LANTHANIDE-ENCODED POLY(STYRENE-CO-METHACRYLIC ACID) MICROSPHERES: SYNTHESIS AND CHARACTERIZATION

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Lanthanide-Encoded Poly(Styrene-co-Methacrylic Acid) Microspheres:

Synthesis and Characterization

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Abstract

Lanthanide-encoded polystyrene-co-methacrylic acid (P(S-MAA)) microspheres with narrow size distributions were synthesized by two-stage dispersion polymerization. I examined how the amounts of methacrylic acid (MAA) and lanthanide (Ln) salts affect the composition of the particles formed in the reaction. Also, I performed a systematic study of Ln ion release into different aqueous solutions. In normal buffers, these particles were stable against ion leakage, even upon prolonged storage and stirring. When strong chelating agent ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA) were present in buffer, the loss of Ln ions increased to 15% after 8 weeks. A preliminary kinetic study of Ln ion incorporation was performed to help understand the particle formation mechanism.
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Chapter 1
General Introduction

Monodisperse polymer particles in the micrometer-size range are used in a wide variety of applications, such as toners, instrument calibration standards, column packing materials for chromatography, spacers for liquid crystal displays, and biomedical and biochemical analysis. Recently, there have been growing academic and industrial interests in monodisperse polymeric microspheres in the micrometer size range.

In the open and patent literature, the term "monodisperse" has been used very loosely. A group of researchers [1] comment that the spread of particle diameters obtained under one set of conditions in their experiments (particles prepared in a mixture of ethanol and toluene), characterized by a volume geometric standard deviation (GSD) of 1.06, while compressed enough to be considered "monodisperse", is not nearly as narrow as that spread of particle diameters obtained under other conditions (particles prepared in ethanol). This creates a confusing situation for finding ways to prepare polymer particles with the narrowest possible size distribution. In my thesis, I will follow the definition given by the National Institute of Standards and Technology (NIST) for monodisperse particle size distribution: "a particle distribution may be considered monodisperse if at least 90% of the distribution lies within 5% of the median size" (Particle Size Characterization, Special Publication 960-961, January 2001).

There are many expressions that can describe particle size distribution. The most commonly used is the coefficient of variation of the particle diameter ($CV_d$) which is defined by the mathematical expression

$$CV_d = \sqrt{\frac{\sum_{i=1}^{n} (d_i - d_{av})^2}{n-1}} / d_{av}$$

(1.1)

Where $d_{av}$ is the number average diameter of all particles, $d_i$ is the diameter of the $i^{th}$ particle, and $n$ is the total number of particles counted in the analysis.

It should be noted that the $CV_d$ usually is sensitive to the size range selected for the calculation. So the $CV_d$ data should be compared in a similar size range. All the useful particles prepared by dispersion polymerization in my work have $CV_d$ values in the range of 1.8 to 5.5%.
1.1 Microspheres by Dispersion Polymerization

The synthesis of micron-size polymer particles with a narrow size distribution is of a great interest for scientific researchers and for different technologies and applications. Dispersion polymerization is an attractive method to produce these micron-size polymer particles with a narrow size distribution in a single step and is suitable for a variety of monomers. [2] The particles prepared via dispersion polymerization are usually between 1 to 15 microns in diameter. The most significant feature of dispersion polymerization is its superiority in the synthesis of highly monodisperse particles, compared the other particle synthesis techniques. This method has recently received great attention for the wide applications of micron-sized, monodisperse polymer particles industry, health care and other scientific research areas. [3-6]

Dispersion polymerization is a type of precipitation polymerization in which the polymerization of a monomer is carried out in the presence of a suitable polymeric stabilizer soluble in the reaction medium. The solvent selected for dispersion polymerization should be a good solvent for both the monomer and the steric stabilizer polymers, but a non-solvent for the polymer being formed. Dispersion polymerization, therefore, starts as a homogeneous solution polymerization in which sterically stabilized polymer particles are formed by the precipitation of the resulting polymers. As the reaction continues and the monomer conversion increases, the polymerization proceeds in the continuous media and, for higher extent, with the particles.

Dispersion polymerization in organic hydrocarbon media was first developed by Osmond and coworkers at ICI in 1969. [2] Two types of monomers, acrylic and vinylic monomers were polymerized in hydrocarbons with oil soluble polymer stabilizers to produce non-aqueous dispersions (NAD) of polymer particles. Later, this concept was extended to form monodisperse polymeric microspheres in polar solvents by Almog et al. [7] They used partially hydrolyzed polyvinyl alcohol (PVA) as a colloidal stabilizer and a quaternary ammonium salt as an electrostatic co-stabilizer. This extension greatly helped dispersion polymerization become a popular method to synthesize polymer particles for numerous research and industrial applications. [8, 9] In recent years, dispersion polymerization in supercritical carbon dioxide has been a particular focus of research and development in both academia and industry for a clean and environmentally-friendly technology under relatively
mild critical conditions (Tc: 304.25 K; Pc: 7.38 MPa). The stabilizers used in this process are usually fluorinated polymers, silicones, and poly(ether-carbonates).

1.1.1 Mechanism of dispersion polymerization

Free radical dispersion polymerization is the most studied dispersion polymerization system, although the chemical reaction involved in dispersion polymerization could use for other types of polymerization, such as anionic, condensation, and oxidation polymerization. The mechanism of dispersion polymerization is very complicated, since it starts as a solution polymerization and changes to a heterogeneous polymerization very quickly. Polymerization kinetics, particle formation, particle growth and all reaction parameters play important roles in determining the ultimate size and molecular weight of the particles formed. The particle formation in dispersion polymerization can be explained by the following three basic mechanistic steps: (1) self-nucleation, (2) aggregative nucleation, and (3) nucleation from micelles. According to a PhD thesis from Lehigh University, the polymer dispersants used in dispersion polymerization does not play a significant role in the formation of particle nuclei since monomer is completely soluble in the medium.

Barrett and Thomas proposed a mechanism for the dispersion polymerization of methyl methacrylate in \( n \)-dodecane. Tseng et al. presented a mechanism for particle formation and growth in dispersion polymerization of polystyrene in ethyl alcohol with polyvinylpyrrolidone (PVP) along with an anionic, nonionic, or comonomeric co-stabilizer. Shen et al. studied the mechanism for the formation of micron-size polymer particles in the dispersion polymerization of methyl methacrylate (MMA) in methanol with polyvinylpyrrolidone (PVP) as the stabilizer, by applying dynamic light scattering to monitor the evolution of the average particle size in the early stages of the polymerization. Paine et al. developed a multibin kinetic model for dispersion polymerization to predict particle size from first principles. The locus of polymerization and how it changes during the reaction is the key factor affecting the final size distribution. According to the mechanism by Paine et al., it is the polymer-grafted stabilizer rather than the homopolymer that is the true stabilizer. In addition, physically adsorbed chains also contribute to the particle stabilization.

Basically, the mechanism proposed by many researchers can be summarized as the following: at the beginning of the reaction, monomer, stabilizer, co-stabilizer and initiator are present in a homogeneous solution in the continuous phase. Upon heating, the
initiator decomposes; free radicals are generated and react with monomer to initiate chain growth to form oligomeric radicals. The growing chains remain soluble in solution until they reach a critical chain length; at that point, these oligomers precipitate. The precipitated chains aggregate to form nano-sized particle (nuclei), which are unstable. These nuclei further aggregate into larger particles by absorbing stabilizer and co-stabilizer from the medium. Once stable particles have been formed, they grow by capturing small nuclei and oligomeric radicals from the continuous phase. In addition, polymerization occurs within the monomer-swollen particles. This process continues until all of the oligomeric radicals and nuclei generated in the reaction medium are consumed.

There is a general agreement that nuclei are formed throughout the dispersion polymerization process, while the number of particles is determined at an early stage. After the particle formation stage is complete, the number of particles does not change, but the particle size increase as the polymerization proceeds.[23, 24, 26, 37, 38] According to Lok et al. [28] and other researchers, [25, 26, 29] the number of stable particle is determined by the aggregation rate of nuclei and the stabilization kinetics. Each stable particle contains many oligomer and polymer chains. Nuclei formed at later stages of the reaction become incorporated into the larger particles already present in the reaction.

The polymer stabilizer plays a very important role in the particle formation process throughout the dispersion polymerization. [26, 36] In the absence of a polymer stabilizer, coalescence cannot be controlled, and stable particles cannot form, which leads to polymer precipitation from the solution. Therefore, it is possible to state that while nucleation kinetics determines the initial number of particles, the polymer stabilizer exercises control over the particles size and size distribution.

According the literature, polyvinylpyrrolidone (PVP), [26, 37-43] poly(acrylic acid) (PAA), [44, 45] poly(methacrylic acid)[46] and hydroxypropylcellulose (HPC) [47, 48] have been used as stabilizers in polar media for dispersion polymerization. In addition to these conventional stabilizers, poly(vinyl methyl ether) (PVME), [49] partially hydrolyzed poly(vinyl alcohol) (hydrolysis = 35%), [50] and poly(2-(dimethylamino)ethyl methacrylate-b-butyl methacrylate) (PDMAEMA-b-PBMA) [51] in alcohols and polystyrene-b-polybutadiene (PS-b-PB) [52] and polystyrene-b-poly(propylene-alt-ethylene) (PS-b-(PP-alt-E)), [53] linear polystyrene (PS), [54] and poly(methyl methacrylate) (PMMA) [54] in hydrocarbon have been used.
The stabilizer polymer chains are adsorbed on or grafted to the particle surface and act as steric stabilizers to prevent coalescence. [36, 44] Thus, it is critical to select an effective stabilizer: not only must it be soluble in the medium throughout the reaction, but it also must be able to cover the particle surface sufficiently to prevent coalescence in order to obtain colloidally-stable latex particles. According to the mechanistic model proposed by Paine et al. [22, 23, 36] and by others, [27, 55] it is the polymer-grafted stabilizer, which is formed due to the chain transfer from radicals to the stabilizer, instead of the homopolymer, that works as the true stabilizer.

1.1.2 Kinetics of dispersion polymerization

According to literature, [22, 56-59] the dispersion polymerization process can be divided into two major stages: nucleation and growth. Nucleation is the early stage in which the formation of nuclei and aggregation between them are predominant. Particle growth is a later stage where particle growth is predominant.

During the nucleation stage of a dispersion polymerization, the reaction begins as a solution polymerization. The polymerization rate, at this stage, depends on the number of free radicals available in the continuous media, the monomer concentration, and the reaction temperature. The polymerization in this phase simply follows the solution polymerization mechanism except that polymer chains longer than a critical chain length precipitate. Therefore, an oligomer chain, initially of an extended configuration with a small amount of monomer units, collapses into a condensed state as it grows to this certain threshold length. This length is called the critical chain length for nucleation ($L_{cr}$) and depends on the solubility of the type of monomer and solvent used.

As particles are formed, polymerization takes place in both the continuous phase and particle phase in any dispersion polymerization system, to varying degrees. The extent to which each of these occurs depends on the partitioning of the monomer and free radicals between these phases. This partitioning is a function of several variables, particularly the solvency of the medium and the nature of the monomer/polymer system. The partitioning determines the relative rates of polymerization in the two phases. This partitioning of monomer, solvent, initiator, and free radicals are largely governed by thermodynamics. Therefore, the reaction kinetics is considered as a combination of solution polymerization and polymerization inside swollen polymer particles. The latter often becomes more
important for polymers in which the gel effect is notable which can increase the rate substantially over solution kinetics. [60]

- **Kinetics model 1**

Barrett and Thomas [2] treated the dispersion polymerization essentially as a micro-bulk polymerization with monomer conversion largely occurring within the particles. Once polymer particles have been formed from the initially-homogeneous reaction mixture, they absorb monomer from the diluents phase, and polymerization subsequently proceeds within the particles. The high viscosity of the monomer-swollen polymer particles greatly hinders radical termination processes. Consequently, the number of radicals within a single polymer particle increases, and the total polymerization rate increases (*gel effect*). Any polymer radicals initiated in the diluent phase are rapidly swept up by existing particles before they have had time to grow to more than a very few monomer units in length. Consequently, initiation can be considered as taking place as though all the initiator were confined within the particles, even though the types of initiator used are known to be distributed between the polymer particles and the diluents. The polymerization kinetics is expressed as Eq.1.2.

\[
R_p = \alpha c_{md} k_p \left( \frac{\varphi R_i}{k_t} \right)^{0.5}
\]  

(1.2)

Where \( \alpha \) is the monomer partition coefficient between polymer and medium, \( c_{md} \) is the monomer concentration in the medium, \( k_p \) is the propagation rate coefficient, \( \varphi \) is the volume fraction of particles at a given time, \( R_i \) is the overall rate of initiation and \( k_t \) is an average termination rate constant.

- **Kinetics model 2**

Lu *et al.* [61] developed a thermodynamic model for the partitioning, based on Flory-Huggins theory [62] as extended by Morton *et al.* [63] They applied this model to the dispersion polymerization of styrene in ethanol considering the dispersion polymerization as a general heterogeneous polymerization and express the kinetics as Eq.1.3.

\[
R_p = k_p [M_p] \left( \frac{\rho A V_p}{2k_t} \right)^{0.5}
\]  

(1.3)

Where \([M_p]\) is the monomer concentration in the particles, \( \rho_A \) is the rate of the adsorption of oligomeric radicals by the polymer particles, \( V_p \) is the volume fraction of polymer particles in the system, and \( k_p \) and \( k_t \) are the rate constants of propagation and termination, respectively.

- **Kinetics model 3**
To simulate the entire mechanism of the dispersion polymerization, Yasuda et al. [64] proposed a simple model for the particle growth stage in the dispersion polymerization of styrene in ethanol based on the following three assumptions: (i) the termination reaction in the ethanol phase and the chain-transfer reactions in the ethanol phase and particles could be neglected, (ii) the mean volume of the radicals captured by the particles was approximately equivalent to that of the monomeric radicals, and (iii) the termination rate constant in the particles was $\beta_{\text{gel}}$ times that of the ethanol phase. Using this model, they simulated the time courses of the particle diameter and the conversion. They compared the experimental time course of the monomer conversion in the dispersion polymerization of styrene in ethanol with that of the solution polymerization of styrene in cyclohexane at 70 °C and found the rate of dispersion polymerization is higher than that of the solution polymerization, indicating that there is a significant gel effect in the dispersion polymerization.

- **Kinetics model 4**

Yasuda et al. [64] also developed a model that can describe the behavior at the particle formation stage in dispersion polymerization. This model was derived based on the following mechanism: (i) polystyrene molecules whose degree of polymerization is greater than $j_{\text{cr}}$ precipitate and form particles, (ii) particle aggregation due to the Brownian diffusion and the shear stress of the fluid continues until the amount of the PVP-g-PS molecules is enough to cover all the particle surface, and (iii) particles do not aggregate with each other once the entire particle surface is covered with the PVP-g-PS molecules. They used a method similar to that of Paine [23] to calculate the amount of the PVP-g-Ps produced in the ethanol phase, which was defined as “graft available”, and the minimum amount of the PVP-g-PS required to prevent particle aggregation, which was defined as “graft required”. When the value of the “graft available” reaches that of the “graft required”, all the particle surfaces are covered with PVP-g-PS molecules and the particle aggregation ceases. Based on this model, the theoretical particle concentrations calculated under various monomer concentrations and stirring speeds were in good agreement with the experimental data.

1.1.3 Functional particles by dispersion polymerization

The introduction of functional groups on the surface of polymer particles has received extensive interest from researchers in the fields of biology, medical analysis, protein synthesis, chromatography, coatings and others. [65-69] For sub-micrometer polymer microspheres, emulsifier-free emulsion polymerization is widely used to prepare particles
with various functional groups at the surface. [70] Emulsifier-free emulsion polymerization and other heterogenous polymerization techniques cannot be employed to prepare micrometer-size particles in a non-complicated manner. These micrometer-size particles are desired for many applications due to the ease of separation and recognition in medical diagnostics. Unfortunately, functional particles in this size range are more difficult to synthesize.

The preparation of micrometer-sized monodisperse homopolymer particles by dispersion polymerization has been extensively studied, especially for PS and PMMA systems. [28, 30, 36, 71] Functionality can be introduced into colloidal particles in various ways: by dispersion copolymerization of monomer and another functional monomer. Alternatively, one might employ functional dispersants, initiators, chain transfer agents, or macromonomers to incorporate desired functionality to the particle surface. However, only a few studies on dispersion polymerization using a combination of monomers have been reported. [29, 38, 44, 71, 72] This lack of reports might be attributed to the high sensitivity of dispersion polymerization to small changes in any of the numerous reaction parameters involved in the process. When small amounts of functional co-monomers are present in the reaction, the particle size changed significantly. In addition, using co-monomers in dispersion polymerization usually leads to particles with a broad size distribution.

In 1986, Tseng et al. [29] employed dispersion copolymerization to prepare polystyrene particles with 1 wt % functional co-monomers like methacrylic acid, hydroxyethyl acrylate, acrylamide, allyltriethoxysilane, vinyl polydimethylsiloxane, and 1-vinyl-1-methyl-sila-14-crown-5. Upon using these different co-monomers, the particle size varied over a wide range (2.5 to 4.0 um). In their experiments, the authors did not try to understand how the different co-monomers changed the particle size, nor did they study the location in the particles of the functional groups introduced via the polar monomers.

In 1987, large monodisperse copolymer particles of styrene and n-butyl methacrylate (n-BMA) were prepared by Ober and Lok [71] by batch dispersion copolymerization in ethanol/water. Further study on this system was carried out by Horák et al. [44] in 1995. Recently, dispersion copolymerization of styrene and butyl acrylate in other polar solvents was also reported. [40]

In 1999, the batch dispersion copolymerization of styrene and glycidyl methacrylate (GMA) in ethanol/water medium was employed to prepare functionalized micron-sized
monodisperse polymer particles by Yang et al. [73]. They investigated the influence of various polymerization parameters such as the initial solubility parameter, stabilizer concentration, initiator concentration, and the composition of the monomer mixture on the particle size and size distribution. They found with decreasing initial solubility parameter, the particle size increased and the size distribution broadened. The particle size decreased with increasing stabilizer concentration, the amount of styrene in the monomer mixture, and decreasing initiator concentration.

In 2000, Cao et al. [74] studied the dispersion copolymerization of methyl methacrylate (MMA) as the major monomer with up to 8 wt % acrylic acid (AA), methacrylic acid (MAA), glycidyl methacrylate (GMA), hydroxyethyl methacrylate (HEMA), or N-dimethylaminoethylmethacrylate (DMAEMA) as different co-monomers using PVP as the stabilizer in methanol-water medium. Particles with a narrow size distribution could be prepared only when small amounts of these functional co-monomers were used (up to 3 wt % based on MMA). With further increase of these functional co-monomers, particles with much broader size distribution were obtained. Larger particles were obtained when they added a co-monomer with a higher polarity and a greater hydrophilicity.

In 2001, Yang et al. [42] studied the dispersion copolymerization of styrene and other vinyl monomers including water-soluble monomers such as acrylamide (Am) and acrylic acid (AA) and hydrophilic monomers such as GMA, methyl methacrylate (MMA), ethyl methacrylate (EMA), and 3-(trimethoxysilyl) propyl methacrylate (TMSPMA); and the hydrophobic monomer N-vinyl carbazole (NVC) in polar solvents. Upon increasing acrylamide concentration in the monomer feed, they found the particle size first decreased and then increased. At a higher fraction of acrylamide, only a gel-like polymer was obtained. The particle size also increased with the increase in the contents of the hydrophilic co-monomers in the monomer mixtures, and the copolymer molecular weight also decreased. Although the amount of the hydrophobic co-monomer in the monomer mixture changed, the particle size was hardly affected.

In 2004, Jing-She Song [75] in our research group introduced the concept of two-stage dispersion polymerization (2-DisP), where problematic reagents, such as functional monomers and cross-linkers that commonly disrupt the particle-forming process, were added to the reaction after the nucleation stage was complete. Co-monomers added after this point became incorporated into the particles without disturbing the final particle size and size
distribution. In this way, Dr Song was able to prepare dye-labeled or functional group-containing micrometer-sized particles with a very narrow size distribution. [76-79] For example, in 2006, Song et al. [80] reported synthesis of monodisperse carboxylated micrometer-sized polystyrene particles by two-stage dispersion polymerization of styrene in ethanol and in 95% ethanol-water. The reaction was carried out in the presence of acrylic acid as the functional co-monomer. Coagulum-free particles with a very narrow size distribution containing 2%, 4%, and 6 wt % AA could be obtained by this method.

Based on this two-stage dispersion polymerization process, a group from China [81] reported the synthesis of poly(styrene-co-itaconic acid) (PS-co-PIA) particles with diameters in the range of 600 to 2100 nm in ethanol/water media using PVP as the stabilizer. Among other factors, the authors discussed the effect of IA concentration on the particle size and size distribution. They found that increasing the amount of IA increased particle size and size distribution \(D_n = 1.8 \mu m\) and \(PDI = 1.03\%\) for PS particles; \(D_n = 2.4 \mu m\) and \(PDI = 1.30\%\) for PS-PIA particles with IA/Sty = 0.07 w/w).

1.2 Encoding Strategies

In drug discovery and medical diagnostics, there is a requirement to develop a flexible, economic and efficient method to simultaneously detect multiple molecular targets in a single sample in order to analyze large numbers of biomolecules. One strategy to meet these requirements, which has several advantages over alternative approaches, is based on encoded microcarriers. These microcarriers, each with different types of compounds on the surface, can be utilized to identify the target analyte. In order to distinguish these microcarriers, a unique identifier is necessary for each type of microcarriers. This concept is called “encoding”. Nowadays, there are many techniques have been invented to encode microcarriers. In this section, I briefly present some encoding methods which are commercially available and hold much promise for the future. Namely, I will introduce spectrometric, electronic, graphical and physical encoding strategies.

1.2.1 Spectrometric encoding

The microcarriers, in spectrometric encoding, are decoded by spectrometric analysis. Mass spectrometry, \(^{13}\)C-NMR and F-NMR spectroscopy, energy-dispersive X-ray spectroscopy, and spectral IR absorption measurements can be used to identify chemical (spectrometric) tags attached to the microcarriers. More commonly, microcarriers can be
identified by their characteristic electromagnetic absorption or emission spectrum when they are optically encoded.[82]

1.2.2 Electronic encoding

In electronic encoding of the microcarriers, small electronic devices, so called radio-frequency (rf) memory tags, have also been developed. [83] For example, an rf tag from *Discovery Partners International*[84] consists of an antenna and a memory chip enclosed in a glass capsule that measures 8 mm x 1 mm x 1 mm. When this rf tag placed near the transceiver, the antenna absorbs the receiver’s rf signal and converts it to electrical energy to activate the memory chip. Consecutively, the transceiver receives the code of the memory chip as a radio signal that identifies the electronic tag. This encoding method is able to generate numerous codes (~$10^{12}$). Unfortunately, these electronic tags could not be used as individual microcarriers because of their relatively large dimensions.

1.2.3 Graphical encoding

Graphical encoding is the name given to processes which store information by the spatial modulation of a material or its properties. One of the most common examples of this encoding method is a traffic sign; with a high differential color contrast between background and the symbols, e.g. a red cross with a yellow background prohibit the passing of vehicles, they could be used to enhance traffic safety. A similar idea is employed in microcarrier identification. SmartBead Technologies [85] developed a form whereby bar-coded aluminum rods (100 μm x 10 μm x 1 μm) are created by optical lithography and dry etching to generate more than 10 codes, consisting of a series of holes of different lengths.

1.2.4 Physical encoding

Physical encoding schemes use the ability to discriminate between microcarriers that have different detectable physical properties ‘as a whole’. These properties include: size, [86, 87] density, refractive index, [88] shape, [89] composition [90, 91] and so on. The shortcoming of this method is that the synthesis method needs to vary with different types of particles in order to distinguish them; thus, commonly, it can be considered as a supplementary method for the previous mentioned methods. For example, Canning *et al.* [92] have used eight parameters, such as size and refractive index, which can be detected by modern flow cytometers in addition to their fluorescent optical signatures.
1.3 Lanthanide-Encoded Microspheres

1.3.1 The properties of lanthanides

The lanthanide series (lanthanides, Ln’s) is the group of elements that start with lanthanum (La, atomic number 57) and end with lutetium (Lu, atomic number 71). It includes cerium (Ce), praseodymium (Pr), neodymium (Nd), promethium (Pm), samarium (Sm), europium (Eu), gadolinium (Gd), erbium (Tb), dysprosium (Dy), holmium (Ho), erbium (Er), thulium (Tm), and ytterbium (Yb). The set of Ln’s may be divided into two groups: the light rare earth elements (La, Ce, Pr, Nd, Pm, Sm, Eu) and the heavy rare earth elements (Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu). [93]

The lanthanides series possess similar physical and chemical properties and are therefore difficult to separate. Their common properties are associated with their particular atomic structure. Each has two outer electrons and eight or nine in the next inner shell [94]. The configurations of outer electrons for lanthanide atoms and lanthanide trivalent ions are given in Table 1-1. No other group of elements in the periodic system displays such similarity as the lanthanide series. The 14 stable lanthanides are strongly electropositive (electronegativity less than 1.2), and they all usually favour the formation of trivalent cations by losing two electrons from the 6s shell and one electron from the 4f shell. [95] However, the divalent or the tetravalent oxidation state is also known for most of these elements in their chemical compounds. [96]

In atoms of the lanthanide elements, the 4f orbitals are progressively filled, with increasing atomic numbers. As a consequence, the configuration of the valence electrons of the outermost shell is the same for all the lanthanide species. The addition of electrons to the inner shells makes little change in many of their physical and chemical properties. The effective ionic radius of the trivalent ions decreases gradually from La (1.16 Å) to Lu (0.97 Å). [95]

Lanthanide ions display a well-defined luminescence characterized by narrow and highly structured emission bands with lifetimes on the order of a millisecond. [97-99] Lanthanide complexes are valuable alternative probes to conventional dyes because the emission is observed in the green-red region of the spectrum, in which few compounds in natural water and biological systems emit light. Undoubtedly, lanthanide’s luminescent properties make these compounds especially useful for the design of luminescent labels. [100-103] One of the
main difficulties in the use of luminescent lanthanide complexes is their low absorption coefficient. [104]

**Table 1-1** Configuration of outer electrons of Ln atoms and Ln$^{3+}$ ions

<table>
<thead>
<tr>
<th>Lanthanide</th>
<th>$Z'$</th>
<th>Configuration of outer electrons</th>
<th>Ion radius</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Atom 4f 5s 5p 5d 5f 6s 4f 5s 5p</td>
<td>Å</td>
</tr>
<tr>
<td>La</td>
<td>57</td>
<td>2 6 1 2 2 6</td>
<td>1.06</td>
</tr>
<tr>
<td>Ce</td>
<td>58</td>
<td>1 2 6 1 2 2 6</td>
<td>1.03</td>
</tr>
<tr>
<td>Pr</td>
<td>59</td>
<td>3 2 6 2 2 6</td>
<td>1.01</td>
</tr>
<tr>
<td>Nd</td>
<td>60</td>
<td>4 2 6 2 3 2 6</td>
<td>0.995</td>
</tr>
<tr>
<td>Pm</td>
<td>61</td>
<td>5 2 6 2 4 2 6</td>
<td>0.979</td>
</tr>
<tr>
<td>Sm</td>
<td>62</td>
<td>6 2 6 2 5 2 6</td>
<td>0.964</td>
</tr>
<tr>
<td>Eu</td>
<td>63</td>
<td>7 2 6 2 6 2 6</td>
<td>0.950</td>
</tr>
<tr>
<td>Gd</td>
<td>64</td>
<td>7 2 6 1 2 7 2 6</td>
<td>0.938</td>
</tr>
<tr>
<td>Tb</td>
<td>65</td>
<td>9 2 6 2 8 2 6</td>
<td>0.924</td>
</tr>
<tr>
<td>Dy</td>
<td>66</td>
<td>10 2 6 2 9 2 6</td>
<td>0.908</td>
</tr>
<tr>
<td>Ho</td>
<td>67</td>
<td>11 2 6 2 10 2 6</td>
<td>0.894</td>
</tr>
<tr>
<td>Er</td>
<td>68</td>
<td>12 2 6 2 11 2 6</td>
<td>0.881</td>
</tr>
<tr>
<td>Tm</td>
<td>69</td>
<td>13 2 6 2 12 2 6</td>
<td>0.869</td>
</tr>
<tr>
<td>Yb</td>
<td>70</td>
<td>14 2 6 2 13 2 6</td>
<td>0.858</td>
</tr>
<tr>
<td>Lu</td>
<td>71</td>
<td>14 2 6 1 2 14 2 6</td>
<td>0.848</td>
</tr>
</tbody>
</table>

$Z'$: atomic number

1.3.2 Analysis of lanthanides

1.3.2.1 Current techniques

There are many analytical methods to determine the presence and content of lanthanides. [105] Their applications depend on the sensitivity and accuracy required as well as on the availability of instrumentation. Isotopic dilution mass spectrometry (IDMS) is an example. It has high accuracy and sensitivity, but measurements are costly. The major analytical techniques employed for the determination of lanthanides are complexometric methods, graphite-furnace/flame atomic absorption spectrometry (GF-AAS, FAAS), inductively coupled plasma atomic emission spectrometry (ICP-AES), X-ray fluorescence (XRF) and neutron activation analysis (NAA). [106, 107]
In this work, the lanthanide ion content of solutions was measured by inductively coupled plasma mass spectrometer (ICP-MS). Most importantly, ICP-MS is an essential part of the mass cytometry instrument; it constitutes the analyzer and the detector in that instrument. Because of the significant importance of the ICP-MS as an analytical tool in my projects, I will give a brief introduction to the ICP-MS instrument.

1.3.2.2 Inductively Coupled Plasma Mass Spectrometer

ICP-MS is a type of mass spectrometry that is highly sensitive, less interferential and capable of determining a range of metals and several non-metals at concentrations below one part in 10^{12} (part per trillion). ICP-MS is also capable of monitoring isotopic species and ratios for the elements of choice.

In general, the instrument consists of three basic units: 1) a conventional argon ICP operating at temperatures of 6000 to 10000 K with a nebulizer, spray-chamber, work coil and associated power supplies, 2) a conventional quadrupole mass spectrometer and associated data collection electronic and 3) an interface consisting of two water cooled nickel cones, each containing a small orifice at the center which allows sampling of the plasma gases and transfer of the ion beam into the mass spectrometer. [106] ICP instruments traditionally have a quadrupole mass analyzer. Scientist at DVS Sciences (Markham, ON, Canada) built the first ICP-MS with a time of flight (TOF) mass analyzer.

 Basically, the samples in solution are injected into the ICP-torch, which has a very high temperature, that is enough to vaporize, atomize and ionize the sample. Then the ion cloud produced by plasma is extracted through a series of cones into the mass spectrometer. The ions are separated on the basis of their mass-to-charge ratio and the detector receives an ion signal proportional to the concentration. The concentration of a sample can be determined through calibration with certified reference materials such as single or multi-element reference standards.

1.3.2.3 Mass cytometry

The lanthanide ion content of the Ln-encoded particles in this work was measured by mass cytometry: flow cytometry-time-of-flight inductively coupled plasma mass spectrometry. Because mass cytometry plays a major role in this work, I will give a general description of this instrument.
Mass cytometry combines a regular cytometer injector and a TOF mass spectrometer. It was developed as a new technique that relies on ICP-MS analyzer that detects metal labeled particles or proteins etc. Instead of fluorescent tags that are used in traditional flow cytometry, mass cytometry technique is based on attaching metal tags to biomolecules and/or polymer particles. The technique of mass cytometry is designed to benefit from the high resolution, sensitivity, and speed of analysis of ICP-TOF mass spectrometry. Many Ln-encode particles can be simultaneously detected in individual measurement. This is due the wide availability of stable Ln isotopes that can be incorporated into these polymer particles. More details about mass cytometry technique and detail description of the instrument can be found in references. [108-110]

1.4 Research Objectives

When I started my research work in September 2009, I was asked to synthesize functional monodisperse micro-size polymer particles for bead-based biological assays. I found that the two-stage dispersion copolymerization of styrene and acrylic acid developed by Ahmed Abdelrahman was successful for obtaining particles with a narrow size distribution and high lanthanide content. However, these particles had two major shortcomings, broad lanthanide content distribution and a lack of the ability to undergo bio-conjugation. I proposed the use of methacrylic acid (MAA) as a co-monomer for the dispersion polymerization of styrene to prepare Ln-encoded particles.

I synthesized the particles using two and three stage dispersion polymerization and employed different levels of MAA. Once I mastered the synthesis techniques, the objective of my M.Sc. research became to study the lanthanide ion content, their incorporation and release from the PS particles. I chose to focus on the following objectives:

[1] To synthesize surface functionalized monodisperse polystyrene microspheres.

[2] To investigate the particles stability and ability to maintain their Ln content in different media

[3] To understand the kinetics of the lanthanide ions incorporation into the PS particles during the synthesis.
1.5 Thesis Outline

This thesis contains four chapters including this introductory chapter. In Chapter 2, I describe the synthesis and characterization of Ln-encoded polystyrene particles with methacrylic acid as the co-monomer. In Chapter 3, I present a systematic study on the stability of the polystyrene particles against the ion leakage. In Chapter 4, I introduce some preliminary kinetics experiments about the Ln incorporation into the polystyrene particles. In Chapter 5, I give a brief summary about my work.

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Chapter 2
The Synthesis and Characterization of Lanthanide-Encoded Poly(Styrene-\textit{co}-Methacrylic Acid) Microspheres

2.1 Introduction

Multiplexed assays are in demand for biological assays, [1, 2] drug delivery research [3] and many other applications. In bioassays, screening large numbers of biomolecules (with antibodies, oligonucleotides) requires a technology that allows multiple analyte assays to be performed simultaneously on a single sample. In this way, the time required for an assay is reduced significantly, and the volume of sample required for the multiplexed assay would be significantly reduced.

Over the past decades, bead-based assays, involving the use of polymer particles, have become increasingly prominent for multiplexed biological assays. Polymer beads are used as solid supports to bind to different affinity products that can target different molecules. By encoding the beads and creating a set of beads for each analyte, the captured analyte can be tracked by decoding and identifying individual beads. Using a set of beads functionalized with different affinity molecules, multi-analyte analysis can be performed simultaneously in a single assay. This approach has seen applications in immunological and gene expression assays. [3-5]

There are many methods available to encode polymer beads, and the design of new encoding strategies is itself a field of research. Among the methods that have been reported are chemical tags that utilize mass spectrometry to identify polypeptides synthesized on bead supports, [6] NMR encoding, [7] electronic encoding using radio frequency tags [8] and even graphical encoding using laser etching to produce a barcode. [9] Most of the commercially available bead-based assays depend on fluorescent dyes as tags to label polymer beads with diameters of a few micrometers. These beads are normally examined at high throughput by flow cytometry. A prominent example of this kind of technology is sold by Luminex®. Luminex® beads consist of a library of 100 different encoded polystyrene beads containing 2 different dyes at 10 different levels of concentration, with a surface functionality for attaching antibodies. [10, 11]

In our group, we are interested in multiplexed bead-based assays based on the new technique of mass cytometry. In mass cytometry, [12] beads (or cells) are injected
individually but stochastically into the plasma torch of an inductively coupled plasma mass spectrometer (ICP-MS) equipped with time-of-flight (TOF) detection. In the plasma at 7000 K, the beads are vaporized, atomized, and ionized to form an ion cloud for which 20 to 30 TOF mass spectra are taken. ICP-MS is a very powerful method for the quantitative detection of metals. It enables the use of metal atoms or individual isotopes as the encoding species. For instruments that are tuned to detect masses in the m/z range of 100 to 200, a much higher multiplicity of simultaneous detection is possible than with fluorescence detection. [13] The lanthanide (Ln) elements are particularly interesting as encoding species. They have low natural abundance and thus low background signals. Meanwhile, they have similar chemistry, and there are in principle 54 natural non-radioactive isotopes available for encoding. [14]

We are interested in the synthesis and characterization of Ln-encoded polymer particles intended for mass cytometry-based bioassays. We focus on polystyrene (PS) particles of narrow size distribution and with diameters in the range of 1 to 3 µm. There are a variety of strategies one can envision for obtaining polymer microspheres labeled with Ln ions. One type of strategy would be to synthesize beads of the appropriate size and then use a swelling method to introduce a liganded lanthanide. Matsuya et al. [15] used this approach to introduce photoluminescent Eu$^{3+}$ complexes into (smaller) PS beads. Vancaeyzeele et al. [16] reported the synthesis of Ln-containing PS nanoparticles by miniemulsion polymerization. These particles, which are too small for mass cytometry, were designed for cell uptake by non-specific endocytosis and employed in bulk assays of cell adhesion by ICP-MS. Thickett et al. [17] reported the preparation of ca. 1 µm diameter PS beads by a two-step process in which particles of uniform size were synthesized by surfactant-free emulsion polymerization. These precursor beads were then swollen with a styrene solution of styrene-soluble lanthanide derivatives, which were subsequently polymerized.

To date, the most useful approach to the synthesis of Ln-encoded PS microspheres for mass cytometry applications was that reported by Abdelrahman et al. [13] This contribution from our group described the synthesis of PS microparticles with a very narrow size distribution by two-stage (and by three-stage) dispersion copolymerization of styrene with acrylic acid (AA) in ethanol in the presence of LnCl$_3$ salts. [13] A variety of different Ln elements could be introduced into the particles. In one set of experiments, they were able to obtain particles containing four different Ln ions (La, Tb, Tm and Ho) at five resolvable levels of concentration, for an encoding variability of 624.
These reactions were carried out in the presence of polyvinylpyrrolidone (PVP) as a polymeric stabilizer. The role of the acrylic acid was to serve as the ligand for the Ln ions. The term “two-stage” refers to a synthesis strategy [18] in which the polymerization of styrene itself was initiated in the first stage, and the addition of acrylic acid [19] and the Ln salts was delayed until well after the nucleation stage was complete. This strategy avoids complications of the co-monomer interfering with particle nucleation. After the end of particle nucleation, the number of growing polymer particles in the reaction remains constant. While nucleation is thought to be complete in a few minutes, we typically waited 1 hour after the onset of the reaction (ca. 5 % to 10 % styrene conversion) to add the AA co-monomer. The Ln content of the particles synthesized in this way could be varied from $10^6$ to $10^8$ Ln ions per particle.

In a variation of this reaction, referred to as 3-stage dispersion polymerization (3-DisP), we added 2 wt % AA based upon styrene in the second stage and then, after an additional 8 hours (60% styrene conversion), added an additional 2 wt % AA + the Ln salts plus a small amount of a cross-linking agent to the reaction. This third stage gave somewhat higher incorporation of the added Ln salts into the particles and an improvement in the uniformity of Ln content per particle.

While this approach appeared to work, we need a better understanding of this chemistry in order to optimize the particle synthesis for mass cytometry applications. We would like to simplify the reaction so that narrow particle size distributions, high Ln ion incorporation, and uniform bead-to-bead Ln ion content could be achieved in a two stage reaction. We also need to increase the −COOH group content at the particle surface to facilitate attachment of bioaffinity reagents to the particles.

Here, I examine the use of methacrylic acid (MAA) as a co-monomer instead of AA in two-stage dispersion polymerization (2-DisP) reactions of styrene in ethanol in the presence of PVP. For example, MAA has a more favorable reactivity ratio for copolymerization with styrene ($r_{sty} = 0.2$, $r_{MAA} = 0.5$) than acrylic acid ($r_{sty} = 0.15$, $r_{AA} = 0.9$). This may lead to a more efficient and uniform incorporation of Ln ions into the particles.

In this chapter, I describe the synthesis of poly(styrene-co-methacrylic acid) (P(S-MAA)) particles. These particles were characterized by scanning electron microscope (SEM), traditional inductively coupled plasma mass spectrometry (ICP-MS) and mass cytometry. Most of the results given in this chapter were published in Ref 20. In addition, I characterized
P(S-AA) particles synthesized by Ahmed Abdelrahman to compare them with the P(S-MAA) particles.

2.2 Experimental Section

2.2.1 Instrumentation

A Hitachi S-5200 field emission–scanning electron microscope (SEM) was utilized at operation voltages from 2 to 5 kV to measure particle size. Titrations were carried out with simultaneous conductometric and potentiometric measurements using a Fisher Scientific digital conductivity, resistivity, and temperature meter (Model # 09-326 2) and an EcoMet pH/mV/TEMP Meter (P25).

Traditional inductively coupled plasma mass spectrometry (ICP-MS) measurements were carried out on an Elan 9000 instrument (Perkin-Elmer SCIEX) operating under normal Ar plasma 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 (Perkin-Elmer SCIEX) was used in all instances. All the experiments were performed using an auto-sampler (Perkin-Elmer AS 93) modified for operation with Eppendorf 1.5 mL tubes. Sample volume was fixed at 1 mL. The sample uptake rate was adjusted depending on the particular experiment, typically 100 µL/min. Standards were prepared from 1000 µg/mL Perkin-Elmer pure Single-Element Standard solutions (Perkin-Elmer, Shelton, CT) by sequential dilution with high purity HNO₃. High purity HNO₃ was measured as well in each experiment to be used as blank signal. The blank signals were subtracted from the sample signals, which were then normalized to the signals of standard solutions (1 ppb).

Mass cytometry measurements were carried out with a CyTOF™ model C2 instrument from DVS Sciences (Markham ON Canada, www.dvssciences.com). [12] Samples were examined in this work at a rate of ca. 1000 particles per second, which corresponds to 60 µL/min slurry uptake rate. The figures of merit of the instrument were measured under standard ICP operating conditions (< 3 % oxide ratio for CeO / Ce). At mass resolution (full width at half maximum) for m/z = 159 M/ΔM > 900, sensitivity with a standard sample aspiration is $1.4 \times 10^8$ ion counts per second per mgL⁻¹ of Tb typically, and abundance sensitivity $6\times10^{-4} – 1.4\times10^{-3}$ (trailing and leading masses, respectively). The mass range (variable, but fixed at m/z = 125 – 215 for this work) and the abundance sensitivity are
sufficient for elemental encoding with up to 60 distinct isotopes. More details about this instrument can be found in Tanner et al. [21] and Bandura et al. [12]

2.2.2 Materials

Styrene (Aldrich), methacrylic acid (MAA, 99 %, Aldrich), absolute ethanol, polyvinylpyrrolidone (PVP) (PVP55, M = 55,000, Aldrich), Triton-X305 (TX305, 70% solution in water, Aldrich), 2, 2’-azobis (2-methylbutyronitrile) (AMBN, DuPont USA), ethylene glycol dimethacrylate (EGDMA, Aldrich), lanthanide (III) chloride hexahydrate salts (LnCl₃·6H₂O, Aldrich) were used as received. 2-(N-morpholino)ethanesulfonic acid (MES, Aldrich, pH 6.5) and ammonium acetate (10 mM, pH 9) were prepared as aqueous solution. Phosphate buffered saline solution (PBS, 150 mM NaCl, 1.2 mM Ca²⁺, 0.8 mM Mg²⁺, 20 mM sodium phosphate, pH 7.2) were from DVS Science. Deionized water (DIW) was purified through a Milli-Q purification system. High purity HNO₃ for ICP-MS analysis was purchased from Seastar Chemical Inc.

2.2.3 Dispersion Polymerization of Styrene with Methacrylic Acid as a Co-monomer

The recipe for the two-stage dispersion polymerization of styrene with MAA in ethanol is listed in Table 2-1. All of the stabilizer (PVP), the co-stabilizer (TX305), initiator (AMBN), and the monomer (styrene) and half of the ethanol were added to a 250 mL three-necked round-bottom flask equipped with a condenser, a mechanical stirrer connected to a (PTFE) half-moon stirring paddle and a gas inlet. The bottom of the hole of the stirring paddle was fixed at the same height as the surface of the solution. After a homogeneous solution formed at room temperature, the solution was deoxygenated by bubbling nitrogen gas at room temperature for 30 min through a syringe needle with the tip under the surface of the solution and stirred mechanically at 100 rpm. The flask was then placed in a preheated 70 °C oil bath after pulling the needle to bring the tip above the surface of the solution. The MAA (and the Ln salts) were dissolved in the remaining ethanol and preheated in the 70 °C oil bath for 5 min. Then 1.5 hours after the polymerization reaction started, this warm solution was added to the reaction flask by syringe over 1 min. The reaction was continued for 24 hours. A list of the MAA and Ln salt content of all reactions described here is presented in Table 2-2.
An aliquot of the as-synthesized particles was examined by SEM. Then the solution was centrifuged (3000 rpm, 15 min) to sediment the particles. Aliquots of the supernatant were reserved for further analysis. The particles were resuspended in water and washed by two additional sedimentation-resuspension steps. Under most circumstances, the particles could be resuspended by shaking the tube containing the particles. When sedimentation was carried out in small (15 mL) falcon centrifuge tubes, redispersion required 15 min sonication (Branson® ultrasonic cleaner, 1210R-MT, 60 W).

**Table 2-1** Standard recipe for the dispersion polymerization of styrene with MAA in ethanol

<table>
<thead>
<tr>
<th>Materials</th>
<th>1st stage</th>
<th>2nd stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Styrene (g)</td>
<td>6.25</td>
<td>--</td>
</tr>
<tr>
<td>PVP55 (g)</td>
<td>1.0</td>
<td>--</td>
</tr>
<tr>
<td>TX305 (g)</td>
<td>0.35</td>
<td>--</td>
</tr>
<tr>
<td>AMBN (g)</td>
<td>0.25</td>
<td>--</td>
</tr>
<tr>
<td>Ethanol (g)</td>
<td>18.75</td>
<td>18.75</td>
</tr>
<tr>
<td>Methacrylic acid (g)</td>
<td>--</td>
<td>a</td>
</tr>
<tr>
<td>LnCl₃·6H₂O(g)</td>
<td>--</td>
<td>b</td>
</tr>
</tbody>
</table>

a. Different amount of MAA was used for different samples. For specific amounts, see Table 2-2;
b. Some combination of LaCl₃·6H₂O, EuCl₃·6H₂O, TbCl₃·6H₂O, HoCl₃·6H₂O, and TmCl₃·6H₂O. For specific amounts, see Table 2-2.
Table 2-2 Methacrylic acid and Ln content of the reaction mixture for synthesis of P(S-MAA)

<table>
<thead>
<tr>
<th>Sample</th>
<th>MAA</th>
<th>$^{139}$La</th>
<th>$^{152}$Eu</th>
<th>$^{169}$Tm</th>
<th>$^{165}$Ho</th>
<th>$^{159}$Tb</th>
<th>Ln total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAA-2</td>
<td>2.0</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>MAA-4</td>
<td>4.0</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>MAA-6</td>
<td>6.0</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>MAA-2-Tb(0.2)</td>
<td>2.0</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.30</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>MAA-4-Tb(0.3)</td>
<td>4.0</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.20</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>MAA-2-Tb(0.3)</td>
<td>2.0</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.30</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>MAA-2-Tb(0.4)</td>
<td>2.0</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.40</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>MAA-2-Tb(0.5)</td>
<td>2.0</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.50</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>MAA-2-Tb(0.6)</td>
<td>2.0</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.60</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>MAA-2-Tb(0.7)</td>
<td>2.0</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.70</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>MAA-(2+2)-Eu(0.1)</td>
<td>4.0 b</td>
<td>--</td>
<td>0.10</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.10</td>
</tr>
<tr>
<td>MAA-4-Eu(0.1)</td>
<td>4.0</td>
<td>--</td>
<td>0.10</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.10</td>
</tr>
<tr>
<td>MAA-2-I</td>
<td>2.0</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
<td>0.2</td>
<td>0.208</td>
</tr>
<tr>
<td>MAA-2-II</td>
<td>2.0</td>
<td>0.005</td>
<td>0.005</td>
<td>0.005</td>
<td>0.005</td>
<td>0.2</td>
<td>0.22</td>
</tr>
<tr>
<td>MAA-2-III</td>
<td>2.0</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.2</td>
<td>0.24</td>
</tr>
<tr>
<td>MAA-2-IV</td>
<td>2.0</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.2</td>
<td>0.28</td>
</tr>
<tr>
<td>MAA-2-V</td>
<td>2.0</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>MAA-2-VI</td>
<td>2.0</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>MAA-2-VII</td>
<td>2.0</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.2</td>
<td>1.00</td>
</tr>
</tbody>
</table>

a. wt % relative to styrene;  
b. 2 wt % relative to styrene was added in the 2nd stage and an additional 2 wt % was added in the 3rd stage.

2.2.4 Particle Characterization

The diameters and size distribution of microspheres were determined by analysis of micrographs obtained by SEM using the software package ImageJ, developed by the National Institute of Health. The samples for SEM imaging were prepared by placing a drop of diluted washed suspension on a Formvar/carbon coated-300 mesh copper grid. Particle-size histograms were constructed from measurements of at least 500 individual particles in the SEM images.

Acid titration analysis was carried out as described by Kawaguchi et al.[22] A 1.00 mL sample of the as prepared particle solution was spun down by centrifugation (14000 rpm, 30
min), and the supernatant (500 µL) was collected for later analysis. About 0.3 g (carefully weighed samples with 0.001 g precision) of each supernatant was first mixed with an excess of freshly prepared aqueous NaOH solution (200 µL, 0.100 M), and then was immediately back-titrated with HCl (0.025 M) to determine the amount of MAA groups that remained in the supernatant. For particle titrations, about 2 g (weighed with 0.001 g precision) washed beads were dispersed in freshly prepared aqueous NaOH (200 µL, 0.100 M), and then, after 30 min, were back-titrated with HCl (0.025 M) to determine the amount of acid groups on the particle surface. Prior to each titration, the pH electrode was calibrated using three buffer solutions (pH = 4.0, 7.0, 10.0). The conductivity and pH of the solutions were monitored continuously. Titrations (20-50 µL per step) were carried out under magnetic stirring with standard HCl until the pH value was about 3. Quantitative information was acquired from the titration plot using the standard extrapolation/intersection method to determine the titration end points.

The Ln content of the polystyrene particles was measured by mass cytometry. A small amount of washed particles was redispersersed in deionized water and diluted to approximately 10⁶ particles per mL. The number of metal ions per particle was calculated as described in Refs. 12 and 20. One calculation example is shown in the section 2.3.4.

Following each particle synthesis, the supernatant liquid was analyzed by ICP-MS to determine the amount of Ln ions that were not incorporated into the PS particles. A 1.00 mL sample of the as prepared particle solution was spun down by centrifugation (14000 rpm, 30 min), and the supernatant (500 µL) was collected for later analysis. A 20 µL aliquot of the supernatant was transferred to a 1.5 mL 1 kDa MWCO Millipore Amicon spin filter and diluted with 380 µL DIW and then centrifuged at 14000 rpm, 30 min. Then a 20 µL aliquot of the flow-through solution was diluted with 980 µL DIW (50-fold dilution). Finally, a 100 µL of this diluted solution was further diluted with 900 µL aqueous HNO₃ (3.0 %) and analyzed by ICP-MS. The approximate dilution factor is 10000.

2.3 Results and Discussion

I begin with a description of the synthesis and characterization of carboxylated PS particles with MAA as the co-monomer prepared by two-stage dispersion polymerization. I use titrations to identify the locations of the carboxyl groups, on the particle surface, buried in the interior, or remaining in the supernatant, and compare these results to previous reports.
that used AA as the co-monomer. Then I describe the introduction of Ln salts into the reaction and examine the incorporation efficiency and determine the Ln content.

2.3.1 Particle synthesis by two-stage dispersion polymerization

I begin by describing the synthesis of poly(styrene-co-methacrylic acid) particles by two stage dispersion polymerization without adding Ln salts. As shown in Table 2-1, polymer synthesis reactions were carried on a scale of 6.25 g styrene monomer, all present in the first stage of the reaction. In addition, the first stage of the reaction contained polyvinylpyrrolidone (PVP55, M = 55 KDa, 16 wt. % based on styrene) as a steric stabilizer, a small amount of non-ionic surfactant TX305, and the azo initiator AMBN, all dissolved in 18.75 g absolute ethanol. After heating the reaction for 1.5 hours at 70 °C (ca. 10% monomer conversion), the co-monomer (MAA) was added as a pre-heated solution in ethanol (18.75 g) as a second stage. In corresponding reactions carried out with Ln salts, various amounts of these salts were mixed with the MAA in ethanol and added to the reaction in the second stage. Table 2-2 indicates the amounts of Ln salts added to each reaction. The reactions were then continued for 24 hours, leading to around 95 % conversion of styrene measured by gas chromatography (GC). The characteristics of all the particles discussed here are collected in Table 2-3.
| Notebook notation | Sample name  
|-------------------|-----------------|
| Table 2-3 Ln content of the reaction, particle size and size distribution of P(S-AA) and P(S-MAA) particles synthesized by two-stage dispersion polymerization  
| Notebooks notation |                  |
| Sample name a & Ln content of the feed b & Diameter, µm & CV d, % c  
| AA150 | AA-2-I d | La, Eu, Ho, Tm (0.005) Tb (0.1) | 2.4 | 3.2  
| AA152 | AA-2-II d | La, Eu, Ho, Tm (0.02) Tb (0.1) | 2.7 | 6.8  
| AA155 | AA-2-III d | La, Eu, Ho, Tm (0.2) Tb (0.1) | 2.4 | 3.8  
| SL005 | MAA-2 | 0 | 1.9 | 4.2 (3.7)  
| SL006 | MAA-4 | 0 | 2.2 | 4.3 (3.3)  
| SL008 | MAA-6 | 0 | 2.0 | 18  
| SL010 | MAA-2-Tb(0.2) | 0.2 | 1.9 | 5.6 (2.9)  
| SL011 | MAA-4-Tb(0.3) | 0.3 | 2.0 | 3.0 (2.8)  
| SL077 | MAA-2-Tb(0.3) | 0.3 | 1.9 | 6.9 (3.9)  
| SL078 | MAA-2-Tb(0.4) | 0.4 | 1.8 | 3.5  
| SL080 | MAA-2-Tb(0.5) | 0.5 | 1.9 | 2.6  
| SL082 | MAA-2-Tb(0.6) | 0.6 | 2.4 | 4.5 (3.8)  
| SL081 | MAA-2-Tb(0.7) | 0.7 | 2.0 | 10.5 (5.5)  
| SL029 | MAA-2-I d | La, Eu, Ho, Tm (0.002) Tb (0.2) | 2.6 | 5.9 (3.7)  
| SL030 | MAA-2-II d | La, Eu, Ho, Tm (0.005) Tb (0.2) | 2.5 | 3.9 (2.6)  
| SL031 | MAA-2-III d | La, Eu, Ho, Tm (0.01) Tb (0.2) | 3.0 | 4.9 (2.6)  
| SL032 | MAA-2-IV d | La, Eu, Ho, Tm (0.02) Tb (0.2) | 2.6 | 6.9 (2.6)  
| SL033 | MAA-2-V d | La, Eu, Ho, Tm (0.05) Tb (0.2) | 2.4 | 2.0 (1.8)  
| SL034 | MAA-2-VI d | La, Eu, Ho, Tm (0.1) Tb (0.2) | 2.5 | 2.3 (2.1)  
| SL035 | MAA-2-VII d | La, Eu, Ho, Tm (0.2) Tb (0.2) | 2.6 | 4.0 (2.8)  

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a. AA: acrylic acid; MAA: methacrylic acid. The value after the co-monomer indicates the amount of AA or MAA (wt % / styrene) added in the synthesis. The values in parentheses after each Ln indicates the amount of LnCl$_3$·6H$_2$O (wt % / styrene) added in the reaction; for example, MAA-2 refers to the PS particles synthesized with 2 wt % methacrylic acid and no Ln salts added in the synthesis. MAA-2-Tb(0.2) refers to PS particles with 2 wt % methacrylic acid and 0.2 wt % TbCl$_3$·6H$_2$O;

b. wt % of LnCl$_3$·6H$_2$O based on styrene;

c. $CV_d$ = coefficient variation of the particle diameter. The values in parentheses refer to particles washed by centrifugation-redispersion to remove small particles in the sample;

d. The Roman numerals indicate that a combination of different Ln salts was used in the reaction.

For the reactions carried out in the absence of Ln salts, I compared reactions in which three different levels of MAA (2 %, 4 % and 6 wt % / styrene) added in the second stage. After 24 hours at 70 °C, the reaction mixtures were cooled to room temperature. I analyzed the particles and the supernatant at this stage. Then the particles were sedimented by centrifugation, redispersed in water and washed by two additional cycles of centrifugation and redispersion in water.

Sample MAA-2 (prepared in the presence of 2 wt % MAA) was colloidally stable in ethanol and in water. No coagulum was observed in the reaction. A scanning electron microscope (SEM) image of the as-prepared (unwashed) particles is presented in Figure 2-1. Although most of the particles in the many images examined were uniform in size, a small number of smaller particles were observed. In the image in Figure 2-1, some such particles are highlighted by red dashed circles. The unwashed sample had a number-average diameter ($D_n$) of 1.9 µm and a coefficient of variation in diameter ($CV_d$) of 4.2 %. After washing these particles by three centrifugation and redispersion cycles in water, most of the small particles were removed. Figure 2-2A and 2-2B show SEM images for these washed particles at low and high magnification, respectively. Figure 2-2C shows the histogram of the size distribution of washed sample MAA-2, constructed from analysis of 300 particles in multiple SEM images. Although, the diameters of the washed particles range from 1.4 to 2.4 µm, the vast majority (98.9%) of these particles have a tight distribution of diameters between 1.7 and 2.1 µm. The washed particles have the same $D_n$ as that of the unwashed particles ($D_n = 1.9$ µm), but a narrower $CV_d$ (3.3%).
Figure 2-1 SEM images of as-prepared polystyrene microspheres (unwashed) synthesized by 2-stage dispersion polymerization with PVP as the stabilizer in the presence of different amounts of MAA. Sample MAA-2 has 2 wt % MAA relative to styrene: $D_n = 1.9 \mu m$, $CV_d = 4\%$. 
Figure 2-2 SEM images and diameter distribution histogram of polystyrene microspheres synthesized by 2-stage dispersion polymerization with PVP as the stabilizer in the presence of different amounts of MAA. Sample MAA-2 has 2 wt % MAA relative to styrene: $D_n = 1.9 \mu$m $CV_d = 4.2 \%$ (3.3 \%) (A, B, C); Sample MAA-4 has 4 wt % MAA relative to styrene: $D_n = 2.2 \mu$m $CV_d = 4.3 \%$ (3.3 \%) (D, E, F); Sample MAA-6 has 6 wt % MAA relative to styrene: Polydisperse and some coagulation, $CV_d = 17.9 \%$ (G, H, I). A, D, G images are low magnification SEM images; B, E, H images are high magnification SEM images and C, F, I plots are histograms of the diameter distribution of washed particles. For I, the insert shows the data on a smaller sensitive scale. The data start from 2.5 to 7 \mu m.

SEM images of sample MAA-4 (prepared in the presence of 4 wt % MAA) show that a larger number of smaller particles were formed than in the synthesis of sample MAA-2. Figure 2-1 shows SEM images of the as-prepared particles. These unwashed particles have a mean diameter of 2.2 \mu m, with $CV_d$ of 4.3 \%. Figure 2-2D and 2-2E show SEM images with low and high magnification for a washed sample MAA-4, and the histogram of diameters is presented in Figure 2-2F. Most of the small particles were removed and the washed particles look monodisperse, with $D_n$ of 2.2 \mu m and $CV_d$ of 3.3 \%. Again the mean diameter was preserved in the washing step, but most of the small particles were removed.

The reaction carried out with 6 wt % MAA (MAA-6) gave a much broader particle size distribution. Some coagulum was observed in the reaction. Figure 2-2G and 2-2H present SEM images of low and high magnification for washed particles of this sample. Figure 11
shows the size distribution histogram of sample MAA-6. The particle sizes ranged from 1.5 \( \mu m \) to 6.5 \( \mu m \). The broad size distribution is reflected in the magnitude of \( CV_d = 17.9 \% \). The population of bigger particles is very low (see the insert in Figure 2-2I).

From the results described above, I infer that uniform PS particles can be synthesized in the presence of 2 and 4 wt % MAA, but larger amounts of MAA in the reaction lead to loss of control of the particle size distribution. From the analysis of SEM images of the particles before and after washing by sedimentation-redispersion, I learned that the number of small particles (possibly generated by secondary nucleation during the reaction) is too small to affect the (number average) mean particle diameter but broadens the calculated breadth of the distribution. Most of the small particles were removed by the washing step.

In copolymer particle synthesis, the carboxylic acid co-monomer can end up in one of three locations, as a soluble polymer in the continuous medium, on the particle surface, and buried in the particle interior. These distributions of carboxylic acid groups can be distinguished by titration experiments. The soluble polymer can be titrated in the reaction medium after sedimenting the particles and removing them. Titrating the washed particles in aqueous media detects –COOH groups readily accessible to aqueous base, and one normally assumes that these acid groups are at (or very close to) the particle surface. Buried acid groups are not titrated in this way. If one wants to do a full accounting for the acid groups introduced in the reaction, one can dissolve the particles in a solvent such as tetrahydrofuran, and titrate the total acid content with strong base in methanol.

Here, I titrated the ethanol phase of the reaction mixture to determine the soluble acid not incorporated into the particles, and I titrated the washed particles in water. I calculated the amount of buried acid groups by subtracting the first two amounts from the amount of MAA added to the reaction. In each instance, I added a known excess of NaOH to the solution and then back-titrated with 0.025 M HCl.

A representative titration curve is presented in Figure 2-3 for the titration of MAA-2 particles. The distribution of carboxyl groups can be calculated based on the following equations as an example:

\[
N_{\text{MAA-meas}} = C_{\text{HCl}} \times V_{\text{HCl}} \times N_A \\
= 0.025 \times (80 \times 10^{-6}) \times (6.02 \times 10^{23}) = 1.20 \times 10^{18}
\]
where $V_{\text{HCl}}$ is the volume (L) of the HCl needed to titrate the surface bound acid and $C_{\text{HCl}}$ is concentration of the standard HCl (0.025 M). For this sample, $V_{\text{HCl}} = 760 – 680 = 80 \mu\text{L}$

$$S_{\text{particle}} = 4 \times \pi \times R^2$$

$$= 4 \times 3.14 \times (1.881/2/1000)^2 = 1.11 \times 10^7 \text{nm}^2$$

$$V_{\text{particle}} = 4/3 \times \pi \times R^3$$

$$= 4/3 \times 3.14 \times (1.881/2/10000)^3 = 3.48 \times 10^{-12} \text{cm}^3$$

$$m_{\text{particle}} = \rho \times V_{\text{particle}}$$

$$= 1.04 \times (3.48 \times 10^{-12}) = 3.62 \times 10^{-12} \text{g}$$

$$N_{\text{particle}} = m_{\text{sample}} \times \text{solids content \%} / m_{\text{particle}}$$

$$= 2.0237 \times 1.14 \% / (3.62 \times 10^{-12}) = 6.37 \times 10^9$$

$$N_{\text{MAA/particle}} = N_{\text{MAA-meas}} / N_{\text{particle}}$$

$$= (1.20 \times 10^{18}) / (6.37 \times 10^9) = 1.89 \times 10^8$$

$$N_{\text{MAA/nm2}} = N_{\text{MAA/particle}} / S_{\text{particle}}$$

$$= (1.89 \times 10^8) / (1.11 \times 10^7) = 17$$

### Table of parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N_{\text{MAA-meas}}$</td>
<td>Number of $\text{–COOH}$ groups titrated</td>
</tr>
<tr>
<td>$R$ (µm)</td>
<td>Mean particle radius</td>
</tr>
<tr>
<td>$S_{\text{particle}}$ (nm²)</td>
<td>Mean surface area per particle</td>
</tr>
<tr>
<td>$V_{\text{particle}}$ (cm³)</td>
<td>Mean particle volume</td>
</tr>
<tr>
<td>$\rho$ (= 1.04 g/cm³)</td>
<td>Density of PS particles</td>
</tr>
<tr>
<td>$N_A$</td>
<td>Avogadro number</td>
</tr>
<tr>
<td>$m_{\text{particle}}$ (g)</td>
<td>Mean particle mass</td>
</tr>
<tr>
<td>$m_{\text{sample}}$ (g)</td>
<td>Mass of sample to be titrate</td>
</tr>
<tr>
<td>$N_{\text{particle}}$</td>
<td>Number of PS particles in the titrated sample</td>
</tr>
<tr>
<td>$N_{\text{MAA/particle}}$</td>
<td>Number of surface $\text{–COOH}$ groups per particle</td>
</tr>
<tr>
<td>$N_{\text{MAA/nm2}}$</td>
<td>Number of surface $\text{–COOH}$ groups per nm²</td>
</tr>
</tbody>
</table>
For this sample with 2 wt % MAA in the feed, 12 % of the MAA groups (likely as a PMAA-rich copolymer with styrene) was found in the supernatant and not incorporated into the particles. By titration of the washed particles, I determined that 37 % of the MAA was located on the particle surface. Based on the assumption that the MAA was distributed either inside the particles, on the particle surface, or remained in the serum, the fraction of MAA buried in the particles was obtained by subtraction. In this way, I calculated that half of the polymerized MAA (51 %) became buried inside the particles. For sample MAA-4, a similar distribution of MAA was found: 11% of the MAA remained in the serum; 37 % of the MAA groups were located on the particle surface, and 52 % of the MAA was buried inside the particles.

Figure 2-3 Potentiometric and conductometric titration of washed samples from MAA-2 synthesized by 2-DisP in the presence of MAA (2.0 wt % / styrene). The red box represents the starting point and then end point of the titration.

From the high conversion of styrene monomer, the uniform size of the particles obtained, the density of polystyrene (1.04 g/mL) and the number of titratable acid groups at the particle surface, I calculated the mean number of surface –COOH groups per particle and the number of –COOH groups per nm² of particle surface area. The latter calculation assumes a smooth and uniform particle surface. These values are collected in Table 2-4.

These results can be compared to those reported by Song et al. [19] who were the first to examine the copolymerization of AA with styrene by two-stage dispersion polymerization.
Both Ahmed Abdelrahman and I modeled our reaction conditions on their report. In my experiments, except for the use of MAA instead of AA, the two sets of reaction conditions are essentially the same. Song et al. reported that 2-DisP worked well with 2 wt % AA under these conditions, but to carry out the reaction in the presence of 4 or 6 wt % AA based upon styrene, substantial changes were required in the reaction. For 2 wt % AA, they reported that all of the AA were incorporated into the particles (no significant titratable –COOH groups in the serum), with 11% of the carboxylic acid groups on the surface and 89 % buried inside. They calculated a mean value of $4.2 \times 10^7$ acid groups per particle, corresponding to $6 – \text{COOH}$ groups per nm$^2$.

**Table 2-4** Surface properties of carboxyl functional P(S-MAA) particles prepared with different amounts of MAA in the second stage

<table>
<thead>
<tr>
<th></th>
<th>MAA-2</th>
<th>MAA-4</th>
<th>MAA-2-Tb(0.2)</th>
<th>MAA-4-Tb(0.3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>particle diameter (µm)</td>
<td>1.9</td>
<td>2.2</td>
<td>1.9</td>
<td>2.0</td>
</tr>
<tr>
<td>MAA in the feed (wt % / sty)$^a$</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>TbCl$_3 \cdot 6\text{H}_2\text{O}$ in the feed (wt % / sty)</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>surface -COOH / nm$^2$</td>
<td>17</td>
<td>38</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>surface -COOH / particle</td>
<td>$1.89 \times 10^8$</td>
<td>$5.73 \times 10^8$</td>
<td>$1.32 \times 10^8$</td>
<td>$2.65 \times 10^8$</td>
</tr>
</tbody>
</table>

$^a$ based on the total styrene in the reaction

In the results reported here, I found several interesting differences in the particles obtained by replacing AA with MAA. First, I observed that the reaction remained well controlled with both 2 and 4 wt % MAA. Second, I found a significantly larger number of -COOH groups on the particle surface. For 2 wt %, in spite of the smaller number of moles of MAA compared to AA, and the presence of substantial amounts of PMAA-rich polymer in the serum, I obtained PS particles with 17 –COOH groups per nm$^2$ on the surface compared to 6 / nm$^2$ for AA. For 4 wt % MAA, this value increased to 38 / nm$^2$. These are large values.

These numbers of –COOH groups seem to be higher than those can be loaded on the surface area of the PS particles. A paper by Goodwin *et al.* [23] cites a value for the area occupied (“parking area”) by a carboxylic acid group at the surface of a latex particle of 25 Å$^2$. Thus there should be a maximum of 4 -COOH per nm$^2$ of surface area. Presuming a smooth particle surface, as appears to be the case here, as inferred from the SEM images of my particles, these beads have higher density of –COOH groups on the surface. A likely explanation for this result is the presence of water-swollen PMAA-rich polymer chains.
grafted to the surface of the particles. This conclusion is consistent with our finding of PMAA-rich polymer in the serum phase.

2.3.2 Particle synthesis in the presence of Lanthanide salts

To synthesize Ln-encoded PS-PMAA particles, I introduced lanthanide chloride salts in the second stage of the dispersion polymerization along with the MAA. This synthesis was modeled on reactions reported by Abdelrahman et al., [13] in which AA was used as the co-monomer to bind Ln ions in the particle interior. I first consider two examples in which 0.2 wt % TbCl₃·6H₂O (based on styrene) was added along with 2 wt % MAA for sample MAA-2-Tb(0.2) and 0.3 wt % TbCl₃·6H₂O was added along with 4 wt % MAA for sample MAA-4-Tb(0.3) (see recipes in Table 2-1). Colloidally stable dispersions were obtained with no coagulum for both samples. Figure 2-4 show SEM images of the as-prepared MAA-2-Tb(0.2) particles. These unwashed particles had a similar size and size distribution to those prepared without Tb salt ($D_n = 1.9 \mu m$ and $CV_d = 5.6 \%$). SEM images with low and high magnification and a size distribution histogram for the washed sample are shown in Figure 2-5A, 2-5B and 2-5C, respectively. Most of the small particles were washed away, and $CV_d$ was reduced to 2.9 \%, with the $D_n$ of 1.9 $\mu m$, the same as that of the unwashed particles. Figure 2-5D and 2-5E show SEM images with low and high magnification for washed MAA-4-Tb(0.3) particles, and the histogram of diameters is presented in Figure 2-5F. These particles look monodisperse, with $D_n$ of 2.0 $\mu m$ and $CV_d$ of 2.8 \%.
Figure 2-4 SEM images of as-prepared Tb-containing polystyrene microspheres (unwashed) synthesized by 2-stage dispersion polymerization with PVP as the stabilizer in the presence of different amounts of MAA. Sample MAA-2-Tb(0.2) has 2 wt % MAA and 0.2 wt % Tb salts relative to styrene: $D_n = 1.9 \ \mu m$, $CV_d = 5.6 \ %$ (A and B); Sample MAA-4-Tb(0.3) has 4 wt % MAA and 0.3 wt % Tb salts relative to styrene: $D_n = 2.0 \ \mu m$, $CV_d = 3.0 \ %$ (C and D). Some small and big particles are highlighted by red dash circles.
Figure 2-5 SEM images and diameter distribution histogram of Ln-encoded polystyrene microspheres synthesized by 2-stage dispersion polymerization with PVP as the stabilizer in the presence of different amounts of MAA. Sample MAA-2-Tb(0.2) has 2 wt % MAA and 0.2 wt % Tb salts relative to styrene: $D_n = 1.9 \mu m$ $CV_d = 5.6 \% (2.9 \%)$ (A, B, C); Sample MAA-4-Tb(0.3) has 4 wt % MAA and 0.3 wt % Tb salts relative to styrene: $D_n = 2.0 \mu m$ $CV_d = 3.0 \% (2.8 \%)$ (D, E, F). A, D images are low magnification SEM images; B, E images are high magnification SEM images and C, F plots are histograms of the diameter distribution of the washed particles.

In Table 2-4, I report titration results for these two samples. I see that adding small amounts of TbCl$_3$·6H$_2$O to the reaction led to somewhat smaller numbers of acid groups on the particle surface. For sample MAA-2-Tb(0.2), I found an average of 12 –COOH/nm$^2$ compared to 17 for the particles (MAA-2) synthesized in the absence of TbCl$_3$·6H$_2$O. For
sample MAA-4-Tb(0.3), I found that the presence of the Ln salt decreased the mean number of –COOH / nm² from 38 to 21.

These results are similar to those reported by Abdelrahman et al., [13] for dispersion polymerization with AA as the co-monomer and TmCl₃ as the Ln salt. For the particles synthesized with 2 wt. % AA plus 0.2 wt. % TmCl₃ ($D_n = 2.1 \, \mu m$), they found $1.9 \times 10^8$ surface –COOH groups per particle and 15 –COOH per nm². I found somewhat smaller numbers per particle ($1.3 \times 10^8$ / particle) with 2 % MAA as the co-monomer in this work. To carry out the reaction with 4 wt % AA and maintain control over particle size, they added 2 wt % AA in the second stage and an additional 2 wt % AA in a third stage. For this 3-DisP sample with 4 wt % AA and 0.2 wt % Tm ($D_n = 2.2 \, \mu m$), they found that the number of titratable surface –COOH groups per particle doubled to $4.3 \times 10^8$ and the number of –COOH per nm² increased from 15 to 28, which is also higher than the value ($2.7 \times 10^8$ / particle and 21/nm²) for particles with 4 % MAA. The slightly larger particle size of particles prepared in presence of AA (2.1 µm) compared to that of particles prepared in presence of MAA (1.9 µm) probably contributed to that larger amount of –COOH groups on the particle surface. All these results demonstrate that the number of –COOH groups per particle doubled when the amount of co-monomer added in the reaction doubled from 2 % to 4 %.

2.3.3 Efficiency of metal ion incorporation into the particles

To determine the efficiency of Ln ion incorporation into the PS particles, I separated the particles from the serum and analyzed the serum for its Ln ion content. Basically, 1.00 mL of the “as-prepared” particle dispersion was sedimented at 14,000 rpm for 30 minutes. The supernatant was collected, diluted with 3 % high purity HNO₃ solution, filtered, and then analyzed by ICP-MS. The instrument was calibrated with standard lanthanide solutions and corrected with a 3 % HNO₃ solution as a background signal. I begin our discussion with results for Eu as a representative example. These data are presented in Table 2-5. Table 2-6 presents the full set of analysis results for three samples each containing five different Ln metals (La, Eu, Tb, Ho and Tm), which were prepared in the presence of 2 wt % MAA. One should keep in mind two factors. First, this analysis assumes that all the Ln ions not detected in the filtered serum were incorporated into the PS particles. Second, that the Ln ions incorporated into the particles were distributed between the large particles of interest for future immunoassays and the small particles removed by sedimentation and redispersion as
well as by the filtration step. The Ln ion content of the larger particles will be addressed below when I describe their analysis by mass cytometry.

In sample MAA-2-II, 0.99 µmol EuCl₃ (0.005 wt % Eu / styrene) was added to the reaction mixture and 0.020 µmol Eu ions were detected in the supernatant. In other words, ca. 98 % of the Eu ions are assumed to be incorporated into the polystyrene particles. For sample MAA-2-IV 4.01 µmol EuCl₃ (0.02 wt % Eu / styrene) was introduced into the reaction, and 0.050 µmol Eu ions was detected in the supernatant. More than 98.8 % Eu ions were incorporated into the particles. However, for sample MAA-2-VII, in which a much larger amount of EuCl₃ (30.66 µmol, 0.2 wt % based on styrene) was introduced into the reaction, 7.96 µmol Eu ions were found in the supernatant. This result indicates that ca. 26% of the Eu ions in the reaction feed remained in the supernatant, and was not incorporated into the particles.

Table 2-5 Incorporation efficiency of Eu for particles prepared with different amount of Ln in the presence of 2 % MAA (wt. % / styrene)

<table>
<thead>
<tr>
<th></th>
<th>[Eu] calc a</th>
<th>[Eu] meas b</th>
<th>Incorp (%) c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ppm</td>
<td>µmol</td>
<td>ppm</td>
</tr>
<tr>
<td>MAA-2-II</td>
<td>3.63</td>
<td>0.99</td>
<td>0.06</td>
</tr>
<tr>
<td>MAA-2-IV</td>
<td>14.74</td>
<td>4.01</td>
<td>0.17</td>
</tr>
<tr>
<td>MAA-2-VII</td>
<td>112.7</td>
<td>30.7</td>
<td>29.3</td>
</tr>
</tbody>
</table>

a Total Eu concentration according to the recipe
b Eu concentration in the supernatant measured by ICP-MS
c Percentage of Eu added in the recipe incorporated into the particles (100 – [Eu] meas b / [Eu] calc a × 100)

Similar results were obtained for the other metal ions (Tm, Ho, La and Tb) studied in this experiment (see Table 2-6). For MAA-2-II (0.005 wt % Ln / styrene) and MAA-2-IV (0.02 wt % Ln / styrene), the incorporation efficiencies of Tm, Ho and La ions were high; with more than 96 % of Ln ions incorporated into the particles. For MAA-2-VII (0.2 wt % LnCl₃), the incorporation efficiency dropped significantly; especially for La, only 47 % of the La ions were incorporated into the particles.
Table 2-6 Incorporation efficiency of Ln for particles prepared with different amount of Ln in the presence of 2 % co-monomer (wt. % / styrene)

<table>
<thead>
<tr>
<th></th>
<th>La</th>
<th>Eu</th>
<th>Ho</th>
<th>Tm</th>
<th>Tb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µmol)</td>
<td>(%)</td>
<td>(µmol)</td>
<td>(%)</td>
<td>(µmol)</td>
</tr>
<tr>
<td>MAA-2-II</td>
<td>1.03</td>
<td>96.8</td>
<td>0.99</td>
<td>98.3</td>
<td>0.98</td>
</tr>
<tr>
<td>MAA-2-IV</td>
<td>4.17</td>
<td>96.3</td>
<td>4.01</td>
<td>98.9</td>
<td>3.97</td>
</tr>
<tr>
<td>MAA-2-VII</td>
<td>31.9</td>
<td>57.8</td>
<td>30.7</td>
<td>74.0</td>
<td>30.3</td>
</tr>
<tr>
<td>MAA-4-Tb(0.3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MAA-2-Tb(0.3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MAA-2-Tb(0.4)</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
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<td>MAA-2-Tb(0.5)</td>
<td>-</td>
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<td>-</td>
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<td>MAA-2-Tb(0.6)</td>
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<td>MAA-2-Tb(0.7)</td>
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<td>-</td>
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<td>-</td>
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<tr>
<td>AA-2-I</td>
<td>0.88</td>
<td>92.2</td>
<td>0.85</td>
<td>98.9</td>
<td>0.82</td>
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<tr>
<td>AA-2-II</td>
<td>3.54</td>
<td>97.93</td>
<td>3.40</td>
<td>98.8</td>
<td>3.29</td>
</tr>
<tr>
<td>AA-2-III</td>
<td>35.36</td>
<td>58.39</td>
<td>34.0</td>
<td>70.4</td>
<td>32.9</td>
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</table>
These results can be summarized as follows: I found high incorporation efficiency (>95%) of Ln ions into the particles synthesized in the presence of 2 wt % MAA as long as the amount of LnCl₃ salts in the reaction mixture was sufficiently small. Useful insights are provided by comparing results for the three samples containing 0.2 wt % Tb. For MAA-2-II and MAA-2-IV, the incorporation efficiency of Tb was high (98%). On the other hand, for sample MAA-2-VII, the Tb incorporation efficiency dropped significantly (to ca. 80%). For this sample, the total LnCl₃ amount was much higher, 1 wt %. I conclude that the efficiency of LnCl₃ incorporation into the particles is high under these reaction conditions, except for the case where the total Ln salt amount approaches 1 wt % based upon styrene. Table 2-6 also suggests that when the total Ln salt concentration reaches a high level, there is a lower efficiency of La incorporation compared to other Ln ions of higher atomic number.

As another test, I examine the results for sample MAA-4-Tb(0.3), in which only one Ln metal (Tb) was employed in the reaction, here with the relatively high concentration of 0.3 wt % / styrene. As shown in Table 2-6, 99.5 % Tb ions were incorporated into these particles. Although the Tb concentration in the feed was higher (48.5 µmol) than that of sample MAA-2-VII (33.5 µmol), sample MAA-4-Tb(0.3) maintained a high Tb incorporation efficiency instead of the decrease seen for sample MAA-2-VII. I explain this result by noting that the total Ln concentration in sample MAA-4-Tb(0.3) was 48.5 µmol, much lower than that of MAA-2-VII (155 µmol). This result supports the idea that the most important factor in determining incorporation efficiency is the total Ln concentration in the reaction.

I also examined the efficiency of Ln ion incorporation for P(S-AA) particles prepared with 2 wt % acrylic acid. These particles were prepared in the presence of five different Ln ions. For this set of particles, the TbCl₃ concentration in the feed was fixed at 0.1 wt %. The data in Table 2-6 shows that the incorporation efficiency of these different Ln elements was over 90% when the total Ln concentration was 0.12 wt % and 0.18 wt % (samples AA-2-I and AA-2-II, respectively). When the total Ln content of the reaction mixture was 0.9 wt % (sample AA-2-III), there was a substantial drop in the incorporation efficiency (ca. 58 - 87 %) that was more pronounced for La than for the other Ln ions.

As one more test of the efficiency of ion incorporation during the dispersion polymerization, I carried out a series of reactions with TbCl₃ as the only Ln, with various levels ranging from 0.3 to 0.7 wt % / styrene. All these particles were prepared in the presence of 2 wt. % MAA, based on styrene. The results were not entirely consistent with
those described above. For sample MAA-2-Tb(0.3), prepared with 50.2 µmol TbCl₃ in the reaction, I found 96 % Tb incorporated into the particles. However, for sample MAA-2-Tb(0.7), prepared with 117 µmol TbCl₃ in the reaction, only 30.6 % Tb ions were incorporated into the particles. Although I see that the incorporation efficiency decreased with the increasing Ln feed concentration, our main conclusion is that one has to monitor carefully the supernatant from these two-stage dispersion polymerization reactions to have confidence in the degree of ion incorporation into the particles.

2.3.4 Metal ions per particle determined by mass cytometry

The metal ion content per particle and their distributions were determined by mass cytometry. [12, 21] The particle sample dispersion was washed by three cycles of centrifugation and resuspension in water. The resultant slurry (ca. 10⁶ beads/mL) was nebulized into the mass cytometer, directly delivering particles into the inductively coupled plasma torch. The ion stream is automatically introduced into the time-of-flight (TOF) mass analyzer. The transient signals corresponding to each bead ionization event were recorded by the detector and stored.

I begin by showing a screen capture of the raw data collected by mass cytometry for a sample of MAA-2-IV (prepared in the presence of La, Tb, Tm, Ho and Eu salts) showing three particles containing the five Ln elements La, Eu (2 isotopes), Tm, Ho, and Tb (Figure 2-6). The instrument captures sequential mass spectra taken at ca. 13 μs intervals. The y-axis reports the start time of acquisition of each mass spectrum. An encoded particle is registered as a signal in 10-30 consecutive spectra over 0.1-0.4 ms and is characterized by simultaneous transient signals for all of the encoding elements or isotopes. Each horizontal grey-blue bar highlights a series of signals for one particle event. Each particle event has 5 types of ions. Approximately 1000 microspheres per second were analyzed in this way. The x-axis represents the pre-selected isotopes in order of increasing m/Z. As a measure of signal background, I note that no Lu and Pr were detected, consistent with not having been added in the synthesis of these particles. The black spots for Tb are darker than for the other elements. This stronger intensity agrees well with the fact that I added ten times more Tb (0.2 %) in the synthesis than other elements (0.02 % for each).
Figure 2-6 A mass cytometry screen capture for sample MAA-2-IV. The x-axis represents pre-selected isotopes in order of increasing m/Z. The y-axis (inverted) reports successive TOF mass spectra. Each horizontal bar highlights one particle event.

One way to examine the data for a large number of measurements on a single type of particle is in terms of two dimensional projections (“dot-dot” or bivariate plots) that reveal the relationship between two variables such as element or isotopic concentrations. [24] Figure 2-7 shows four bivariate plots from the multidimensional data set obtained from a mass cytometry measurement for sample MAA-2-IV. The bottom x-axis and the left-hand y-axis represent the original counts (mean intensity, \(I\)) of any two isotopes of interest measured by mass cytometry directly. Each point on the plot shows the x and y isotopic-intensities for a single particle. The color of the dots in this diagram reflect the magnitude of the number of the particles detected with that specific Ln loading, with blue the lowest and red the highest. The top x-axis and the right-hand y-axis represent the calculated numbers of Ln ions per particle. The relationship between the mean intensity measured by the TOF detector and the number of metal atoms per particle was determined by the instrument calibration with a standard (0.5 ppb) solution containing a mixture of lanthanides. Basically, I calculated the transmission efficiency of Ln using the data from the standard solution. Then the mean intensity of Ln can be converted to the number of ions per particle using corresponding Ln transmission efficiency. Here, I show a sample calculation. The number of counts for Tm for the standard solution on the same measurement day was 154000. The number of counts that I obtained for Tm for sample SL030 was 258. Thus, the calculated transmission efficiency of Tm is \(8.6 \times 10^{-5}\) (= counts for standard solution \(\times (10^{12} / \text{concentration of standard solution}) \times (1 / N_A) / \times M_{\text{Tb}} = 154000 / 0.5 / (6.02 \times 10^{11}) / \times 169\)). And the number of Tm ions per particle is \(3.0 \times 10^6\) (= counts for Tm / transmission efficiency of Tm = 258 / \((8.6 \times 10^{-5}))\).
Figure 2-7A presents a logarithmic Ho/Tm bivariate plot. Although the measured intensities of Ho and Tm content vary from ca. 10 to 1000 counts per particle, we believe that the relatively small number of dots that appear as low-intensity signals may be due to occasional small particles in the sample, to particles that fragment during nebulization and injection into the plasma torch, and to low concentrations of free ions in the aqueous solution. The vast majority (around 89 %) of the particles exhibit a very tight distribution of intensities ca. 258 counts for Tm and 228 counts for Ho. Instrument calibration with the standard (0.5 ppb) solution led to the conclusion that the particles contain around \(3.0 \times 10^6\) Tm ions and \(2.8 \times 10^6\) Ho ions. This behavior in the bivariate plot reflects the characteristic feature of particles prepared by 2-DisP, in presence of MAA, which is their relatively low bead-to-bead variability of the metal (Ln) content, as well as the similar incorporation efficiency for different Ln ions into these beads.

Figure 2-7B presents a logarithmic Tb/Tm bivariate plot. The content of Tb is around \(5.3 \times 10^7\) ions per particle; much higher than that for Tm \((3.0 \times 10^6)\). This intensity difference reflects the higher Tb content of the beads, related to the higher concentration of TbCl\(_3\) (0.2 wt %) compared to TmCl\(_3\) (0.02 wt %) in the particle synthesis.

An example of the mass dependency of the instrument is seen in Figure 2-7C, where I present a logarithmic bivariate plot for La/Tm with the measured intensity values on the bottom x-axes and left-hand y-axes. Although the feed concentrations of LaCl\(_3\) and TmCl\(_3\) in the synthesis were same (0.02 wt. % / styrene), and both ions had high incorporation efficiencies (96.3 % for La and 98.3 % for Tm), the mass cytometry response was different for both elements. The intensity for La is around 90 counts whereas that for Tm is around 258 counts (Tm on the bottom x-axes and La on the left-hand y-axes). The main reason is that the mass analyzer used here has a significantly lower sensitivity for elements at the lower end of the mass range, and the detection efficiency is lower for \(^{139}\)La than for \(^{169}\)Tm. Using the standard solution, the number of Ln ions per particles was calculated to be equal to \(2.6 \times 10^6\) La ions and \(3.0 \times 10^6\) Tm ions per particle.

Figure 2-7D presents a dot-dot plot for two isotopes of Eu \((^{151}\)Eu and \(^{153}\)Eu) of approximately equal natural abundance. Because every Eu-labeled particle should contain almost equal numbers of these two isotopes, this type of plot reveals the spread of Eu loading from particle to particle.
Figure 2-7 Dot-dot plots of mass cytometry results for sample MAA-2-IV synthesized by 2-DisP in presence of multiple Ln salts (0.02 % La, Eu, Ho, Tm and 0.2 % Tb) and MAA: 2.0 wt%/styrene. (A) $^{165}$Ho/$^{169}$Tm bivariate plot; (B) $^{159}$Tb/$^{169}$Tm bivariate plot; (C) $^{139}$La/$^{169}$Tm bivariate plot; (D) $^{151}$Eu/$^{153}$Eu bivariate plot.

Quantitative information about the particle population can be drawn from a histogram representation of the frequency distribution of signal intensities (or number of ions) for individual particles. In Figure 2-8, the population distribution is presented for the Tb ion signal collected for 2 min (ca. $4 \times 10^5$ particles). The x-axis of this plot is the number of Tb ions per particle. Figure 2-8A and 2-8B show the histogram of Tb signal distribution of sample MAA-2-Tb(0.2) determined by mass cytometry. Figure 2-8A is the ungated Tb signal intensity obtained from FlowJo software based on the original data from mass cytometry. An average of $9.1 \times 10^6$ Tb ions per particle was calculated from the measured ion intensities and the standard calibration solution, with a coefficient of variation of Ln distribution ($CV_{Ln}$) of 34 %. There are small populations on the both sides of the main peak. Choosing the main
part of this raw-data peak, and using the data processing capabilities in the FlowJo software package, a gated peak representing 88.7 % of the particles was obtained with a same mean value ($9.1 \times 10^6$ Tb ions per particle). The gated peak has a much narrower Ln ion distribution ($CV_{Ln} = 15\%$), which I believe is the value characteristic of the particle synthesis.

As mentioned above, I attribute signals at the low end of the intensity distribution peak to particle fragmentation. There may also be a contribution of some small particles that were not removed during the particle purification step and free ions in the continuous media. I also attribute weak signals on the high end to occasional formation of particle dimers (and larger aggregates) during injection into the plasma torch. These signals often appear as a peak with two-times the number of Ln ions per bead. Both of these populations contribute to the apparent broad Ln distribution ($CV_{Ln} = 34\%$) calculated from the ungated data. Although the $CV_{Ln}$ for gated peak was reduced to 15 %, this value was still high compared to the variation in particle diameter and volume. The value of $CV_d$ for the washed sample MAA-2-Tb(0.2) is 2.9 %, which translates to a variation in the particle volume ($CV_V$) of 8.7 %. One anticipates that for a given synthesis, the Ln ion content of particles should scale with particle volume.

Figure 2-8C and 2-8D show the histogram of Tb signal distribution of sample MAA-4-Tb(0.3), with an average of $1.47 \times 10^7$ Tb ions per particle with $CV_{Ln}$ of 36% for the ungated peak and 14% for the gated peak. The gated signal captured 85.5 % of the total events. Again, $CV_{Ln}$ was higher than that $CV_V$ (8.3 %).

This is an important result for two reasons. First, the Tb response from the mass cytometry measurement is proportional to the Tb added in the synthesis. The signal intensity increased from $9.1 \times 10^6$ to $1.47 \times 10^7$ Tb ions per particle, a factor of 1.6, when the amount of Tb added in the synthesis was increased from 0.2 to 0.3 wt. %, based on styrene. Second, using double amount of MAA in the synthesis did not affect the level of metal incorporation. Based upon this result, I focused our attention on syntheses carried out with 2 wt % MAA.
Figure 2-8 Distribution of Tb$^{3+}$ (A) ungated and (B) gated signal intensity measured by mass cytometry for MAA-2-Tb(0.2) microspheres synthesized by 2-DisP in presence of Tb: 0.2 wt%/styrene and MAA: 2.0 wt%/styrene. As indicated in A, the gated signal in B captures 88.7 % of the total events. Distribution of Tb$^{3+}$ (C) ungated and (D) gated signal intensity measured by mass cytometry for MAA-4-Tb(0.3) microspheres synthesized by 2-DisP in presence of Tb: 0.3 wt%/styrene and MAA: 4.0 wt%/styrene. As indicated in C, the gated signal in D captures 85.5 % of the total events.
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<th>Diameter (µm)</th>
<th>CV_d (%)</th>
<th>Ln incorporation efficiency (%) ²</th>
<th>Ln incorporation efficiency (10⁻⁶ Ln atoms/particle, calculated) ³</th>
<th>Ln incorporation efficiency (10⁻⁶ Ln atoms/particle, measured) ⁴</th>
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a. wt. % of LnCl₃·6H₂O based on styrene;
b. From ICP-MS measurements on the filtered reaction supernatant;
c. Calculated from the efficiency of Ln ion incorporation and the measured particle diameters, assuming 100 % styrene conversion;
d. From mass cytometry measurements, using a standard solution containing La, Tb, Tm for instrument calibration;
e. MAA: methacrylic acid. The value after the co-monomer indicates the amount of MAA (wt. % / styrene) added in the synthesis. The Roman numerals indicate that a combination of different Ln salts was used in the reaction. For example, MAA-2-II refers to PS particles with 2 wt % methacrylic acid plus 0.005 wt. % LaCl₃·6H₂O, EuCl₃·6H₂O, HoCl₃·6H₂O, TmCl₃·6H₂O and 0.2 % TbCl₃·6H₂O (all based on styrene) added in the reaction.
To examine our ability to control the level of ion incorporation into PS-PMAA particles loaded with a combination of Ln metals at different levels of concentration, I synthesized a series of samples (MAA-2-I to MAA-2-VII). Each type of particle contains a different combination of 5 Ln metals (La, Eu, Tm, Ho and Tb), see Table 2-7. All these particles have a similar size and size distribution. Figures 2-9 to 2-15 show the SEM images and histograms of size distribution for these seven samples. Using mass cytometry, I determined the mean numbers of Ln ions per particle. These values are compared in Table 2-7 to values calculated from the particle size and the efficiency of Ln ion incorporation determined by ICP-MS analysis of the reaction serum as reported in Table 2-5. This calculation assumes 100% conversion of styrene in the particle synthesis reaction.

**Figure 2-9** SEM images and size distribution histogram of as-prepared Ln-containing polystyrene microspheres (unwashed) synthesized by 2-stage dispersion polymerization with PVP as the stabilizer in the presence of 2 wt % MAA, based on styrene. Sample MAA-2-I: $D_n = 2.6 \text{ \mu m}$, $CV_d = 5.9 \% (3.7 \%)$. 

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Figure 2-10 SEM images and size distribution histogram of as-prepared Ln-containing polystyrene microspheres (unwashed) synthesized by 2-stage dispersion polymerization with PVP as the stabilizer in the presence of 2 wt % MAA, based on styrene. Sample MAA-2-II: $D_n = 2.5 \, \mu m$, $CV_d = 3.9 \% \; (2.6 \%)$. 
Figure 2-11 SEM images and size distribution histogram of as-prepared Ln-containing polystyrene microspheres (unwashed) synthesized by 2-stage dispersion polymerization with PVP as the stabilizer in the presence of 2 wt % MAA, based on styrene. Sample MAA-2-III: $D_n = 3.0 \, \mu m$, $CV_d = 4.9 \%$ (2.6 \%).
Figure 2-12 SEM images and size distribution histogram of as-prepared Ln-containing polystyrene microspheres (unwashed) synthesized by 2-stage dispersion polymerization with PVP as the stabilizer in the presence of 2 wt % MAA, based on styrene. Sample MAA-2-IV: $D_n = 2.6 \, \mu m$, $CV_d = 6.9 \%$ (2.6 \%).
Figure 2-13 SEM images and size distribution histogram of as-prepared Ln-containing polystyrene microspheres (unwashed) synthesized by 2-stage dispersion polymerization with PVP as the stabilizer in the presence of 2 wt % MAA, based on styrene. Sample MAA-2-V: $D_n = 2.4 \, \mu m$, $CV_d = 2.0 \% \,(1.8 \%)$. 
Figure 2-14 SEM images and size distribution histogram of as-prepared Ln-containing polystyrene microspheres (unwashed) synthesized by 2-stage dispersion polymerization with PVP as the stabilizer in the presence of 2 wt % MAA, based on styrene. Sample MAA-2-VI: $D_n = 2.5 \, \mu m$, $CV_d = 2.3 \%$ (2.1 \%).
In Table 2-7, I note that for sample MAA-2-II, the $CV_{\text{Ln}}$ values are large (30 to 50%) for La, Eu, Ho and Tm, but narrow (14%) for Tb. For samples MAA-2-IV and MAA-2-VII, I also find smaller $CV_{\text{Ln}}$ values associated with higher Ln content. For example, in sample MAA-2-VII, the $CV_{\text{Ln}}$ values are smaller than in the other samples for ions present in larger numbers. Here, $CV_{\text{Tb}}$ (23%) is somewhat larger than for the other two samples. Although the TbCl$_3$ concentration in the reaction feed was constant (0.2 wt % based on styrene) for all three reactions, for sample MAA-2-VII, the measured number of Tb/particle ($8.5 \times 10^6$) was smaller.

Another important point raised by the data in Table 2-7 is the difference between calculated the number of ions per particle and the experimental data from the mass cytometry. The calculated values are based on the Ln incorporation efficiency measured for
the filtered reaction serum by ICP-MS. In most cases, the calculated values are larger than the experimental values. One possible explanation is that this difference is due to Ln ions incorporated into the smaller PS particles separated from the sample before analysis by mass cytometry. There are some inconsistencies with this conclusion. For sample MAA-2-VII, the measured value for Tm (2.8×10⁷ ions/particle) is essentially identical with calculated value (2.7×10⁷ ions/particle). There is one result in which the value measured by the mass cytometry is actually larger than the calculated value: for sample MAA-2-IV, I calculated 3.0×10⁷ Tb ions/particle, but the mass cytometry measurement yielded 5.3×10⁷ Tb ions/particle. At this point I have no explanation for these differences.

Figure 2-16B shows the relation between the mass cytometry response and the corresponding amount of Tm added during the particle synthesis. The x-axis represents the number of Tm ions per particle determined by mass cytometry after correction. The left-hand y-axis represents the Tm concentration added in the synthesis (wt % based on styrene). The right-hand y-axis represents the mean diameter of the particles. With the increasing amounts of Tm added in the synthesis, from 0.002 to 0.2 wt %, the mean value determined by mass cytometry increased linearly from 1.9 × 10⁵ to 2.7 × 10⁷ Tm ions per particle, with a R² of 0.999. In the top half of this figure (Figure 2-16A), I artificially superimpose the gated peaks taken from Figure 2-17 on this x-axis, positioned to correspond to the particles in Figure 2-16B. The left-hand peak (sample MAA-2-IV) had an average of 3.0 × 10⁶ Tm ions per particle, whereas the right-hand peak (sample MAA-2-VII with a ten-fold higher TmCl₃ content in the reaction feed) had an average of 2.7 x 10⁷ Tm ions per particle. Similar results were obtained for the other Ln metals used in this experiment. Results for Ho are presented in Figure 2-18 as an example.
Figure 2-16 The Relation between the mass cytometry response (on the x-axis) and the corresponding amount of Tm added during the particle synthesis (feed on the left-hand y-axis). The blue horizontal curve indicates the particle diameter. The straight line through the data points has the slope of $8.51 \times 10^{-5}$ ($R^2 = 0.999$). The right-hand y-axis represents the mean diameter of the particles. The upper plot is artificially constructed by transferring the distribution vs log (signal intensity) plots obtained by FlowJo to correspond to the mean number of Tb ions per particle shown in B for two samples (MAA-2-IV and MAA-2-VII) highlighted by the blue dashed circles.
Figure 2-17 Distribution of Tm$^{3+}$ (A) ungated and (B) gated signal intensity measured by mass cytometry for sample MAA-2-IV (Tm: 0.02 wt%/styrene, left blue peak) and MAA-2-VII (Tm: 0.2 wt%/styrene, right red peak) microspheres synthesized by 2-DisP in the presence of MAA (2.0 wt%/styrene). As indicated in A, the gated signal in B captures more than 88% of the total events.
Figure 2-18 (A) The relation between the number of Ho ions per particle (on the $x$-axis) and the corresponding amount of Ho added during the particle synthesis (feed on the left-hand $y$-axis). A linear fit to the data gave a slope of $7.38 \times 10^{-5}$ ($R^2 = 0.993$). The right-hand $y$-axis represents the mean diameter of the particles. (B) The relation between the number of Ho ions per $\mu$m$^3$ (on the $x$-axis) and the corresponding amount of Ho added during the particle synthesis (feed on the left-hand $y$-axis). This is the same set of particles shown in Figure 2-16B and Figure 2-18 but different elements.

Another feature of the data in Figure 2-16B merits a comment. One of the curious aspects of dispersion polymerization of styrene in ethanol in the presence of PVP is that the particle size is difficult to control. One can always obtain a narrow size distribution but there
are often run-to-run variations in particle diameter.[18, 25] For 6 of the 7 samples presented in Figures 2-16B, 2-18 and 2-19, the diameters are similar, but one sample had a significantly larger diameter. This type of variability has been attributed to the sensitivity of the nucleation step to small changes in reaction conditions that are normally beyond the experimenter’s ability to control. Where there are large changes in particle volume in otherwise identical syntheses, one expects the Ln ion content to scale with particle volume. In Figure 2-19, I replot the data in Figure 2-16B against the Tm content per \( \mu \text{m}^3 \) of particle volume and find a similar linear relationship.

![Graph showing the relation between Tm feed (wt %) and Tm content per \( \mu \text{m}^3 \)](image)

**Figure 2-19** The relation between the number of Tm ions per \( \mu \text{m}^3 \) (on the x-axis) and the corresponding amount of Tm added during the particle synthesis (feed on the left-hand y-axis). This is the same set of particles shown in Figure 2-16B and Figure 2-18. The blue curve indicates the particle diameter. A linear fit to the data gave a slope of \( 7.38 \times 10^{-5} \) (\( R^2 = 0.993 \)). The right-hand y-axis represents the mean diameter of the particles.

### 2.4 Summary

In this chapter, I described the synthesis and characterization of a series of Ln-encoded poly(styrene-co-methacrylic acid) particles prepared by two-stage dispersion polymerization and compared them to analogous particles that copolymerized with acrylic acid as the co-
monomer. These P(S-MAA) particles reported here, with diameters on the order of 2 µm, with narrow size distribution, were designed to have applications in bead-based bioassays using mass cytometry for sample detection.

Different amounts of MAA (2 %, 4 % and 6 wt % / styrene) were added during the synthesis to study the effect of MAA on the particle size and number of carboxylic group. I found monodisperse particles could be obtained in the presence of 2 and 4 wt % MAA. Larger amount of MAA in the reaction led to loss of control of the particle size distribution. The distributions of carboxylic group were determined by potential and conductivity titration experiments. I found that half of MAA added in the reaction formed –COOH groups that were buried inside the particles, 38 % of MAA acid groups were located on the particle surface, and 12 % of the MAA remained in the supernatant and not incorporated into the particles. Titration results also show that the number of carboxylic acid group on the surface of the particles doubled when the amount of co-monomer added in the reaction doubled from 2 % to 4 %.

The introduction of Ln ions into the particles was achieved by adding LnCl3 salts along with MAA at the 2nd stage of dispersion polymerization. The incorporation efficiency of Ln salts was determined by analyzing the supernatant of particle dispersion by ICP-MS. I found high incorporation efficiency (> 95 %) of Ln ions into the particles synthesized in the presence of 2 wt % MAA as long as the total amount of LnCl3 salts in the reaction mixture was sufficiently small. The most important factor in determining incorporation efficiency is the total Ln concentration in the reaction instead of the individual Ln concentration. The incorporation efficiency of Ln ions decreased with the increasing amounts of Ln salts added in the reaction. I also studied the Ln incorporation efficiency for P(S-AA) particles prepared with 2 % AA by Ahmed Abdelrahman and found similar results.

The Ln ion content per particle and their distributions were determined by mass cytometry analysis. This instrument design allows for the introduction of individual particles and their analysis by ICP-MS detection. Ln signals were measured for particles loaded with individual Ln ions, as well as with the mixtures of Ln complexes. The Ln content distributions ($CV_{Ln}$) were quite broad before the gating, however, the value of $CV_{Ln}$ decreased a lot after the gating. The average Ln content per particle by mass cytometry was in excellent agreement with the theoretically predicted loading value. I would be able to control the Ln
content of the particles by tailoring the particle size and the adjusting the \( \text{Ln} \) feed concentration.

### 2.5 References


Chapter 3
The Release and Extraction of Lanthanide Ions from Metal-Encoded Poly(Styrene-co-Methacrylic Acid) Microspheres

3.1 Introduction

For encoded beads, the stability of the particles against degradation is critical for successful applications in bead based assay. For fluorescent particles, one concern is photobleaching. In this sense, quantum dots are superior to fluorescent dyes because of their higher photostability. For element-encoded beads to be detected by mass cytometry, the main concern is the stability during storage and during biofunctionalization against leakage of ions from the beads into the continuous medium. Thus I am interested in the stability of the Ln-encoded particles against the ion leakage upon storage in different media.

Our group reported the synthesis of Ln-encoded polystyrene particles by two stage dispersion polymerization (2-DisP), using acrylic acid (AA) as the carboxylic acid containing co-monomer. [1] We showed that polystyrene-co-acrylic acid (P(S-AA)) particles containing 2 wt % acrylic acid could be obtained with a very narrow diameter distribution, with control over Ln ion labeling levels between $10^5$ to nearly $10^9$ Ln ions per bead. In that synthesis, the AA and LnCl$_3$ salts were added after about 10 % of styrene conversion. At the highest labeling levels, we encountered signal saturation from the time of flight (TOF) detector, indicating that with the current mass cytometer instrument, it is possible to have too high content of an individual element or isotope. A curious feature of this synthesis was that the bead-to-bead variation of the Ln ion content was significantly larger ($CV_{Ln} = 40\%$) than the corresponding variation in particle volume ($CV_V = 10\%$). This problem could be overcome if additional AA and a small amount of a cross-linking agent (ethylene glycol dimethacrylate) were added in a third stage after about 60% styrene conversion. This approach reduced the magnitude of $CV_{Ln}$ to about 15%. These particles were very stable against ion leakage to the continuous medium in traditional buffers (sodium carbonate/bicarbonate, ammonium acetate, sodium acetate) over a pH range of 3 to 10, with less than 0.1% ion loss after 3 weeks.

Recently, we reported the synthesis of Ln-encoded polystyrene particles by 2-DisP in ethanol using methacrylic acid (MAA) as the co-monomer, [2] and showed that polystyrene-co-methacrylic acid (P(S-MAA)) particles with a narrow size distribution could be obtained for up to 4 wt % MAA based on styrene. In these reactions, only about 88% of the MAA was
incorporated into the particles. The remaining 12 % of the MAA added in the reaction ended up as ethanol-soluble material that could be quantified by titration after sedimentation of the particles. An interesting feature of this reaction was that the Ln ion incorporation efficiency (>95% in most cases) was higher than that for 2-DisP of styrene/acrylic acid (ca. 85%). These particles were also shown to be stable with respect to storage in normal buffer media 2-(N-morpholino) ethanesulfonic acid (MES, 100 mM, pH 6.5), phosphate buffered saline solution (PBS, 100 mM, pH 7.2), and ammonium acetate (AmAc, 10 mM, pH 9.0), with less than 0.5 % ion leakage after 8 weeks under continuous stirring, as described in Chapter 2.

In this chapter, I report a more systematic study of ion release from both (P(S-AA)) particles and (P(S-MAA)) particles. I compare the behavior in normal buffers with that in buffers containing strong chelators, ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA). One issue I wished to explore was whether the strong chelating molecules could extract Ln ions from these particles.

3.2 Experimental Section

3.2.1 Instrumentation

Traditional inductively coupled plasma mass spectrometry (ICP-MS) measurements were carried out on an Elan 9000 instrument (Perkin-Elmer SCIEX) operating under normal Ar plasma 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 MicroFlow PFA-ST concentric nebulizer (Elemental Scientific, Inc) was used in all instances. All the experiments were performed using an autosampler (Perkin-Elmer AS 93) modified for operation with Eppendorf 1.5 mL tubes. Sample size was fixed at 1 mL. The sample uptake rate was adjusted depending on the particular experiment, typically 100 µL/min. Standards were prepared from 1000 µl/mL PE pure Single-Element Standard solutions (Perkin-Elmer, Shelton, CT) by sequential dilution with high purity HNO₃. High purity HNO₃ was measured as well in each experiment to be used as blank signal. Sample signals deducted the blank signals first and then were normalized to the signals of standard solutions (1 ppb).

3.2.2 Materials

2-(N-morpholino)ethanesulfonic acid (MES, Aldrich), Bis(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane (bistris, Aldrich), diethylenetriaminepentaacetic acid (DTPA, minimum 98 % titration, Aldrich), ethylenediaminetetraacetic acid (EDTA, Aldrich) were
used as received. Deionized water (DIW) was purified through a Milli-Q purification system. The MES buffer solution was prepared by dissolving MES solid in water and adjusting the pH to 6.5 by adding several drops of sodium hydroxide aqueous solution (5 M). The MES/DTPA solution was prepared by adding a mixture of DTPA and MES solid into water and dissolving them with the addition of 5M NaOH solution. The bistris buffer solution was prepared by dissolving bistris solid in water and adjusting the pH to 6.3 by adding several drops of 1 M HCl. Bistris/DTPA solution was prepared by first dissolving DTPA in 5 M NaOH and then mixing this solution with bistris buffer to obtain a solution with pH 6.9. Phosphate buffered saline solution (PBS, 150 mM NaCl, 1.2 mM Ca$^{2+}$, 0.8 mM Mg$^{2+}$, 100 mM sodium phosphate, pH 7.2) was a gift from DVS Sciences. PBS/EDTA solution was prepared by dissolving solid EDTA disodium dihydrate solution in the PBS buffer. The ammonium acetate buffer solution (AmAc, 10 mM, pH 9.0) was prepared by Dr. A. Abdelrahman in our laboratory.[1] High purity HNO$_3$ for ICP-MS analysis was purchased from Seastar Chemical Inc. The polystyrene-co-methacrylic acid (P(S-MAA)) and polystyrene-co-acrylic acid (P(S-AA)) particles described here were synthesized by two-stage dispersion polymerization and are the same samples described in Chapter 2.

3.2.3 Ion release experiments

All the particles examined here were first washed by 6 centrifugation-resuspension cycles using deionized water and then redispersed in different buffer solutions, namely MES (0.1 M, pH 6.5), MES/DTPA (0.1 M/0.01 M, pH 6.5), PBS (0.1 M, pH 7.2), PBS/EDTA (0.1 M/0.005 M, pH 7.2), ammonium acetate (0.01 M, pH 9.0) and bis-tris/DTPA (0.03 M/0.01 M, pH 6.94), to make solutions with a solids content of 0.5 wt %. The solutions were stirred continuously under room temperature. Aliquots (100 µL) were taken at different time intervals over an eight-week period. Each sample was mixed with deionized water (900 µL) and spun down by centrifugation (5000 rpm, 30 min) immediately to separate the particles from the supernatant. At the end, the supernatant was collected and diluted 10 times with HNO$_3$ aqueous solution (3.0 %) and then analyzed by ICP-MS.

3.3 Results

3.3.1 Particle Synthesis and characterization

The polymer particle samples examined here are the same samples described in Chapter 2. Because our notation for the samples is based on the amount of reactants used in the
synthesis, we briefly describe how the reactions were carried out. Most of these particles were prepared by two-stage dispersion polymerization (2-DisP) in ethanol. A mixture containing all of the styrene, the polyvinylpyrrolidone (PVP) stabilizer, the initiator, a small amount of Triton-X305 surfactant in half of the ethanol was deoxygenated and then heated at 70 °C to initiate free radical polymerization. In parallel, we prepared a solution containing the acrylic acid (AA) or methacrylic acid (MAA) co-monomer plus the lanthanide (Ln) chloride salts, dissolved in the remaining alcohol, deoxygenated and heated at 70 °C. After about 10% styrene conversion, we added the warm solution containing the co-monomer and Ln salts, and the reaction was continued for 24 h, to >95% styrene conversion. Some particles were prepared by three-stage dispersion polymerization (3-DisP) in which additional AA or MAA was added to the reaction after about 60% styrene conversion. The particles were separated from the reaction mixture by sedimentation. The supernatant was analyzed for residual Ln ion content. The particles were then washed by successive sedimentation-redispersion cycles in deionized water.

The diameters and size distribution of particles were determined by the analysis of micrographs obtained by scanning electron microscopy (SEM). Some representative SEM images of sample AA-(2+2)-Eu(0.1), MAA-(2+2)-Eu(0.1), MAA-4-Eu(0.1), AA-2-I, AA-2-II and AA-2-III are listed in Figure 3-1. The SEM images of sample MAA-2-II, MAA-2-IV and MAA-2-VII can be found in Chapter 2. The mean number of surface –COOH groups per particle was determined by a combination of conductometric and potentiometric titration according to the method described by Kawaguchi et al. [3] The Ln ion content of each particle and the distribution of Ln ions per particle were determined by mass cytometry. The characteristics of all the particles examined here are collected in Table 3-1 and Table 3-2. In my notation, AA refers to P(S-AA) particles and MAA refers to P(S-MAA) particles. Further details about the notation are explained in footnotes to the two tables.
Figure 3-1 SEM images of Ln-encoded polystyrene particles synthesized by multiple-stage dispersion polymerization with PVP as the stabilizer in the presence of different co-monomer. (A) AA-(2+2)-Eu(0.1); (B) MAA-(2+2)-Eu(0.1); (C) MAA-4-Eu(0.1); (D) AA-2-I; (E) AA-2-II; (F) AA-2-III.
Table 3-1 Lanthanide content of the reaction, particle size and size distribution of P(S-AA) and P(S-MAA) particles with single element (Eu) synthesized by two-stage and three-stage dispersion polymerization

<table>
<thead>
<tr>
<th>Notebook notation</th>
<th>Sample name</th>
<th>Diameter (µm)</th>
<th>$CV_d$ (%)</th>
<th>Eu content of the feed $^a$</th>
<th>Eu incorporation efficiency (%) $^b$</th>
<th>Eu atoms/particle, calculated $^c$</th>
<th>Eu atoms/particle, measured $^d$</th>
<th>$CV_{Eu}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA120</td>
<td>AA-(2+2)-Eu(0.1) e</td>
<td>2.2</td>
<td>1.6</td>
<td>0.1</td>
<td>99.6</td>
<td>9.5×10$^6$</td>
<td>2.1×10$^7$</td>
<td>25</td>
</tr>
<tr>
<td>SL021</td>
<td>MAA-(2+2)-Eu(0.1) e</td>
<td>2.1</td>
<td>2.8</td>
<td>0.1</td>
<td>99.4</td>
<td>8.2×10$^6$</td>
<td>5.0×10$^6$</td>
<td>28</td>
</tr>
<tr>
<td>SL022</td>
<td>MAA-4-Eu(0.1) e</td>
<td>2.4</td>
<td>2.8</td>
<td>0.1</td>
<td>99.7</td>
<td>1.2×10$^7$</td>
<td>8.5×10$^6$</td>
<td>22</td>
</tr>
</tbody>
</table>

a. wt. % of LnCl$_3$·6H$_2$O based on styrene;
b. From ICP-MS measurements on the filtered reaction supernatant;
c. Calculated from the efficiency of Ln ion incorporation and the measured particle diameters, assuming 100 % styrene conversion;
d. From mass cytometry measurements, using a standard solution containing La, Tb, Tm for instrument calibration;
e. AA: acrylic acid; MAA: methacrylic acid. The value after the co-monomer indicates the amount of AA or MAA (wt. % / styrene) added in the synthesis. (2+2) indicates that this reaction was run in three stages, with 2 wt % AA or MAA added in the second stage and an additional 2 wt % AA or MAA was added in the third stage. The values in parentheses after each Ln indicates the amount of LnCl$_3$·6H$_2$O (wt. % / styrene) added in the reaction; for example, AA-(2+2)-Eu(0.1) refers to PS particles with 2 wt % acrylic acid plus 0.1 wt. % EuCl$_3$·6H$_2$O added in the 2nd stage and additional 2 wt % acrylic acid added in the 3rd stage. MAA-4-Eu(0.1) refers to the PS particles synthesized with 4 wt % methacrylic acid and 0.1 wt. % EuCl$_3$·6H$_2$O.
Table 3-2 Lanthanide content of the reaction, particle size and size distribution of P(S-AA) and P(S-MAA) particles with multiple elements synthesized by two-stage dispersion polymerization

<table>
<thead>
<tr>
<th>Notebook notation</th>
<th>Sample name</th>
<th>Ln content of the feed</th>
<th>Diameter (µm)</th>
<th>CVd (%)</th>
<th>Ln incorporation efficiency (%)</th>
<th>10^-6 Ln atoms/particle, calculated</th>
<th>10^-6 Ln atoms/particle, measured (CV_Ln, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>La</td>
<td>Eu</td>
<td>Tb</td>
</tr>
<tr>
<td>AA150</td>
<td>AA-2-I e</td>
<td>La, Eu, Ho, Tm (0.005)</td>
<td>2.4</td>
<td>3.2</td>
<td>92</td>
<td>0.59</td>
<td>0.87 (74)</td>
</tr>
<tr>
<td></td>
<td>AA-2-II e</td>
<td>La, Eu, Ho, Tm (0.02)</td>
<td>2.7</td>
<td>6.8</td>
<td>98</td>
<td>3.6</td>
<td>4.5 (34)</td>
</tr>
<tr>
<td></td>
<td>AA-2-III e</td>
<td>La, Eu, Ho, Tm (0.2)</td>
<td>2.4</td>
<td>3.8</td>
<td>58</td>
<td>15</td>
<td>1.2 (56)</td>
</tr>
<tr>
<td>SL030</td>
<td>MAA-2-II e</td>
<td>La, Eu, Ho, Tm (0.005)</td>
<td>2.5</td>
<td>2.6</td>
<td>97</td>
<td>0.70</td>
<td>0.28 (52)</td>
</tr>
<tr>
<td></td>
<td>MAA-2-IV e</td>
<td>La, Eu, Ho, Tm (0.02)</td>
<td>2.6</td>
<td>2.6</td>
<td>96</td>
<td>3.1</td>
<td>2.4 (29)</td>
</tr>
<tr>
<td>SL035</td>
<td>MAA-2-VII e</td>
<td>La, Eu, Ho, Tm (0.2)</td>
<td>2.6</td>
<td>2.8</td>
<td>58</td>
<td>19</td>
<td>18 (21)</td>
</tr>
</tbody>
</table>

a. wt. % of LnCl₃·6H₂O based on styrene;
b. From ICP-MS measurements on the filtered reaction supernatant;

c. Calculated from the efficiency of Ln ion incorporation and the measured particle diameters, assuming 100 % styrene conversion;

d. From mass cytometry measurements, using a standard solution containing La, Tb, Tm for instrument calibration;

e. AA: acrylic acid; MAA: methacrylic acid. The value after the co-monomer indicates the amount of AA or MAA (wt. % / styrene) added in the synthesis. (2+2) indicates that this reaction was run in three stages, with 2 wt % AA or MAA added in the second stage and an additional 2 wt % AA or MAA was added in the third stage. The values in parentheses after each Ln indicates the amount of LnCl₃·6H₂O (wt. % / styrene) added in the reaction; for example, AA-(2+2)-Eu(0.1) refers to PS particles with 2 wt % acrylic acid plus 0.1 wt. % EuCl₃·6H₂O added in the 2nd stage and additional 2 wt % acrylic acid added in the 3rd stage. MAA-4-Eu(0.1) refers to the PS particles synthesized with 4 wt % methacrylic acid and 0.1 wt. % EuCl₃·6H₂O;

f. The Roman numerals indicate that a combination of different Ln salts was used in the reaction.
3.3.2 Particle stability upon storage in water and in buffer

In Ref 1, we reported preliminary studies of the stability toward ion leakage of two samples of Ln-encoded P(S-AA) copolymer particles, one synthesized by 2-DisP, the others synthesized by 3-Disp. We used traditional ICP-MS to follow the loss of Tm$^{3+}$ ions from the particles to the continuous medium. Experiments were carried out in three buffer solutions at pH values of 3, 7, and 10.6. At pH 3, there was no detectable level of Tm$^{3+}$ ions in the continuous medium over the entire 3 weeks of the experiment. In the 2-DisP sample, we detected about 3 ppb of Tm$^{3+}$ ions in the continuous medium at the onset of stirring, but this value did not increase with prolonged stirring. For the 3-DisP sample, no Tm$^{3+}$ could be detected at pH 7. Ion leakage was more prominent at pH 10.6 reaching 4.5 ppb for the 2-DisP sample and 1 ppb for the 3-DisP sample after 3 weeks. Nevertheless, in the most severe cases, the total loss of Tm from the sample represented only 0.1% of its Tm content.

In this chapter, I report a more systematic study of ion release, comparing particle samples with different levels of Ln ion content and particles containing several different Ln ions. I was particularly interested in phosphate buffer, because Ln ions and phosphate interact strongly. I was also interested to see what the consequences might be of having strong chelating molecules like EDTA and DTPA in the buffer solutions. As in Ref 2, I took aliquots from time to time, diluted the samples, sedimented the particles by centrifugation, and used ICP-MS to monitor the concentration of Ln ions in the continuous medium. I monitored solutions that were stirred at room temperature over a period of 8 weeks. These experiments were carried out on the 9 particle samples listed in Table 3-1 and Table 3-2 in the 6 different buffer media listed in Table 3-3.
Table 3-3 Buffer solutions employed in the ion release experiments

<table>
<thead>
<tr>
<th>Buffer</th>
<th>MES</th>
<th>MES/DTPA</th>
<th>PBS</th>
<th>PBS/EDTA</th>
<th>Ammonium acetate</th>
<th>Bistris/DTPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.5</td>
<td>6.5</td>
<td>7.2</td>
<td>7.2</td>
<td>9.0</td>
<td>6.9</td>
</tr>
<tr>
<td>c (buffer)</td>
<td>0.1 M</td>
<td>0.1 M</td>
<td>0.1 M</td>
<td>0.1 M</td>
<td>0.01 M</td>
<td>0.03 M</td>
</tr>
<tr>
<td>c (chelator)</td>
<td>0</td>
<td>0.01 M</td>
<td>0</td>
<td>0.005 M</td>
<td>0</td>
<td>0.01 M</td>
</tr>
</tbody>
</table>

Figure 3-2 shows the ion release behavior of $\text{Eu}^{3+}$ in samples MAA-(2+2)-Eu(0.1), MAA-4-Eu(0.1) and AA-(2+2)-Eu(0.1) dispersed in different buffer media. The x-axis represents the times when aliquots were taken from the particle samples, and zero time is defined as the onset of stirring. The y-axis describes the Ln ion content found in the continuous media, reported as the percentage of Ln originally present in the particles. Information about the Ln ion content of each of the particle samples is presented above in Tables 1 and 2. The main message from the data in Figure 3-2 is that in MES and PBS buffers at neutral pH, and ammonium acetate buffer at pH 9.0, there is essentially no significant loss of Eu ions from the particles to the buffers. The amount of Eu detected in the buffer media was less than 0.1 ppb ($6.5 \times 10^{-13}$ M). This can be compared to the Eu content of the particle-containing buffer solutions (20.7 ppb or $1.4 \times 10^{-8}$ M) due to Eu content of the particles. When EDTA and DTPA were present in the buffer, the loss of Eu ions was more prominent. Differences among the samples were small. Most of the ion release occurred in the first 20000 min (ca. 2 weeks), and the loss for these samples was approximately 7% of the initial Eu content. One aliquot (bistris/DTPA, 8 wk) showed a higher Eu content in the supernatant, but this is a single point and may not be representative of the sample as a whole.
Figure 3-2 Release profiles of Eu³⁺ ions from different particles at 0.5 % solids content into different buffer media determined by ICP-MS:

- MES buffer: [MES] = 0.1 M, pH = 6.5;
- MES/DTPA buffer: [DTPA] = 0.01 M, [MES] = 0.1 M, pH = 6.5;
- PBS buffer: [PBS] = 0.1 M, pH = 7.2;
- PBS/EDTA buffer: [EDTA] = 0.005 M, [PBS] = 0.1 M, pH = 7.2;
- Ammonium acetate buffer: [AmAc] = 0.01 M, pH = 9;
- Bistris/DTPA buffer: [DTPA] = 0.01 M, [bistris] = 0.03 M, pH = 6.9.

(A) Leakage of Eu³⁺ ions from particles MAA-(2+2)-Eu(0.1), which was synthesized by 3-stage dispersion polymerization with 4 % MAA as co-monomer (2 % was added in the 2nd stage and 2 % was added in the 3rd stage) and containing 0.1 % Eu;

(B) Leakage of Eu³⁺ ions from particles MAA-4-Eu(0.1), which was synthesized by 2-stage dispersion polymerization with 4 % MAA as co-monomer and containing 0.1 % Eu;

(C) Leakage of Eu³⁺ ions from particles AA-(2+2)-Eu(0.1), which was synthesized by 3-stage dispersion polymerization with 4 % AA as co-monomer and containing 0.1 % Eu.
Figure 3-3 presents ion release data from sample MAA-2-II, prepared with 2 wt % MAA as a co-monomer. This sample contains La, Eu, Ho, Tm and Tb ions, with the Tb ion content about 4 times higher than that of the other 4 elements. The five panels in Figure 3-3 present the data for each of these ions. For each of these ions, the extent of loss to the buffer was negligible in media that did not contain EDTA or DTPA. The curious result for La$^{3+}$ of a negative percentage of released ions was caused by the fact that the La signals in the ICP-MS measurements was actually smaller than that of the blank. For the buffers containing EDTA or DTPA, one detects ions in the supernatant of the initially prepared samples (i.e., at t = 0). The level of ion release remained relatively constant for ca. 1000 min (1 day) and then began to increase. It is likely that further ion loss would occur at times longer than the 8 weeks in these experiments, but I have no data to support this idea. There are two other features of the data that merit comment. First, there is a greater fractional loss of La$^{3+}$ in the presence of EDTA or DTPA than of the other ions. Even here, in the most severe case, only 15% of the...
La content was released after 8 wk. Second, the fractional loss of Tb, which is present at higher concentration in the particles, is smaller than for the other ions. Similar results were obtained for samples MAA-2-IV and MAA-2-VII, and their release profiles are presented in Figure 3-4 and 3-5.

![Figure 3-4](image)

**Figure 3-4** Release profiles of different Ln$^{3+}$ ions (La, Eu, Ho, Tm and Tb) from particle MAA-2-IV at 0.5 % solids content into different buffer media determined by ICP-MS: The sample MAA-2-IV was synthesized by 2-stage dispersion polymerization in the presence of 2 % MAA, loaded with 0.05 % La, Eu, Ho, Tm and 0.2 % Tb. MES buffer: $[\text{MES}] = 0.1 \text{ M}$, pH = 6.5; MES/DTPA buffer: $[\text{DTPA}] = 0.01 \text{ M}$, $[\text{MES}] = 0.1 \text{ M}$, pH = 6.5; PBS buffer: $[\text{PBS}] = 0.1 \text{ M}$, pH = 7.2; PBS/EDTA buffer: $[\text{EDTA}] = 0.005 \text{ M}$, [PBS] = 0.1 M, pH = 7.2; Ammonium acetate buffer: $[\text{AmAc}] = 0.01 \text{ M}$, pH = 9.
I was concerned that the experiments described above involving ICP-MS analysis of the supernatant might not detect Ln ions released into PBS buffer if the Ln ions precipitated in the form of LnPO₃. To investigate this issue, I used mass cytometry to compare two samples of MAA-2-II that had been allowed to age for one year at room temperature. One sample had been stored in DI water; the other had been stored in the PBS buffer used for the experiments presented in Figure 3-3. Data obtained for both particle samples showed essentially identical content of the five different Ln ions. I conclude that on the time scale of one year, there is no significant ion release from these PS particles in PBS buffer.

In Figure 3-6, I compare the release data for individual ions in chelator-containing buffers for samples MAA-2-II, MAA-2-IV and MAA-2-VII. These samples differ in the ion content of the particles. Figure 3-6A shows relatively little loss of Ho³⁺ in MES/DTPA for particles prepared with 0.005, 0.05, and 0.2 wt % HoCl₃. For these same particles in PBS/DTPA (Figure 3-6B), I see a higher fractional loss of Ho ions from the sample with the
lowest Ho content. Corresponding data for Tm$^{3+}$ are presented in Figures 3-6C and 3-6D. The Ho and Tm content are similar in each of the three samples. There was a greater fractional loss of Tm ions in PBS/DTPA for the particle sample with the lowest Tm content.

![Graphs showing release patterns of Ho$^{3+}$ and Tm$^{3+}$](image)

**Figure 3-6** Comparison between the release patterns of particles at 0.5 % solids content with different amount of Ln determined by ICP-MS: All the three samples were synthesized by 2-stage dispersion polymerization in the presence of 2 % MAA, with different amount of Ln salts. MAA-2-II: 0.005 % La, Eu, Ho, Tm and 0.2 % Tb; MAA-2-IV: 0.05 % La, Eu, Ho, Tm and 0.2 % Tb; MAA-2-VII: 0.2 % La, Eu, Ho, Tm and Tb; MES/DTPA buffer: [DTPA] = 0.01 M, [MES] = 0.1 M, pH = 6.5; PBS/EDTA buffer: [EDTA] = 0.005 M, [PBS] = 0.1 M, pH = 7.2; (A) Leakage of Ho$^{3+}$ ions from different particles into MES/DTPA aqueous buffer; (B) Leakage of Ho$^{3+}$ ions from different particles into PBS/EDTA aqueous buffer; (C) Leakage of Tm$^{3+}$ ions from different particles into MES/DTPA aqueous buffer; (D) Leakage of Tm$^{3+}$ ions from different particles into PBS/EDTA aqueous buffer. Note that the sample in B was examined over a longer time (45 days).
**Figure 3-7** Comparison of Eu$^{3+}$ ions release from different particles at 0.5 % solids content into different media determined by ICP-MS: All these particles contain 0.1 % Eu salts. MES/DTPA buffer: [DTPA] = 0.01 M, [MES] = 0.1 M, pH = 6.5; PBS/EDTA buffer: [EDTA] = 0.005 M, [PBS] = 0.1 M, pH = 7.2; Bistris/DTPA buffer: [DTPA] = 0.01 M, [bistris] = 0.03 M, pH = 6.9; MAA-4-Eu(0.1) was synthesized by 2-stage dispersion polymerization with 4 % MAA as co-monomer; MAA-(2+2)-Eu(0.1) was synthesized by 3-stage dispersion polymerization with 4 % MAA as co-monomer (2 % was added in the 2nd stage and 2 % was added in the 3rd stage); AA-(2+2)-Eu(0.1) was synthesized by 3-stage dispersion polymerization with 4 % AA as co-monomer; (A) Leakage of Eu$^{3+}$ ions from different particles into MES/DTPA aqueous buffer; (B) Leakage of Eu$^{3+}$ ions from different particles into PBS/EDTA aqueous buffer; (C) Leakage of Eu$^{3+}$ ions from different particles into Bistris/DTPA aqueous buffer.

Figure 3-7 presents the data from Figure 3-2 in a different way. Here I compare particles with a similar Eu and carboxylic acid content but synthesized in different ways. AA-(2+2)-Eu(0.1) and MAA-(2+2)-Eu(0.1) were synthesized by 3-stage dispersion polymerization, with 2 wt % AA or MAA added both in the second stage and in the third stage. Sample MAA-4-Eu(0.1) was prepared by 2-DisP with 4 wt % MAA added in the second stage. What is important here is to note that in all three chelators-containing buffers, there is less release of Eu ions in the MAA-4-Eu(0.1) sample. Here the ion release is very small, and amounts to only 2% of the ion content after 8 wk in these buffer.
3.4 Discussion

I am surprised by the ability of EDTA and DTPA to remove Ln ions from the particles. The minimal release of ion in the presence of PBS buffer suggests to us that the ion loss does not involve passive dissociation of ions bound to acrylate or methacrylate groups in the particles, followed by diffusion into the continuous phase. Phosphate ions should bind strongly to Ln ions, and I imagine that if there was passive loss of Ln ions from the particles, that the presence of excess phosphate in the PBS buffer would prevent the re-absorption of these ions into the particles. I can also rule out precipitation of the Ln salts as LnPO₃. Mass cytometry experiments on a sample aged one year in PBS buffer showed the same particle-by-particle Ln content as a corresponding sample aged one year in DI water. Thus I infer that the ion loss is a more active process involving the EDTA or DTPA molecules.

At this time I do not know much about the location of the Ln ions within the particle or the nature of binding in the polystyrene matrix. Metal salts of carboxylate groups bound to hydrophobic polymers are a type of ionomer. In ionomers, one has ion clusters of metal carboxylates that are locally phase separated from the matrix polymer.[4] In the future, I hope to obtain a better understanding of the internal structure of these metal-encoded polystyrene particles. I can, however, draw a firm conclusion that in the absence of strong chelating agents, these particles are stable against ion leakage, even upon prolonged storage. This is an important property for their use in bead-array biological assays based on mass cytometry detection.

3.5 Summary

In this chapter, I described a systematic study of ion release, carried on the lanthanide-encoded polystyrene-co-methacrylic acid (P(S-MAA)) and polystyrene-co-acrylic acid (P(S-AA)) copolymer particles, which were synthesized by two-stage or three-stage dispersion polymerizations. These particles with different levels of lanthanide (Ln) ion content and containing several different types of Ln ions were dispersed in normal buffer media, namely 2-(N-morpholino)ethanesulfonic acid (MES), phosphate buffered saline solution (PBS), ammonium acetate (AmAc) and buffers containing strong chelating molecules like ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA). Traditional inductively coupled plasma mass spectrometry (ICP-MS) was employed to follow the loss of ions into the aqueous medium as a function of time. The release behaviors
of Ln ions were compared. In MES and PBS buffers at neutral pH, and AmAc at pH 9.0, there is essentially no significant loss of Ln ions from the particles to the buffers. When the chelating agent EDTA and DTPA were present in the buffer, the loss of Ln ions was more prominent, less than 15 % after 8 weeks under stirring. The differences among the different samples were small. Considering the ability of EDTA and DTPA to remove Ln ions from the particles and the fact of minimal ion release in the presence of PBS buffer, we infer that ion loss is a more active process involving the EDTA or DTPA molecules. The main conclusion is that in the absence of strong chelating agents, these particles are stable against ion leakage, even upon prolonged storage and stirring. This is of great importance for their application in bead-array biological assays based on mass cytometry detection.

3.6 References


Chapter 4
Kinetic study of Lanthanide Incorporation: Preliminary Results

4.1 Introduction

We are interested in determining when the lanthanide (Ln) salts become incorporated into the PS particles during the synthesis of Ln-encoded PS particles by multiple-stage dispersion polymerization and how the Ln ions are distributed in the PS particles. As described in Chapter 2 and Chapter 3, for the synthesis of polystyrene particles in ethanol, the Ln ions were introduced in the second stage as LnCl₃ salts along with the co-monomer (methacrylic acid). If the feed concentration of lanthanide salts was not too high, almost 100% incorporation was obtained. In other words, all of the Ln ions added in the reaction were incorporated into the final particles. Because of the good solubility of the lanthanide salts and their acrylate complexes in ethanol, it is possible that incorporation of the Ln ions may not occur uniformly during the reaction. Kinetic studies should allow us to examine this aspect of the reaction.

Depending upon when Ln ions become incorporated into the particles, there are three possible profiles that one might expect for the lanthanide ion incorporation into the PS particles. As depicted from Scheme 4-1, the Ln ions may reside on the shell of the PS particles, or concentrate in the core of the PS particles or distribute homogeneously all over the particles.

Scheme 4-1 The possible distribution of Ln ions incorporated into the PS particles

In this chapter, I describe initial experiments that were designed to understand how the lanthanide ions are incorporated into the PS particles when added in the second stage of the two stage dispersion polymerization (2-DisP). During the synthesis, aliquots of the reaction mixture were taken over different time intervals and analyzed by traditional inductively
coupled plasma mass spectrometry (ICP-MS), mass cytometry and scanning electron microscope (SEM). I used two different types of particle samples. Both of them were synthesized using MAA as a co-monomer. I employed two different levels of lanthanide concentration. The results presented in this chapter can be considered as preliminary results and further experiments are being carried out in our laboratory to attain more understanding.

4.2 Experimental Section

4.2.1 Instrumentation

A Hitachi S-5200 field emission–scanning electron microscope (SEM) was utilized at operation voltages from 1 to 5 kV to measure particle size. Traditional inductively coupled plasma mass spectrometry (ICP-MS) measurements were carried out on an Elan 9000 instrument (Perkin-Elmer SCIEX) as described in Chapter 2. Mass cytometry measurements were carried out with a CyTOF™ model C2 instrument from DVS Sciences (Markham ON Canada, www.dvssciences.com). The details have been described in Chapter 2.

4.2.2 Materials

Deionized water (DIW) was purified through a Milli-Q purification system. High purity HNO₃ for ICP-MS analysis was purchased from Seastar Chemical Inc. Polystyrene-co-methacrylic acid (P(S-MAA)) particles were synthesized by two-stage dispersion polymerization and the synthesis details were reported in Chapter 2.

4.2.3 Kinetic study of Ln ion incorporation

Two different PS particle samples were synthesized and studied, as shown in Table 4-1. They were prepared in the presence of MAA as the co-monomer and two different levels of lanthanide concentration were employed. The reaction temperature is 70 °C and the half-life time (t₁/₂) for the initiator AMBN at this temperature is 7 hours. During the synthesis, aliquots (ca 200 µL) were collected from the reaction by a micro-syringe at different time intervals for ICP-MS, mass cytometry and SEM analysis.

First, for ICP-MS analysis, 10 µL from the collected aliquot was immediately diluted with 990 µL HNO₃ aqueous solution (3.0 %), and spun down by centrifugation (13000 rpm, 30 min) to obtain a supernatant sample. The clear solutions were then collected. To remove any small particles that may still remain in the collected supernatant solution, 500 µL supernatant was transferred to a 15 mL 3 KDA MWCO Millipore Amicon spin filter and diluted
with 4500 µL HNO₃ (3.0 %) and then centrifuged at 4000 rpm for 45 min. Then a 100 µL aliquot of the flow-through solution was diluted with 900 µL HNO₃ (3.0 %) and analyzed by ICP-MS. The approximate dilution factor is 10000.

Second, a small amount of each collected sample was diluted to approximately 10⁶ particles per mL and analyzed by mass cytometry to determine the Ln content of the polystyrene particles prepared at different times. The number of metal ions per particle was calculated as described in Chapter 2.3.4.

Third, the collected aliquots were analyzed by SEM. The diameters and size distribution of microspheres were determined by analysis of micrographs obtained by SEM using the software package ImageJ, developed by the National Institute of Health. The samples for SEM imaging were prepared by placing a drop of diluted suspension of each collected aliquot on a Formvar/carbon coated-300 mesh copper grid. Particle-size histograms were constructed from measurements of at least 300 individual particles in the SEM images.

### Table 4-1 Recipe for two-stage dispersion polymerization of styrene

<table>
<thead>
<tr>
<th>Materials</th>
<th>1st stage</th>
<th>2nd stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAA-2-Low</td>
<td>MAA-2-High</td>
</tr>
<tr>
<td>Styrene (g)</td>
<td>6.25</td>
<td>-</td>
</tr>
<tr>
<td>PVP55 (g)</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>TX305 (g)</td>
<td>0.36</td>
<td>-</td>
</tr>
<tr>
<td>AMBN (g)</td>
<td>0.25</td>
<td>-</td>
</tr>
<tr>
<td>EtOH (g)</td>
<td>18.75</td>
<td>18.75</td>
</tr>
<tr>
<td>MAA (g)</td>
<td>-</td>
<td>0.13 (2 %)</td>
</tr>
<tr>
<td>LnCl₃·6H₂O (g) Tb</td>
<td>0.007 (0.1 %)</td>
<td>0.035 (0.5 %)</td>
</tr>
<tr>
<td></td>
<td>Tm</td>
<td>0.007 (0.1 %)</td>
</tr>
</tbody>
</table>

### 4.3 Results and Discussion

In order to obtain information about the timing of Ln salts incorporation into the PS particles, I carried out a survey study of the size and lanthanide-content evolution of the particles during the synthesis. During each reaction, I took aliquots of the particles formed at that point in the reaction at different time intervals. Each aliquot was then analyzed for its metal content and for the size. Mass cytometry was used to determine the Ln content in the particles. Complementary ICP-MS measurements were utilized to determine the residual Ln
content in the supernatant. SEM was employed for monitoring the size evolution during the particle synthesis.

- **MAA-2-Low**

Figure 4-1 shows SEM images and size distribution histograms for aliquots collected during the synthesis of sample MAA-2-Low (SL085), which was prepared in the presence of 2% MAA and the lower concentration of TbCl₃·6H₂O and TmCl₃·6H₂O, each at 0.1 wt. % / Styrene. For simplicity, I will denote particles with diameters smaller than 1.0 µm as “small-diameter particles” and particles with diameters larger than 2.5 µm as “large-diameter particles”. In order to have a consistent standard for gating and calculating the average diameter, I plot the histogram of diameters from all the measurements in Origin software. Then, a Gaussian distribution was fit on the histogram to determine the gating range of the data. Then the average diameters (Dₙ), standard deviation (σₖ) and coefficient of variation in diameter (CVₖ = σₖ / Dₙ) were calculated based on the selected data within this gating range from the Gaussian curve.

Figures 4-1A and 4-1B show the SEM images for the aliquots taken at 3 hours after the initiation of the reaction, and the histogram of diameters is presented in Figure 4-1C. At this early reaction time (3 hours after initiation), there is a broad distribution of particle sizes with lots of small-diameter particles and large-diameter particles ranging from 0.6 to 2.2 µm. But the main population was located at ca. 1.41 µm, with the CVₖ of 3.38 %, based on the data from 1.25 to 1.55 µm.

Two hours later (5 hours after initiation), the number of small-diameter particles decreased a bit. The average diameter of the main population evolved to 1.58 µm. And the size distribution decreased to 3.30 %, based on the data from 1.4 to 1.75 µm (Figure 4-1D, 4-1E and 4-1F).

Eleven hours after initiation, the average diameter of the main population grew to ca 1.92 µm, while there were few small-diameter particles and large-diameter particles (Figures 4-1G, 4-1H and 4-1I). Thus the CVₖ was decreased to 2.5 %, as calculated from the gated data ranging from 1.75 to 2.10 µm.

Toward the end of the reaction (25.5 hours after initiation), as one can see in Figures 4-1J, 4-1K and 4-1L, the average particle size of the major population was ca. 2.12 µm with
very few smaller particles. The $CV_d$ was further decreased to 2.03 %, based on the gated data from 2.0 to 2.25 µm.

We extended the reaction time for this experiment. After the reaction was over (31.5 hours after initiation), the final particle size was still 2.12 µm, without any increase. However, the $CV_d$ increased a bit to 2.18 %, based on the data from 1.95 to 2.25 µm. compared to the sample taken at 25.5 hours (Figures 4-1M, 4-1N and 4-1O).
Figure 4-1 SEM images and diameter distribution histogram of polystyrene microspheres at different times after the initiation of the reaction. This sample (MAA-2-Low) was synthesized by 2-stage dispersion polymerization with PVP as the stabilizer in the presence of 2 % MAA, 0.1 % TbCl₃·6H₂O and 0.1 % TmCl₃·6H₂O, relative to the weight of styrene. 3 hours (A, B, C); 5 hours (D, E, F); 11 hours (G, H, I); 23.5 hours (J, K, L); 31.5 hours (M, N, O). Time zero refers to initiation of the reaction. The second stage reactants were added at t= 1.5 hours.
• MAA-2-High

To further investigate the size evolution of Ln-encoded polystyrene particles prepared by two-stage dispersion polymerization, I used SEM to examine another sample (MAA-2-High), which was prepared in the presence of 2% MAA and the high concentration of TbCl₃·6H₂O and TmCl₃·6H₂O, each at 0.5 wt.% / Styrene. Figure 4-2 shows SEM images and size distribution histograms for aliquots collected during the synthesis of sample MAA-2-High.

Figures 4-2A and 4-2B show the SEM images for the aliquots taken at 2 hours after the initiation of the reaction and the histogram of diameters is presented in Figure 4-2C. At this early reaction time (2 hours after initiation), there is a broad distribution of particle sizes with two main populations at ca. 0.6 and 1.2 µm in addition to few large-diameter particles.

One hour later (3 hours after initiation), the particle diameter of the two main populations evolved to ca. 0.7 and 1.3 µm. In addition, one sees fewer large-diameter particles, as shown in Figure 4-2D, 4-2E and 4-2F. One can note that the particle population at 1.3 µm became the major population with an obvious decrease in the number of small- and large-diameter particles.

Eight hours after initiation, the average diameter of the main population grew to ca. 1.85 µm, while there were few small-diameter particles and almost no large-diameter particles. Based on the selected data ranging from 1.7 to 2.0 µm, the average diameter was 1.85 µm and $CV_d$ decreased to 2.29 % (Figures 4-2G, 4-2H and 4-2I).

Toward the end of the reaction (23.5 hours after initiation), as one can see in Figures 4-2J, 4-2K and 4-2L, the average particle size of the major population was 2.15 µm with very few small-diameter particles. And the $CV_d$ further decreased to 2.05 %, based on the data from 2.0 to 2.3 µm.

We extended the reaction time for this experiment. After the reaction was over (31.5 hours after initiation), the final particle size was 2.15 µm. Surprisingly, there was an increase in the population of small-diameter particles in this sample compared to the sample taken at 23.5 hours. Here the $CV_d$ increased a slightly (3.50 %), based on the data from 1.85 to 2.5 µm (Figures 4-2M, 4-2N and 4-2O).
Figure 4-2 SEM images and diameter distribution histogram of polystyrene microspheres at different times after the initiation of the reaction. This sample (MAA-2-High) was synthesized by 2-stage dispersion polymerization with PVP as the stabilizer in the presence of 2 % MAA, 0.5 % TbCl₃·6H₂O and 0.5 % TmCl₃·6H₂O, relative to the weight of styrene. 2 hours (A, B, C); 3 hours (D, E, F); 8 hours (G, H, I); 23.5 hours (J, K, L); 31.5 hours (M, N, O). Time zero refers to initiation of the reaction. The second stage reactants were added at t = 1.5 hours.
Figure 4-3 shows the change of particle size and particle size distribution with the polymerization time of sample MAA-2-Low and MAA-2-High. In this figure, the x-axis shows the reaction time, the y-axis represents the average particle diameter of the major population (selected data based on the Gaussian curve). The standard deviation of particle size ($\sigma_d$) of the same population is presented in the form of the error bars. The average particle size increased with the reaction time while the particle size distribution ($CV_d$) had an overall descending trend. The particle sizes for sample MAA-2-Low are a bit smaller than that of sample MAA-2-High.

![Graph showing particle size distribution over reaction time](image)

**Figure 4-3** The change of particle diameter and distribution with the reaction time for sample MAA-2-Low (○) and MAA-2-High (●), synthesized by two-stage dispersion polymerization. The average diameter and size distribution presented here were based on the gated data from the Gaussian curve.

According to my literature survey, there are three papers that follow the evolution of particle size in dispersion polymerization. [1-3] Two of these papers were presented by the Poehlein group. In the first article, they developed a model for the kinetics of dispersion polymerization; and in their second article, they validated the mathematical model through simulations and measurements of the polymerization rate and particle size evolution during seeded batch and continuous polymerization experiments.
In their first article, [1] Poehlein group developed a mathematical model to predict the rate of polymerization and particle growth for seeded dispersion polymerization in polar organic media. In their study, they chose to work with seeded dispersion polymerization system using monodisperse seeds because polymerization in the presence of monodisperse seed particles avoids the complication of the nucleation process, and also simplifies modeling the particle growth.

In their second article, [2] the Poehlein group validated the model they developed by comparing simulations and measurements of the polymerization rate and particle size evolution during seeded batch polymerization experiments. For their experiments, they used seeded dispersion of styrene in ethanol using PVP360K ($M_w = 360,000$) as the stabilizer and 1.1 µm PS seed particles (made by dispersion polymerization). Reasonable fits of the experimental monomer conversion data were obtained from the simulation. Examination of polymerization rates versus monomer conversion curves for different runs at various temperature levels (60, 70 and 80 °C), showed that solution phase polymerization became more important at higher temperatures. In all cases, the relative contribution from polymerization inside particles increased with conversion. Using SEM to measure the particle diameter, they show that the experimental values of seed particle volumes after growth were as predicted by the simulation modeling.

Soini et. al. [3] developed a mathematical model of the basic polymerization kinetic scheme for dispersion polymerization, which involves the initiation, propagation, and termination reactions. They assumed that the propagation and termination rate constants were independent of the chain length. They derived the following formula to estimate the particle size at any time during the polymerization reaction:

$$V(i_0, t) = \frac{m_0 k_d l(0)}{2 \rho} \int_0^t e^{-k_d t} \times \left( i_0^2 + i_0 \frac{2}{\delta(t)} + \frac{2}{\delta^2(t)} \right) \delta(t) e^{-\delta(t) l_0} dt$$

(4.2)

Where $V$ is the total volume of the insoluble polymer component with critical chain length $i_0$ and at time $t$, $m_0$ is the weight of the monomer molecule, $\rho$ is the density of polymer, $k_d$ is decomposition rate constant of the initiator and $\delta(t)$ is a function of rate constants.
They demonstrated the applicability of this mathematical model to the dispersion polymerization reaction of styrene in isopropanol with polyvinylpyrrolidone (PVP40K) as the stabilizer at 70 °C. They measured the particle size and size distribution at half hourly intervals by the Flying Light-Scattering Indicatrix (FLSI, angular dependency of the intensity of light scattered by a moving individual particle) method. This technique is based upon Mie scattering from individual particles in an instrument resembling a flow cytometer, and is sensitive to particles in the size range of 1 to 10 µm. Fitting data to Equation 4.1 allowed them to obtain the following parameters from the experimental data of $V(i_0 , t)$: $k_d$, $k_{rm}/k_{1/2rm}$ and $i_0$. They reported the total volume of insoluble material, which is the product of the volume of each particle times the number of particles, to fit their model. As depicted in Figure 4-5, they found that the total volume of the polystyrene particles increased with the reaction time, which agreed well with their mathematical model as expressed in Equation 4.2. Since the number of particles was constant after the nucleation stage, this model also indicates that the volume of each particle increased with the reaction time. While they did not present SEM images, their FLSI data showed relatively narrow size distributions.

In our system, I used SEM images of at least 300 individual particles to measure the change of particle diameter with the reaction time. For the sake of simplicity in describing
the images, I classified all the particles into three populations: small-particle population, the main population and a large-particle population. I found that the average diameters of the main population of the particles grew with the reaction time (Figure 4-3). However, I also saw some small-diameter particles and large-diameter particles at the early stages of the reaction, as shown in Figure 4-1A, E, G and Figure 4-2A, E, G. As the reaction proceeded, the number of small-diameter particles decreased. In parallel, the number of large-diameter particles disappeared at later stages of the reaction. At the end of the reaction, the particles size distributions were quite narrow, as shown in Figure 4-1J, K, M, N and Figure 4-2J, K.

The evolution of the main population of particles in my experiments is similar to that reported by the Poehlein group and by Soini group. Soini et al. did not report the presence of small particles, but they did not show any SEM images. Their light scattering method would not detect any small particles in the sample. Poehlein et al. did not mention any small particles in their sample, but one can see in the SEM images in their paper that their particle size distribution was relatively broad. There were some small particles present. Thus my observation of a small particle population at the early stages of the reaction is important. Why these particles formed and why they disappeared at higher monomer conversion in my experiments raise a topic for future study.

I also observe a population of large particles at low monomer conversions. I do not believe that these particles were formed during the reaction. Dr. Jingshe Song in our group discussed this phenomenon in his thesis. When he used a chain transfer agent (CTA) in his 2-DisP reactions, low molecular weight polymers were formed. Some of these low mass polymers remained soluble in ethanol at 70 °C, but precipitated when the reaction was cooled, forming new rather large particles in an uncontrolled fashion.[5] When he changed the composition of the solvent to reduce the solubility of the polymer at 70 °C, the big particles disappeared.

I believe that the large-diameter particles formed at early times in my experiments might be the result of the precipitation of PS oligomer chains that were soluble in hot ethanol but phase separated on cooling. According to the literature, the critical molar mass is ca. 12,000 for polystyrene itself in ethanol at 70 °C.[6] For PS copolymers with MAA, this critical molar mass for solubility in hot ethanol could be significantly higher. In my samples, the aliquots taken during the first 5 hours probably contained some polymer with a molecular weight less than the critical value. So these low mass polymer chains were not captured by
the existing particles during the polymerization reaction, but precipitated as large-diameter particles once the reaction was cooled.

Figure 4-5 shows the change of metal content of supernatant and particles with the reaction time. The x-axis represents the reaction time of 2-DisP polymerization. The left-hand y-axis represents the percentage of Tm content in supernatant detected by ICP-MS to the total Tm content added in the reaction. We assume that there was no precipitation of the lanthanide ions during the polymerization reaction. Thus, the decrease in Ln ion concentration in the continuous medium corresponds to Ln ions incorporated into the particles. The right-hand y-axis of Figure 4-5 shows the number of Tm ions per particle determined by mass cytometry calibrated with the standard solution containing a mixture of lanthanides of known concentration.

As shown in Figure 4-5A, for sample MAA-2-Low, after 2 hours of the polymerization reaction (30 min after the addition of the lanthanide salts), the amount of Tm ions found in the continuous media was about 40 % of that added to the reaction. This means that, at this point of time, 60 % of the Tm ions were already incorporated into the particles. One can see that the amount of Tm ions in the media detected by ICP-MS decreased continuously with the reaction time, until \( t = 11 \) h. After ca. 11 hours of reaction, the Tm content in the supernatant decreased essentially to zero. At the end of the reaction, almost all the Tm ions added in the reaction were incorporated into the PS particles. Similar results were obtained for Tb in this reaction: a decrease of Tb\(^{3+}\) concentration in the supernatant as measured by ICP-MS (Figure 4-5B).

In parallel, mass cytometry was used to measure the evolution of lanthanide content of the individual particles. Unfortunately, the mass cytometry results for the aliquots collected in the early stages of the reaction are not reliable. Mass cytometry is not sensitive enough to measure the low Ln ion content for the large number of small-diameter particles. In Figure 4-5A, for sample MAA-2-Low, after 8 hours of the polymerization, each particle contained only \( 1.4 \times 10^6 \) Tm ions. As the reaction proceeded, the Tm content of the PS particles measured by mass cytometry increased gradually, leveling off at \( t = 13 \) h. At the end of the reaction, the final particles contained on average \( 2.8 \times 10^7 \) Tm ions per particle. The growth in Ln ion content over time complements the ICP-MS measurements showing a decrease in the Ln ion content in the supernatant. Similar results were obtained for Tb shown in Figure 4-5B: an increase in the Tb\(^{3+}\) content per particle measured by mass cytometry.
Figure 4-5C shows a somewhat different profile for the Tm content change for the sample MAA-2-High. Two hours after the initiation of the reaction (30 min after the addition of the lanthanide salts), almost 40% of the Tm ions became incorporated into the particles. After that, additional consumption of Tm and Tb ions was much slower. Eight hours later, about 50% of Tm ions was found in the continuous medium, i.e., half of the Tm ions had been incorporated into the particles. At this stage of the reaction, each particle contained on average $1.1 \times 10^7$ Tm ions. With the increase of reaction time, the Tm content in the continuous medium decreased slowly, while the Tm content in the particles increased. After ca. 24 hours of reaction, the Tm content of the supernatant leveled off, as did the Tm content in the particles. At the end of the reaction, there was ca. 25% of the Tm remaining in the continuous medium. The mean number of Tm ions per particle was $7.0 \times 10^7$. As explained in Chapter 2, at this high Ln concentration level, I found less than 100% incorporation efficiency in the particle synthesis.

For high Ln concentration, not all the Ln ions were incorporated into the particles, i.e., some Ln ions were left in the continuous medium (low incorporation efficiency as described in Chapter 2). However, comparing Figure 4-5C and Figure 4-5D, one can notice that the incorporation efficiency of Tb (62%) is lower than that of Tm (75%). The number of Tb ions per particle is only half of that of Tm ions.

According to these two samples, we know that at short reaction times, a substantial fraction of the Ln ions were already consumed: 60% at 2 hours for MAA-2-Low and 40% at 2 hours for MAA-2-High. There are some discrepancies in the metal content results presented above. Based on these preliminary experiments, I cannot explain what might be the reason for that. These experiments are being repeated in a more systematic way by other members in our group.
Figure 4-5 The change of metal content of supernatant and particles with the reaction time for sample MAA-2-Low (A) (B) and MAA-2-High (C) (D), synthesized by two stage dispersion polymerization. The Ln content in supernatant (■) was determined by traditional ICP-MS and the Ln content in particles (□) was measured by mass cytometry.

4.4 References


In this thesis, I described the synthesis of lanthanide-encoded polystyrene particles with methacrylic acid as the co-monomer by multiple stage dispersion polymerization in ethanol. Different lanthanide ions were loaded into these particles during the synthesis through the addition of lanthanide trichloride salts and excess methacrylic acid to the reaction well after the particle nucleation stage was complete. These poly(styrene-co-methacrylic acid) particles obtained were characterized in terms of their size, size distribution, lanthanide content, lanthanide content distribution and surface acid groups.

Using variations of lanthanide salts and methacrylic acid concentrations, I could synthesize particles with a diameter of ca. 2.0 µm with narrow size distribution (less than 5.0 %, by SEM). Mass cytometry measurements revealed that individual particles contain ca. $10^5 - 10^8$ chelated lanthanide ions, either a single element or a mixture of elements. Potentiometric titrations showed that each particle had ca. $10^7 - 10^8$ carboxylic acid groups on the surface.

I monitored the incorporation efficiency of lanthanide ion into the particles by inductively coupled plasma mass spectrometry. I found high incorporation efficiency (> 95 %) of lanthanide ions into the particles when the total amount of LnCl$_3$ salts in the reaction mixture was sufficiently small. The Ln incorporation efficiency decreased with the increasing amount of LnCl$_3$ salts.

A systematic study of the stability of these particles against the ion leakage was performed. There was no significant loss of Ln ions from the particles to regular buffer solutions. When strong chelating agents were present in the buffer, the loss of Ln ions was more prominent, but still less than 15 % after 8 weeks.

Preliminary kinetics experiments about the timing of Ln incorporation into the polystyrene particles were carried out. Based on these experiments, I could not draw a firm conclusion regarding the kinetics of lanthanide ions incorporation into the particles. It is nevertheless clear that this novel method of incorporating lanthanide into poly(styrene-co-methacrylic acid) particles through the second stage of two-stage dispersion polymerization yields microspheres suitable for the highly multiplexed detection of biomolecules.