscript{4}@SiO\textsubscript{2}-SH NPs and obtained four different NaHoF\textsubscript{4}@SiO\textsubscript{2}-PEG NPs: NaHoF\textsubscript{4}@SiO\textsubscript{2}-PEG2k-OMe, NaHoF\textsubscript{4}@SiO\textsubscript{2}-
PEG5k-OMe, NaHoF₄@SiO₂-PEG2k-COOH and NaHoF₄@SiO₂-PEG5k-COOH NPs. The colloidal stability of the above four NPs was examined by DLS.

In DI-water, NaHoF₄@SiO₂-PEG2k-OMe NPs showed a main peak with a mean diameter $d_{\text{mean}} = 104$ nm. After the dispersion medium was changed to PBS, this $d_{\text{mean}}$ value remained unchanged at 105 nm. However, after 24 h of storage in PBS, not only the $d_{\text{mean}}$ value further increased to 155 nm but also a considerable aggregation peak appeared (Figure 6-5). This indicates that the colloidal stability of NaHoF₄@SiO₂-PEG2k-OMe NPs eventually decreased upon storage in PBS.

The behaviour of NaHoF₄@SiO₂-PEG2k-COOH NPs was different from that of than NaHoF₄@SiO₂-PEG2k-OMe NPs in PBS. When then dispersion was changed from water to PBS, the $d_{\text{mean}}$ value of the main peak on the DLS CONTIN plot of NaHoF₄@SiO₂-PEG2k-COOH NPs decreased from 118 nm to 101 nm. After 24 h of storage in PBS, the $d_{\text{mean}}$ value increased to 112 nm. A slight increased aggregation peak was observed compared to the CONTIN plot in water. Nevertheless, the increase was much less than the one observed for NaHoF₄@SiO₂-PEG2k-OMe NPs (Figure 6-5). This difference indicates that NaHoF₄@SiO₂-PEG2k-COOH NPs are more colloidal stable than NaHoF₄@SiO₂-PEG2k-OMe NPs in PBS.

It is worth mentioning that many researchers use number-weighted instead of intensity-weighted size distribution plots to report their DLS results. This can conceal aggregation peaks and lead to an overestimation of NP colloidal stability. An example based on the two CONTIN plots corresponding to the two NaHoF₄@SiO₂-PEG2k NPs after 24 h of storage in PBS is presented in the appendix of this chapter, showing that inappropriate expression of DLS data may cause a biased conclusion.
Figure 6-5. DLS CONTIN plots of NaHoF$_4$@SiO$_2$-PEG2k-OMe and NaHoF$_4$@SiO$_2$-PEG2k-COOH NPs in different media. Both NPs were colloidally stable in water. However, after 24 h of storage in PBS, NaHoF$_4$@SiO$_2$-PEG2k-OMe NPs showed a higher extent of aggregation than NaHoF$_4$@SiO$_2$-PEG2k-COOH NPs. This difference indicates that the NaHoF$_4$@SiO$_2$-PEG2k-COOH NPs are more colloidally stable than NaHoF$_4$@SiO$_2$-PEG2k-OMe NPs in PBS.

On the contrary, both NaHoF$_4$@SiO$_2$-PEG5k-OMe and NaHoF$_4$@SiO$_2$-PEG5k-COOH NPs showed no aggregation peak at all even after 24 h of storage in PBS (Figure 6-6), which suggests that PEG5k can stabilize NaHoF$_4$@SiO$_2$ NPs more effectively in PBS than PEG2k. Since both NaHoF$_4$@SiO$_2$-PEG2k and NaHoF$_4$@SiO$_2$-PEG5k NPs were prepared from the same batch of NaHoF$_4$@SiO$_2$-SH NPs that had a surface thiol density of 0.58 nm$^{-2}$ (determined by Ellman’s assay), the two NPs should have a same surface PEG density $\sigma = 0.58$ nm$^{-2}$. However, their reduced tethering density $\sigma \pi R_g^2$ differs from each other. By using the equation $R_g = 0.181N^{0.58}$ (nm), where $N$ is the number of repeat units, we have $R_g = 1.6$ nm for PEG2k ($N = 44$) and 2.8 nm for PEG5k ($N = 110$), respectively. According to the discussion in Section 3.3.4, for PEG2k, the reduced tethering density $\sigma \pi R_g^2 = 3.3$ when $\sigma = 0.58$ nm$^{-2}$, which corresponds to a squeezed conformation.
For PEG5k, the reduced tethering density $\sigma R_g^2 = 9.7$, which corresponds to a more extended brush conformation. This difference may explain why PEG5k can provide better colloidal stability for the NaHoF$_4$@SiO$_2$ NPs than PEG2k.

![DLS CONTIN plots of NaHoF$_4$@SiO$_2$-PEG5k-OMe NPs and NaHoF$_4$@SiO$_2$-PEG5k-COOH NPs in different media.](image)

Figure 6-6. DLS CONTIN plots of NaHoF$_4$@SiO$_2$-PEG5k-OMe and NaHoF$_4$@SiO$_2$-PEG5k-COOH NPs in different media. After 24 h of storage no aggregation peak was observed for both NPs, indicating a good colloidal stability.

In summary, the two NaHoF$_4$@SiO$_2$-PEG5k NPs were more colloidally stable in PBS than the two NaHoF$_4$@SiO$_2$-PEG2k NPs. Among the two NaHoF$_4$@SiO$_2$-PEG2k NPs, NaHoF$_4$@SiO$_2$-PEG2k-COOH NPs showed better colloidal stability than NaHoF$_4$@SiO$_2$-PEG2k-OMe NPs. The z-average hydrodynamic diameter ($d_z$) and dispersity values of all the four different NaHoF$_4$@SiO$_2$-PEG NPs are listed in Table 6-2.
Table 6-2. Z-average diameters of different NPs in aqueous media (PDI in parentheses).

<table>
<thead>
<tr>
<th></th>
<th>(d_z) in water</th>
<th>(d_z) in PBS at 24 h</th>
<th>(d_z) in PBS after 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHoF(_4)@SiO(_2) -SH NPs</td>
<td>111 nm (0.22)</td>
<td>(unstable)</td>
<td>(unstable)</td>
</tr>
<tr>
<td>NaHoF(_4)@SiO(_2) -PEG2k-OMe NPs</td>
<td>97 nm (0.22)</td>
<td>98 nm (unstable)</td>
<td>-- (^a)</td>
</tr>
<tr>
<td>NaHoF(_4)@SiO(_2) -PEG2k-COOH NPs</td>
<td>103 nm (0.21)</td>
<td>114 nm (0.34)</td>
<td>104 nm (0.57)</td>
</tr>
<tr>
<td>NaHoF(_4)@SiO(_2) -PEG5k-OMe NPs</td>
<td>87 nm (0.16)</td>
<td>86 nm (0.16)</td>
<td>83 nm (0.10)</td>
</tr>
<tr>
<td>NaHoF(_4)@SiO(_2) -PEG5k-COOH NPs</td>
<td>97 nm (0.15)</td>
<td>95 nm (0.21)</td>
<td>95 nm (0.20)</td>
</tr>
</tbody>
</table>

\(^a\) The \(d_z\) value is not reported due to the high polydispersity.

6.3.3 Non-specific Cell Binding of NaHoF\(_4\)@SiO\(_2\) -SH and NaHoF\(_4\)@SiO\(_2\) -PEG5k NPs Measured by Mass Cytometry

Non-specific cell binding of NaHoF\(_4\)@SiO\(_2\) -SH NPs, NaHoF\(_4\)@SiO\(_2\) -PEG5k-OMe and NaHoF\(_4\)@SiO\(_2\) -PEG5k-COOH NPs was tested on KG1a cells, a variant subline derived from the human acute myelogenous leukemia cell line KG1.\(^{113}\) All the NPs were incubated with KG1a cells with three different NP concentrations: 33, 170 and 830 NPs per cell, respectively. The incubation was performed at 4 °C in PBS containing 0.5% (w/v) BSA for 2 hours. Then the cells were repeatedly washed 3x with PBS to remove unbound NPs. After fixation by paraformaldehyde and staining with the Ir-intercalator, the cells were finally suspended in DI-water and analyzed by mass cytometry.

An example of the gating procedure used in this mass cytometry data analysis is shown in Figure 6-7. First, the cytometry data containing all the cell events were gated on a \(^{103}\)Rh-\(^{191}\)Ir bivariate plot to identify live cells against dead cells. Since the Rh-intercalator selectively stains dead cells, the cell population showing high Ir intensity but low Rh intensity was gated as live cells. Second, the live cells were gated on a \(^{193}\)Ir-\(^{191}\)Ir bivariate plot to exclude events of cell aggregates (the high intensity end of the trace) and debris (the low intensity tail of the trace),
leaving only events of cell singlets. Third, the cell singlets were further gated on a $^{145}\text{Sm}-^{166}\text{Er}$ plot. This gating was employed because in this experiment, the KG1a cells were also stained with two Ln-MCP-tagged antibodies, $^{145}\text{Sm}$-anti-CD45 and $^{166}\text{Er}$-anti-CD34. Only cells expressing both CD45 and CD34 antigens were subjected to further analysis. Finally, the cell binding of NaHoF$_4$@SiO$_2$ NPs to those CD45- and CD34-overexpressed live cell singlets was shown by an intensity histogram of $^{165}$Ho in Figure 6-7(D).

Figure 6-7. Gating procedure in mass cytometry data processing. (A) Gating of live cells. The Rh-intercalator selectively stains dead cells, so cells stainable by only the Ir-intercalator but not the Rh intercalator are identified as live cells. (B) Gating of cell singlets. The cell singlets are gated from aggregates and debris (the big tail with low Ir intensity) on the $^{191}$Ir-$^{193}$Ir bivariate plot. (C) Gating of cells expressing desired biomarkers. The cells are stained by two MCP-tagged antibodies ($^{145}\text{Sm}$-anti-CD45 and $^{166}\text{Er}$-anti-CD34), so only cells showing a high signal intensity in both $^{145}\text{Sm}$ and $^{166}\text{Er}$ channels are gated as the ones expressing enough CD34 and CD45. (D) The final histogram of $^{165}$Ho intensity after all the gatings, which yields the binding extent of NaHoF$_4$@SiO$_2$ NPs to the cells.
The Ho intensity histograms showing non-specific cell binding for the three different types of NPs are displayed in Figure 6-8. At relatively low dosages (33 and 170 NPs/cell), cells incubated with all the three NPs showed hardly any detectable Ho signal by mass cytometry, indicating that the extent of non-specific binding was minimal. When the dosage was increased to 830 NPs/cell, a higher level of non-specific binding was observed for NaHoF$_4$@SiO$_2$-SH NPs compared to NaHoF$_4$@SiO$_2$-PEG5k-OMe and NaHoF$_4$@SiO$_2$-PEG5k-COOH NPs. These results demonstrate that PEGylation of the NPs is important for suppressing non-specific binding to cells.

![Figure 6-8](image.png)

Figure 6-8. Ho intensity histograms showing non-specific cell binding of the three different NPs (top) NaHoF$_4$@SiO$_2$-SH NPs (middle) NaHoF$_4$@SiO$_2$-PEG5k-OMe NPs (bottom) NaHoF$_4$@SiO$_2$-PEG5k-COOH NPs at different dosages.

One major advantage of mass cytometry over flow cytometry is that mass cytometry is inherently quantitative without any external calibration standards. In mass cytometry, the average
number of Ln ions per cell $N_{Ln \text{ per cell}}$ and the mean intensity of Ln histogram $I_{Ln}$ are related by the equation

$$N_{Ln \text{ per cell}} = \frac{I_{Ln}}{t_{Ln}}$$

Here $t_{Ln}$ is the transmission coefficient. Using the transmission coefficient of $^{165}$Ho $t_{Ho} = 2.57 \times 10^{-4}$ one can convert the mean Ho intensity values to the numbers of Ho ions per cell. Also, one spherical $d = 12.0$ nm NaHoF$_4$ NP is calculated to contain 12,400 Ho ions. In this way the number of Ho ions per cell can be further converted to the number of NPs bound to each cell $N_{\text{NPs per cell}}$. The corresponding percentage of NPs bound to cells $\%_{\text{NPs per cell}}$ can be calculated with the equation

$$N_{\text{NPs per cell}} = \frac{I_{Ho}}{t_{Ho} \times 12,400} = \frac{I_{Ho}}{3.19}$$

$$\%_{\text{NPs on cell}} = \frac{N_{\text{NPs per cell}}}{\text{Dosage}_{\text{NPs per cell}}}$$

The calculated results are summarized in Table 6-3. In this table, I assumed that the mean Ho intensity observed at 0 NP/cell was due to background signal of the mass cytometry instrument, so all the mean Ho intensity values $I_{Ho}$ are corrected to $I_{Ho,\text{corr}}$ by subtracting this background signal

$$I_{Ho,\text{corr}} = I_{Ho} - I_{Ho}(0 \text{ NP/cell}) = I_{Ho} - 1.02$$
Table 6-3. Calculated non-specific cell binding results of NaHoF$_4$@SiO$_2$-SH, NaHoF$_4$@SiO$_2$-PEG5k-OMe and NaHoF$_4$@SiO$_2$-PEG5k-COOH NPs.

<table>
<thead>
<tr>
<th>Type of NP</th>
<th>Dosage</th>
<th>$I_{Ho}$</th>
<th>$I_{Ho, corr}$</th>
<th>N NPs per cell</th>
<th>% NPs on cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 NP/cell</td>
<td>1.02</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NaHoF$_4$@SiO$_2$-SH</td>
<td>33 NPs/cell</td>
<td>1.27</td>
<td>0.25</td>
<td>0.080</td>
<td>0.24%</td>
</tr>
<tr>
<td></td>
<td>170 NPs/cell</td>
<td>3.55</td>
<td>2.53</td>
<td>0.80</td>
<td>0.48%</td>
</tr>
<tr>
<td></td>
<td>830 NPs/cell</td>
<td>18.3</td>
<td>17.3</td>
<td>5.5</td>
<td>0.66%</td>
</tr>
<tr>
<td>NaHoF$_4$@SiO$_2$-PEG5k-OMe</td>
<td>33 NPs/cell</td>
<td>1.07</td>
<td>0.05</td>
<td>0.015</td>
<td>0.05%</td>
</tr>
<tr>
<td></td>
<td>170 NPs/cell</td>
<td>1.51</td>
<td>0.49</td>
<td>0.15</td>
<td>0.09%</td>
</tr>
<tr>
<td></td>
<td>830 NPs/cell</td>
<td>4.16</td>
<td>3.14</td>
<td>1.0</td>
<td>0.12%</td>
</tr>
<tr>
<td>NaHoF$_4$@SiO$_2$-PEG5k-COOH</td>
<td>33 NPs/cell</td>
<td>1.18</td>
<td>0.13</td>
<td>0.040</td>
<td>0.12%</td>
</tr>
<tr>
<td></td>
<td>170 NPs/cell</td>
<td>2.07</td>
<td>1.05</td>
<td>0.33</td>
<td>0.20%</td>
</tr>
<tr>
<td></td>
<td>830 NPs/cell</td>
<td>6.57</td>
<td>5.55</td>
<td>1.8</td>
<td>0.21%</td>
</tr>
</tbody>
</table>

From Table 6-3 we can see that for dosages up to 830 NPs/cell, the two PEGylated NaHoF$_4$@SiO$_2$ NPs showed less than 0.2% of non-specific binding to KG1a cells. Those NPs provide very low background signals for cell analysis by mass cytometry, making them promising candidates for the detection of low-abundance cellular biomarkers.

6.3.4 Preparation of NaHoF$_4$@SiO$_2$-PEG NP-GaM-Ab Conjugate

Specific targeting of cellular biomarkers requires conjugation of antibodies to the Ln NPs. Considering the relatively high cost of primary antibodies ($500 – 1,000 / mg), a secondary antibody, goat anti-mouse IgG Fc antibody (GaM Fc Ab) ($120 / mg) was used to optimize the protocol for NP-Ab conjugation. For antibody conjugation, NaHoF$_4$@SiO$_2$-PEG5k-COOH NPs were first activated by EDC and sulfo-NHS and then reacted with N-(2-aminoethyl)maleimide to convert the surface –COOH groups to maleimides. At the same time, the GaM Fc Ab was reacted with Traut’s reagent (30-fold excess) to introduce thiol groups onto the antibody. Since maleimide can eventually hydrolyze and thiol groups are susceptible to oxidation, both maleimide-activated NPs and thiolated antibodies have to be freshly prepared and reacted with each other right away.
In addition, the buffer medium used for both the thiolation reaction of the Abs and the thiol-maleimide coupling reaction between the NPs and the Abs contained a few mM of EDTA to chelate any possible divalent cations (e.g. Ca\(^{2+}\)) that could promote the oxidation of the thiols. The coupling reaction was stirred at room temperature for 16 h. Then the NP-GaM-Ab conjugate was purified from unbound antibodies by centrifugation, washed and stored in PBS containing 0.5% (w/v) BSA at 4 °C for further use (Scheme 6-1).

Scheme 6-1. Preparation of the NP-GaM-Ab conjugate via thiol-maleimide coupling. The COOH groups on the NP surface were first activated by EDC and sulfo-NHS, then the sulfo-NHS esters were reacted with N-(2-aminoethyl)maleimide to convert COOH groups to maleimide groups. At the same time, the GaM Fc Ab was reacted with Traut’s reagent to introduce thiol groups onto the Ab. The thiolated Abs were then reacted with the maleimide-activated NPs for 16 h to form the NP-GaM-Ab conjugate.

The number of Ab per NP was quantified according to the following procedure. Before the conjugation reaction, the concentration of thiolated Ab was 13.3 μM (determined by \(A_{280}\)) in 100 μL of C-buffer, corresponding to a total amount of 1.33 nmol antibodies. After the conjugation reaction, the NPs were sedimented by centrifugation at 16,000 × g for 20 min. The concentration of unbound Ab in the supernatant (200 μL) was determined (by a Bradford assay) to be 5.0 μM, so the total amount of unbound Ab was

\[
5.0 \text{ μM} \times 200 \text{ μL} = 1.00 \text{ nmol}
\]

and total amount of Ab bound to the NPs was calculated to be
1.33 nmol – 1.00 nmol = 0.33 nmol

which corresponds to a total number of $2.2 \times 10^{14}$ Ab molecules.

I previously calculated that each $d = 12.0$ nm NaHoF$_4$ NP has a mass of $5.4 \times 10^{-15}$ mg. The total mass of NPs in the conjugation reaction was determined by ICP-MS to be 0.054 mg NaHoF$_4$, which corresponded to $1.0 \times 10^{13}$ NPs. Thus the average number of Abs per NP was

$$\frac{2.2 \times 10^{14}}{1.0 \times 10^{13}} = 22$$

Figure 6-9 shows the TEM image and DLS CONTIN plot of the NP-GaM-Ab conjugate. The Ab conjugation did not affect the size and morphology of the NPs under TEM. Nevertheless, DLS analysis showed a considerable increase of $d_z$ (173 nm) in PBS compared to the precursor NaHoF$_4$@SiO$_2$-PEG5k-COOH NPs ($d_z = 95$ nm in PBS). Given that IgG only has a small hydrodynamic diameter of 11 nm (measured by DLS in pH 7.6 Tris-buffer saline), the large increase of $d_z$ may be due to some extent of NP aggregation caused by the Ab conjugation.

![Figure 6-9](image)

6.3.5 Non-specific and Specific Cell Binding of the NP-GaM-Ab Conjugate Measured by Mass Cytometry

Non-specific and specific cell binding experiments of the NaHoF$_4$@SiO$_2$-PEG NP-GaM-Ab conjugate were performed on Ramos cells, a human lymphoma B cell line that overexpresses CD20 on the cell surface. In the specific binding experiment, the Ramos cells were first
incubated with a mouse anti-human CD20 primary antibody and then stained with NP-GaM-Ab that specifically targets the Fc stem of the primary antibody. In the non-specific binding experiment, the cells were directly incubated with the NP-GaM-Ab conjugate without adding any primary antibody. This experiment evaluates the extent of non-specific cell binding of NP-GaM-Ab to Ramos cells (Scheme 6-2). The cells were analyzed by mass cytometry and the Ho intensity histograms were used to calculate the number and percentage of NPs bound to cells.

Scheme 6-2. Non-specific and specific binding experiment of the NaHoF₄@SiO₂-PEG NP-GaM-Ab conjugate to Ramos cells. In the non-specific binding experiment, the Ramos cells were directly incubated with the NP-GaM-Ab conjugate. In the specific binding experiment, the Ramos cells were first stained with a mouse anti-human CD20 primary Ab and then incubated with the NP-GaM-Ab conjugate. In the mass cytometry analysis, the negative cell population referred to the cells in the non-specific binding experiment and the positive cell population referred to the cells in the specific binding experiment.

The mass cytometry Ho intensity histograms showing non-specific and specific cell binding profiles of NP-GaM-Ab to Ramos cells are displayed in Figure 6-10. In this figure, all the red-colored histograms correspond to negative cell events (non-specific binding experiments) and all the cyan-colored histograms correspond to positive cell events (specific binding experiments). Even at the lowest dosage (130 NPs/cell), a clear separation between the negative and positive cell
population can be observed. With the increase of NP dosage, the two cell populations became more and more separated. More importantly, at the highest dosage (16,700 NPs/cell) the signal intensity of the negative cell population was only slightly higher than the zero-intensity baseline, providing a very low level of background signal.
Figure 6-10. Ho intensity histograms showing non-specific and specific binding of NP-GaM-Ab to Ramos cells. Red-colored histograms refer to negative cell populations from the non-specific binding experiments (Ramos cells + NP-GaM-Ab). Cyan-colored histograms refer to positive cell populations from the specific binding experiments (Ramos cells + mouse anti-human CD20 + NP-GaM-Ab). The mean Ho intensity $I_{Ho}$ values of every histogram are shown on the left side of the histograms.

The calculated number of NPs bound to each cell as well as the percentage of NPs bound to cells are listed in Table 6-4. The number of NPs bound to each cell $N_{NPs\ per\ cell}$ and percentage of
NPs bound to cells $\%_{\text{NPs on cell}}$ are calculated from mean Ho intensity values $I_{\text{Ho}}$ and robust standard deviation values $rSD_{\text{Ho}}$ of the Ho intensity histograms in Figure 6-10 using the following equations. Each $d = 12.0 \text{ nm}$ NaHoF$_4$ NP generates a Ho intensity value of 3.19 in the mass cytometry measurement.

$$N_{\text{NPs per cell}} = \frac{I_{\text{Ho}} \pm rSD_{\text{Ho}}}{3.19}$$

$$\%_{\text{NPs per cell}} = \frac{N_{\text{NPs per cell}}}{\text{Dosage}} \times 100$$

With all the NP dosages, the amount of non-specific binding was less than 0.1%, even lower than the results of NaHoF$_4@\text{SiO}_2$-PEG5k-OMe and NaHoF$_4@\text{SiO}_2$-PEG5k-COOH NPs with no antibody attached (Table 6-3). On the other hand, the NP-GaM-Ab conjugate presented very high level of specific binding to Ramos cells stained with mouse anti-human CD20 antibody. At the lowest dosage (130 NPs/cell), almost 100% of the NPs were bound to cells. When the NP dosage increased, the mean Ho intensity kept increasing. At the highest dosage (16,700 NPs/cell) the Ho intensity ($I_{\text{Ho}} = 9,600$) was close to the saturation value ($\sim 10,000$ counts per cell event) of the TOF detector in the mass cytometer.
Table 6-4. Calculated results of non-specific and specific binding of the NP-GaM-Ab conjugate to Ramos cells.

<table>
<thead>
<tr>
<th>Type of Experiment</th>
<th>Dosage</th>
<th>$I_{Ho}$</th>
<th>$rSD_{Ho}$</th>
<th>$N_{NPs \text{ per cell}}$</th>
<th>$%_{NPs \text{ on cell}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Specific Binding</td>
<td>130 NPs/cell</td>
<td>0.3</td>
<td>0</td>
<td>0.1</td>
<td>0.06%</td>
</tr>
<tr>
<td>(Ramos Cell + NP-GaM-Ab)</td>
<td>670 NPs/cell</td>
<td>0.8</td>
<td>0</td>
<td>0.3</td>
<td>0.04%</td>
</tr>
<tr>
<td></td>
<td>3,300 NPs/cell</td>
<td>3.0</td>
<td>1.7</td>
<td>$1.0 \pm 0.6$</td>
<td>0.03%</td>
</tr>
<tr>
<td></td>
<td>16,700 NPs/cell</td>
<td>20</td>
<td>6.6</td>
<td>$6.3 \pm 2.0$</td>
<td>0.04%</td>
</tr>
<tr>
<td>Specific Binding</td>
<td>130 NPs/cell</td>
<td>440</td>
<td>310</td>
<td>$(1.4 \pm 1.0) \times 10^2$</td>
<td>100%</td>
</tr>
<tr>
<td>(Ramos Cell + mouse anti-human CD20 + NP-GaM-Ab)</td>
<td>670 NPs/cell</td>
<td>1,700</td>
<td>1,000</td>
<td>$(5.4 \pm 3.6) \times 10^2$</td>
<td>81%</td>
</tr>
<tr>
<td></td>
<td>3,300 NPs/cell</td>
<td>5,400</td>
<td>2,600</td>
<td>$(1.7 \pm 0.8) \times 10^3$</td>
<td>51%</td>
</tr>
<tr>
<td></td>
<td>16,700 NPs/cell</td>
<td>9,600</td>
<td>3,100</td>
<td>$(3.1 \pm 1.0) \times 10^3$</td>
<td>18%</td>
</tr>
</tbody>
</table>

Previous studies based on quantitative flow cytometry revealed a value of $3.7 \times 10^5$ copies of CD20 per Ramos cell. In my experiment, the highest dosage was 16,700 NPs/cell. My calculation describe above gives an average number of 22 antibodies per NP. So, at the highest NP dosage, the number of GaM Fc Ab per cell was $16,700 \times 22 = 3.7 \times 10^5$, which is almost the same as the number of CD20 antigens per cell. As a result, we can derive that only at the highest NP dosage there was a chance for the surface CD20 antigens to be saturated, assuming that the antibodies on the backside of the NPs could also bind to the surface antigens. At lower NP dosages, the surface CD20 antigens were far from being saturated, and the mean Ho intensity continued to increase when the NP dosage was increased. However, the percentage of NPs bound to cells showed a decreasing trend with increasing NP dosages.

Nevertheless, even at the highest dosage (16,700 NPs/cell) where the percentage of NPs bound to cells was the lowest, the ratio of specific to non-specific binding still reached a high value of 480. When the dosages were lower (130, 670 and 3,300 NPs/cell), the corresponding ratios became even higher, ranging from 1,600 to 2,100 (Table 6-5). In short, the NP-GaM-Ab conjugate exhibited outstanding properties for specifically targeting CD20 on the surface of Ramos cells.
Table 6-5. The ratio of specific (positive) to non-specific (negative) binding of the NP-GaM-Ab conjugate to Ramos cells at different dosages.

<table>
<thead>
<tr>
<th>Dosage</th>
<th>Type of Experiment</th>
<th>Mean Ho Intensity</th>
<th>Positive to Negative Intensity Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>130 NPs/cell</td>
<td>Specific Binding</td>
<td>440</td>
<td>1,600</td>
</tr>
<tr>
<td></td>
<td>Non-specific Binding</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>670 NPs/cell</td>
<td>Specific Binding</td>
<td>1,700</td>
<td>2,100</td>
</tr>
<tr>
<td></td>
<td>Non-specific Binding</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>3,300 NPs/cell</td>
<td>Specific Binding</td>
<td>5,400</td>
<td>1,800</td>
</tr>
<tr>
<td></td>
<td>Non-specific Binding</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>16,700 NPs/cell</td>
<td>Specific Binding</td>
<td>9,600</td>
<td>480</td>
</tr>
<tr>
<td></td>
<td>Non-specific Binding</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

6.3.6 Discussion: Does Steric Hindrance Between NPs Impede NP Binding to the Cell Surface?

In Table 6-4, the percentage of NPs bound to cells showed a decreasing trend with increasing NP dosages. Although we do not yet have a clear explanation for this trend, we consider if steric hindrance between NPs can impede NP binding to the cell surface when there are already a large number of NP-GaM-Ab conjugates bound to the cells.

To provide an insight into this issue, I developed a model to calculate how many NP-GaM-Ab conjugates are needed to fully cover the surface of one Ramos cell. Ramos cell is a type of human B cell with a diameter of about 7 μm. DLS studies showed a hydrodynamic diameter $d_z = 173$ nm for the NP-GaM-Ab conjugate in PBS (Figure 6-9(B)). If we consider both the cell and the NP-GaM-Ab conjugate as hard spheres, in which the size of the NP-GaM-Ab small sphere is assumed to be represented by its hydrodynamic diameter, this question can be converted to a 3-dimensional sphere coverage problem: how many $d_s = 173$ nm small spheres are needed to achieve a maximum coverage on the surface of a $d_L = 7$ μm large sphere without any overlapping between the small spheres? This type of question seems simple and has been extensively studied for over 100 years, yet it still lacks a universal solution. Nevertheless, it can be simplified to a 2-
dimensional close-packing of equal spheres in a finite space. Figure 6-11(A) shows a scheme of small spheres with a radius $r_S$ covering the surface of a large sphere $r_L$. The overall area of the 2-dimensional space is the surface area of a spherical shell with a radius of $r_S + r_L$, which equals to $4\pi(r_S + r_L)^2$. Also, it is known that the greatest fraction of space occupied by spheres is achieved by hexagonal close-packing, which is shown in Figure 6-11(B). In hexagonal close-packing, the space fraction occupied by spheres is about 0.74. So the area occupied by each sphere with a radius $r_S$ is $\pi r_S^2 / 0.74$. Thus the total number $N$ of small spheres to achieve a maximum coverage on the surface of a large sphere is expressed as

$$N = \frac{4\pi(r_S + r_L)^2}{\pi r_S^2 / 0.74}$$

![Figure 6-11](image)

Figure 6-11. (A) An example of small spheres with a radius $r_S$ covering the surface of a large sphere with a radius $r_L$. (B) Hexagonal close-packing of spheres on a surface.

In my case, $r_S = 87$ nm is the hydrodynamic radius of the NP-GaM-Ab conjugate, $r_L = 3.5$ μm is half the size of one Ramos cell. Substituting these numbers to the above equation yields

$$N = \frac{4\pi \times (3500 + 87)^2}{\pi \times 87^2 / 0.74} = 5100$$

which means that about 5,000 NP-GaM-Ab conjugates are needed to achieve a maximum coverage on the surface of one Ramos cell. In my experiments, at the highest dosage (16,700 NPs/cell) there were average 3,000 NP-GaM-Ab conjugates bound to each cell, which is about 60% of a maximum coverage. Based on this (perhaps oversimplified) model, I infer that it is possible that steric
hindrance between NPs can impede NP binding to the cell surface at high NP dosages, given that the NP-GaM-Ab conjugates cover almost the whole cell surface.

6.4 Summary

In this chapter, I described preparation and application of NaHoF$_4$@SiO$_2$-PEG NPs as an elemental tag for cell assays by mass cytometry. OA-capped NaHoF$_4$ NPs with an average $d = 12.0$ nm were coated with a uniform silica shell by a reverse microemulsion method. The resulting NaHoF$_4$@SiO$_2$ NPs were first reacted with MPTMS to introduce thiol groups, then the NaHoF$_4$@SiO$_2$-SH NPs were PEGylated by reacting with maleimide-PEGs that have different lengths (2k and 5k) and end groups (–OMe and –COOH). After PEGylation, the overall diameter of NaHoF$_4$@SiO$_2$-PEG NPs was 21.4 nm characterized by TEM.

A quantitative Ellman’s assay was performed to determine the number of thiols per NaHoF$_4$@SiO$_2$-SH NP. After the PEGylation reaction all the thiols reacted with PEG-maleimide. Thus I assumed that the number of thiols per NaHoF$_4$@SiO$_2$-SH NP was equal to the number of PEGs per NaHoF$_4$@SiO$_2$-PEG NP. For NaHoF$_4$@SiO$_2$-PEG5k-COOH NPs, I calculated an average number of 840 PEG molecules per NP, corresponding to a surface density of 0.58 PEG molecules per nm$^2$ (or a docking area of 1.7 nm$^2$/PEG).

DLS studies showed that all the four PEGylated NaHoF$_4$@SiO$_2$ NPs, including NaHoF$_4$@SiO$_2$-PEG2k-OMe, NaHoF$_4$@SiO$_2$-PEG2k-COOH, NaHoF$_4$@SiO$_2$-PEG5k-OMe and NaHoF$_4$@SiO$_2$-PEG5k-COOH NPs, were colloidal stable in water. When the dispersion medium was changed to PBS, after 24 h of storage, the two NaHoF$_4$@SiO$_2$-PEG5k NPs showed no sign of aggregation whereas the two NaHoF$_4$@SiO$_2$-PEG2k NPs displayed increased aggregation peaks in their DLS CONTIN plots. This difference indicates that PEG5k can better stabilize the NaHoF$_4$@SiO$_2$ NPs than PEG2k in PBS.

Non-specific cell binding of NaHoF$_4$@SiO$_2$-SH, NaHoF$_4$@SiO$_2$-PEG5k-OMe and NaHoF$_4$@SiO$_2$-PEG5k-COOH NPs was tested on KG1a cells by mass cytometry. At relatively low dosages (33 and 170 NPs/cell) there was hardly any detectable signal of NP bound to cells. When the dosage increased to 830 NPs/cell, the non-PEGylated NaHoF$_4$@SiO$_2$-SH NP showed 0.66% of non-specific binding whereas the two PEGylated NPs only showed 0.12% and 0.21% of non-specific binding, respectively. The non-specific binding test confirmed that the PEGylation
did help reducing non-specific cell binding of NPs. Such a low level of non-specific binding guarantees a low background signal in mass cytometry and makes the NaHoF$_4$@SiO$_2$-PEG5k NPs promising candidates for detecting low-abundance cellular biomarkers.

For specific cell binding experiments, the NaHoF$_4$@SiO$_2$-PEG5k-COOH NPs were conjugated with GaM Fc Ab via a thiol-maleimide coupling to prepare the NP-GaM-Ab conjugate. A Bradford assay yielded an average number of 22 antibodies per NP. The NP-GaM-Ab conjugate was then tested on Ramos cells. In the specific binding experiment, the Ramos cells were first incubated with a mouse anti-human CD20 primary antibody, then stained with the NP-GaM-Ab conjugate that specifically targets the primary antibody and analyzed by mass cytometry. In the non-specific binding experiment, the Ramos cells were directly stained with the NP-GaM-Ab conjugate without adding any primary antibody to evaluate non-specific cell binding of the NP-GaM-Ab conjugate. Both non-specific and specific cell binding experiments were performed at four different NP dosages: 130, 670, 3,300 and 16,700 NPs/cell. At the first three dosages the NP-GaM-Ab conjugate exhibited outstanding specific-to-non-specific binding ratios of 1,600, 2,100 and 1,800, respectively. At the highest dosage 16,700 NPs/cell, the ratio decreased to 480. In the specific binding experiment, when the dosage increased, the number of NPs bound to each cell kept increasing, indicating that the CD20 surface antigen on Ramos cell was not saturated. Nevertheless, when the dosage increased, the percentage of NPs bound to cells showed a decreasing trend, while I do not yet have an explanation of this trend. In summary, the NP-GaM-Ab conjugate was able to detect CD20 surface antigens on Ramos cells very efficiently.
6.5 Appendix

6.5.1 Quantification of Thiol Groups on NaHoF$_4$@SiO$_2$-SH NPs

The number of thiol groups on each NaHoF$_4$@SiO$_2$-SH NP was quantified by an Ellman’s assay. The assay was performed in a reaction buffer (R-buffer) containing 0.1 M pH 8.0 sodium phosphate and 1 mM EDTA. A series of standard solution was prepared by dissolving cysteine hydrochloride monohydrate in R-buffer with following concentrations: 1.5, 1.25, 1.0, 0.75, 0.5, 0.25 mM. In a separate microcentrifuge tube, the DTNB (Ellman’s reagent) solution was prepared by dissolving 4 mg of DTNB in 1 mL R-buffer. Both DTNB and cysteine solution were freshly prepared for the assay.

To obtain a calibration curve, a set of 1.5 mL microcentrifuge tubes were filled with 500 μL R-buffer, 10 μL DTNB solution and 50 μL of cysteine standard solution in each tube. The tubes were incubated at room temperature for 20 minutes and then taken to measure absorbance at 412 nm on a PerkinElmer LAMBDA™ 25 UV/vis Spectrometer. Plotting absorbance at 412 nm against concentration of cysteine gives the calibration curve of this Ellman’s assay.

To quantify the number of thiol groups per NP, 500 μL of R-buffer, 10 μL DTNB solution and 50 μL of NaHoF$_4$@SiO$_2$-SH NP solution in water (2.1 mg/mL NaHoF$_4$, accurate concentration determined by ICP-MS) were mixed in a separate tube. The mixed solution was incubated at room temperature for 15 minutes and then taken to measure absorbance at 412 nm. The value was converted to concentration of thiols by using the calibration curve of cysteine (Figure 6-12). The calculation of the average number of thiol groups per NP was described above in section 6.3.1.
To determine the NP concentration by ICP-MS, the NaHoF₄@SiO₂-SH NP solution in water was diluted 1×10⁶ times using 2% HNO₃ (ICP grade, SEASTAR CHEMICALS Inc.). There was no extra digestion step prior to the ICP-MS analysis. ICP-MS analysis provided the concentration of Ho in the diluted solution, which was converted to the concentration of NaHoF₄ in the original solution using the equation

\[ [\text{NaHoF}_4] = [\text{Ho}]_{\text{ICP-MS}} \times 10^6 \times \frac{M_w(\text{NaHoF}_4)}{A_w(\text{Ho})} \]

\[ = 1.3 \, \mu\text{g/L} \times 10^6 \times \frac{263.91}{164.93} \]

\[ = 2.1 \, \text{mg/mL} \]

6.5.2 Comparison between Intensity-, Volume- and Number-Weighted Size Distribution Plots of the Two NaHoF₄@SiO₂-PEG2k NPs in PBS for 24 h

When DLS is used to study NP aggregation, the most meaningful data to examine are intensity-weighted size distribution plots. These plots are yielded by an ill-posed inverse Laplace transform of the autocorrelation function, and the long-time tail in the autocorrelation decay curve can appear as an aggregation peak at large \( R_h \) in the CONTIN plot. Figure 6-13(A) shows an example of transformation from a DLS autocorrelation decay curve to an intensity-weighted size distribution plot. The data come from a sample of NaHoF₄@SiO₂-PEG2k-OMe NPs after 24 h of
storage in PBS. The long-time tail (indicated by the black arrow) at $\tau = 10^3 \mu$s appears as the big aggregation peaks at $d_h > 1,000$ nm on the intensity-weighted size distribution plot. In contrast, in for the sample of NaHoF$_4$@SiO$_2$-PEG2k-COOH NPs after 24 h in PBS (Figure 6-13(B)), there is no obvious long-time tail in the autocorrelation decay curve. Here only a small aggregation peak appears on the intensity-weighted size distribution plot.

Transformation from intensity-weighted to volume- and number-weighted size distribution plots is valid only when all the following requirements are satisfied: 1) all the particles are spherical, 2) all the particles are homogeneous, 3) the optical properties of all the particles are known and 4) there is no error in the intensity-weighted plot. When there is NP aggregation it is almost impossible to satisfy all the above requirements. Thus a presentation of number-weighted size distribution plots can lead to misinterpretation of the DLS data. For example, when one compares the two intensity-weighted plots in Figure 6-13(A) and (B) one can easily conclude that NaHoF$_4$@SiO$_2$-PEG2k-COOH NPs showed a better colloidal stability than NaHoF$_4$@SiO$_2$-PEG2k-OMe NPs after 24 h of storage in PBS. However, on the number-weighted plots the large aggregation peak of NaHoF$_4$@SiO$_2$-PEG2k-OMe NPs completely disappeared. If only the number-weighted plots are presented, one can easily reach the wrong conclusion that the two NaHoF$_4$@SiO$_2$-PEG2k NPs had the same colloidal stability in PBS.
Figure 6-13. DLS autocorrelation function, intensity-, volume- and number-weighted size distribution plots on the two NaHoF₄@SiO₂-PEG2k-OMe NPs after 24 h of storage in PBS. (A) The NaHoF₄@SiO₂-PEG2k-OMe NPs showed a long-time tail (pointed by the arrow) on the autocorrelation decay curve, which was converted to the aggregation peaks in the intensity-weighted size distribution plot. The aggregation peaks disappeared upon transformation from intensity-weighted to number-weighted distribution plot. (B) The NaHoF₄@SiO₂-PEG2k-COOH NPs did not show an obvious long-time tail, thus only a small aggregation peak was observed on the intensity-weighted size distribution plot. This small peak also disappeared on the number-weighted distribution plot.
6.5.3 Quantification of Unbound GaM Fc Ab by the Bradford Assay

After the conjugation reaction between NaHoF₄@SiO₂-PEG NPs and GaM Fc Ab, the amount of unbound GaM Fc Ab left in the supernatant was quantified by a Bradford assay. To obtain a standard curve, a series of GaM Fc Ab standard solutions (8.0, 6.0, 4.0, 2.0, 1.0 μM) in PBS was prepared by sequential dilution from a 16 μM Ab stock solution. In each 1.5 mL microtube, 10 μL of standard solution or the supernatant was mixed with 300 μL of Bradford reagent and incubated at room temperature for 20 min, then the solutions were taken to measure absorbance at 595 nm on a Tecan Infinite® 200 PRO Microplate Reader (Tecan Group, Männedorf, Switzerland). Plotting $A_{595}$ against [Ab] yielded the calibration curve for GaM Fc Ab by Bradford assay. The concentration of unbound GaM Fc Ab left in the supernatant was determined by using the calibration curve (Figure 6-14).

![Figure 6-14. Calibration curve of GaM Fc Ab by Bradford assay (microplate method).](image)
6.5.4 Do Antibody-Antigen Binding Reactions Follow Diffusion-Controlled Kinetics?

It is normally believed that antibody-antigen binding kinetics are not diffusion-controlled. My experiments provide a means of testing this idea. In the theory of diffusion-controlled reactions, the steady-state value of the second-order rate constant $k_{\text{diff}}$ of a reaction $A+B \rightarrow C$ can be expressed as

$$k_{\text{diff}} = 4\pi N A \left( D_A + D_B \right) \left( R_A + R_B \right)$$

where $D_A$ and $D_B$ are the translational diffusion coefficients of the two reactants $A$ and $B$, $R_A$ and $R_B$ are the radii of the two reactants $A$ and $B$, respectively.

If we assume that the binding reaction between the NaHoF$_4$@SiO$_2$-PEG5k NP-Ab conjugate and the anti-CD20 primary antibody attached to the surface of CD20(+) Ramos cells follows a diffusion-controlled kinetics, equation (7) can be used to calculate the second-order diffusion-controlled rate constant for this binding reaction.

In this case, the size of a Ramos cell is much larger than the size of a NP-Ab conjugate, so

$$R_A + R_B = R_{\text{cell}} = 3.5 \, \mu m$$

On the other hand, a large cell diffuses much more slowly than a small NP-Ab conjugate, so the contribution of cell to the diffusion coefficient $D$ can be neglected. Thus we can assume that the $D$ in equation (7) is equal to the diffusion coefficient of the NP-Ab conjugate. DLS measurement on Zetasizer Nano ZS yielded a diffusion coefficient of 2.84 $\mu m^2/s$ for the NP-Ab conjugate, corresponding to the CONTIN plot presented in Figure 6-9(B). Then we have

$$D_A + D_B = D_{\text{NP-Ab}} = 2.84 \, \mu m^2/s$$

Combining these values, we can calculate $k_{\text{diff}}$ for the binding between the NP-Ab conjugate and the CD20(+) Ramos cell to be
However, literature reports indicate that the second-order rate constants of most antibody-antigen binding reactions are in the range of $10^4 – 10^6$ M$^{-1}$s$^{-1}$, much lower than the result calculated above.\textsuperscript{122} This confirms that the antibody-antigen binding reactions do not follow diffusion-controlled kinetics. Nevertheless, this simple calculation can still provide us with some insight for interpreting the binding kinetics between the NP-Ab conjugate and the cell surface antigen. If the binding reaction did follow a diffusion-controlled kinetics, one collision between the NP-Ab conjugate and the cell surface would lead to one binding event. Given that the actual second-order rate constant is $10^4 – 10^6$ times less than the one based on diffusion-controlled kinetics, we can conclude that on average each NP-Ab conjugate undergoes $10^4 – 10^6$ collision events with the cell surface for each successful antibody-antigen binding event.
7 Chapter 7: Future Work

7.1 Overview

In Chapter 6, I showed the first example of NaHoF₄@SiO₂-PEG NPs as an effective mass tag for cell assays based on mass cytometry. Following this state-of-the-art approach, future work may be directed to various ways to further investigate the application of Ln-NP-based mass tags in mass cytometry. In this chapter I would like to discuss three possible future directions: demonstrating the sensitivity of NP-based mass tags for detecting low-abundance biomarkers, extending the application of NP-based mass tags to multiplexed cell assays, and solving potential problems caused by the silica coating on the NPs.

7.2 Demonstrating the Sensitivity of NP-based Mass Tags for Detecting Low-abundance Cellular Biomarkers

In conventional flow cytometry, detection of low-abundance cellular biomarkers relies on antibodies tagged with highly bright fluorophores. Literature studies based on quantitative flow cytometry displayed a detection limit of 3100 copies per cell of HER3 surface antigens on HT29 human colon cancer cell line. As described in Chapter 6, the target biomarker was CD20 on Ramos cells, which has a relatively high expression level of $3.7 \times 10^5$ copies per cell. Since the ultimate goal of using Ln NPs as mass tags for mass cytometry is to utilize them for the detection of low-abundance biomarkers, it is crucial to be able to design future experiments that can demonstrate NP-based tags showing higher sensitivity than polymer-based tags.

When researchers compare the sensitivity of different fluorophores in flow cytometry, they do antibody titration studies. In an antibody titration assay, cells are stained with fluorescent antibodies with a series of antibody concentrations (or dilution factors) and subjected to flow cytometry analysis. The mean fluorescence intensities (MFI) of both positive and negative cell populations are plotted as a function of antibody concentration. Figure 7-1(A) shows an example of antibody titration experiment of CD8 antigen on peripheral blood mononuclear cells (PBMC), in which the antibody was labelled with one of the three fluorophores: Brilliant Violet™ 421 (BV421), Pacific Blue™ (PacBlue) and PE. The sensitivity of fluorophores is illustrated by their staining indices (S.I.), which in this case is defined as
From Figure 7-1(B) we can clearly see that BV421 and PE showed similar sensitivity in detecting CD8 on PBMC, whereas the sensitivity of PacBlue was much lower.\textsuperscript{109}

![Figure 7-1](image-url)

Figure 7-1. Antibody titration experiment of CD8 on PBMC. (A) Staining profile of BV421, PacBlue and PE conjugates of anti-CD8 antibody at different antibody concentrations. Vertical axes represent the fluorescence intensity histograms of CD8(+) and CD8(-) PBMC cell subpopulations. (B) Mean fluorescence intensity (MFI) values of CD8(+) and CD8(-) PBMC cell subpopulations. Comparison of staining indices (S.I.) of three different fluorophores. Reprinted with permission from ref. 104.

Figure 7-2 shows another antibody titration experiment of CD127 on PBMC. Since CD127 is a low-abundance biomarker, only BV421 but not PacBlue showed enough sensitivity to fully discriminate CD127(+) and CD127(-) subpopulations in PBMC.\textsuperscript{109}
For future experiments by mass cytometry, if we can identify a low-abundance biomarker on a certain cell line that can only be detected by NP-tagged antibody but not MCP-tagged antibody, then we can perform an experiment similar to the one in Figure 7-2 to demonstrate that NP-based mass tags do show higher sensitivity than polymer-based mass tags.

7.3 Extending the Application of NP-based Mass Tags to Multiplexed Cell Assays.

In Chapter 6, the NaHoF\textsubscript{4}@Si\textsubscript{2}O\textsubscript{2}-PEG NPs were conjugated with a goat anti-mouse secondary antibody. For simultaneous detection of multiple biomarkers in one single cell assay we need a ‘cocktail’ of NaLnF\textsubscript{4} NPs conjugated with different primary antibodies. The lanthanide (Ln) series contains 14 different elements (excluding the radioactive Pm) and 46 naturally abundant stable isotopes. The Ln isotopes are listed in Table 7-1. Among all the Ln elements, La, Pr, Tb, Ho and Tm have only one stable isotope, which makes them the best candidates for NaLnF\textsubscript{4} NP mass tags. In addition, Ce and Lu have one predominantly abundant isotope (88.4% for \textsuperscript{140}Ce and 97.4% for \textsuperscript{175}Lu). Thus they can also be considered for NP mass tags if we need more channels for multiplexed cell assays.
Table 7-1. Naturally abundant (abundance > 1%) stable isotopes of lanthanide elements.

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<th>Gd</th>
<th>Tb</th>
<th>Dy</th>
<th>Ho</th>
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Another factor we have to consider is the ease of synthesizing NaLnF$_4$ NPs. The fundamental studies by Yan and co-workers pointed out that all the Ln elements, based on its different cubic-to-hexagonal phase transition behaviour during the nucleation and growth of NaLnF$_4$ NPs, can be divided into three groups: light lanthanides (La to Nd), middle lanthanides (Sm to Tb) and heavy lanthanides (Dy to Lu). In the synthesis of NaLnF$_4$ NPs, when the reaction temperature eventually increases from room temperature to the setpoint (between 250 and 330 °C),
middle lanthanides directly form hexagonal-phase NaLnF$_4$ nuclei, which makes them the easiest to form monodisperse NaLnF$_4$ NPs. Heavy lanthanides, however, first nucleate to form cubic-phase NaLnF$_4$ NPs that have to be transformed to the hexagonal phase. Since the energy barrier of cubic-to-hexagonal phase transition for heavy lanthanides is relatively high, the synthesis of monodisperse NaLnF$_4$ NPs for heavy lanthanides typically requires higher reaction temperatures than those for middle lanthanides. The third group, that is the light lanthanides, behave even more differently. Light lanthanides tend to form LnF$_3$ nuclei instead of NaLnF$_4$ nuclei. Increasing the reaction temperature and time can force the transition from LnF$_3$ to NaLnF$_4$, yet the product NPs will often be large and polydisperse in size. In summary, the difficulty of making monodisperse NaLnF$_4$ NPs is in the order of light lanthanides > heavy lanthanides > middle lanthanides.

In the previous chapters, I included the synthesis of NaTbF$_4$ (middle lanthanide), NaYF$_4$ and NaHoF$_4$ (heavy lanthanides) NPs. By using the same reaction conditions one should be able to prepare monodisperse NaTmF$_4$ and NaLuF$_4$ (both heavy lanthanides) NPs. In future, unexpected difficulties may occur when somebody attempts to synthesize NaLaF$_4$, NaCeF$_4$ and NaPrF$_4$ (light lanthanides) NPs. A possible solution to this problem would be to dope 20% of La, Ce or Pr with Y to make doped NaYF$_4$:Ln NPs (Figure 7-3). The element Y only has one stable isotope with an atomic mass of 89, which does not interfere with other Ln isotopes in mass cytometry.

Figure 7-3. Possible NaLnF$_4$ NP mass tags for a 7-plex cell assay in mass cytometry.
7.4 Solving Potential Problems Caused by Silica Coating on NaLnF$_4$ NPs

In Chapter 6, I described the preparation of NaHoF$_4$@SiO$_2$-PEG NPs, in which the silica shell thickness was only 4.7 nm. Nevertheless, since mass cytometry analysis does not employ acid digestion prior to the sample injection to the instrument, long-term usage of silica-coated NaLnF$_4$ NPs may cause eventual deposition of silicon materials inside the sample chamber and ICP cones. To circumvent this potential problem, the NaLnF$_4$ NPs can be coated with a cross-linked polymer shell other than silica. In 2015, Wang and co-workers reported a reverse microemulsion method, similar to the silica coating protocol, but using dopamine instead of TEOS, to coat OA capped NaYF$_4$:Yb,Er UCNPs with a layer of polydopamine (PDA). The shell thickness could be adjusted by changing the amount of dopamine added. The surface of polydopamine shell is reactive toward amine and thiol groups, making it possible to introduce surface functionalities.

Scheme 7-1 shows a proposed surface functionalization strategy to prepare PDA-coated NaLnF$_4$ NPs. NaLnF$_4$@PDA NPs first react with thioglycolic acid (TGA) to introduce –COOH groups on to the PDA shell. The –COOH groups are then activated by EDC/sulfo-NHS followed by reaction with maleimide-PEG-amine (MAL-PEG-NH$_2$) to PEGylate the NaLnF$_4$@PDA NPs. The resulting NaLnF$_4$@PDA-PEG NPs can readily react with thiolated antibodies using the protocol developed in Chapter 6 to form the NP-Ab conjugate. This strategy provides an alternative to silica for coating NaLnF$_4$ NPs to prepare NP-based mass tags for cell assays by mass cytometry.
Scheme 7-1. A planned strategy of preparing NaLnF₄@PDA-PEG NP-Ab conjugate.
7.5 Closing Remarks

In the above sections, I listed three future directions that can advance the development of Ln-NP-based mass tags for mass cytometry. There are also other possible studies that people can investigate to expand the toolbox of mass cytometry, e.g. developing mass tags containing heavy metal ions other than lanthanides. In summary, mass cytometry is an emerging technique yet less mature than the well-established conventional flow cytometry. It requires more collaboration between the developer and the end-users of mass cytometry to exploit the full potential of this new bioanalytical method.
References

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