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## Synthesis of a Functional Metal-Chelating Polymer and Steps towards Quantitative Mass Cytometry Bioassays

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### Abstract

We describe the synthesis and characterization of metal-chelating polymers with a degree of polymerization of 67 and 79, high DTPA functionality,  $M_w/M_n = 1.17$ , and a maleimide as an orthogonal functional group for conjugation to antibodies. The polymeric disulfide form of the  $DP_n = 79$  DTPA polymer was analyzed by thermogravimetric analysis to determine moisture and sodium-ion content, and by isothermal titration calorimetry (ITC) to determine the  $Gd^{3+}$  binding capacity. These results showed each chain binds  $68 \pm 7 Gd^{3+}$  per chain. Secondary goat anti-mouse IgG was covalently labeled with the maleimide form of the DTPA polymer ( $DP_n = 79$ ) carrying  $^{159}Tb$ . Conventional ICP-MS analysis of this conjugate showed each antibody carried an average of  $161 \pm 4 ^{159}Tb$  atoms. This result was combined with the ITC result to show there are an average of  $2.4 \pm 0.3$  polymer chains attached to each antibody. Eleven monoclonal primary antibodies were labeled with different lanthanide isotopes using the same labeling methodology. Single cell analysis of whole umbilical cord blood stained with a mixture of 11 metal-tagged antibodies was performed by mass cytometry.

### Keywords

RAFT; Metal-Chelating Polymer; DTPA; Antibody-Polymer Conjugate; Mass Cytometry; Single Cell Analysis; Highly Multiplex Assay

## INTRODUCTION

One of the goals of modern bioanalytical chemistry is the simultaneous (multiplexed) detection of multiple biomarkers in individual cells. A biomarker can be broadly defined as a characteristic protein, gene, or small molecule that can be objectively measured and evaluated as an indicator of normal biological or pathogenic processes.<sup>1</sup> For example, tumor biomarkers contribute greatly to the selection of appropriate personalized cancer therapy in clinical trials. Immunophenotyping of blood biomarkers using flow cytometry has played an

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**SUPPORTING INFORMATION AVAILABLE.** Experimental procedure for polymer synthesis, table of  $^1H$  NMR  $T_1$  and  $T_2$  relaxation constants for all polymer samples ( $DP_n = 67$ ) analyzed by  $^1H$  NMR in this paper, table of  $^1H$  NMR end-group analysis and GPC data for all polymer samples, GPC chromatographs comparing PtBA-Trithiocarbonate and PtBA-Disulfide ( $DP_n = 67$ ),  $^1H$  NMR spectra of PtBA-Trithiocarbonate and PAA-Disulfide ( $DP_n = 67$ ) showing the calculation of  $DP_n$  by end-group analysis,  $^1H$  NMR spectra of Amino Polymer-Disulfide and P(DTPA)-Disulfide ( $DP_n = 67$ ) demonstrating the calculation of ethylenediamine and DTPA functionalization, GPC (Aqueous, RI) chromatographs comparing P(DTPA)-Disulfide and P(DTPA)-Maleimide ( $DP_n = 67$ ), thermogravimetric analysis results of disodium EDTA  $2H_2O$ , isothermal titration calorimetry thermogram and binding isotherm for the binding of  $Gd^{3+}$ -citrate with DTPA, antibody dilution series for all 11 tagged antibodies, and a description of clusters of differentiation (CD) and the rationale behind the gating strategy in Figure 4. This material is available free of charge via the Internet at <http://pubs.acs.org>.

important role in the diagnosis of leukemia subtypes and selection of therapy. It is well documented that tumor progression in breast, prostate, bladder, and blood cancers, to name a few, invokes changes in the types and numbers of biomarkers expressed at each stage of carcinogenesis.<sup>2,3,4</sup> Moreover, it is now widely accepted that no single biomarker will have the sensitivity and specificity necessary for diagnosis and disease prognosis when measured on its own. One needs a robust analytical technology capable of providing a simultaneous assay for a broad constellation of proteins, small molecules and gene transcripts.

One approach that lends itself to multiplexed analysis is based on the use of antibodies labeled with metal ions as bioaffinity agents in conjunction with inductively coupled mass spectrometry (ICP-MS) detection.<sup>5,6,7</sup> In ICP-MS, a sample is burned in a plasma torch at 7000K, which atomizes the sample and then ionizes all metals with quantitative efficiency. This technique is widely used for elemental analysis because of its large dynamic range and its ability to resolve individual masses. For immunoassays with metal-tagged antibodies, the lanthanide isotopes are particularly useful. These ions have similar chemistry, low natural abundance, and masses in a useful range ( $m/z$  from 100 to 200) for mass cytometry detection. The sensitivity of the method can be enhanced through attachment of metal-chelating polymers to antibodies. For example, we reported a 5-plex bulk immunoassay of three human leukemia cell lines by ICP-MS, using antibodies labeled with a polymer that carried on the order of 30 Ln ions per polymer chain.<sup>5</sup>

Much more powerful single cell analysis, with a high degree of multiplexing, is possible with the new technique of mass cytometry.<sup>8</sup> In this technique, cells are injected individually but stochastically into the argon plasma, where they are vaporized, atomized, and ionized. The ion cloud generated is analyzed by time-of-flight mass spectrometry, and the intensities of each ion are determined by averaging the 20 to 30 mass spectra taken during the 200  $\mu$ s that the ion cloud is sampled by the instrument. On the order of 1000 cells per second can be analyzed in this way. Prior to analysis, live cells are stained with a cocktail of antibodies, each type carrying a different lanthanide isotope. This approach identifies biomarkers on the cell surface. One can also examine intracellular antigens in cells that are fixed and permeabilized prior to treatment with the antibody cocktail.

The sensitivity of mass cytometry depends linearly on the number of lanthanide ions carried by each antibody. This in turn depends upon the number of lanthanide ions bound to the metal-chelating polymer, as well as the number of these polymers attached to each antibody. To increase the sensitivity and range of the methodology, it is important to increase the ion-carrying capacity of the metal-chelating polymers. One imagines that as the length of the polymer is increased, there is greater likelihood that the polymer could interfere with antibody-antigen recognition. Metal-chelating polymer synthesis for bioassays is a young field, however, and one lacks the knowledge necessary to design optimal polymers for this analytical technique.

In this paper, we describe the synthesis and characterization of a second generation metal-chelating polymer intended for antibody labeling. The requirement set for the polymer was to have a metal-binding ligand in essentially every repeat unit, to have a degree of polymerization of 60 or larger, thus increasing the number of metal binding sites per polymer chain, to obtain polymer molecules with a narrow distribution of lengths, and to maximize the yield of polymer with a suitable end functionality for antibody attachment. Radical addition-fragmentation chain transfer (RAFT) polymerization is a useful methodology to synthesize polymers of controlled length, narrow molar mass distribution, and with control over end groups. To meet our requirements, we explored the idea that RAFT polymerization of an inexpensive, commercially available monomer followed by pendant group modification in near quantitative yield would have several advantages. One

of the most important advantages is that the mean chain length and distribution can be characterized at this early stage of the synthesis, and that these characteristics would be largely preserved throughout the subsequent transformations.

One of the objectives of this paper is to describe the synthesis and pendant group transformations with an emphasis on analytical characterization of the polymer at each stage. The functional end group content was characterized by reaction of the free thiol, protected as a disulfide throughout the synthesis, with a bis-maleimide to introduce the reactive group for antibody modification. Isothermal titration microcalorimetry, using  $Gd^{3+}$  citrate as a probe, was employed to compare the number of lanthanide ions bound to the polymer to the number of diethylenetriamine pentacetic acid (DTPA) ligands determined by  $^1H$  NMR. Using goat anti-mouse IgG as a model, we used traditional ICP-MS to establish that antibody modification with the metal chelating polymer introduced an average of 2.4 metal-containing polymer molecules per antibody.

In addition, we describe an application of this metal-chelating polymer for antibody labeling and 11-plex single cell analysis using mass cytometry for the identification and abundance of the different cell populations in human umbilical cord blood.

## EXPERIMENTAL

### Synthesis

Details of polymer synthesis are presented in the Supporting Information.

### Instrumentation and Characterization

**Gel permeation chromatography**—The nominal molecular weights and polydispersities ( $PDI = M_w/M_n$ ) of poly(tert-butyl acrylate) (PtBA) samples was measured with a gel-permeation chromatograph (GPC) system equipped with a Viscotek VE 3219 UV/VIS detector (set to 310 nm), VE 3580 RI detector, and a Polymer Labs gel 5 micrometer Mixed-D (300\*7.5 mm) column and a gel 5 micrometer guard column (kept at room temperature). The flow rate was maintained at 0.6 mL/min using a Waters 515 HPLC Pump. Tetrahydrofuran (THF) was used as the eluent, and the system was calibrated with polystyrene standards.

The nominal molecular weights and polydispersities of all anionic, water-soluble samples were measured with a Viscotek gel-permeation chromatograph (GPC) equipped with a Viscotek VE3210 UV/VIS detector, VE3580 refractive index detector, and Viscotek ViscoGEL G4000PWXL and G2500PWXL columns (kept at 30 °C). The flow rate was maintained at 1.0 mL/min using a Viscotek VE1122 Solvent Delivery System and VE7510 GPC Degasser. An eluent of 0.2 M  $KNO_3$ , 200 ppm  $NaN_3$ , and 25 mM pH 8.5 phosphate buffer was used. The system was calibrated with poly(methacrylic acid) standards. Samples were dissolved in sodium bicarbonate/carbonate buffer (pH 9.4, 200 mM) prior to injection.

**$^1H$  NMR**— $^1H$  NMR (400 MHz) spectra were recorded on a Varian Hg 400 or a Varian 400 spectrometer with a 45° pulse width and at a temperature of 25° C. All small molecule samples as well as poly(tert-butyl acrylate) samples were dissolved in  $CDCl_3$ , with chemical shifts referenced to TMS at 0 ppm, or  $CD_2Cl_2$ , with chemical shifts referenced to the  $CH_2Cl_2$  peak at 5.320 ppm. All water-soluble polymers were dissolved in  $D_2O$ , with chemical shifts referenced to the HDO peak at 4.77 ppm.<sup>9</sup> Small molecules were analyzed with 64 transients and a delay time of 1 second. Polymers were analyzed with 512 transients and a delay time of 10 seconds.  $T_1$  and  $T_2$  relaxation constants were measured for the  $DP_n = 67$  PtBA-Trithiocarbonate, PAA-Disulfide, Amino Polymer-Disulfide, P(DTPA)-Disulfide, and P(DTPA)-Maleimide samples by the standard  $T_1$  inversion recovery and  $T_2$  spin-echo

sequences included with the Varian 400 spectrometer (Supporting Information, Table S1). For P(DTPA)-Maleimide, the maleimide signal (6.88 ppm) had a  $T_1$  relaxation constant of 4.38 s; thus a delay time of 25 s ( $5 \times T_1$ ) is in principle necessary to allow full relaxation. However, spectra collected with delay times of 10 s (512 transients) and 25 s (384 transients) showed no difference in relative integration between the maleimide and phenyl RAFT agent signals. All other signals had  $T_1$  relaxation constants  $\approx 2$  s. Thus a delay time of 10 s was chosen. We assume an inherent  $\pm 5\%$  error in the integration values from all  $^1\text{H}$  NMR measurements.

**Thermogravimetric Analysis (TGA)**—TGA measurements were performed on a TA SDT Q600 instrument. The ceramic sample and reference cups were extensively cleaned with a Bunsen burner prior to every run. Once cooled, the cups were placed into the instrument, the instrument's balance was tared, sample (6–10 mg) was added to the sample cup, and the sample analysis was initialized. Data was collected from 22 to 800 °C at a constant heating rate of 1 °C/min, except for the  $\text{Na}_2\text{CO}_3$  sample which was run at a rate of 10 °C/min. All analyses were run under a stream of air.

**Isothermal Titration Calorimetry (ITC)**—The lanthanide ion binding capacity of the DTPA-containing polymers was determined via isothermal titration calorimetry using  $\text{Gd}^{3+}$  as a probe following an approach slightly modified from that reported for characterizing DTPA groups attached to polysaccharides.<sup>10, 11</sup> For this purpose, P(DTPA)-Disulfide (sample  $\text{DP}_n = 79$ ) was dissolved in citrate buffer (100 mM, pH 5.5) to a concentration lower than 0.5 mM. Calorimetry titrations were performed on a Microcal VP-ITC instrument (Microcal, Inc., Northampton, MA, USA). The sample cell (1.40 mL) was filled with a solution of the analyte (e.g. polymer or DTPA) in citrate buffer and the syringe (300  $\mu\text{L}$ ) was filled with  $\text{Gd}^{3+}$  (5.0 mM) in citrate buffer (100 mM, pH 5.5). For each titration, the samples were continuously stirred (400 rpm), while the titrant was injected (5 to 10  $\mu\text{L}$ ) in the thermostated cell (25 °C) with a 300 second delay between injections and a 2 second data collection interval. Titrations were performed in triplicate for statistical purposes, and the data was processed with OriginPro 7.0 software.

### Antibody labeling with metal-chelating polymers

Metal-labeled antibodies were prepared as follows. An antibody at 1 mg/mL in 150 mM sodium phosphate buffer, pH 7.2 and in the absence of bovine serum albumin (BSA) or gelatin was subjected to mild reduction by TCEP (tris(2-carboxyethyl)phosphine) to convert the disulfides in the Fc fragment to thiols. The reduction and subsequent Ab-polymer conjugation steps were performed in 1.5 mL 50K MWCO centrifugal devices (Millipore YM-50). Concurrently and separately, the DTPA sites of the metal-chelating polymer were loaded with an isotopically enriched lanthanide solution in 20 mM ammonium acetate buffer, pH 5.2. Metal-loading and subsequent purification of the polymer was performed in 1.5 mL 3K MWCO centrifugal devices (Millipore YM-3). Finally, the maleimide group of the purified, lanthanide-loaded metal-chelating polymer was bound to the thiol groups of the partially-reduced Ab in Tris-buffered saline (TBS, 25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4). The metal-tagged Ab was washed several times in EDTA-free TBS and stored at +4 °C. These polymers are resistant to leaching, and we have observed no exchange of lanthanide ions between differently tagged Abs when deployed in a multiple antibody staining cocktail.<sup>5</sup>

The number of metal atoms carried by each antibody was determined quantitatively through a combination of UV/VIS spectroscopy and ICP-MS analysis. Goat anti-mouse immunoglobulins (Pierce #31168) labeled with P(DTPA)-Maleimide ( $\text{DP}_n = 79$  sample) chelating  $^{159}\text{Tb}$  were resuspended in Tris-buffered saline (pH 7.4, 150 mM NaCl). Protein

concentration was measured using a Nanodrop ND-1000 UV/VIS spectrometer (Thermo Fisher Scientific, USA). An aliquot of labeled antibody was diluted 1:100000 in 2% HCl, and 0.1 mL was analyzed by ICP-MS using an ELAN DRCPlus™ instrument (PerkinElmer SCIEX). A 0.50 ppb terbium standard was prepared from 1 mg/mL PE Pure single-element standard solution (PerkinElmer, Shelton, CT) and used for metal quantification in antibody samples. The ICP-MS data quantifies the number of terbium atoms per mL, and the Nanodrop determines the number of antibody molecules per mL. Thus, the number of terbium atoms per one antibody molecule was calculated. When combined with the ITC data that gave the number of lanthanide ions per polymer chain, it was possible to calculate the number of polymer chains per antibody.

### Standard Error Calculation

Standard errors were calculated using standard error propagation expressions assuming an uncertainty of  $\pm 5\%$  in NMR integrations, an inherent  $\pm 5\%$  error for each water/DTPA value and sodium/DTPA value from the TGA measurements,  $\pm 2\%$  precision on absorbance values determined with the Nanodrop instrument (manufacturer's specification), and  $\pm 2\%$  error in the metal ion intensities determined by ICP-MS.

### 11 Antibody Whole Cord Blood Analysis

Antibodies for a simultaneous 11-plex assay of whole cord blood were obtained from BioLegend Inc. (San Diego, US). The sample of umbilical whole core blood was obtained from the University Health Network, Toronto. Each of the 11 antibodies was labeled as described above with P(DTPA)-maleimide **79** following binding to the polymer of a different lanthanide isotope (see Table 1) for each antibody. For cell staining all eleven antibodies were mixed together at 10 mg/ml of each antibody in 1% BSA, and a titration series of 0.1, 1 and 10 mg/ml antibody was prepared. Whole heparinized umbilical cord blood was treated with RBC lysis buffer (BioLegend Inc., San Diego). The leukocytes were washed once with 5% BSA/PBS and were stained with the 11 metal-tagged antibody cocktail. Washed cells were fixed in 3.7% formaldehyde and counter-stained with an Ir-intercalator (the same sample described in Ref. <sup>8</sup>) for nucleated cell identification.

Labeled cells were analyzed by mass cytometry<sup>8</sup> using a CyTOF™ instrument from DVS Sciences, Richmond Hill, Ontario. Mass cytometry is a real-time analytical technique whereby cells or particles are individually introduced into an inductively-coupled plasma flame, and each resultant ion-cloud is analyzed by time-of-flight mass spectrometry. Dual-counting, the combination of digital counting and analog modes of ion detection, allows a much wider range of ion signal (simultaneous detection of very small and very large signals). Data was collected in FCS 3.0 format and was processed by FlowJo™ (DVS Sciences) software.

## RESULTS AND DISCUSSION

### Polymer synthesis

We began with the synthesis of poly(*t*-butyl acrylate)<sup>12</sup> using di-1-phenylethyl trithiocarbonate (CTA) as the RAFT agent. The polymer, as a concentrated (20 wt %) solution in THF, was treated in air with a small excess of aminoethanol to aminolyze the trithiocarbonate group and oxidize the resulting thiol groups to an amine-stable disulfide linkage. GPC (THF) chromatograms using RI and UV/VIS (310 nm) detectors demonstrate the transformation was successful. (Figure S1, Supporting Information).

As shown in Scheme 1, the next step in the synthesis involved deprotection of the *t*-butyl ester to form poly(acrylic acid) (PAA).<sup>13</sup> This was followed by a coupling step with *t*-BOC-

ethylenediamine using DMTMM.<sup>14</sup> Next, the BOC groups were removed using TFA in a mixture of DCM and anisole<sup>15</sup> (3:6:1 v:v). <sup>1</sup>H NMR analysis was performed at different points in the synthesis to demonstrate that a) the degree of polymerization remained constant, b) deprotection reactions proceeded efficiently, and c) quantitative functionality was achieved in amide-coupling reactions. <sup>1</sup>H NMR spectra and a summary of <sup>1</sup>H NMR and GPC data are gathered in Supporting Information (Figures S2-S5, Table S2). To provide additional confidence in the values of peak integrations, we carried out measurements of NMR T<sub>1</sub> and T<sub>2</sub> relaxation times (Table S1, Supporting Information).

**Introducing the ligand**—The penultimate step of the polymer transformation involved the introduction of DTPA units as metal-chelating groups. Reactions with DTPA dianhydride were unsuccessful in that GPC traces of the modified polymer (not shown) contained an additional broad, more rapidly eluting peak likely due to coupling of two or more polymer chains. Instead, we used a large excess of DTPA with a moderate excess of DMTMM as an amide coupling agent (Scheme 1). <sup>1</sup>H NMR demonstrates complete DTPA functionality, and aqueous GPC shows no additional higher molecular weight peaks (Supporting Information, Figure S5-S6, Table S2).

**Disulfide reduction and end-group functionality**—To add a linker for antibody attachment, we reduced the disulfide of the DTPA-containing polymer with an excess of dithiothreitol (DTT) and then, following a washing step, immediately reacted the thiols generated with an excess of the 2,2'-(ethylenedioxy)bis(ethylmaleimide). The aqueous GPC chromatographs of P(DTPA)-disulfide (sample **67**) and its corresponding maleimide polymer demonstrate the expected transformation from a dimeric polymer to a unimer (Supporting Information, Figure S6). Additionally, the protons on the double bond of the maleimide provide a <sup>1</sup>H NMR signal at 6.88 ppm that can be used for quantifying the reactive end-group content of the polymer. Figure 1 shows that the DP<sub>n</sub> = 67 sample (pH 7.0 reaction) has a terminal maleimide on 92% of the chains.

### Metal binding capacity and end-group content

While <sup>1</sup>H NMR is an effective methodology for determining the mean degree of polymerization and the mean number of DTPA groups per chain, it is important to have an independent measure of the number of lanthanide metal ions bound per polymer under conditions used for metal-ion incorporation during bioassays. For this purpose we turn to isothermal titration calorimetry (ITC). ITC is an analytical technique used to measure the binding affinity and stoichiometry for substrate/ligand interactions in biological systems where the affinity is lower than nanomolar. This technique has been used effectively to analyze the binding of Gd<sup>3+</sup> ions to sodium hyaluronate<sup>10</sup> or chitosan<sup>11</sup> modified to incorporate pendant DTPA units. Polymer carriers for multiple Gd<sup>3+</sup> ions are important as contrast agents for magnetic resonance imaging. In the sodium hyaluronate polymer, <sup>1</sup>H NMR resonances for DTPA were not well resolved from polymer backbone peaks. In the chitosan polymer, however, the NMR resonances were better resolved. In addition, only a fraction of the hyaluronic acid carboxyl groups or chitosan amino groups were converted to DTPA pendants. Thus these authors assumed that each DTPA would bind a Gd<sup>3+</sup> ion and the number of Gd<sup>3+</sup> ions bound was a reasonable measure of the number of DTPA groups present. The data reported were entirely consistent with this assumption. There are several important distinctions between our RAFT polymers and their polysaccharide derivatives. The most important is that we have a much higher density of DTPA groups. Thus it is possible that as the polymer approaches saturation with Gd<sup>3+</sup> ions, the binding affinity of the remaining DTPA groups may decrease, so that the number of Gd<sup>3+</sup> ions per polymer may be fewer than the number of DTPA groups.

For immunoassays by mass cytometry, we commonly treat metal-chelating polymers with excess lanthanide ions at pH 5.5. Thus the ITC experiments reported below were carried out at pH 5.5. To facilitate a comparison of our results with those reported in refs 10 and 11, we use  $Gd^{3+}$  as a probe of the Ln binding capacity of our polymers. A more subtle problem with our system arises from the fact that we have to weigh out polymer samples for ITC analysis. These polymer samples may contain small amounts of water, and because the dried samples are obtained from partially neutralized dialyzed polymer,<sup>13</sup> the number of sodium ions per repeat unit needs to be determined independently. For this purpose, we turn to thermal gravimetric analysis (TGA).

**Thermal gravimetric analysis**—In Figure 2, we compare TGA traces obtained for a commercial sample of the disodium salt of EDTA, known to contain 2 waters of hydration ( $EDTA^{2-}2Na^+2H_2O$ ), with traces obtained for both P(DTPA)-disulfide polymers (**67** and **79**). The first mass loss of the EDTA salt was complete at 150 °C, and accounted for 9.7% of the total mass. Next, a series of mass losses began at 225 °C, with a subsequent remaining mass (ceramic yield) of 27.4% mass at 800 °C.

The mechanism of thermal decomposition of  $EDTA^{2-}2Na^+2H_2O$  has been elucidated previously by Gonzales-Vilchez and coworkers.<sup>16</sup> They showed that the first observed mass loss (9.7%, complete by 150 °C) corresponds to the loss of the two waters of hydration. The subsequent mass loss beginning at 225 °C is due to decarboxylation (loss of two  $CO_2$  molecules). At 800 °C, the remaining 27.4% mass represents stable sodium carbonate. To confirm the thermal stability of sodium carbonate, we performed a TGA analysis (10 °C/min to 800 °C) of a sample of anhydrous sodium carbonate and observed no mass loss.

The corresponding polymer traces in Figure 2 show much broader transitions. Some water molecules in these non-crystalline polymer samples will be bound less tightly than the waters of hydration in the EDTA crystals, and it is likely that the higher density of  $-COOH$  groups leads to a lower onset temperature for decarboxylation. Thus only a fraction of the mass loss up to 200 °C is due to possible moisture content. The fact that both polymer samples obtained from freeze-drying gave identical traces also suggests that the moisture content in these samples is small. In our view, if the samples were highly hygroscopic, the amount of moisture they contained would vary with small differences in handling or delays between the freeze-drying and the TGA measurements. Our initial assumption in the data analysis is that the ca. 5% mass loss up to 150 °C is due to loss of moisture. At 800 °C, the samples had ceramic yields of 21.2% (sample **67**) and 20.7% (sample **79**) which we assign to the formation of  $Na_2CO_3$ .

To proceed with our analysis, we make three distinct assumptions about water content of the samples: (i) no significant moisture, (ii) water loss complete by 150 °C, corresponding to the behavior seen for the EDTA salt, and (iii) mass loss to 200 °C due only to moisture. The values calculated with these assumptions for sodium ion content of the polymer and the corresponding polymer molecular weights are collected in Table 2. In compiling this table, we calculate molecular weights ( $M_n$ ) for the DTPA-disulfide polymer, based upon  $DP_n = 67$  and 79, respectively, for the case in which each pendant group contains a DTPA unit that is fully protonated (i.e., not partially neutralized). Based on the TGA results, we then also calculate an apparent or adjusted  $M_n$  that includes both the sodium ions and the water molecules per polymer. An error of  $\pm 5\%$  is assumed for the  $H_2O$  and  $Na^+$  per DTPA unit values obtained from this analysis.

One can see in Table 2 that the full range of our assumptions about water content leads to only small changes in the number of calculated  $Na^+$  ions per DTPA. If we take 150 °C as the most likely water-loss temperature, we find that there are on average 2.3  $Na^+$  ions per

DTPA. This is a reasonable result, given that the DTPA was attached to the polymer backbone in the presence of DMTMM at pH 8.5 in a sodium DTPA buffer. Subsequent purification of a macromolecule by dialysis against DI water, as was performed here, is not expected to remove the counter-ions.<sup>13</sup> The reported  $pK_a$  values of a DTPA monamide are reported to be 1.8, 3.8, 6.4, and 9.9.<sup>17</sup> There will be a broader range of  $pK_a$  values for a polymer containing multiple DTPA-amide units. Our finding of 2.3  $Na^+$  per DTPA is not inconsistent with this range of  $pK_a$  values. The most useful result from Table 2 is the value of the *adjusted* polymer molecular weight suitable for use in interpreting the ITC data presented below.

### Isothermal Titration Calorimetry

The ITC measurements reported in refs <sup>10</sup> and <sup>11</sup> of the binding of  $Gd^{3+}$  to DTPA-groups attached to a polysaccharide backbone were carried out for samples in acetate buffer. These experiments showed that the titration end points were easily identified from the isotherms plotted from the data.<sup>10,11</sup> A quantitative analysis of these data in terms of binding constants was more difficult because of the very large binding constant ( $\log K = 22.5$ ) of the DTPA-Gd pair. In this study, we employed citrate buffer with the dual role of maintaining constant pH as well as serving as a competitive ligand of lower binding affinity ( $\log K = 7.83$ ) to  $Gd^{3+}$ . To test the reasonableness of this hypothesis, we first carried out titrations of DTPA itself, adding solutions of  $Gd^{3+}$  in citrate buffer (100 mM, pH 5.5) into citrate buffer (blank), and into solutions of DTPA (0.5 mM) in the same buffer. These thermograms are presented in the top part of Figure S7 in Supporting Information. For the background titration of  $Gd^{3+}$  into citrate buffer (blank), small exothermic signals ( $\sim 0.2 \mu\text{cal/sec}$ ) correspond to the heat of dilution of the Gd-citrate complex in citrate buffer. In contrast, the titration of  $Gd^{3+}$  into a solution of DTPA in citrate buffer shows strong endothermic peaks ( $6 \mu\text{cal/sec}$ ). Smaller injections were performed near the equivalence point to improve the accuracy of the titrations. The titration isotherm calculated from the data is plotted in the lower part of Figure S7, and the end point occurs for a 1:1 molar equivalence.

A representative binding isotherm for the titration of the P(DTPA)-disulfide polymer (sample **79**) with  $Gd^{3+}$  is shown in Figure 3. The binding curve shows a slight decrease in the enthalpy of binding for the injections before 1.0 mmol of  $Gd^{3+}$  per gram of polymer. After this, a rapid change in the signal is observed for the following injections. The curve reaches a constant value after saturation of the binding sites in the polymer chain. Unlike the case of DTPA itself, these data cannot be fitted successfully to a one-site binding model characterized by a single binding equilibrium constant. The fits are better to a two-site model, but the values of the numbers of  $Gd^{3+}$  per binding site ( $n_1, n_2$ ) from the fitted data varied from titration to titration. This result suggests the existence of multiple sites with different binding strengths along the polymer backbone. The sums of the stoichiometric values for the two-site model fits ( $n_{\text{tot}}$ ) were similar for the titrations, and this result provides a quantitative measure of the number of  $Gd^{3+}$  that bind to the polymer chain regardless of the binding energetics for each site. From triplicate titrations, we find that the polymer sample contains  $1.48 \pm 0.03$  mmol  $Gd^{3+}$  per gram of polymer.

The quantity of interest to us is the mean number of  $Gd^{3+}$  ions per polymer, where we consider that the disulfide bond in P(DTPA)-disulfide links *two* polymer chains. Another way of thinking about this value is in terms of the number of  $Gd^{3+}$  ions per phenyl end group. To determine this value, we begin by calculating (eq 1) the amount of polymer (in mmol/g) in a weighed sample of this polymer. This calculation (eq 1) relies on the values of the “adjusted molecular weight” reported in Table 2.



$$\text{PolymerMolecules}(mmol/g) = \frac{2(\text{chains/disulfide}) \bullet 1000 (mmol/mol)}{\text{AdjustedMolecularWeight} (g/mol)} \quad (1)$$

This value, in combination with the ITC result of 1.48 mmol DTPA/g polymer leads to the values of Gd<sup>3+</sup> per phenyl end group listed in Table 3. For the **79** sample, we see that assuming that the freeze-dried disulfide polymer contains no water leads to a value of 63 ± 7 of Gd<sup>3+</sup> per phenyl end group, whereas the finding of 2.2 H<sub>2</sub>O molecules per DTPA leads to a value of 68 ± 7 of Gd<sup>3+</sup> per phenyl end group. This value 68 ± 7 is consistent with the value of DP<sub>n</sub> = 79 for the precursor polymer as the P(tBA)-trithiocarbonate and PAA-disulfide, and the <sup>1</sup>H NMR result indicating full DTPA functionalization of the polymer backbone.

For the purposes of this paper, we assume the binding capacity of these metal-chelating polymers is identical for all trivalent lanthanide ions. In the experiments described below, we use Tb<sup>3+</sup> as a single isotope lanthanide ion to determine how many polymers are attached per antibody under our normal antibody labeling protocol. This is an assumption that can be checked in the future by ITC experiments with different metal ions.

### Covalent attachment of metal-chelating polymers to monoclonal antibodies

In this section we discuss reaction conditions for attachment of P(DTPA)-maleimide to antibodies (Abs) and describe experiments to quantify the mean number of polymers per antibody. As described in detail in the Experimental section, Ab modification begins with reduction of disulfide bonds to thiols in the hinge region of the antibody using TCEP in 150 mM sodium phosphate buffer, pH 7.2. In parallel, the DTPA sites of the P(DTPA)-maleimide were loaded with an isotopically enriched lanthanide solution in 20 mM ammonium acetate buffer, pH 5.2. Finally, the maleimide group of the purified, lanthanide-loaded polymer was bound to the thiol groups of the partially-reduced Ab in tris-buffered saline (TBS, 25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4). The metal-tagged Ab was washed several times in EDTA-free TBS and stored at +4 °C. The strong chelation of the lanthanide by DTPA is resistant to leaching, and we have observed no exchange between differently tagged Abs when deployed in a multiple antibody staining cocktail.<sup>5</sup>

In order to interpret mass cytometry data quantitatively in terms of the number of biomarkers per cell, it is necessary to know the number of metal ions (i.e., the number of metal-chelating polymers) carried per antibody.<sup>18, 19</sup> In order to obtain this information, we carried out model experiments on goat anti-mouse secondary antibody labeled with <sup>159</sup>Tb via reaction with the P(DTPA)-maleimide sample **79**, which we assume contains an average of 68 ± 7 Tb<sup>3+</sup>/polymer. After purification of the functionalized Ab to remove excess polymer and metal ions, the antibody concentration was measured with a Nanodrop UV/VIS spectrometer, yielding 2.73 × 10<sup>10</sup> molecules Ab/mL. In addition, an aliquot of this solution was diluted and analyzed by conventional ICP-MS, from which the metal concentration was determined to be 4.4 × 10<sup>12</sup> atoms <sup>159</sup>Tb/mL. Dividing the metal concentration by the antibody concentration yields an average of 161 ± 4 metal atoms per antibody. Finally, dividing 161 metal atoms per antibody by an assumed 68 Tb<sup>3+</sup> ions per chain shows that there are an average of 2.4 ± 0.3 polymer chains bound to each antibody. Note that using values of 63 or 71 binding sites per chain changes this value only slightly, to 2.6 ± 0.3 or 2.3 ± 0.2 polymer chains, respectively, per antibody.

### Simultaneous 11-plex Antibody staining and analysis of Whole Cord Blood

Metal chelating polymer P(DTPA)-maleimide **79** was used to label 11 antibodies with different lanthanide isotopes. This antibody cocktail was used for identification of the major

cell populations found in umbilical cord blood. As described in the Experimental section, all eleven antibodies for cell staining were mixed together at 10 mg/ml of each antibody in 1% BSA, and a titration series of 0.1, 1 and 10 mg/ml antibody was prepared. Whole heparinized umbilical cord blood (100  $\mu$ L per sample) was treated with RBC lysis buffer to disintegrate the majority of red blood cells. The leukocytes were washed once with 5% BSA/PBS and were stained with the mixture of 11 metal-tagged antibodies. Washed cells were fixed in 3.7% formaldehyde and counter-stained with an Ir-intercalator for nucleated cell identification. Samples were analyzed by mass cytometry,<sup>8</sup> and the data in FCS 3.0 format were processed by FlowJo™ software.

The major cell types (Lymphocytes, Granulocytes, Monocytes and subsets of CD3 T-cells and B-cells) are shown on the two-dimensional smoothed dot-plots presented in Figure 4. A description of clusters of differentiation (CD) and the rationale behind the gating strategy in Figure 4 are presented in the Supporting Information. Mean isotope signal values for cells within each gate were used to create titration curves. Optimal antibody concentrations for each metal-tagged Ab were determined from the dilution series (Supporting Information, Figures S8 & S9). The graphs clearly show that at the appropriate concentration of metal-tagged antibody, the cell population is defined by several specific markers. The mean percentage of cells in the granulocyte (16.0%), monocyte (12.3%) and lymphocyte (51.1%) populations is within normal values.<sup>20</sup> It is interesting to note that all T cells expressed high levels of CD38, which is consistent with previous findings.<sup>21</sup>

Figure 5 shows the characteristic immunophenotypes of six major cell populations found in umbilical cord blood. Data is presented in a radial plot format.<sup>22</sup> Each spoke of the plot is a logarithmic measure of a single antigen's expression. Eleven antigens were identified by the eleven respective metal-tagged antibodies; therefore, eleven spokes are featured in the plot. Antibodies are arranged in an arbitrary order. The 12 o'clock spoke is the decimal fraction of the population of each cell type. Each cell type is assigned a color line; for example the B cells are recognized by the red line. We find the radial plot format useful because it visually represents how one particular cell differs from another in immunophenotype. Thus, the B-cells show high CD20, CD38, CD45 expression, while granulocytes are negative for CD20, CD3, are low for CD38 and CD45, but express high levels of specific markers such as CD15, CD16, CD11b. These results demonstrate that by using a metal-chelating polymer with different lanthanide isotopes to create an 11 antibody cocktail, it is possible to perform phenotypic analysis of the major cell types in whole cord blood in a multiplex format in a single mixture. The absence of compensation in mass cytometry permits the analysis of 11 biomarkers in a single sample of 100  $\mu$ L of whole blood.

## SUMMARY

We report the synthesis of a metal-chelating polymer based on a polyacrylate backbone with DTPA ligands attached to each of the repeat units. The synthesis strategy involves initial preparation of poly(*t*-butyl acrylate) by RAFT polymerization, followed by aminolysis and oxidation to a dimeric disulfide polymer as a means of protecting the terminal thiol groups. Following pendant group transformation, the disulfide bond was reduced with DTT and reacted with a large excess of a bismaleimide to introduce reactive functionality (with ca. 90% efficiency) to one end.

The polymer was characterized <sup>1</sup>H NMR to determine the mean degree of polymerization and end-group content and by size exclusion chromatography to determine the polydispersity. We used thermal gravimetric analysis to estimate moisture and sodium ion content of the partially neutralized polymer and isothermal titration calorimetry (ITC) to measure the lanthanide binding capacity. For sample **79**, we found a number average degree

of polymerization of **79** for the initial formation of P(tBA) and this value remained in the range of 75 to 79 ( $\pm 7\%$ ) following subsequent pendant group transformations. This value is within experimental error of the number of  $\text{Gd}^{3+}$  ions found by ITC to bind to the polymer.

This polymer was attached to a series of antibodies to biomarkers for human cord blood cell components as well as to a sample of goat anti-mouse secondary antibody. Using traditional ICP-MS analysis, we determined that this secondary antibody on average contained  $161 \pm 4$   $^{159}\text{Tb}$  ions. Assuming that  $\text{Tb}^{3+}$  and  $\text{Gd}^{3+}$  bind equally well to metal chelating polymer sample **79**, we infer that the intact antibodies are labeled with an average  $2.4 \pm 0.3$  polymer molecules. The correspondingly labeled monoclonal primary metal-tagged antibodies were used in a 11-plex mass cytometry bioassay to determine the main types of cells present in a small amount of whole cord blood.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

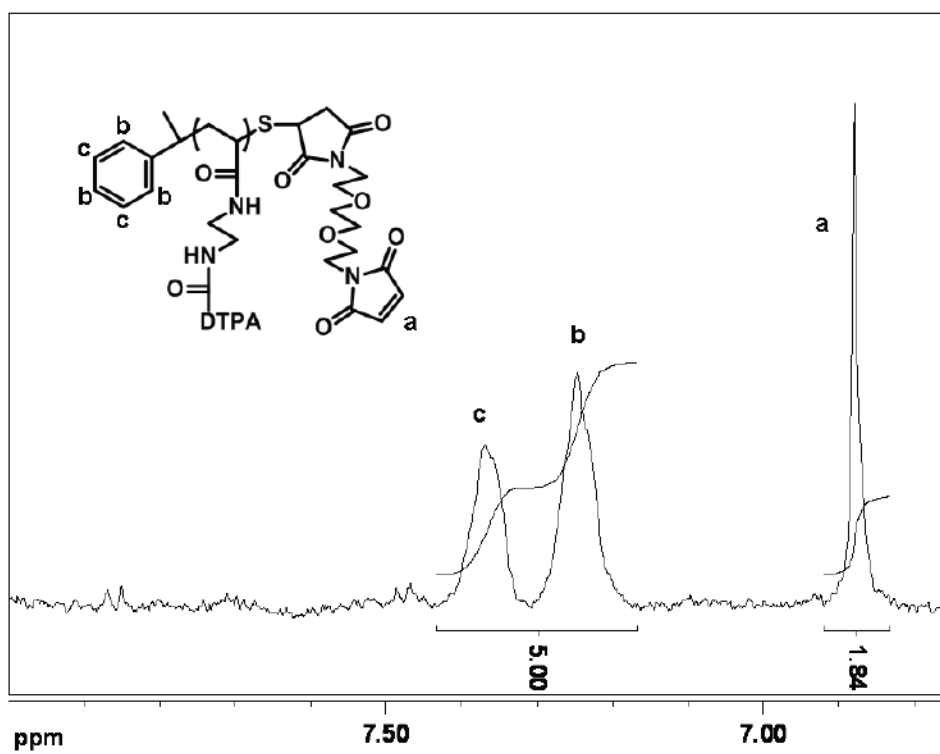
## Acknowledgments

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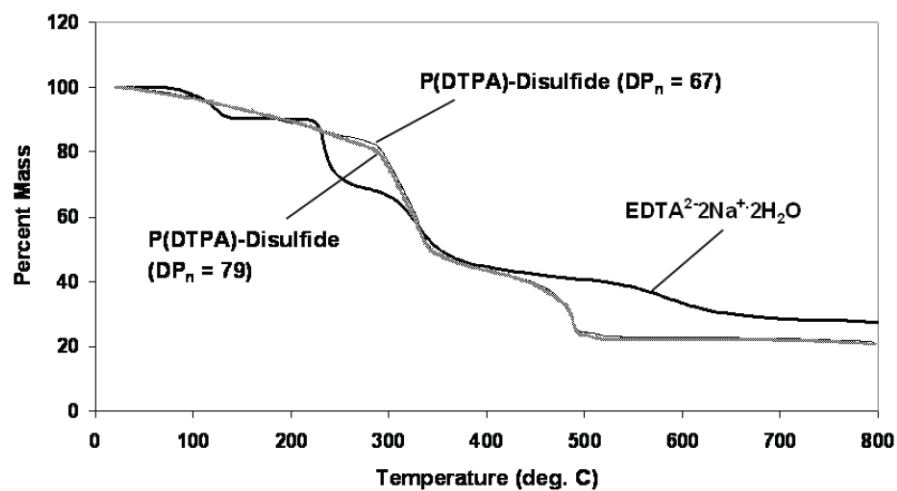
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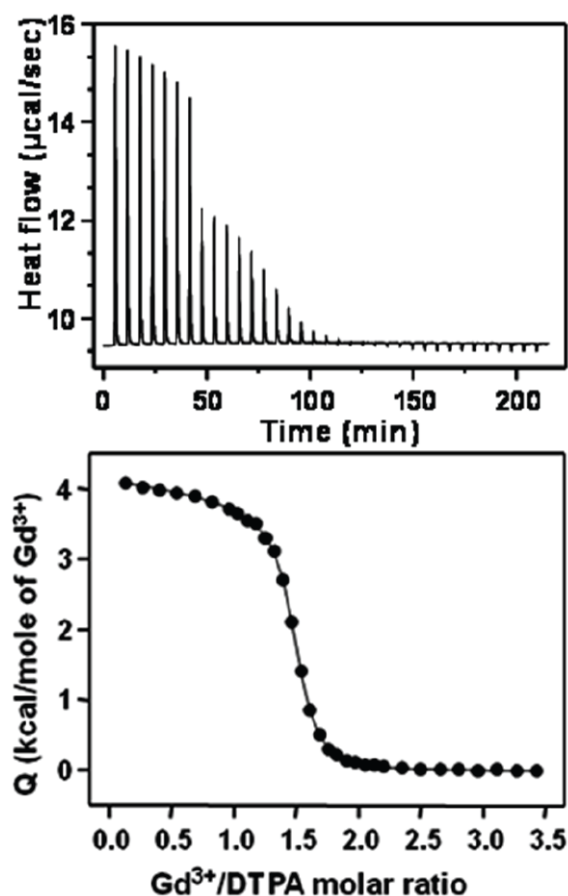
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**Figure 1.**  $^1\text{H}$  NMR Spectrum ( $\text{D}_2\text{O}$ ) of P(DTPA)-maleimide (sample **67**, reaction pH = 7.0). Comparing the 5H phenyl end-group with the 2H vinylic protons of the maleimide linker shows end-functionalization =  $(1.84/2) \times 100\% = 92\%$ .

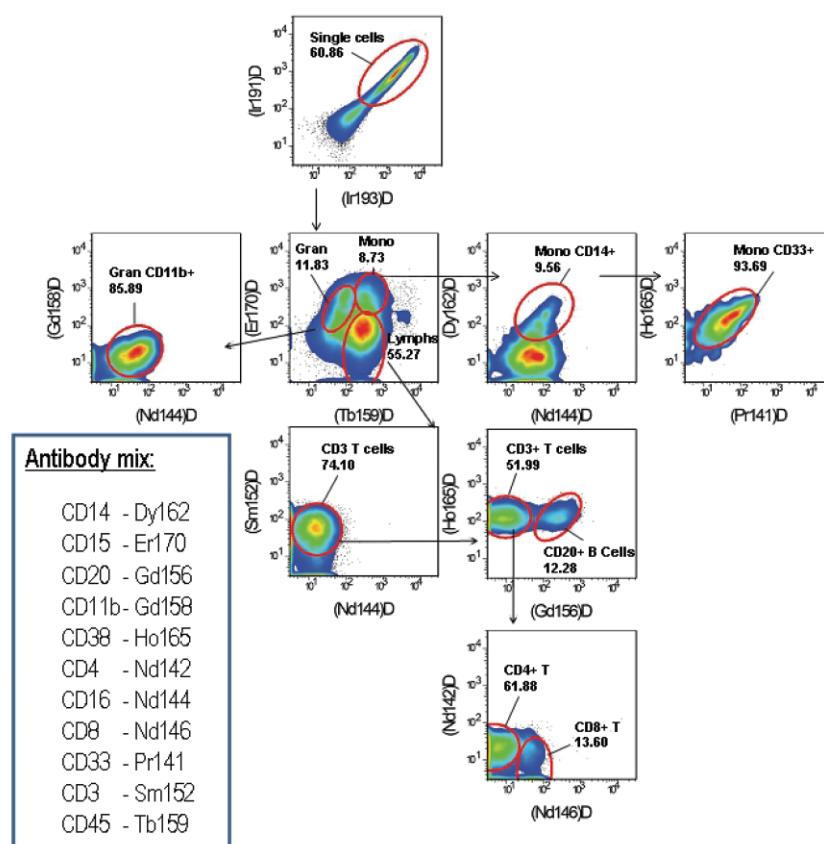


**Figure 2.** TGA traces of EDTA<sup>2-</sup>·2Na<sup>+</sup>·2H<sub>2</sub>O and DTPA-disulfide polymers (**67** and **79**). These TGA traces were collected in the presence of air at a heating rate of 1 °C/min.



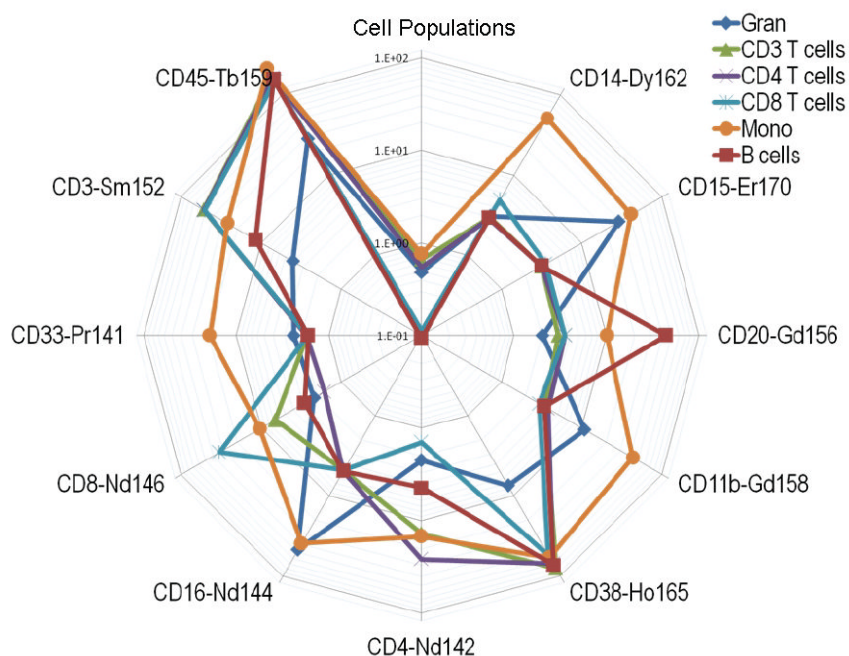
**Figure 3.**

Top: Isothermal titration calorimetric thermogram of P(DTPA)-Disulfide polymer (sample **79**, 0.27 mg of polymer per mL) with  $\text{Gd}^{3+}$  (5.0 mM) in citrate buffer (100 mM, pH 5.5) at 25 °C. Endothermic signals correspond to the dissociation of  $\text{Gd}^{3+}$ -citrate and binding to a DTPA pendant group. Bottom: Binding isotherm calculated from the titration above. The solid line was obtained from nonlinear least squares regression of a two binding site model and used to extract the equivalence points for each binding site ( $n_1$ ,  $n_2$ ). The total equivalence point ( $n_{\text{tot}}$ ) corresponds to the sum of the individual equivalence points and represents the mmol of  $\text{Gd}^{3+}$  required to saturate the DTPA pendant groups in one gram of polymer. This value is situated at the inflection point of the titration curve ( $n_{\text{tot}} = 1.48 \pm 0.3$  mmol of  $\text{Gd}^{3+}$  per gram of polymer).]

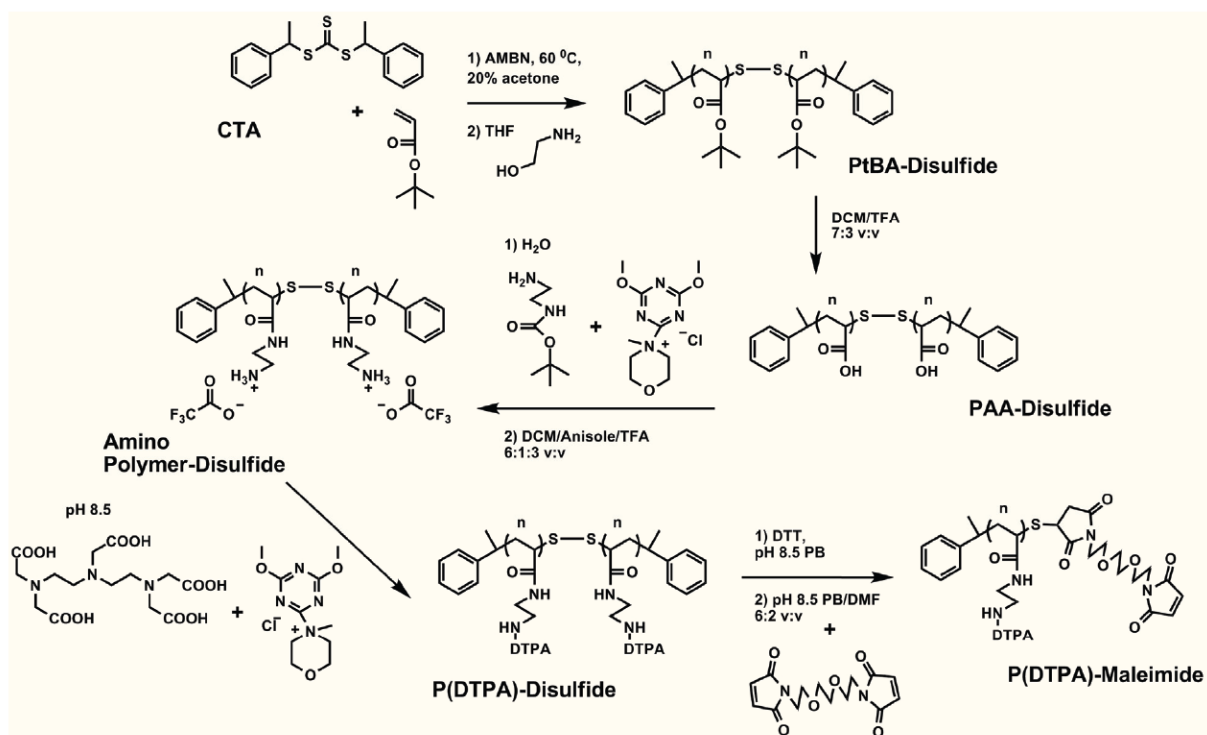


**Figure 4.** Cell population gating strategy for umbilical cord blood stained with a mixture of 11 metal-tagged antibodies. Whole heparinized umbilical cord blood was treated with RBC lysis buffer. The leukocytes were washed once with 1% BSA/PBS and stained with a mixture of 11 antibodies conjugated to MCP preloaded with different lanthanide isotopes. Optimal antibody concentrations for each metal-tagged Ab were determined from the dilution series (Supporting Information, Figures S8 and S9). Washed cells were fixed in 3.7% formaldehyde and counter-stained with the Ir-intercalator for nuclear cell identification. Samples were analyzed by mass cytometry, and the data in FCS 3.0 format were processed by FlowJoTM software. The axes reflect dual-counting (D), which is the combination of counting and analog modes of ion detection, and allows simultaneous detection of very small and very large signals. The major cell types (Lymphocytes, Granulocytes, Monocytes and subsets of CD3 T-cells and B-cells) are shown on two dimensional smoothed dot-plots.





**Figure 5.** Biomarker analysis of whole umbilical cord blood cells stained with a mixture of 11 metal-tagged antibodies. Optimal antibody concentrations for each metal-tagged Ab were determined from the dilution series (Supporting Information, Figures S8 & S9). Samples were analyzed by mass cytometry and data in FCS 3.0 format were processed by FlowJo™ software. Cell population gating strategy is presented in Figure 4. Mean values for gated populations are presented on the logarithmic radial diagram. Six main cell types are shown as individual color lines.

**Scheme 1.**

Synthesis of Metal-Chelating Polymers. AMBN = 2,2'-azobis(2-methylbutyronitrile), THF = tetrahydrofuran, DCM = dichloromethane, TFA = trifluoroacetic acid, DTT = dithiothreitol, PB = phosphate buffer, DMF = dimethylformamide.  $n \approx 67$  or 79.

**Table 1**

Antibodies used for whole cord blood analysis and the lanthanide isotopes used to label them

Ab	isotope	Ab	isotope
CD33	<sup>141</sup> Pr	CD11b	<sup>158</sup> Gd
CD4	<sup>142</sup> Nd	CD45	<sup>159</sup> Tb
CD16	<sup>144</sup> Nd	CD14	<sup>162</sup> Dy
CD8	<sup>146</sup> Nd	CD38	<sup>165</sup> Ho
CD3	<sup>152</sup> Sm	CD15	<sup>170</sup> Er
CD20	<sup>156</sup> Gd		

H<sub>2</sub>O and Na<sup>+</sup> Content and Adjusted Molecular Weights<sup>a</sup> for P(DTPA)-Disulfide Polymer Samples calculated from TGA Analysis.

**Table 2**

Sample <sup>b</sup>	H <sub>2</sub> O loss Temp.	H <sub>2</sub> O per DTPA unit	Na <sup>+</sup> per DTPA unit	<sup>1</sup> H NMR Molecular Weight <sup>c</sup>	Adjusted Molecular Weight <sup>d</sup>	Gd <sup>3+</sup> ion per polymer <sup>e</sup>
67	No H <sub>2</sub> O	0	2.1	65,900 Da ± 12%	72,200 Da ± 11%	---
	150 °C	2.2	2.3		78,100 Da ± 11%	
	200 °C	3.7	2.4		82,100 Da ± 10%	
79	No H <sub>2</sub> O	0	2.1	77,600 Da ± 12%	84,900 Da ± 10%	63 ± 7
	150 °C	2.2	2.3		91,700 Da ± 10%	
	200 °C	3.5	2.4		95,800 Da ± 10%	

<sup>a</sup> All molecular weights presented in this table are for the dimeric disulfide form: two chains bound together through a disulfide bond. The standard error calculation is described in the experimental section.

<sup>b</sup> DP<sub>n</sub> per phenyl end group determined by <sup>1</sup>H NMR at the PtBA stage by comparing the integration of the C<sub>6</sub>H<sub>5</sub> end group at 7.2 ppm to that of the backbone methine protons.

<sup>c</sup> Calculated from DP<sub>n</sub>/phenyl end group at the PtBA stage, attachment of a DTPA unit to each pendant group and assuming that the DTPA groups are fully protonated (not partially neutralized by Na<sup>+</sup> ions).

<sup>d</sup> An apparent molecular weight that includes the mass contribution of attendant water molecules and sodium counter-ions.

<sup>e</sup> These values indicate the number of Gd<sup>3+</sup> ions per phenyl end group of the polymer, calculated from the ITC result of 1.48 ± 0.3 mmol Gd<sup>3+</sup>/g polymer and the adjusted molecular weight.