Macromolecules

ARTICLE

pubs.acs.org/Macromolecules

Surface Functionalization Methods To Enhance Bioconjugation in Metal-Labeled Polystyrene Particles

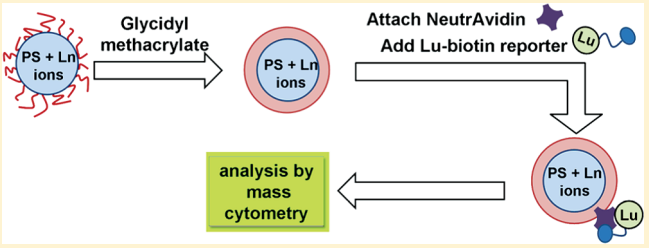
Ahmed I. Abdelrahman,[†,§] Stuart C. Thickett,[†,‡,§] Yi Liang,[†] Olga Ornatsky,[†] Vladimir Baranov,[†] and Mitchell A. Winnik*[,†]

[†]Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, ON M5S3H6, Canada

[‡]School of Chemistry F11, The University of Sydney, NSW 2006, Australia

Supporting Information

ABSTRACT: Lanthanide-encoded polystyrene particles synthesized by dispersion polymerization are excellent candidates for mass cytometry based immunoassays; however, they have previously lacked the ability to conjugate biomolecules to the particle surface. We present here three approaches to postfunctionalize these particles, enabling the covalent attachment of proteins. Our first approach used partially hydrolyzed poly(*N*-vinylpyrrolidone) as a dispersion polymerization stabilizer to synthesize particles with high concentration of −COOH groups on the particle surface. In an alternative strategy to provide −COOH functionality to the lanthanide-encoded particles, we employed seeded emulsion polymerization to graft poly(methacrylic acid) (PMAA) chains onto the surface of these particles. However, these two approaches gave little to no improvement in the extent of bioconjugation. In our third approach, seeded emulsion polymerization was subsequently used as a method to grow a functional polymer shell (in this case, poly(glycidyl methacrylate) (PGMA)) onto the surface of these particles, which proved highly successful. The epoxide-rich PGMA shell permitted extensive surface bioconjugation of NeutrAvidin, as probed by an Lu-labeled biotin reporter (ca. 7×10^5 binding events per particle with a very low amount of nonspecific binding) and analyzed by mass cytometry. It was shown that coupling agents such as EDC were not needed, such was the reactivity of the particle surface. These particles were stable, and the addition of a polymeric shell did not affect the narrow lanthanide ion distribution within the particle interior as analyzed by mass cytometry. These particles represent the most promising candidates for the development of a highly multiplexed bioassay based on lanthanide-labeled particles to date.



INTRODUCTION

In addition to applications in the coatings, microelectronics, and environmental protection industries,[1−5] there is now tremendous interest in using polymer nano- and microparticles as platforms for bioassays,[6−8] particularly multiplexed immunoassays.[9−20] In these types of biological applications, polymer particles must satisfy several criteria, including appropriate size, narrow particle size distribution, and the attachment of labels for particle detection. Various substances can be used to label polymer particles for immunoassays, including fluorescent dyes,[21,22] quantum dots,[23,24] Cornell dots,[25] surface-enhanced Raman scattering (SERS) active materials,[26−28] or metal ions.[29−31] In addition, the particles must have surface functionality for attaching bioaffinity agents such as antibodies to the particles. Many different functional groups have been used for this purpose, such as amino,[32] aldehyde,[33] hydrazide,[34] hydroxyl,[35] epoxide,[33] and carboxyl groups.[36,37]

In our group, we are interested in fabricating metal-encoded particles for mass cytometry-based bioassays.[29,30,38] Mass cytometry[39] is a new technique based on inductively coupled plasma time-of-flight mass spectrometry (ICP-TOF MS) that can measure the metal content of individual particles. We chose lanthanide (Ln) elements as metal labels for our particles due to their low natural abundance in biological systems and hence low background levels in analytical measurements. Additionally, most members of the Ln family have a similar chemical reactivity, allowing the use of similar loading and tagging protocols. We have explored several methods for the synthesis of Ln-labeled particles for mass cytometry applications; as yet none of these approaches have been appropriately optimized. Using a combination of surfactant-free emulsion polymerization (SFEP) and seeded emulsion polymerization,[30] we prepared Ln-encoded polystyrene (PS) particles with diameters on the order of 800 nm and with carboxyl groups on the surface. NeutrAvidin, a deglycosylated form of avidin, was conjugated to the surface of these particles using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) chemistry.[36] As a proof-of-concept immunoassay, the particles were further functionalized

Received: March 15, 2011
Revised: April 27, 2011
Published: May 20, 2011

via exposure to biotin-labeled immunoglobulin (IgG). These IgG-functionalized particles were able to capture goat antimouse-IgG, itself labeled with a metal chelating polymer as a reporter group carrying multiple copies of a Ln ion different from that encoded in the PS particles. While this demonstration bioassay appeared to work, the particles themselves suffered from a broad particle-to-particle distribution of lanthanide ion content. This broad distribution limits their resolving power and applicability in highly multiplexed immunoassays.

In a second approach, we used two-stage dispersion copolymerization (2-DisP) of styrene and acrylic acid (AA) in ethanol in the presence of poly(*N*-vinylpyrrolidone) (PVP).[29] Dispersion polymerization of styrene in ethanol in the presence of polymeric stabilizers like PVP is effective for the synthesis of particles with micrometer diameters and an exceptionally narrow size distribution. In our reactions, we obtained 2 μm diameter monodisperse Ln-encoded particles, with coefficients of variation of the diameters (CV_d) on the order of 1% and with narrow Ln ion distributions. In our copolymerization reactions, the AA was added to the reaction mixture 1 h after initiation along with much smaller amounts of the relevant lanthanide salt(s) in order to chelate the Ln ions within the particles. The AA comonomer also provided surface carboxyl functionality, which could be quantified by conductometric titration. These reactions led to good control over the Ln loading (10^5-10^8 ions per particle) with a relatively narrow particle-to-particle Ln distribution—an improvement on the seeded emulsion polymerization process. We were able to load multiple metal labels into each particle, and each label was readily resolved by mass cytometry, highlighting the potential of this procedure. This method is particularly effective for synthesizing beads of constant and known Ln ion composition that can be used to calibrate the mass cytometer.[38]

We encountered difficulty, however, in attaching significant numbers of biomolecules to the particles for use in model bioassays. For example, only small amounts of NeutrAvidin could be attached to the particle surface in spite of a large number of surface −COOH groups determined by titration. These particles were sterically stabilized by a corona of PVP. While this polymer is useful in that it is effective at suppressing nonspecific protein adsorption to the particles,[40] these chains also appear to interfere with bioconjugation to the carboxyl groups under the corona at the particle surface.

In order to use particles synthesized by the 2-DisP method in immunoassays, the issue of the protein-repellent PVP surface layer must be addressed. There are numerous potential solutions to this problem. The most obvious, such as using an active dispersant like poly(acrylic acid) (PAA) to provide colloidal stability, appears to interfere with incorporation of the Ln ions into the particle core. Other potential approaches include modifying the PVP surface layer by opening (hydrolyzing) the pyrrolidone ring (potentially yielding numerous carboxyl and amino groups within the corona) or creating a functional polymer shell over the pre-existing surface of the particles. There are examples in the literature of building polymer shells onto dispersion polymerization particles. Zhang et al.[41] reported seeded dispersion polymerization using a PS particle seed, with a mixture of styrene and methacrylic acid (MAA) in the second stage, to form carboxylated particles in ethanol. In their work, seed particles of diameter 1.9 μm were grown to a final size of 2.2 μm with a narrow particle size distribution (PDI = 1.02) with a very high accessible acid content by titration (up to 97 mg of acid monomer per gram of polymer). However, the reaction appeared to be delicate, and very specific conditions were required to obtain monodisperse particles in a coagulum-free system.

There are other examples in the literature describing particles made by dispersion polymerization in ethanol and subsequently used as seed latexes for emulsion polymerization after redispersion (via centrifugation) into water. Three publications are of particular interest. Omer-Mizrahi et al.[42] synthesized a shell of poly(glycidyl methacrylate) (PGMA) onto PS particles in water. The shell was extremely thick (∼360 nm) and the particle morphology was raspberry-like, but the particle size distribution remained narrow. This synthesis employed a concentration of sodium dodecyl sulfate well above its critical micelle concentration, with the resultant morphology suggesting that the nucleated PGMA particles coagulate and stick to the surface of the significantly larger PS particles, forming a shell with functional epoxide groups. Alam et al.[43] synthesized pH-responsive particles by seeded emulsion polymerization, using 1.7 μm PS seed particles, with a mixture of MAA, N-isopropylacrylamide (NIPAM), and a cross-linking agent. Finally, Ahmad et al.[44] reported seeded emulsion copolymerization of styrene and hydroxyethyl methacrylate (HEMA) with a PS seed at different comonomer ratios to form functionalized particles. All of these methods are potential candidates for functionalization of our metal-encoded dispersion polymerization particles.

In this paper, we present three approaches to synthesize lanthanide-encoded polymer particles with functional surfaces suitable for further bioconjugation, while retaining the narrow particle size and lanthanide content distributions only attainable with particles synthesized by dispersion polymerization. In the first approach, we preactivated the PVP via basic hydrolysis[45] to provide functional groups on the PVP polymer and then used the "activated" PVP as dispersants in our established 2-DisP method. For the second approach, we used the particles obtained by 2-DisP in the presence of PVP as seeds for second stage polymerization of methacrylic acid (MAA) with the idea of generating PMAA grafts on the PVP corona of the particles. In the third approach, we used the dispersion particles as seeds for second-stage polymerization with GMA to grow an epoxy-rich shell of PGMA over the PVP corona of the particles.

We examined the particle size and particle size distribution of these particles by scanning electron microscopy (SEM). The metal content of these particles was measured by mass cytometry, and their stability was monitored in different aqueous media over different time intervals. Results of both techniques were evaluated with respect to satisfying the criteria necessary for reproducible bioassays as measured by mass cytometry.

EXPERIMENTAL SECTION

Reagents. Absolute ethanol, polyvinylpyrrolidone (PVP55) (Aldrich, molecular weight = 55 000 Da), Triton-X305 (TX305, 70% solution in water, Aldrich), 2,2′-azobis(2-methylbutyronitrile) (AMBN, Dupont USA), lanthanum(III) chloride hydrate ($LaCl_3$, Fluka), thulium(III) chloride hexahydrate ($TmCl_3$, Aldrich), europium(III) chloride hexahydrate ($EuCl_3$, Aldrich), terbium(III) chloride hexahydrate ($TbCl_3$, Aldrich), holmium(III) chloride hexahydrate ($HoCl_3$, Aldrich), and praseodymium(III) chloride hexahydrate ($PrCl_3$, Aldrich) were used without further purification. NeutrAvidin was obtained in lyophilized form from Thermo Scientific (PI-3100), and bovine serum

Table 1. Recipes for the Synthesis of D2($PVP_{1.6}$) and D3-($PVP_{3.2}$) by Dispersion Polymerization of Styrene with Activated PVP

materials (g added)	first stage	second stage[a]
styrene	6.25	
PVP55	0.9[b]	
activated PVP[c]	0.1[d]	
TX305	0.35	
AMBN	0.25	
ethanol	18.75	18.75
acrylic acid		0.125
lanthanide salts		0.0156[e]

[a] 1.0 h after the reaction was initiated. [b] 0.8 g was used in the synthesis of D3($PVP_{3.2}$). [c] Prepared according to Scheme 2. [d] 0.2 g was used in the synthesis of D3($PVP_{3.2}$). [e] ($LaCl_3 \cdot 6H_2O$ + $EuCl_3 \cdot 6H_2O$ + $TbCl_3 \cdot 6H_2O$ + $HoCl_3 \cdot 6H_2O$ + $TmCl_3 \cdot 6H_2O$) 3.1 mg of each.

albumin (BSA) was from Sigma-Aldrich (A2153). Water was purified through a Milli-Q purification system. High purity HCl and HNO_3 for ICP-MS analysis were purchased from Seastar Chemical Inc. Styrene (S, Sigma-Aldrich) was washed with a 5% w/w KOH solution and subsequently passed through a column of basic alumina (Sigma-Aldrich) prior to storage under refrigeration at 4 °C. Monomer was never kept for more than 1 week. Glycidyl methacrylate (GMA, >99% pure, Sigma-Aldrich) and methacrylic acid (MAA, Sigma-Aldrich) were used as received. Potassium persulfate (KPS, Sigma-Aldrich) and sodium dodecyl sulfate (SDS, Sigma-Aldrich) were used as received. 2,2′-Azobis(2-methylbutyronitrile) (AMBN, Wako) was recrystallized from ethanol. All water used was Milli-Q grade (Millipore).

PVP55 Pyrrolidone Ring-Opening (PVP Activation). The method used here is adopted from the method of von Specht et al.[45] in which 1.0 g of PVP55 was dissolved in 50 mL of 0.1 N NaOH, and the solution was heated for 36 h in an autoclave at 140 °C. In order to methylate the γ-aminobutyric of the opened pyrrolidone ring and to prevent its closing, 2.86 mL of 35% formaldehyde solution was added, the pH was adjusted to 9, and the solution was cooled to 0 °C; 1.5 M sodium tetrahydroborate was added, and the solution was stirred for 45 min. The amount of ring-opening obtained by von Specht et al. was 15%. This extent of ring-opening was determined based on a method described by Frank et al.[46] in which an excess of 1.5 mL of 1 N HCl is added to a 10 mL portion, and the amino acid formed is titrated in ethanol with 39.4 mM ethanolic KOH.

Dispersion Polymerization Using Activated PVP. The recipe for the two-stage dispersion polymerization (2-DisP) of styrene with AA in ethanol is listed in Table 1. The procedure used is similar to the one described in ref 29, in which the PS-*co*-PAA particles were obtained after 24 h reaction under ethanol-saturated nitrogen. Instead of using untreated PVP55 as a dispersant, we doped the PVP55 with activated PVP at two different levels, 1.6 and 3.2 wt % with respect to styrene. In these reactions, AA and the lanthanide chloride salt were added in the second stage, 1 h after initiating the polymerization. Fractional conversion was measured by gravimetry and was typically 95−99%.

Redispersion of Dispersion Polymerization Particles into Water. Particles previously made by 2-DisP in ethanol were redispersed into water by the following method: the desired mass of PS dispersion in ethanol was weighed out, and this dispersion was divided among polypropylene centrifuge tubes (2 mL capacity, Eppendorf tubes) and spun in a desktop centrifuge (Eppendorf centrifuge, 5415 C) at 13 000 rpm for 1 min. After this period of time the PS particles had settled at the bottom of the tube, with a clear ethanol supernatant above the polymer. The supernatant was carefully removed by Pasteur pipet, leaving only a wet solid at the bottom of the centrifuge tube. The total volume of the dispersion was re-established by adding the appropriate volume of Milli-Q water. The particles were easily redispersed into water by gentle shaking of the centrifuge tube. This process was repeated three times to ensure effective redispersion into water.

Seeded Emulsion Polymerization with Methacrylic Acid (MAA). The method presented here is analogous to that of Alam et al.[43] A sample of PS-*co*-PAA particles made by 2-DisP[29] (denoted D1, 2.25 g, solids content 8.71% w/w, d_{avg} = 2.1 μm, CV_d = 1.8%) was redispersed into water by the method described above. These particles were used as a seed for emulsion polymerization with MAA with a 2:1 (D1:MAA) ratio by mass. 0.1 g of MAA was added to the particles, along with additional water (8.33 g). In the work of Alam et al., a mixture of hydrophilic monomers was used; however, in this case only MAA was chosen—no additional hydrophobic monomer was added. Polymerization was initiated by KPS (5 mg, corresponding to an initiator concentration of 0.7 mM) and was allowed to proceed for 24 h under magnetic stirring.

Seeded Emulsion Polymerization with Glycidyl Methacrylate (GMA). The method presented here represents conditions chosen from the work of Omer-Mizrahi et al.[42] to create poly(glycidyl methacrylate) (PGMA) shells on the surface of PS particles made by dispersion polymerization. A variety of procedures were performed in this work to coat a range of different Ln-labeled particles. A representative method is as follows:

A sample of D1 was taken (equivalent to 0.15 g of solid polymer based on the solids content of the dispersion) and redispersed into pure water as described above. The aqueous dispersion was then placed in a small round-bottom flask, and GMA (0.6 g) was added to the system. The weight ratio of GMA (shell) to PS (core) was close to 4:1. The latex volume was made up to a total 10 mL with Milli-Q water, and 0.1 g of SDS (~36 mM, CMC = 8.2 mM in water at 25 °C) was then added. The system was agitated and allowed to stir magnetically for 30 min to allow for swelling and establishment of monomer-swollen micelles. The system was then degassed using high purity N_2 prior to the sample being raised to the reaction temperature (343 K) and the injection of the initiator (potassium persulfate, 0.012 g) in a small volume of water. Polymerization took place for 6 h. Unless otherwise mentioned, certain ratios were kept constant in this work: the volume fraction of GMA in the system was 6%, the ratio of SDS to water was 1% w/v, and the KPS to GMA ratio was 2% w/v monomer. Conversion was measured by gravimetry and was typically close to 100%. A 4 mL (2 × 2 mL) sample of the latex was cleaned by three centrifugation and redispersion cycles into water. Another sample of PS particles (denoted D1′) was used as seeds for growing PGMA shells (sample D5(GMA-4)). D1′ sample was synthesized also by 2-DisP and has an average diameter (d_{avg}) of 1.6 μm and a size distribution (CV_d) of 2.9%.[29]

Mass Cytometry. The lanthanide ion content of each sample was measured by mass cytometry. Data reported in this article refer to the analysis of 10 000−20 000 individual particles. Mass cytometry experiments were carried out using a CyTOF instrument from DVS Sciences Inc. (Toronto, ON, Canada). The instrument is commercially available and has been described in detail elsewhere.[39]

Calculation of the Number of Metal Ions per Particle. The number of ions per particle (N) was calculated using eq 1, based on the intensity values (I) obtained by mass cytometry for each metal, using the instrument transmission coefficient (T).

$$N = I/T \quad (1)$$

The value of the transmission coefficient T represents the ratio of the number of ions detected (in counts) by the time-of-flight (TOF) detector to the number of ions generated by the inductively coupled plasma (ICP) torch. The exact value of T differs slightly from day to day and also varies across the spectrum of early to late lanthanide elements. In addition, different lanthanide ions have small differences in their susceptibility to form other compounds (e.g., oxides) in the plasma,

which reduces the portion of the ions that can reach the TOF detector.

The instrument transmission coefficient is calculated by instrument calibration (done on a daily basis) using standard solutions of known metal concentrations. The difference in the value of T calculated at different times for the same metal ion is ascribed to the variation in the ionization efficiency of the plasma and to slight differences in the detector sensitivity. For lanthanide ions, T values are typically in the range of 3.3×10^{-5}–2.0×10^{-3} counts/metal ion. The magnitude of T means that approximately one ion in every 10 000–30 000 generated by the ICP torch is detected by the TOF detector. A typical value of T is 0.91×10^{-4} counts/metal ion for ^{165}Ho, for example. In sample D2($PVP_{1.6}$) (see below), the ^{165}Ho counts measured by mass cytometry was 201. From eq 1, the number of ^{165}Ho ions per particle was calculated to be 2.2×10^6. In the section below, we report directly the number of ions per particle.

ICP-MS. Sample analysis by ICP-MS employed an ELAN 9000 instrument (Perkin-Elmer SCIEX). Typical operating conditions of the instrument are based on stable Ar plasma optimized to provide less than a 3% CeO^+/Ce^+ ratio in a 1 μg L^{-1} standard multielement solution diluted in a 1:10 dilution of concentrated (33–36 wt %) HCl, i.e., 3.3–3.6% HCl. This requirement was achieved by applying 1400 W forward plasma power, 17 L/min Ar plasma gas flow, 1.2 L/min auxiliary Ar flow, and 0.95 L/min (Perkin-Elmer cross-flow nebulizer) Ar flow. Under these operating conditions, the typical sensitivity is 4×10^4 cps for 1 μg L^{-1} Ir standard solution in 3.3–3.6% HCl. The detection limits for lanthanide elements were less than 1 ng L^{-1}. The sample uptake rate was adjusted depending on the particular experiment and sample size, typically 100 μL/min. Experiments were performed using an autosampler (Perkin-Elmer AS 93) modified for operation with Eppendorf 1.5 mL tubes. Sample sizes varied from 150 to 300 μL. Standards were prepared from 1000 μg/mL PE Pure Single-Element Standard solutions (Perkin-Elmer, Shelton, CT) by sequential dilution with 2% HNO_3 in high-purity deionized water (DIW) produced using an Elix/Gradient (Millipore, Bedford, MA) water purification system.

Surface Acid Titration. Surface acid titration analyses were performed in a method analogous to Kawaguchi et al.[47] Samples of latex were diluted in high purity water to a solids content of 0.05% w/w. To this, 200 μL of a standardized 0.1 M NaOH solution was added by micropipet (Eppendorf) and stirred magnetically. This solution was then back-titrated using a 0.0098 M HCl solution (standardized separately against an 0.998 M solution of Na_2CO_3) added in volume increments ranging from 20 to 200 μL. Equivalence points corresponding to the titration of free base and the titration of deprotonated surface acid groups were monitored by both potentiometric and conductometric methods (using a Fisher Scientific conductivity meter and an Ecomet pH probe). A blank solution (no latex) was titrated under the same conditions to account for dissolved carbon dioxide in the solution.

Infrared Spectroscopy. Fourier transform infrared spectroscopy (FTIR) was performed on solid PS/PGMA samples. Samples were dried in an oven and then pelleted with KBr. IR spectra were collected between 4000 and 400 cm^{-1} (32 scans) on the Perkin-Elmer Spectrum 1000 instrument. Data collection was performed using SPECTRUM software (Perkin-Elmer).

SEM. Latex samples were prepared for scanning electron microscopy (SEM) by creating an approximately 1:500 dilution of the as-prepared latex sample in water and placing one drop of this solution on a formvar/carbon copper electron microscopy grid (Pelco). Samples were analyzed in the SE mode on the Hitachi S-5200 scanning electron microscope (Centre for Nanostructure Imaging, University of Toronto). The accelerating voltage was 1 kV and current 20 mA. For each sample, a series of images were taken; particle size distributions were constructed on the basis of counting at least 200 particles to provide meaningful statistics with regards to average size and polydispersity. Image analysis was performed using ImageJ.[48]

DLS. The diameters of submicrometer particles were measured by dynamic light scattering (DLS) with a BI90 particle sizer (Brookhaven Instruments Corp.) at a fixed scattering angle of 90°.

Bioconjugation with Proteins. The following protocol was used for all bioconjugation reactions presented in this article:

Aqueous samples of 10^9 polymer particles per mL were washed in Polylink Coupling buffer (50 mM MES, pH 5.2, 0.05% Proclin300) at 1000g for 5 min, twice. 170 μL of MES buffer was then added to the particles. A 0.2 g mL^{-1} solution of EDC in MES buffer was prepared, and 20 μL of this solution was added to the particles and mixed with gentle vortexing. The sample was then split into two separate centrifuge tubes, containing either 25 μL of NeutrAvidin (10 mg mL^{-1} solution) or 25 μL of a 10 mg mL^{-1} solution of BSA in the case of control samples. The tubes were mixed gently and allowed to react overnight at 4 °C. The particles were then pelleted at 5000g for 20 min, and the blocking buffer 1% BSA in PBS (Phosphate Buffered Saline) was added. The particles were then sonicated for 30 min in a sonication bath and pelleted. The supernatant was then aspirated.

At this point, the introduction of a "reporter tag" for mass cytometry analysis took place. For example, the Lu biotin-conjugated reporter tag (Figure S1 in the Supporting Information) will bind to any NeutrAvidin bound to the particle surface. The particles were incubated with 100 μL of 500 nM of Lu in 1% BSA for 40 min at room temperature, then washed once with PBS, and finally redispersed in a 0.26% w/w NaCl solution in 10 mM tris buffer. The particles were then analyzed by mass cytometry.

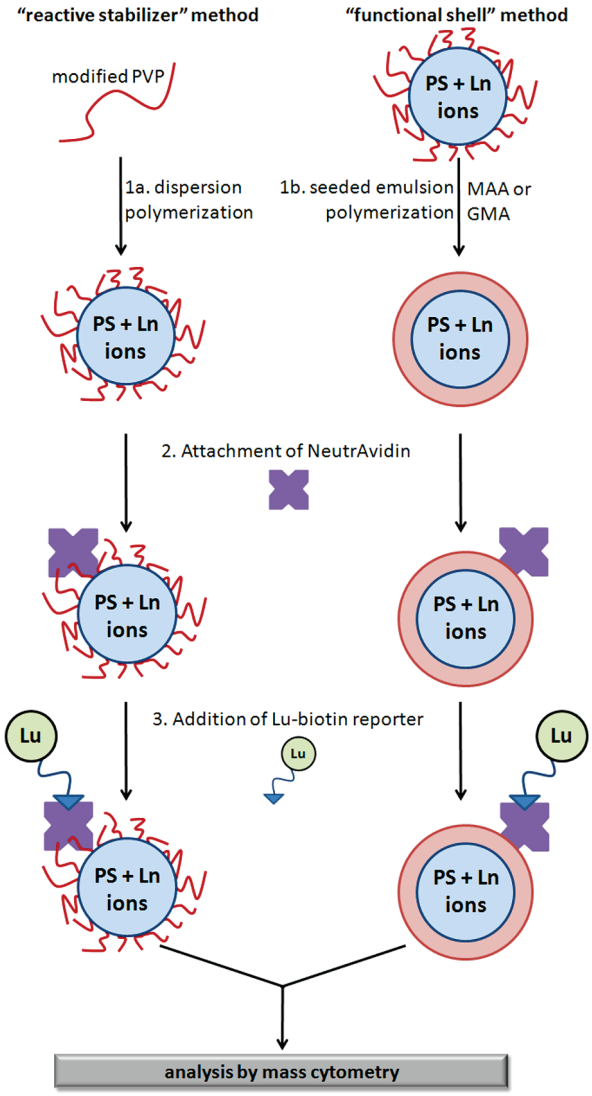
Particles coated with PGMA such as sample D5(GMA-2) (see Table 4) were treated with NeutrAvidin or with BSA as described above, in the presence of EDC, and without pretreatment of the beads with EDC. In the latter case, the bead solution was reacted directly with the protein in question. The results from these two experiments are denoted D5(GMA-2)-EDC-NA and D5(GMA-2)-NA in Table 5 for the presence and absence of the EDC coupling agent, respectively, during the bioconjugation protocol.

Testing Particle Stability upon Aging in Buffer. The stability of the PGMA coating on PS particles containing various Ln ions was tested using the following method: Three samples of experiment D5-(GMA-4) were diluted to 0.5% w/w solids content in two different buffers: a pH 3 buffer (50 mM sodium acetate/acetic acid) and a pH 7 buffer (10 mM ammonium acetate). These samples were stirred magnetically at room temperature in sealed scintillation vials. Aliquots were taken after 2 h, 2 days, and 11 days, and SEM images were collected to determine both particle size and morphology. From these aliquots, the supernatant was collected via centrifugation, and the elemental composition was determined by ICP-MS in order to determine the extent of Ln leaching from these particles.

RESULTS AND DISCUSSION

The objective of the experiments described here is to explore methods to modify the surface of metal-encoded polystyrene microparticles prepared by two-stage dispersion polymerization to improve the ease and effectiveness of attaching bioaffinity agents to the particle surface. As mentioned in the Introduction, particles prepared by dispersion polymerization in the presence of poly(*N*-vinylpyrrolidone) (PVP) as a polymeric stabilizer are protected against nonspecific protein adsorption by a solvent-swollen corona of PVP chains. This corona also interferes with the attachment of biomolecules such as NeutrAvidin to the surface of the particles, in spite of a substantial concentration of carboxylic acid groups (ca. 10^9 per particle) detected by titration. We will use the effectiveness of NeutrAvidin conjugation as a

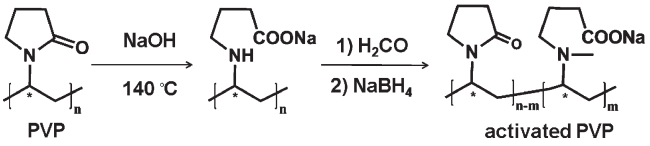
Scheme 1. Bioconjugation Conditions Used To Attach NeutrAvidin to the Particle Surface



measure of the ease of attachment of bioaffinity agents to the particles following surface modification.

We describe three approaches to this surface modification. In one strategy, we carry out chemical modification of PVP to convert some of the pyrrolidone groups to carboxylic acids and introduce some of this modified PVP into the synthesis of PS particles by two-stage dispersion polymerization. In the second and third strategies, we use samples of metal-containing PS particles prepared by 2-DisP as seeds for subsequent polymerization reactions in water. Most of these experiments were carried out on a sample labeled D1, in which La, Tb, Ho, and Tm were loaded into the particle interior by copolymerization with acrylic acid. We have reported previously that metal-encoded PS particles synthesized in this way have a very narrow diameter distribution and a particle-to-particle variation in the distribution of lanthanide ions that is only slightly larger than the particle-to-particle variation in volume.[29] For example, as we will see below, these particles show a coefficient of variation in their Tb content (CV_{Tb}) of 15.1% and a similar variation in the other elements.

Scheme 2. Ring-Opening of PVP by Basic Hydrolysis



Our approach to bioconjugation is summarized in the lower half of Scheme 1. The particles were treated with an appropriate reagent (here we primarily use EDC to activate surface −COOH groups) and then coupled to NeutrAvidin. In a second step, the modified particles were exposed to a biotin−polypeptide conjugate reporter group (whose structure is shown in Figure S1, Supporting Information) containing a single DTPA-Lu ion at the end opposite to biotin. The particle suspension was subsequently analyzed by mass cytometry. The high binding affinity of biotin with NeutrAvidin ($K \sim 10^{15}$ M^{-1})[49−51] ensures that each surface-bound NeutrAvidin unit will be "labeled" with 3 or 4 Lu ions, which can be detected by their mass. In our design, successful biofunctionalization would lead to attachment of a minimum of 10^5 Lu ions per particle.

As a negative control, we carried out parallel experiments in which we used the same EDC chemistry to attach BSA instead of NeutrAvidin to the particle surface. We used BSA-modified particles to measure the extent of nonspecific reporter group binding to the surface of our particles relative to our protein of interest (NeutrAvidin). Following the addition of the Lu-biotin reporter and analysis by mass cytometry, the measured Lu intensity of the BSA modified particles would indicate the amount of nonspecific reporter group binding to the particles. For the NeutrAvidin-labeled particles, we report the mean number of Lu atoms per particle, but for the BSA-labeled particles, we report the Neut/BSA ratio, the ratio of the mean number of Lu atoms per NeutrAvidin particle to the mean number of Lu atoms per BSA particle (see Table 3). High values of the Neut/BSA ratio indicate a particle population with very low amounts of nonspecific reporter-group binding. Conversely, a Neut/BSA ratio that is close to 1 indicates extensive nonspecific binding to the particle surface and the inability to differentiate the mass cytometric signal of a target protein compared to background adsorption events.

Dispersion Polymerization with Activated PVP. We initially examined the use of activated PVP as a dispersant in two-stage dispersion polymerization (2-DisP) experiments to synthesize Ln-labeled polymer particles. To activate chains of PVP55 (PVP with an average molecular weight of 55 kDa), the polymer was treated with 1.0 N KOH at 140 °C for 36 h (see Scheme 2). The amino group of the opened pyrrolidone ring was then methylated by treatment with formaldehyde followed by $NaBH_4$ to prevent its reclosing in solution. Back-titration against KOH, using the method described in Frank et al.,[46] indicated that 18% of the pyrrolidone rings were opened under these reaction conditions (see titration curve in Figure S2 of the Supporting Information).

The activated PVP (in a mixture with unactivated PVP55) was then used as a dispersant in the preparation of lanthanide-encoded PS-*co*-PAA particles by 2-DisP in absolute ethanol (Table 1). We prepared two samples, denoted D2($PVP_{1.6}$) and D3($PVP_{3.2}$). Both samples were synthesized in the presence of five Ln chloride salts (La, Eu, Tb, Ho, and Tm), each at the

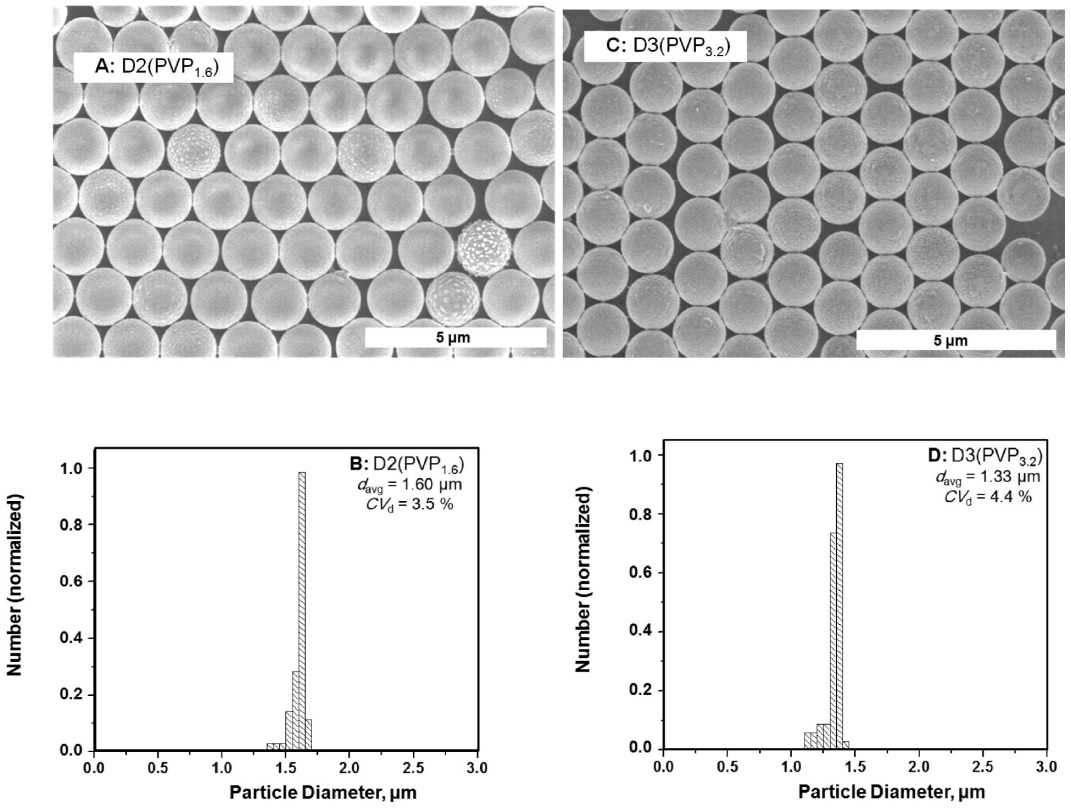


Figure 1. SEM images (A, C) and particle distribution histograms (B, D) for particles synthesized by 2-DisP. D2($PVP_{1.6}$) (A, B) and D3($PVP_{3.2}$) (C, D) were prepared in presence of 1.6 and 3.2 0 wt % styrene, respectively.

concentration level of 0.05 wt % styrene. The salts were dissolved in the ethanolic solution of AA (2.0 wt %/styrene) and added 1 h after initiating the polymerization. D2($PVP_{1.6}$) was prepared in the presence of activated PVP (1.6 wt %/styrene) while D3-($PVP_{3.2}$) sample was prepared at a higher activated PVP concentration (3.2 wt %/styrene). Stable, coagulum-free dispersions were obtained in both experiments after 24 h of polymerization.

Figure 1 shows representative SEM images and histograms of the particle size distribution for experiments D2($PVP_{1.6}$) and D3($PVP_{3.2}$). Sample D2($PVP_{1.6}$) has an average diameter of 1.60 μm, which is smaller than the diameter (ca. 2.0 μm) of particles prepared with a similar recipe in the presence of only unactivated PVP55.[29] The modified reaction maintained a narrow size distribution of the particles (CV_d = 3.5%). Sample D3($PVP_{3.2}$), which was prepared in the presence of a higher concentration of the activated PVP, had a much smaller average diameter (1.33 μm) and a broader size distribution (CV_d = 4.4%).

We explain the small particle size produced in the presence of the modified PVP in terms of the role of grafting of the dispersant to PS in the dispersion polymerization reaction. The grafting of polystyrene from chains of PVP is essential for the anchoring of PVP onto the growing particles and its ability to act as a dispersant. If more PVP−PS grafting is possible, newly nucleated particles will be more effectively stabilized, resulting in more initial particles and a smaller overall final particle size. We now recognize that the activated PVP has sites with more labile hydrogen atoms along the polymer backbone (labeled with the asterisk in Scheme 2) due to the higher electron density on the nitrogen atom, which can more effectively stabilize the tertiary

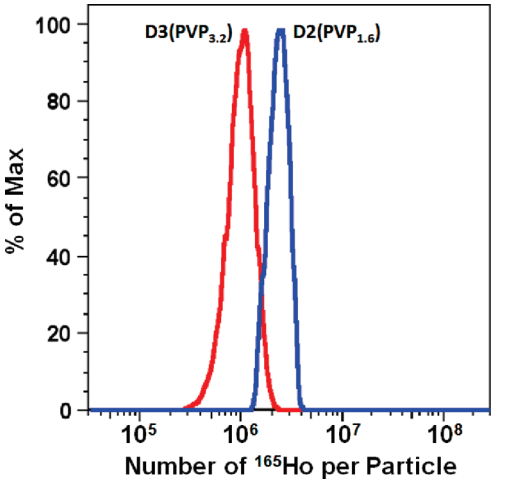


Figure 2. Ho content of D2($PVP_{1.6}$) (CV_{Ho} = 20.6%) and D3($PVP_{3.2}$) AA139 (CV_{Ho} = 27.1%) prepared by two-stage dispersion polymerization in presence of activated PVP.

radical. The higher electron density on the nitrogen atom is a consequence of the replacement of the electron-withdrawing amide group, in the PVP55, with the electron-donating methyl group in the activated PVP.

The metal content for these particles was measured by mass cytometry. Figure 2 shows the ^{165}Ho distribution in samples D2($PVP_{1.6}$) and D3($PVP_{3.2}$). The average Ho atom content of sample D2($PVP_{1.6}$) was 2.2 × 10^6 Ho atoms per particle, and the coefficient of variation of the Ho content distribution (CV_{Ho})

was 20.6%, while sample D3($PVP_{3.2}$) was found to contain 1.24×10^6 Ho atoms per particle, with CV_{Ho} equal to 27.1%. The ratio of Ho content between the two samples [D2($PVP_{1.6}$) to D3($PVP_{3.2}$)] was 1.77, which is nearly identical to the ratios found for the other four metals incorporated into the interior of these particles (see Figure S3 in the Supporting Information for additional histograms of the Tb content of D2($PVP_{1.6}$) and D3($PVP_{3.2}$). We note that this ratio (1.77) is very close to the ratio of particle volumes of D2($PVP_{1.6}$) and D3($PVP_{3.2}$). Consequently, the difference in the metal content can be ascribed to the difference in particle size, and the use of differing amounts of activated PVP as stabilizers did not affect the ability to incorporate Ln ions into the particle interior.

The ability of particles synthesized in the presence of activated PVP to undergo bioconjugation was then determined by covalent attachment of NeutrAvidin to the surface of these particles using EDC chemistry. Quantification of the extent of binding was measured through the use of the Lu biotin-conjugated reporter tag with subsequent measurement by mass cytometry. Unfortunately, the reporter signal was as low as that obtained with particles made by dispersion polymerization without the activated PVP (Figure S4 in the Supporting Information).

In this paragraph, we attempt to explain the low level of Lu signal measured, from which we infer that little NeutrAvidin was covalently bound to the particle surface. From the decrease in particle size, we know that the modified PVP is selectively incorporated into the PS particles. Thus, the problem with limited NeutrAvidin coupling is not due to the absence of modified PVP on the particle surface but, rather, the locus of the carboxyl groups that they add to the particle composition. If grafting of PS to activated PVP takes place at the ring-opened sites, then these sites will be located close to or at the surface of the PS core of the particle. From this perspective, these new carboxyl groups are hidden by the PVP corona from functionalization by proteins. It is also possible that the additional concentration of carboxyl groups generated by this method is insufficient to make an appreciable difference to the measured extent of bioconjugation. Given these results, no further analysis of this system was considered.

Table 2. Recipe for the Synthesis of Functional Monomer Coatings onto the Surface of the PS Seed Latex D1 by Seeded Emulsion Polymerization[a]

	amount added (g)	
materials	D4(MAA)	D5(GMA-2)
methacrylic acid	0.1	
glycidyl methacrylate		0.67
seed latex D1 (as solid polymer)	2.25 (0.2)	1.71 (0.15)
water	8.33	9.48
sodium dodecyl sulfate		0.1
potassium persulfate	0.005	0.012

[a] Experiments were performed with methacrylic acid (D4(MAA)) or with glycidyl methacrylate in the presence of excess surfactant (D5(GMA-2)).

Seeded Emulsion Polymerization with Methacrylic Acid. There are a number of reports in the literature describing the use of particles prepared by dispersion polymerization as seed particles for emulsion polymerization.[42−44] In the first set of experiments described below, samples of PS particles synthesized by dispersion polymerization in the presence of PVP55[29] were sedimented by centrifugation, redispersed in water, and used as seeds for subsequent surface modification by polymerization with methacrylic acid. Our experimental design was based on the work of Alam et al.[43] In their work, PS seed particles prepared by dispersion polymerization were transferred to water and subsequently reacted in a seeded emulsion polymerization process with a mixture of monomers, MAA, *N*-isopropylacrylamide, and an acrylamide-based cross-linker.

We used MAA as the sole second stage monomer with the idea of incorporating chains of the moderately water-soluble PMAA into the seed particles. In this way, we hoped to generate particles with an increased acid content at the surface for subsequent bioconjugation experiments, following separation of the particles from the PMAA homopolymer formed in the reaction and present in the aqueous phase. The ratio (by mass) of polystyrene to MAA was chosen to be 2:1. Seed particles were swollen overnight at room temperature with the added MAA under constant stirring prior to polymerization. The experimental parameters used for these syntheses are shown in Table 2. The seed latex utilized was the redispersed sample D1, which is shown in Figure 3A.

SEM analysis of the particle population (denoted D4(MAA)) after polymerization and three subsequent centrifugation and redispersion cycles into pure water indicated that the particles remained smooth and monodisperse (d_{avg} = 2.1 μm, CV_d = 1.6%; see Figure 3B). There is evidence in the SEM micrograph in Figure 3B that some particles are slightly deformed after polymerization with MAA, in contrast to the uniform PS spheres in the seed latex (Figure 3A), but the origin of this effect is unclear. The fractional conversion of MAA in D4(MAA) was determined by gravimetry to be 70%; however, no increase in average particle size was observed relative to the seed latex D1. This curious

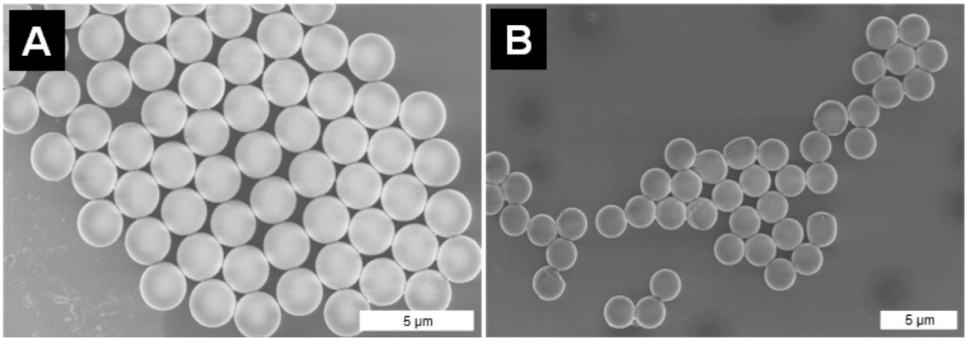


Figure 3. SEM image of D1 seed particles (d_{avg} = 2.11 μm and CV_d = 1.3%) synthesized by dispersion polymerization (A) and sample D4(MAA) (d_{avg} = 2.1 μm and CV_d = 1.6%) after seeded emulsion polymerization of D1 with MAA in a 2:1 (D1:MAA) ratio (B).

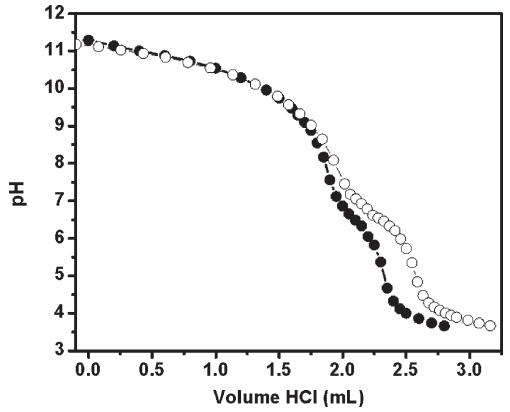


Figure 4. Surface acid titration results of D1 (black circles, seed latex) and D4(MAA) (open circles, after polymerization with MAA) after back-titration with 0.01 M HCl performed under identical conditions.

observation is similar to the results reported by Alam et al.,[43] who saw little increase in particle size after polymerization but verified the presence of acid groups on the surface of their particles by demonstrating a strong particle size dependence on the pH of the continuous phase.

Upon centrifugation and redispersion of our particles into pure water, we found that ~48% of the newly formed solids had been washed away in the supernatant. The large reduction in the solids content of the latex was attributed to aqueous-phase polymerization of MAA occurring at the same time as grafting of PMAA chains onto the particles. Despite the large amount of aqueous-phase PMAA formed during this process, the mass fraction of acidic polymer incorporated into the particles increased significantly relative to the D1 seed particles: in sample D4(MAA), the mass of PMAA was ca. 15% of the total mass of polymer, while in D1, the mass of incorporated acrylic acid comonomer was only 1.6% of the total polymer mass.

The concentration of surface acid groups was measured for the particles D1 and D4(MAA) by conductometric and potentiometric surface acid titration. While gravimetric analysis of D4(MAA) demonstrated an increase in the acid content of the particles, titrations were needed to determine the location of these additional acid groups. From our synthesis protocol (preswelling the particles with MAA prior to polymerization), it is likely that some of the added PMAA is buried within the particle interior where it would not be detected by titration.[52] We observed an increase in titratable acid groups by approximately a factor of 2 (from 1×10^9 to 2.1×10^9 acid groups per particle) after polymerization with MAA and purification of the particles. Under identical dilution conditions, both equivalence points (representing the titration of free base and surface carboxylate groups respectively) occur at the same pH (see Figure 4) while a greater number of titratable acid groups is clearly evident on D4(MAA) (open circles) compared to D1 (filled circles). On the basis of the particle number density (i.e., the number of polymer particles per unit volume of latex) and the PMAA content of our particles by gravimetry, we predict 2.4×10^9 acid groups per particle from the added PMAA. If we assume that the increase in titratable surface acid groups (i.e., an additional 1.1×10^9 acid groups per particle) is solely from the PMAA now present in the system, we can conclude that nearly 50% of the PMAA present in D4(MAA) is titratable and hence resides at the particle surface.

Table 3. Mass Cytometry Results for the Bioconjugation of NeutrAvidin to the Surface of PS Dispersion Polymerization Particles before Further Polymerization with MAA Probed with a Lu−Biotin Reporter

sample	no. of Lu ions per particle	Neut/BSA[a]
D1 (seed PS particles)	6.7×10^4	1.1
D4(MAA)[b]	1.0×10^5	1.4

[a] Neut/BSA refers to the ratio of the average number of Lu ions per particle of the sample with NeutraAvidin bound to the surface compared to the equivalent experiment where BSA was attached to the surface. [b] Synthesized by seed emulsion polymerization with MAA using D1 seeds.

To test the effectiveness with which the PMAA particles (D4(MAA)) could be biofunctionalized, we used EDC chemistry in an attempt to attach NeutrAvidin to the particles. In parallel, we carried out identical experiments on the precursor D1 particles. After washing the particles by successive centrifugation−redispersion steps, the particles were treated with an excess of the biotin−polypeptide−Lu reagent and analyzed by mass cytometry.[53] As a negative control, we used similar chemistry to bind BSA to both D1 and D4(MAA) particles and, after washing, treated these particles with an excess of the biotin−polypeptide−Lu reagent. The results for these experiments are presented in Table 3. In the middle column, we report the number of Lu ions per particle detected by mass cytometry for the D1 particles and for the D4(MAA) particles following the surface binding of NeutrAvidin and treatment with the Lu reporter as described above. The Lu signal from the D4(MAA) particles was measured to be ~50% higher (1.0×10^5 vs 6.7×10^4 Lu ions per particle) than the D1 particles, suggesting a slight increase in the attachment of NeutrAvidin to the modified particles.

The data presented in the middle column of Table 3 also consist of a contribution that is due to nonspecific adsorption of the reporter group to the particles. As a control, we carried out similar experiments on both particles treated with EDC and then reacted with BSA prior to exposure to the Lu-reporter group. The right-hand column of Table 3 is the "Neut/BSA ratio" for the two sets of particles studied. The data in Table 3 indicate there is only a 10% increase in the Lu signal above that measured for nonspecific binding to the D1 seed particles and only a 40% increase for binding to D4(MAA). We infer from these results that introducing grafted PMAA chains onto the surface of our seed particles did not greatly improve the amount of specific protein binding, with the Neut/BSA ratio being only slightly above unity.

These disappointing results mentioned above can be contrasted with other experiments in our laboratory, with the PS particles synthesized by a combination of surfactant free emulsion polymerization and seeded emulsion polymerization to obtain Ln-encoded PS particles. This synthesis employed azocyanopentanoic acid as an acid-containing initiator and gave much more encouraging results in terms of the signal from the biotin−polypeptide reporter group. In those experiments, we found a typical Lu reporter signal of over 2.8×10^5 Lu ions per particle and a Neut/BSA ratio of over 100 under bioconjugation conditions identical to those reported here.[30] Those particles had other problems (a tendency to aggregate in solution and broader particle-to-particle distribution of loaded Ln ions compared to particles made by dispersion polymerization) that led us to carry

out the experiments reported in this paper. However, as the grafting of PMAA to D1 seed particles did not lead to a sufficient improvement in the amount of bioconjugation to the particles, we turned to other approaches to meet our goals.

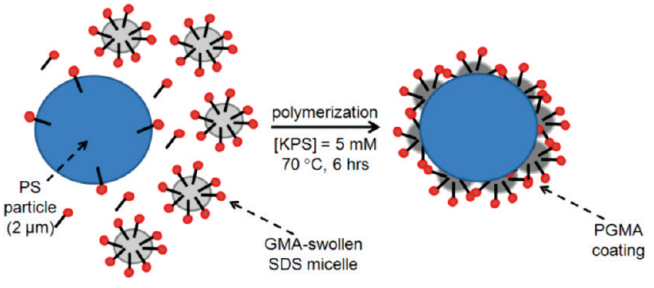
Seeded Emulsion Polymerization with Glycidyl Methacrylate. In a second approach, we followed the methodology described by Omer-Mizrahi et al.[42] for the growth of a poly(glycidyl methacrylate) (PGMA) shell onto the surface of PS particles by seeded emulsion polymerization. This procedure is unusual compared to most seeded emulsion polymerization experiments in that the polymerization was performed with a surfactant concentration well above its CMC. These authors obtained particles with a raspberry-like surface morphology. We infer from this result that small, SDS-stabilized PGMA particles (of the order of 50 nm) are nucleated in the reaction and then coalesce onto the surface of the much larger PS particles (of the order of 2 μm) to build a PGMA shell as the polymerization proceeds (see Scheme 3). Such a coating of PGMA would potentially cover up the protein-repellent PVP layer, and the presence of reactive epoxide groups on the surface should permit subsequent bioconjugation.

In our experiments, the D1 PS seed particles were transferred from ethanol to water via three centrifugation and redispersion cycles. The ratio of GMA to PS was set at approximately 4:1 by mass, and the recipe used to prepare sample D5(GMA-2) is presented in Table 2. A number of experiments were performed in order to determine the ability to form PGMA shells on the surface of our PS particles (see Table 4). The SEM image shown in Figure 5 demonstrates an increase in particle size compared to the smooth D1 PS seed particles (Figure 3A), with a final roughened spherical morphology. A large number of very small PGMA particles, with diameters in the range of 50−100 nm, were observed in the background of these SEM images. A sample of the supernatant fluid (after centrifugation of the larger PS particles) was examined by dynamic light scattering and showed the presence of particles with a mean diameter of 52 nm. In the larger PS particles, the PGMA shell was approximately 60−100 nm thick, with only a small percentage of the added GMA actually grafted to the particle surface. Washed, redispersed, and dried particles were analyzed by FTIR, confirming the presence of PGMA grafting from characteristic epoxide peaks at 845 and 910 cm^{-1}. The previously reported "raspberry" morphology was seen in only a few experiments (see Figure S5 in the Supporting Information for the SEM image of sample D5(GMA-1)), suggesting that the resultant particle morphology is sensitive to system parameters such as stirring effects and the concentration of added surfactant. This is feasible given the unusual polymerization mechanism employed in these experiments.

We examined the lanthanide (Ln) content distribution of PGMA-coated particles by mass cytometry and compared the results to those obtained with the D1 precursor particles. Recall that the D1seed latex contained La, Tb, Ho, and Tm and that the particle-to-particle variation of these elements was very low (e.g., CV_{Tb} = 15.1%). Mass cytometry data for sample D5(GMA-2) containing a PGMA shell are presented as an isotopic "dot-plot" diagram in Figure 6A. The color intensity in Figure 6A represents a 2-dimensional map of the distribution of both the ^{159}Tb and ^{165}Ho isotopes. A one-dimensional projection of Figure 6A onto the ^{159}Tb axis is shown in Figure 6B as a single isotope distribution. This distribution is both monomodal and narrow. The narrow variation in number of ions found for each isotope demonstrates that the construction of the PGMA shell on the particle surface preserves the narrow distribution of metal ions in the particle interior.

Following the formation of a PGMA shell on the PS seed latex, we examined the covalent attachment of NeutrAvidin to the surface of these particles. The amount of NeutrAvidin bound to the particle surface was measured by mass cytometry via the analysis of a Lu-tagged biotin−polypeptide reporter. The first set of experiments involved direct reaction of NeutrAvidin with the

Scheme 3. Proposed Polymerization Mechanism Whereby Agglomeration of Small PGMA Particles Takes Place onto the Surface of the Larger PS Particles



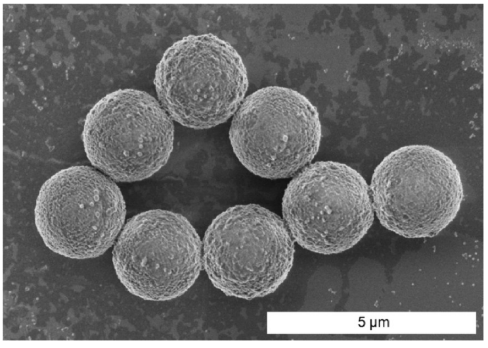


Figure 5. SEM image of PS particles (sample code D5(GMA-2), d_{avg} = 2.23 μm and CV_d = 1.4%) with a shell of glycidyl methacrylate (GMA) by seeded emulsion polymerization of D1 in the presence of excess surfactant (for SEM image of the seed latex D1, see Figure 3A).

Table 4. Reproducibility of Surface Coating Experiments Using an Excess of GMA

sample code (precursor)	GMA:PS ratio	d_{avg} initial, d_{avg} final (μm)	CV_d initial, CV_d final (%)	shell thickness (nm)	% GMA incorporated	comment
D5(GMA-1) (D1)	3.87	2.11, 2.67	1.3, 3.6	280	30	raspberry-like
D5(GMA-2) (D1)	4.41	2.11, 2.23	1.3, 1.4	60	6	rough, spherical
D5(GMA-3) (D1)	5.21	2.11, 2.24	1.3, 3.9	65	6	rough, spherical
D5(GMA-4) (D1′)[a]	3.96	1.61, 1.79	1.8, 2.6	90	29	rough, spherical

[a] Seed particles (d_{avg} = 1.6 μm) synthesized by two-stage dispersion polymerization (see ref 29).

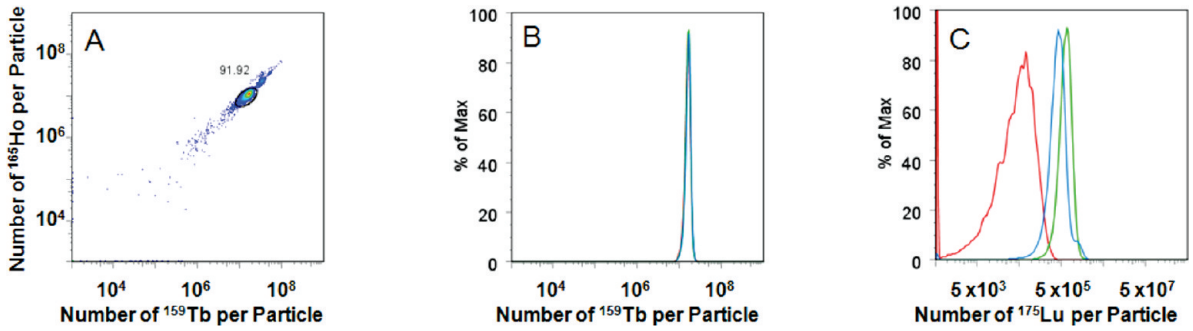


Figure 6. Mass cytometry data for the analysis of sample D5(GMA-2), which consists of a PGMA shell on D1 seed particles. Shown are (A) Tb−Ho isotopic dot−dot diagram; (B) Tb content distribution; (C) Lu content distribution for the detection of the Lu−biotin reporter, which measures the extent of protein binding to the surface. Red curve: nonspecific binding of the Lu−biotin reporter to sample D5(GMA-2)-BSA (covalent attachment of BSA, CV_{Lu} = 65.8%). Blue curve: binding of the Lu−biotin reporter to sample D5(GMA-2)-NA (covalent attachment of NeutrAvidin without EDC, CV_{Lu} = 32.5%). Green curve: binding of the Lu−biotin reporter to sample D5(GMA-2)-EDC-NA (covalent attachment of NeutrAvidin after surface activation with EDC, CV_{Lu} = 27.8%).

Table 5. Mass Cytometry Results for Bioconjugation of NeutraAvidin to the Surface of PS Dispersion Polymerization Particles before and after Seeded Emulsion Polymerization with GMA[a]

sample	no. of Lu ions (reporters)	Neut/BSA[b]
D1 (seed PS particles)	6.7×10^4	1.1
D5(GMA-2)-NA[c]	4.9×10^5	10.4
D5(GMA-2)-EDC-NA[d]	7.1×10^5	15.1
ST120[e]	3.2×10^5	11.5

[a] Binding was probed using an Lu−biotin reporter. [b] Neut/BSA refers to the ratio of the average number of Lu ions per particle of the sample with NeutrAvidin bound to the surface compared to the equivalent experiment where BSA was attached to the surface. [c] PGMA-coated particles were conjugated to NeutrAvidin without EDC activation. [d] PGMA-coated particles were activated with EDC prior to NeutrAvidin attachment to the surface. [e] PS particles made by seeded emulsion polymerization (see ref 30).

PGMA-coated particles (sample D5(GMA-2)). Azizi et al.[54] reported that epoxides readily react with aliphatic and aromatic amines in water at room temperature, and based on this precedent, we examined the possibility that lysine amine groups of NeutrAvidin would react directly with the epoxide groups present on the surface of our particles. This experiment is denoted D5(GMA-2)-NA in Table 5. We observe that there is a significant increase (~8 times greater) in the number of reporter Lu ions per particle for bioconjugation to D5(GMA-2) relative to the seed particles (D1). In addition, we infer from the increase in the Neut/BSA ratio from 1.1 to 10.4 that the amount of nonspecific reporter-group binding to D5(GMA-2) decreased significantly. The distribution of Lu ions per particle is shown in Figure 6C for D5(GMA-2)-NA (blue curve). This peak is much narrower and appears at greater Lu ion values than the signal for the negative control D5(GMA-2)-BSA (red curve). The Lu signal from the negative control measures the tendency of the Lu−biotin polypeptide to bind nonspecifically to the BSA-labeled particles. These results demonstrate a significant improvement in the extent of bioconjugation to our particles following the construction of a PGMA shell.

In a second set of experiments, we pretreated the PGMA-coated particles with EDC prior to exposure to NeutrAvidin. We denote these particles as D5(GMA-2)-EDC-NA in Table 5. While this experiment was designed as a kind of control, since one would not expect the hydrolysis of surface epoxy groups[55] in water to form carboxylic acids, we in fact found a 50% increase in the measured number of reporter Lu ions per particle. The data in Table 5 show not only the 50% increase in Lu signal compared to the particles reacted with NeutrAvidin without treatment with EDC but also that the Neut/BSA ratio increased from 10.4 to 15.1, indicating a further reduction in nonspecific protein binding. The Lu content distribution for D5(GMA-2)-EDC-NA is shown in Figure 6C (green curve); the distribution is monomodal and narrow (CV_{Lu} = 27%) with a significantly greater number of Lu ions per particle than seen in the data for nonspecific reporter-group binding (red curve). These results suggest that there are carboxylic acid groups present on the surface of our PGMA-coated particles, which are accessible to activation with EDC and subsequent reaction with NeutrAvidin. While we have no unambiguous explanation for this result, it may be that some of the −COOH groups present on the surface of the seed D1 particles migrated to the surface of the particles during the polymerization with GMA.

We compare the results for bioconjugation of NeutrAvidin to PGMA-coated particles presented here to the results obtained with the Ln-encoded PS particles mentioned above that were prepared by a combination of surfactant-free emulsion polymerization and seeded emulsion polymerization.[30] The acid groups in these particles come from the ACVA initiator, which generates particles with an even higher titratable surface acid concentration than present in D1. In Table 5 we compare bioconjugation data from ref 30 for the covalent attachment of NeutrAvidin to these particles (denoted ST120) with data obtained here for PGMA-coated particles. Significantly more conjugation of NeutrAvidin to the surface of PGMA-coated particles was measured relative to that found for sample ST120, at an equivalent Neut/BSA ratio. The data in Table 5 show that an average of 7×10^5 Lu ions were detected per particle, indicating effective reporter group binding and effective covalent attachment of NeturAvidin to the particles. Thus, sample D5(GMA-2)-EDC-NA represents the best demonstration of surface bioconjugation to date.

Particle Stability upon Storage. In the past, we have carried out extensive studies of particle stability toward ion leakage for Ln-encoded PS particles similar to sample D1. By centrifuging samples and analyzing the supernatant by ICP-MS, we showed that less than 0.1% of Ln ions were lost for samples stored in aqueous buffer solutions with pH values ranging from 3 to

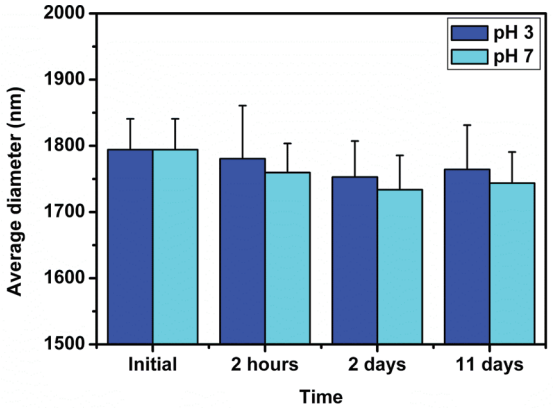


Figure 7. Average particle size (determined by SEM) for samples of D5 (GMA-4) exposed to buffers of 50 mM sodium acetate/acetic acid buffer, pH 3, and 10 mM ammonium acetate buffer, pH 7, for different lengths of time.

10.[29,30,38] Here we carried out a small number of similar experiments on the PGMA-coated particles to see if coating the particles might somehow and unexpectedly lead to an increase in the rate of loss of Ln ions buried in the PS core of the precursor particles. No such increase was found.

The PGMA-coated particles described above maintained their colloidal stability following centrifugation and redispersion and under the conditions used to attach NeutrAvidin to the PGMA-coated PS particles. The particles also remained colloidally stable during long-term (months) storage in deionized water. There was also no evidence for particle aggregation or coagulation under the conditions used to test the chemical stability of the particles in the presence of buffer. These experiments were carried out on magnetically stirred solutions of particles from sample D5-(GMA-4) diluted to 0.5 wt % with 50 mM sodium acetate/acetic acid buffer at pH 3 and 10 mM ammonium acetate buffer at pH 7.

Changes in particle morphology under these conditions were examined by SEM. Aliquots from these samples were taken at short (2 h), intermediate (2 days), and long (11 days) exposure times, followed by SEM image analysis. The average particle diameters measured under these conditions are presented in Figure 7. We observe a slight decrease in particle size from 1.79 to 1.74 μm, with the coefficient of variation remaining between 3 and 3.5%. The original PGMA shell in sample D5(GMA-4) was measured to be ~95 nm thick; thus, this change in dimensions likely reflects partial degradation of the PGMA shell during storage under the conditions tested. Some of the particles in the sample exhibited distorted shapes, as can be seen in the SEM images in Figure 8. These changes were most prominent in particles exposed to pH 3 and even after only 2 days storage (Figure 8B) showed some flattened particles that we refer to as pancakes. As seen in Figure 8C, these features are particularly prominent in the acid-treated particles after 11 days. At this time, some of the particles exposed to ammonium acetate also show signs of shape deformation. These changes are almost certainly related to hydrolytic degradation of the PGMA layer, through reaction with acid at pH 3 and with ammonia at pH 7 in the ammonium acetate buffer.[55]

There is less indication of hydrolytic instability of particles stored in deionized water. While epoxy groups are known to undergo slow hydrolysis in neutral water,[55] we found that the particles could be stored in water at room temperature for several weeks without interfering with surface reactivity. Indeed, the experiments described above on the attachment of NeutrAvidin to PGMA-coated particles were carried out on particles stored in unbuffered deionized water for 2 weeks prior to reaction with the protein.

SUMMARY

Previous experiments reported by our laboratory described the synthesis of polystyrene microparticles encoded with lanthanide ions, designed to be used in multiplexed bead-based assays with detection by mass cytometry. The PS particles were synthesized by two-stage dispersion copolymerization of styrene, acrylic acid, and lanthanide salts in ethanol in the presence of poly(*N*-vinylpyrrolidone) (PVP) as a polymeric stabilizer. These particles were characterized by a narrow particle size distribution and a controllable number, ranging from 10^5 to 10^8, of Ln ions per particle. Although surface titration of these particles in water showed the presence of substantial numbers of surface carboxylic acid groups (ca. 10^9 per particle), covalent coupling of bioaffinity agents to the particle surface was inefficient. It appears that the water-swollen PVP corona grafted to the PS particle surface, which likely extends a few nanometers into the aqueous medium, screens the surface against the approach by molecules as large as antibodies and NeutrAvidin to the PS surface, where the −COOH groups are located. Thus, only low levels of biofunctionalization could be detected.

In this paper, we described three strategies designed to overcome this problem. In the first strategy, we carried out new particle syntheses using a mixture of PVP and partially hydrolyzed PVP as the polymeric stabilizer. Ring-opening hydrolysis of PVP followed by methylation of the secondary amino group produced a polymer with pendant carboxylic acid groups. If these −COOH groups were located at the edge of the corona, they should be accessible for attaching biomolecules such as NeutrAvidin. The particle syntheses were successful in that PS particles with a narrow size distribution were obtained. The decrease in particle size in the presence of the partially hydrolyzed polymer indicates that these polymer molecules are more reactive toward grafting than PVP itself: more nuclei were formed, leading to a larger number of smaller particles. Bioconjugation experiments, however, demonstrated that the attachment of NeutrAvidin was only marginally improved by the presence of reactive stabilizers, This result suggested that the −COOH groups on the partially hydrolyzed PVP are located close to the inner PS surface of the particles and are not accessible for the covalent attachment of biomolecules.

In the second approach, we used Ln-encoded PS microparticles obtained by 2-DisP as seeds for second-stage emulsion polymerization with methacrylic acid (MAA). The particles were first swollen with MAA, and then polymerization was initiated with potassium persulfate. After purification by centrifugation−resuspension to remove PMAA homopolymer, we could show that the particles remained narrow in size distribution, increased in mass by 13%, and the number of titratable acid groups had doubled to 2.1×10^9 acid groups per particle. Nevertheless, we had difficulty in coupling significant amounts of NeutrAvidin to the particle surface via EDC chemistry.

In the third approach, we used the same Ln-encoded PS particles as seeds for emulsion polymerization of glycidyl methacrylate (GMA), with the idea of coating the particles with a layer of PGMA. Polymerization in the presence of excess surfactant led

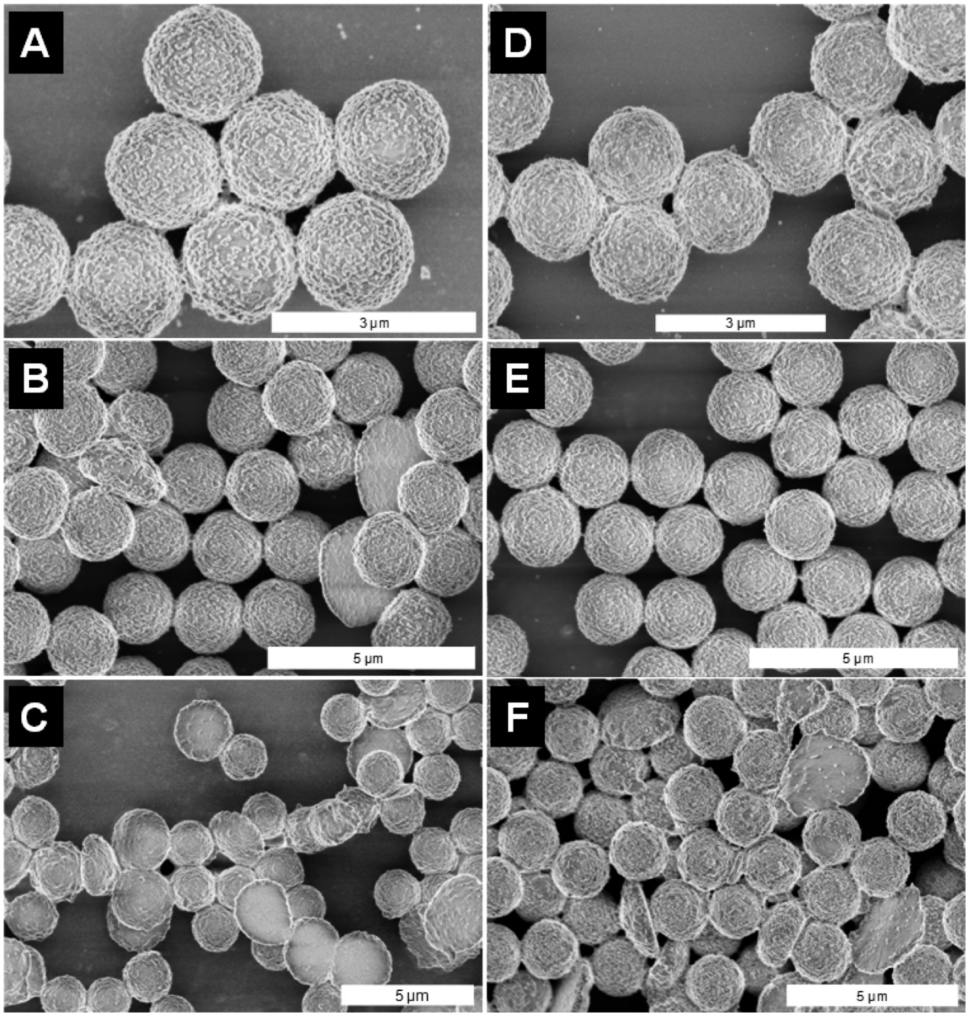


Figure 8. SEM images from stability experiments where samples of D5(GMA-4) were exposed to buffered solutions of different pH for differing periods of time; pH 3 (A, B, and C) and pH 7 (D, E, and F). A and D are D5(GMA-4) samples after 2 h. B and E are D5(GMA-4) samples after 2 days. C and F are D5(GMA-4) samples after 11 days.

to a roughened particle morphology in which a shell of PGMA ca. 60−100 nm thick was deposited onto the surface of the seed particles. These PGMA particles were stable to centrifugation and redispersion into water. The PGMA provided reactive sites to attach NeutrAvidin directly to the particle surface via the reaction of the epoxide group with primary amine residues. As a negative control, BSA was used in place of NeutrAvidin.

The extent of coupling was tested with a biotin−polypeptide−Lu reporter probe and monitored by mass cytometry. For the direct labeling of the particles with NeutrAvidin, we detected an average of 4.9×10^5 Lu ions per particle, more than 8 times greater than for the seed particles themselves. More importantly, the enhanced signal was accompanied by a substantial reduction in nonspecific binding. Taking the Lu signal for the BSA-functionalized beads as a measure of nonspecific binding, we found a Neut/BSA ratio of 10 for the GMA particles, nearly an order of magnitude improvement on the seed particles themselves. Further increases to the amount of bioconjugation were achieved when EDC was used as an activating agent, indicating that carboxylic acid groups are also present at the particle surface. For these particles, we found an average of 7×10^5 Lu ions per particle, with a Neut/BSA ratio of 15.

In previous work,[29] we have shown that two-stage dispersion copolymerization for the synthesis of PS particles provides excellent control over particle size, size distribution, and Ln ion content. Improvements were needed to enhance the biofunctionalization of these particles. In this paper, we have shown that the formation of PGMA shells by seeded emulsion polymerization is a very promising approach for solving this problem. The incorporation of PGMA onto the surface of our particles increased the amount of protein conjugation by a factor of 10 or more relative to the original particles, while retaining particle colloidal stability and a narrow particle size distribution. Given the ability to resolve numerous metal tags simultaneously, we believe that this methodology is a viable route for the development of particles for use in multiplexed bioassays based on mass cytometry detection.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Structure of the Lu biotin-conjugated reporter, the titration curve of activated PVP55, the Tb content of D2($PVP_{1.6}$) and of D3($PVP_{3.2}$), the mass cytometry results for bioconjugation of NeutrAvidin to D2($PVP_{1.6}$),

and a SEM image of D5(GMA-1) particles. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: mwinnik@chem.utoronto.ca.

Author Contributions

§These authors contributed equally to this work.

ACKNOWLEDGMENT

The authors thank NSERC Canada, the National Institutes of Health (R01-GM076127), DVS Sciences Inc., the Province of Ontario, and Amgen for their support of this research.

REFERENCES

(1) Ouyang, J.; Chu, C. W.; Szmanda, C. R.; Ma, L.; Yang, Y. *Nature Mater.* **2004**, *3*, 918.
(2) Sulitzky, C.; Rückert, B.; Hall, A. J.; Lanza, F.; Unger, K.; Sellergren, B. *Macromolecules* **2002**, *35*, 79.
(3) Feng, J.; Winnik, M. A.; Shivers, R. R.; Clubb, B. *Macromolecules* **1995**, *28*, 7671.
(4) Klapper, M.; Clark, C. G.; Mullen, K. *Polym. Int.* **2008**, *57*, 181.
(5) Berber, H.; Alpdoan, G.; Asci, B.; Yildirim, H.; Sungur, S. *Anal. Lett.* **2010**, *43*, 2331.
(6) Elimelech, H.; Nedelec, J. M.; Hardy-Dessources, A.; Babonneau, F.; Avnir, D. *J. Mater. Chem.* **2010**, *20*, 9515.
(7) Eldering, J. A.; Dulat, A.; Zimmerman, R.; Gupta, V. *Diabetes* **2010**, *59*, A474.
(8) Aytur, T.; Foley, J.; Anwar, M.; Boser, B.; Harris, E.; Beatty, P. R. *J. Immunol. Methods* **2006**, *314*, 21.
(9) Bombalski, L.; Min, K.; Dong, H. C.; Tang, C. B.; Matyjaszewski, K. *Macromolecules* **2007**, *40*, 7429.
(10) Fan, A. P.; Lau, C. W.; Lu, J. Z. *Anal. Chem.* **2005**, *77*, 3238.
(11) Hsu, C. C.; Wobus, C. E.; Steffen, E. K.; Riley, L. K.; Livingston, R. S. *Clin. Diagn. Lab. Immunol.* **2005**, *12*, 1145.
(12) McHugh, T. M.; Miner, R. C.; Logan, L. H.; Stites, D. P. *J. Clin. Microbiol.* **1988**, *26*, 1957.
(13) Pavlov, I.; Martins, T. B.; Delgado, J. C. *Clin. Vaccine Immunol.* **2009**, *16*, 1327.
(14) Pickering, J. W.; Martins, T. B.; Greer, R. W.; Schroder, M. C.; Astill, M. E.; Litwin, C. M.; Hildreth, S. W.; Hill, H. R. *Am. J. Clin. Pathol.* **2002**, *117*, 589.
(15) Sato, K.; Tokeshi, M.; Kimura, H.; Kitamori, T. *Anal. Chem.* **2001**, *73*, 1213.
(16) Sun, M.; Manolopoulou, J.; Spyroglou, A.; Beuschlein, F.; Hantel, C.; Wu, Z.; Bielohuby, M.; Hoeflich, A.; Liu, C.; Bidlingmaier, M. *Steroids* **2010**, *75*, 1089.
(17) Tang, D. P.; Yu, Y. L.; Niessner, R.; Miro, M.; Knopp, D. *Analyst* **2010**, *135*, 2661.
(18) Volper, E. M.; Olekszak, H.; Cropp, B.; Meeks, J.; Johnson, A. J.; Imrie, A.; Gubler, D. J.; Nerurkar, V. R. *Am. J. Trop. Med. Hyg.* **2009**, *81*, 91.
(19) Xia, H.; Liu, L.; Nordengrahn, A.; Kiss, I.; Merza, M.; Eriksson, R.; Blomberg, J.; Belak, S. *J. Virol. Methods* **2010**, *168*, 18.
(20) Yu, X.; Hartmann, M.; Wang, Q.; Poetz, O.; Schneiderhan-Marra, N.; Stoll, D.; Kazmaier, C.; Joos, T. O. *PLoS One* **2010**, 5.
(21) Hennig, A.; Bakirci, H.; Nau, W. M. *Nature Methods* **2007**, *4*, 629.
(22) Narayanan, N.; Patonay, G. *J. Org. Chem.* **1995**, *60*, 2391.
(23) Bruchez, M., Jr; Moronne, M.; Gin, P.; Weiss, S.; Alivisatos, A. P. *Science* **1998**, *281*, 2013.
(24) Lin, C. A. J.; Liedl, T.; Sperling, R. A.; Fernández-Argüelles, M. T.; Costa-Fernández, J. M.; Pereiro, R.; Sanz-Medel, A.; Chang, W. H.; Parak, W. J. *J. Mater. Chem.* **2007**, *17*, 1343.
(25) Sen, D. J.; Prajapati, M. K.; Prajapati, P. M. *Int. J. Drug Dev. Res.* **2010**, *2*, 164.
(26) Grabar, K. C.; Griffith Freeman, R.; Hommer, M. B.; Natan, M. J. *Anal. Chem.* **1995**, *67*, 735.
(27) Moskovits, M. *Rev. Mod. Phys.* **1985**, *57*, 783.
(28) Nie, S.; Emory, S. R. *Science* **1997**, *275*, 1102.
(29) Abdelrahman, A. I.; Dai, S.; Thickett, S. C.; Ornatsky, O.; Bandura, D.; Baranov, V.; Winnik, M. A. *J. Am. Chem. Soc.* **2009**, *131*, 15276.
(30) Thickett, S. C.; Abdelrahman, A. I.; Ornatsky, O.; Bandura, D.; Baranov, V.; Winnik, M. A. *J. Anal. At. Spectrom.* **2010**, *25*, 269.
(31) Vancaeyzeele, C.; Ornatsky, O.; Baranov, V.; Shen, L.; Abdelrahman, A.; Winnik, M. A. *J. Am. Chem. Soc.* **2007**, *129*, 13653.
(32) Grabarek, Z.; Gergely, J. *Anal. Biochem.* **1990**, *185*, 131.
(33) Hermanson, G. T. *Bioconjugate Techniques*, 2nd ed.; Academic Press: San Diego, 2008.
(34) Horák, D.; Karpíšek, M.; Turková, J.; Beneš, M. *Biotechnol. Prog.* **1999**, *15*, 208.
(35) Miron, T.; Wilchek, M. *Bioconjugate Chem.* **1993**, *4*, 568.
(36) Staros, J. V.; Wright, R. W.; Swingle, D. M. *Anal. Biochem.* **1986**, *156*, 220.
(37) Borque, L.; Maside, C.; Rus, A.; Del Cura, J. *J. Clin. Immunoassay* **1994**, *17*, 160.
(38) Abdelrahman, A. I.; Ornatsky, O.; Bandura, D.; Baranov, V.; Kinach, R.; Dai, S.; Thickett, S. C.; Tanner, S.; Winnik, M. A. *J. Anal. At. Spectrom.* **2010**, *25*, 260.
(39) Bandura, D. R.; Baranov, V. I.; Ornatsky, O. I.; Antonov, A.; Kinach, R.; Lou, X.; Pavlov, S.; Vorobiev, S.; Dick, J. E.; Tanner, S. D. *Anal. Chem.* **2009**, *81*, 6813.
(40) Wu, D.; Zhao, B.; Dai, Z.; Qin, J.; Lin, B. *Lab-on-a-Chip* **2006**, *6*, 942.
(41) Zhang, H.; Huang, H.; Sun, R.; Huang, J.-X. *J. Appl. Polym. Sci.* **2006**, *99*, 3586.
(42) Omer-Mizrahi, M.; Margel, S. *J. Colloid Interface Sci.* **2009**, *329*, 228.
(43) Alam, M. A.; Miah, M. A. J.; Ahmad, H. *Polym. Adv. Technol.* **2008**, *19*, 181.
(44) Ahmad, H.; Miah, M. A. J.; Rahman, M. M. *Colloid Polym. Sci.* **2003**, *281*, 988.
(45) Von Specht, B. U.; Seinfeld, H.; Brendel, W. *Hoppe-Seyler's Z. Physiol. Chem.* **1973**, *354*, 1659.
(46) Frank, H. P. *J. Polym. Sci.* **1954**, *12*, 565.
(47) Kawaguchi, S.; Yekta, A.; Winnik, M. A. *J. Colloid Interface Sci.* **1995**, *176*, 362.
(48) Rasband, W. S. National Institutes of Health, Bethesda, MD, 1997−2008; http://rsb.info.nih.gov/ij/.
(49) Hong, C. Y.; Pan, C. Y. *Macromolecules* **2006**, *39*, 3517.
(50) Sankaran, N. B.; Rys, A. Z.; Nassif, R.; Nayak, M. K.; Metera, K.; Chen, B. Z.; Bazzi, H. S.; Sleiman, H. F. *Macromolecules* **2010**, *43*, 5530.
(51) Weber, P. C.; Ohlendorf, D. H.; Wendoloski, J. J.; Salemme, F. R. *Science* **1989**, *243*, 85.
(52) Rios, L.; Hidalgo, M.; Cavaille, J. Y.; Guillot, J.; Guyot, A.; Pichot, C. *Colloid Polym. Sci.* **1991**, *269*, 812.
(53) Lathia, U. S.; Ornatsky, O.; Baranov, V.; Nitz, M. *Anal. Biochem.* **2010**, *398*, 93.
(54) Azizi, N.; Saidi, M. R. *Org. Lett.* **2005**, *7*, 3649.
(55) Kalal, J.; Svec, F.; Marousek, V. *J. Polym. Sci., Part C: Polym. Symp.* **1974**, 155.