SUPPORTING INFORMATION

Lanthanide-Containing Polymer Microspheres by Multiple-Stage Dispersion Polymerization for Highly Multiplexed Bioassays

Ahmed I. Abdelrahman, Sheng Dai, Stuart C. Thickett, Olga Ornatsky, Dmitry Bandura,
Vladimir Baranov and Mitchell A. Winnik*

Department of Chemistry, University of Toronto, 80 St George Street Toronto ON
M5S3H6, Canada

S1. EXPERIMENTAL SECTION

Materials.

Styrene (Aldrich), acrylic acid (AA, Aldrich), acetoacetyethyl methacrylate (AAEM, Aldrich) absolute ethanol, polyvinylpyrrolidone (PVP) (Aldrich, PVP55, M = 55,000), Triton-X305 (TX305, 70% solution in water, Aldrich), 2,2'-azobis(2-methylbutyronitrile) (AMBN, Dupont USA), ethylene glycol dimethacrylate (EGDMA, Aldrich), bovine serum albumin (BSA, Sigma), mouse IgG, anti-mouse IgG (BD Biosciences), Polylink Coupling kit for COOH Microspheres (Polysciences, Inc.), 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), praseodymium(III) chloride hexahydrate (PrCl_3 , Aldrich) were used without further purification. Avidin labeled with fluorescein isothiocyanate (FITC-Avidin), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Fluka, USA and used as received. Water was purified through a MilliQ purification system. High purity HCl for ICP-MS analysis was purchased from Seastar Chemical Inc.

The anti-mouse IgG was modified by covalent attachment of a metal chelating polymer followed by incorporation of Pr ions as described by Lou et al.¹ We refer to this sample as anti-mouse IgG-X4-Pr.

Two and Three-Stage Dispersion Polymerizations.

The standard recipe for the two-stage dispersion polymerization (2-DisP) of styrene with AAEM or AA in ethanol is listed in Table 1 in the main text. The following procedure was used: All of the stabilizer (PVP), the co-stabilizer (TX305), initiator (AMBN), and the styrene monomer and half of the ethanol were added to a 250 mL three-neck reaction flask equipped with a condenser, a mechanical stirrer and a gas inlet. After a homogeneous solution formed at room temperature, the solution was deoxygenated by purging with ethanol-saturated nitrogen gas at room temperature for 30 min. The flask was then placed in a 70 °C oil bath and stirred mechanically at 100 rpm with an overhead stirring bar connected to a (PTFE) half-moon stirring paddle. The AA (or AAEM) and the lanthanide salt(s) (LnCl_3) were dissolved in the remaining ethanol at 70 °C under nitrogen. After the polymerization reaction had run for 1 h, the hot LnCl_3 plus AA (or AAEM) solution in the remaining ethanol was added to the reaction flask. The amount of lanthanide salts added for each sample is listed in Table 2. The reaction was continued for 24 h. Final conversion was approximately 95-99% as determined gravimetrically. In some experiments (see Table 1) additional styrene was added along with lanthanide salts and AAEM or AA in the second stage of the reaction.

For sample AA105, a mixture of additional AA and a small amount of crosslinking agent, EGDMA, in 10 ml ethanol were heated to 70 °C and added to the reaction after the polymerization reaction had run for 9 hr (ca. 60 % styrene conversion). This can be considered to be a three-stage dispersion polymerization reaction (3-DisP).

Characterization of Particle Sizes and Size Distributions

The microsphere diameters and diameter distributions were determined from micrographs obtained with a Hitachi S-5200 FE-scanning electron microscope (SEM). The samples for SEM imaging were prepared by placing a drop of diluted suspension on a Formvar/carbon coated-300 mesh copper grid. There was no need to purify the microspheres by centrifugation and resuspension in alcohol to obtain clear images. A

particle-size histogram was constructed from measurements of at least 200 individual microspheres for each sample.

Lanthanide Incorporation and Ln Ion Release.

The lanthanide ion content of each sample was measured by FC-MS: flow cytometry-time-of-flight inductively coupled plasma mass spectrometry (ICP-MS). Data reported here refer to analysis of 10,000 to 20,000 individual particles.

The lanthanide ion content of the serum phase was measured by traditional ICP-MS. To monitor the residual ion content following particle synthesis, the PS beads were sedimented by centrifugation at 5000 rpm, and the supernatant was collected and diluted with 3.0 % HCl before injection into the ICP-MS. To test for release of Ln ions from the particles into aqueous media, samples of the particles were prepared in different buffer solutions, namely 10 mM ammonium acetate (pH 7.0), 50 mM sodium acetate (pH 3.0) and sodium carbonate/bicarbonate 200mM (pH 10.6) solutions. The microspheres were first washed by 3 centrifugation-resuspension steps using deionized water and then resuspended in the buffer solutions (solids content 0.5 wt %). Aliquots were taken at regular time intervals over a three-week period and spun down. The supernatant was then subjected to ICP-MS analysis.

Bioconjugation.

Standard conditions were employed to conjugate mouse IgG to the bead surface using water-soluble carbodiimide chemistry.² The lanthanide encoded microspheres were first washed twice in cold Polylink Coupling buffer (50 mM MES, pH5.2) by low speed centrifugation (1000 x g, 10 min). Freshly prepared heterobifunctional cross-linker EDC solution (0.2 g/ml 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride in MES) was then mixed with the bead suspension, followed by gentle vortexing. EDC-activated microspheres were then split into two tubes labeled “IgG” and “BSA”. An aliquot of mouse IgG/PBS (2 μ L, 18 μ g) was added to the tube labeled “IgG”, and an aliquot of 0.5% BSA/PBS (4 μ L, 20 μ g) was added to the tube labeled “BSA”. Both sets of samples were mixed gently and left to react on a shaker for 2 h at room temperature and then washed. Microspheres were then blocked in 0.5% BSA/PBS buffer for 1 h prior to addition of 100 μ g/ml anti-mouse IgG-X4-Pr as the test-antigen reporter to be captured

by the mouse IgG coated microspheres. Non-specific binding was tested by adding similar amounts of anti-mouse IgG-X4-Pr to PS microspheres without bioconjugation or to BSA coated PS microspheres. After several washes, the microspheres were subjected to bead-by-bead FC-MS analysis for their Ln content as an indication of a successful immunoreaction. Between 30,000 and 60,000 microspheres were analyzed.

The validity of the FC-MS measurement was examined by a similar assay employing fluorimetry in which the same microsphere sample (AA105) was conjugated to FITC-labeled avidin using carbodiimide chemistry. 1.5 mg of washed microspheres were mixed with 50 μ l of 0.02 w% EDC and 0.03w% NHS in 7.4 phosphate buffer solution and left for 15 min in the dark. Then 20 μ g FITC-labeled avidin was added and left to react 2.0 h in dark. After 6 washing cycles in PBS, the microspheres were subjected to fluorescence measurement (λ_{ex} 485 nm) using front face fluorescence spectra were recorded with a SPEX Fluorolog 3 spectrometer (Jobin Yvon/SPEX, Edison, New Jersey) in a 1 cm quartz cuvette.

Instrumentation.

FC-MS. Mass cytometry experiments were carried out using a model C2 instrument (CyTOF™) from DVS Sciences (Toronto ON Canada, www.dvssciences.com). The instrument has an inductively coupled plasma source. Time-of-flight mass resolving technology provides high spectrum generation frequency of 76.8 kHz (normally) and the capability of collecting multiple spectra from each particle-induced transient ion cloud, typically of 200-300 microseconds duration with a mass resolution of 800. The transients can be resolved and characterized individually at a peak frequency of 1100 particles per second. A fast TOF ion detector (Model 14882, ETP Electron Multipliers, SGE International Pty. Ltd., Ringwood, Victoria, Australia) is used for ion detection.

ADC data from the PDA1000 digitizer (Signatec, Inc., Newport Beach, CA, USA) used in two ways: to provide pseudo-ion counting mode for low intensity signals (counting mode) and analog data collected for high intensity signals (analog mode). In counting mode, distinct peaks from single ions within each m/z time-of-flight interval are detected separately in each single push-out spectrum. Detector is calibrated employing the best linear fit correlation between the analog and pseudo-counting signals.

The figures of merit of the instrument are measured under standard ICP operating conditions (< 3 % oxide ratio). At mass resolution (full width at half maximum) for $m/z = 159$ $M/\Delta M > 900$, sensitivity with a standard sample aspiration is 1.4×10^8 ion counts per second per mgL^{-1} of Tb typically, and abundance sensitivity $6 \times 10^{-4} - 1.4 \times 10^{-3}$ (trailing and leading masses, respectively). The mass range (variable, but fixed at $m/z = 125 - 215$ for this work) and the abundance sensitivity are sufficient for elemental encoding with up to 60 distinct isotopes.

More details about this instrument can be found in refs.3-5.

ICP-MS. Sample analyses by traditional ICP-MS employed an ELAN DRC^{Plus} instrument (Perkin-Elmer SCIEX). Typical operating conditions of the instrument are based on a stable Ar plasma optimized to provide less than a 3% CeO^+/Ce^+ ratio in a 1 ppb standard multielement solution diluted in 10% 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 nebulizer (Burgener Micromist or MicroFlow PFA-ST concentric nebulizers) Ar flow. Under these operating conditions, the typical sensitivity is 4×10^4 cps for 1 ppb Ir standard solution in 10% HCl. The detection limits for lanthanide elements were less than 1 ppt. The sample uptake rate was adjusted depending on the particular experiment and sample size, typically 100 $\mu\text{L}/\text{min}$. 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 $\mu\text{g}/\text{mL}$ PE Pure Single-Element Standard solutions (Perkin-Elmer, Shelton, CT) by sequential dilution with high-purity deionized water (DIW) produced using an Elix/Gradient (Millipore, Bedford, MA) water purification system.

Figures S1 and S2 present scanning electron microscope (SEM) images of particle samples AA050 (2-DisP, 4% AAEM, Eu) and AA105 (3-DisP, 4% AA, La, Tm)). Conductometric and potentiometric titration curves for sample AA087 are presented in Figure S3.

Figure S1. SEM image of PS microspheres from sample AA050 synthesized in the presence of EuCl_3 (1.0 wt%/styrene) and AAEM (4.0 wt%/styrene). $d = 2.7 \mu\text{m}$, $\text{CV}_d = 1.5\%$. The scale bar is $5.0 \mu\text{m}$.

Figure S2. SEM image of sample AA105 microspheres synthesized in the presence of TmCl_3 (0.1 wt%/styrene) and LaCl_3 (0.1 wt%/styrene). $d = 2.2 \mu\text{m}$, $\text{CV}_d = 1.1\%$. The scale bar is $5.0 \mu\text{m}$

Figure S3. Potentiometric and conductometric titrations of AA087 microspheres synthesized by 2-DisP in presence of AA (2.0 wt%/styrene). The highlighted area corresponds to 1.6 μmol of HCl

S2. CALCULATION OF THE NUMBER OF METAL IONS PER MICROSPHERE FROM FC-MS DATA:

The mean number of metal ion per microsphere (N) can be calculated from the intensity value measured by the FC-MS detector according to the equation:

$$N = I \times I_F \times T$$

Where:

I is the mean intensity measured by the TOF detector

I_F is the analog-to-digital intensity factor for a particular ion (= 0.045 for Eu)

T is the transmission coefficient of the detector (the number of ions that reach the detector per number of ions injected) = 3.86×10^{-5} for the examples given here

Table S1.

Number of Metal Ions per Microsphere determined for PS bead samples AA050

(AAEM), AA068 (AAEM) and AA069 (acrylic acid)

Sample	I	N (10^6 Eu ions)
AA050	6940	8.1
AA068	15108	17.6
AA069	90591	106.0

S3. PS PARTICLES FOR BINARY ENCODING

The suite of binary encoded PS microspheres that we synthesized and characterized is presented below in Table S2. Experimental results for these five different particle samples are presented in Figure S2. These particles contain some combination of La, Tb, Ho, and Tm. The uppermost pair of particles registers the presence of La, Tb and Tm, but not Ho. The next particle contains all four elements. The next particle contains La, Tb, and Ho. The last sample at the bottom of the figure contains La, Ho, and Tm, but not Tb.

Figure S4. Detection of individual element-encoded microspheres by FC-MS. Inset table: determination of the “*encoding number*” of the microspheres whose stacked mass spectra are shown. The encoding number is obtained by assigning arbitrarily binary (0-not detected, 1-detected) values for La (2^3), Tb (2^2), Ho (2^1), and Tm(2^0). For example, the code for AA090 is assigned to be: $2^3 + 2^2 + 0 + 2^0 = 13$, denoted #13.

Our bead recognition/decoding algorithm selects only transient mass spectra containing a bead (indicated as grey horizontal rows to the right of each set of spectra). The data within these bands are then interpreted with a *binary encoding algorithm* to assign numerical values to each type of bead in the set. The algorithm is code based on either the presence (level = 1) or absence (level = 0) of a particular metal. We are able to distinguish the presence of all four elements (see Figure 4). This allows us to construct a binary code of $2^4 - 1 = 15$ unique microspheres.

Table S2. Microbeads of the *Binary* Encoded Library containing monoisotopic-lanthanides synthesized by two-stage dispersion polymerization in the presence of 2 wt%/sty acrylic acid.

	LnCl₃ wt%/styrene			
	Tb	Ho	La	Tm
AA083	0.1	-	-	-
AA084		0.1	-	-
AA085	-	-	0.1	
AA086	-	-	-	0.1
AA087	0.1	0.1	0.1	0.1
AA088	0.1	0.1	0.1	-
AA089	0.1	0.1	-	0.1
AA090	0.1	-	0.1	0.1
AA091	-	0.1	0.1	0.1

S4. RESOLVING POWER OF MASS CYTOMETRY

In order to demonstrate the ability of mass cytometry to resolve multiple isotopes and measure their abundance, we present a mass spectrum in Figure S5 of a mixture of 14 ions (La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb and Lu) in a solution containing 20 pg/mL of each element in 3% HCl.

Figure S5. A typical mass spectrum integrated from mass cytometry measurements on a solution containing 20 pg/mL in 3% HCl of each of La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb and Lu ions at natural isotopic abundance. The spectrum shown was accumulated over a 0.52 s integration time (40,000 single push-out spectra). Only the most abundant isotopes are identified in the spectrum. The ^{136}Xe detected is from the plasma gas. This figure is reproduced from ref. 3.

S5- DISTRIBUTION OF FC-MS SIGNAL INTENSITY FOR AA105

The signal intensity was measured by mass cytometry for a population of ca. 20,000 AA105 microspheres. The signal intensity distribution for ^{139}La and for ^{169}Tm ions is presented in Figure S5. This sample was prepared by 3-stage dispersion polymerization as described in the main text in the presence of AA (4.0 wt%/styrene), EGDMA (1.0 wt%/styrene), $\text{LaCl}_3 \cdot 6\text{H}_2\text{O}$ (0.1 wt%/styrene) and $\text{TmCl}_3 \cdot 6\text{H}_2\text{O}$ (0.1 wt%/styrene)

Figure S6. Signal intensity distribution for A) ^{139}La and B) for ^{169}Tm for sample AA105. this sample is characterized by $\text{CV}_{\text{La}} = 13\%$ and $\text{CV}_{\text{Tm}} = 11\%$.

S6. BIOCONJUGATION EXPERIMENTS

Attempts to conjugate proteins to the surface of particles prepared by 2-DisP were unsuccessful. Attempts to bind FITC-avidin to the surface of AA087 particles (data not shown) using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride in MES as described in the Experimental Section above gave particles with no significant fluorescein emission. Figure S6 presents results of an attempt to conjugate mouse IgG to sample AA069. Here anti-mouse-IgG-X4-Pr was used as a reporter tag. The figure compares the ^{141}Pr intensity signals for the interaction of the reporter tag with bare particles, with beads treated with EDC and then reacted with mouse IgG or with BSA. The ^{141}Pr signals were weak and indistinguishable for all three cases.

Figure S7. Mass cytometry analysis (^{141}Pr intensities) of the interaction of anti-mouse-IgG-X4-Pr with bare AA069 microbeads and with samples of these beads subjected to conjugation conditions with BSA and with mouse IgG.

To demonstrate the feasibility of bioconjugation to the surface of particles prepared by 3-DisP, we treated a sample of AA105 with FITC-avidin under EDC coupling conditions. Details are given in the Experimental section above. After 6 centrifugation-resuspension washing cycles with phosphate-buffered saline (PBS), the particle

suspension (ca 0.3 mg/mL) was examined for fluorescence using the “front-face” geometry of the spectrometer. The results are presented in Figure S7. Strong fluorescence from the fluorescein chromophore was observed from the beads, which was much stronger than background and also much stronger than emission from the supernatant obtained by resedimenting the microbeads. Thus bioconjugation to the surface of AA105 is possible.

Figure S8. Fluorescence spectra for AA105 microspheres modified with FITC-avidin (denoted “Beads”), the supernatant serum after centrifugation of the avidin-modified beads and the PBS used in the bioconjugation reaction

COMPLETE CITATION FOR REFERENCES 2 AND 8 OF THE MAIN TEXT

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