Thiol-Reactive Star Polymers Display Enhanced Association with Distinct Human Blood Components

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Supporting Information

ABSTRACT: Directing nanoparticles to specific cell types using nonantibody-based methods is of increasing interest. Thiol-reactive nanoparticles can enhance the efficiency of cargo delivery into specific cells through interactions with cell-surface proteins. However, studies to date using this technique have been largely limited to immortalized cell lines or rodents, and the utility of this technology on primary human cells is unknown. Herein, we used RAFT polymerization to prepare pyridyl disulfide (PDS)-functionalized star polymers with a methoxy-poly(ethylene glycol) brush corona and a fluorescently labeled cross-linked core using an arm-first method. PDS star polymers were examined for their interaction with primary human blood components: six separate white blood cell subsets, as well as red blood cells and platelets. Compared with control star polymers, thiol-reactive nanoparticles displayed enhanced association with white blood cells at 37 °C, particularly the phagocytic monocyte, granulocyte, and dendritic cell subsets. Platelets associated with more PDS than control nanoparticles at both 37 °C and on ice, but they were not activated in the duration examined. Association with red blood cells was minor but still enhanced with PDS nanoparticles. Thiol-reactive nanoparticles represent a useful strategy to target primary human immune cell subsets for improved nanoparticle delivery.

KEYWORDS: star polymers, blood, thiols, platelets, targeting

1. INTRODUCTION

Efficient cellular delivery is a critical hurdle in the development of effective nanotherapeutics. Most targeting approaches employ antibody-based strategies. However, this poses design challenges as antibody constructs are generally relatively large molecules, which may distort the desired nanoparticle (NP) size or structure. This is particularly problematic for very small NPs, which may otherwise have the benefit of deeper penetration into tumor tissues. Exploiting the differential expression of cell-surface (exofacial) thiol groups (−SH) across different cell subtypes is an alternative method to target small molecules and NPs. It has been postulated that targeting exofacial thiols might represent a natural mechanism of cell uptake. Cancer cells have been reported to express higher levels of cell-surface thiol groups than nontransformed equivalents. These include greater levels of glutathione, cysteine, and/or thioredoxin, which may be further upregulated in the hypoxic regions of tumors. In vitro studies using immortalized cell lines and rodent studies have shown that NPs can bind cell-surface thiol groups and can efficiently target some cancer cells. In a murine model of adoptive T cell therapy for cancer, two cytokines known to promote in vivo T cell expansion and antitumor effector function were encapsulated within thiol-reactive liposomes. These liposomes were conjugated to exofacial thiols present on CD8+ T cells in serum-free media ex vivo. After readministering the liposome-conjugated T cells into B16-melanoma-bearing mice, T cell proliferation increased, and established tumors were eradicated. NPs can also be engineered to present pyridyl disulfide (PDS) groups which can potentially exchange with cell-surface thiols, thereby enhancing NP binding. Whether NPs decorated with PDS groups target cells within primary human blood is currently unknown but of considerable interest given the wide range of cell types in blood and the critical role they play in coagulation and immunity.

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Human blood contains myriad cell types that can be broadly divided into (1) phagocytic and nonphagocytic white blood cells (WBC), (2) red blood cells (RBC), and (3) platelets. Phagocytic WBCs include monocytes and granulocytes that act in the primary defense against infection and are essential components of the innate immune response to pathogens. They are primed to clear pathogens and also avidly phagocytose many NP preparations. Dendritic cells are also phagocytic and play a principal role in initiating the adaptive immune response, referred to as antigen presentation. They process pathogens or vaccines for presentation to important cells of the adaptive immune system—B cells and T cells, which generate antibody- and cell-mediated immune responses.\textsuperscript{12,13} Natural killer (NK) cells are innate immune cells primed to kill those cells infected by viruses or transformed into cancers. RBCs are the most common cell type in blood (1000-fold more abundant than WBCs) and play a critical role in oxygen transport. Platelets (or “thrombocytes”) are small (2–3 μm) anuclear cell fragments involved in the process of hemostasis. They form “platelet plugs” that adhere to damaged endothelium and become activated. Activated platelets release factors including adenosine diphosphate (ADP) that attracts additional platelets to the site and forms the foundation of the blood clot. Platelet activation is also associated with the formation of intravascular clots adjacent to atherosclerotic plaques in subjects with cardiovascular disease.\textsuperscript{14}

We and others have previously used whole human blood assays to measure NP association with multiple immune cell subsets.\textsuperscript{15–18} Herein we have modified our previous approaches to also allow examination of RBCs and platelets. Using human blood to evaluate bionano interactions offers numerous benefits over traditional in vitro cell line or purified cell models as it contains: (1) primary human cells, (2) multiple immune cell types, (3) components of the coagulation cascade (e.g., platelets and proteins), and (4) autologous human plasma.

Due to their nanoscale dimensions, controlled polymerization and efficient coupling chemistries, star polymers\textsuperscript{19} are attracting considerable attention as potential carriers for both therapeutic agents\textsuperscript{20} and genetic material.\textsuperscript{21} In particular, the preparation of stars by an arm-first methodology using reversible addition–fragmentation chain transfer (RAFT) polymerization represents a convenient route for preparing materials with both reactive sites for subsequent functionalization and an antifouling MPEG brush corona.\textsuperscript{22,23} By incorporating reactive moieties, star polymers have been subsequently modified with drug\textsuperscript{20}, radiolabel,\textsuperscript{24} and gadolinium chelates for enhancing MRI contrast.\textsuperscript{25} We have recently demonstrated that star polymers exhibit enhanced circulation time (depending on the size)\textsuperscript{23} and that the biodistribution is affected by the route of administration.\textsuperscript{26} However, to date there have been no investigations of the extent to which star polymers associate with human blood cells. This is an essential step toward better understanding these NPs and is an important precursor to future clinical use.

Molecules such as PDS that react with exofacial thiols can readily be incorporated into star polymers to facilitate enhanced binding to target cells or carrier cells in blood. Herein we characterize how PDS modification affects NP interaction with major cellular subsets of human blood: WBCs, RBCs, and platelets. We demonstrate PDS modification enhanced association with transformed cells and some (but not all) WBC subsets, as well as platelets and to a lesser degree RBCs.

2. EXPERIMENTAL SECTION

2.1. Nanoparticle Synthesis. 2.1.1. Materials. The functional RAFT agents (3-(benzylsulfanylthiocarbonylsulfanyl)-propionic acid (BSPA) and 2-(pyridine-2-yl disulfanyl) ethyl 2-(((dodecylthio)-carboxothioyl)thio)propanoate (PDSD) were synthesized using published procedures.\textsuperscript{25} 2-Vinyl-4,4-dimethyl-5-oxazolone (VDM) was synthesized using the method of Li et al.\textsuperscript{26} Cyamine5 amine (Lumiprobe), oligo(ethylene glycol) methyl ether acrylate (Aldrich, OEGA, M, = 480 g mol\textsuperscript{−1}, 99%), N,N′-methylenebis(acrylamide) (Aldrich, 98%) and azobis(isobutyronitrile) (Aldrich, AIBN) were used as received. All other reagents were AR grade.

2.1.2. Synthesis of Core-Functionalized Star Polysteres Using Arm-First RAFT Polymerization. The synthesis of functional PEGylated stars using an arm-first method is detailed in Figure 1.

2.1.3. Arm Synthesis. The typical arm synthesis for benzy1 functional PEOGA using BSPA RAFT agent is given as follows (Entry 1, Table S1): BSPA RAFT agent (16.3 mg, 0.06 mmol), OEGA\textsubscript{180} (1 g, 2.08 mmol), AIBN (1.0 mg, 0.006 mmol), and toluene (3 mL) were all mixed in a 5 mL vial equipped with a magnetic stirrer bar. The vial was then sealed, and the mixture was deoxygenated by sparging with nitrogen for 20 min. The deoxygenated reaction mixture was stirred at 70 °C; after 6 h, the reaction was quenched by placing the vial in ice water. The polymer arms were recovered by precipitating the reaction mixture three times from toluene into a mixture of petroleum ether and diethyl ether (1:1 v/v). For the synthesis of pyridyl disulfide functional arms PDSD RAFT agent (31.2 mg, 0.06 mmol) was substituted for BSPA. The arms were characterized using both gel permeation chromatography (GPC) and ¹H NMR.

2.1.4. Arm-First Star Polymer Synthesis. A typical synthesis procedure is given below for the benzy1 functional arm-first star polymer (Entry 3, Table S1): Benzy1 functional PEOGA arms (Entry 1, Table S1: 608 mg, 0.068 mmol), VDM (13.9 mg, 0.1 mmol), AIBN (2.4 mg, 0.0015 mmol), cross-linker [N,N′-methylenebis(acrylamide)] (53.9 mg, 0.35 mmol), and 5 mL of toluene were mixed in a vial equipped with a magnetic stirrer bar. The vial was then sealed, and the mixture was deoxygenated by sparging with nitrogen at 0 °C for 20 min. The deoxygenated reaction mixture was then stirred at 70 °C for 24 h. An aliquot of the reaction mixture was sampled periodically for GPC analysis to facilitate monitoring of the star formation process. The reaction mixture was then quenched in ice water. For pyridyl disulfide functional stars pyridyl disulfide functional PEOGA arms (Entry 2, Table S1) were substituted for benzy1 functional PEOGA arms. The stars were isolated by precipitating the reaction mixture three times from toluene into diethyl ether/chloroform (90:10 v/v).

2.1.5. Cyamine5 (Cy5)-Labeled Star Polymer Synthesis. A typical Cy5 labeling reaction is as follows: BSPA-POEGA Star (Entry 3, Table S1: 200 mg, 0.0017 mmol), Cyamine5 amine (0.7 mg, 0.001 mmol), and triethylamine (0.5 mg, 0.005 mmol) were dissolved into 2 mL of N,N-dimethylformamide and allowed to react at room temperature for 48 h while being carefully protected from light. The Cy5-labeled star polymer was recovered by exhaustive dialysis in the dark against methanol using SnakeSkin dialysis tubing (MWCO 3000 Da). Successful labeling with Cy5 was confirmed via GPC using dual RI and UV–vis detection, and fluorescence spectroscopy.

2.2. Nanoparticle Characterization. 2.2.1. Gel Permeation Chromatography (GPC). GPC analyses of the polymers were carried out on a Shimadzu modular system composed of an SIL-20AID autosampler, a PL 5.0 mm bead-size guard column (50 × 7.8 mm) followed by four 300 × 7.8 mm linear columns (500, 104, 103, and 105 Å pore size) using N,N-dimethylacetamide [DMAc; w/v LiBr, 0.05% 2, 7-dibutyl-4-methylphenol (BHT)] at 50 °C as the eluent (flow rate = 1 mL min\textsuperscript{−1}). An RID-20A differential refractive-index and UV–vis detector was used. Samples were filtered through 0.45 μm PTFE filters before injection. Calibration was performed with narrow-polydispersity polystyrene standards ranging from 500 to 3 × 10\textsuperscript{4} g mol\textsuperscript{−1}.

2.2.2. Nuclear Magnetic Resonance (NMR). ¹H NMR spectroscopy using a Bruker UltraShield 400 MHz spectrometer (Bruker Daltonics Inc., NSW, Australia) running Topspin, version 1.3. All spectra were recorded in CDCl\textsubscript{3}.
2.2.3. Dynamic Light Scattering (DLS). Dynamic light scattering measurements were performed on a solution of star polymer (1 mg mL$^{-1}$ in PBS) using a Malvern Zetasizer Nano Series running DTS software and operating a 4 mW He–Ne laser at 633 nm at an angle of 173°. Data was collected at a constant temperature of 25 °C and analyzed using Malvern supplied DTS software. The number-average hydrodynamic diameter is reported.

2.2.4. Fluorescence Measurements. Fluorescence spectra were obtained using a fluorescence spectrophotometer (Shimadzu RF-5301 PC). Slit widths were set at 2.5 mm for both excitation and emission of Cyanine 5. The photomultiplier voltage was set at 950 V.

2.2.5. Transmission Electron Microscopy (TEM). TEM figures were recorded using a Tecnai F20 transmission electron microscope operating at an accelerating voltage of 200 kV. TEM grids were prepared as follows: 10 μL of a 0.1 wt % solution was deposited on a Formvar-coated copper grid (GSCu100F-50, Proscitech) and allowed to dry in air at room temperature for at least 12 h.

2.3. Blood Acquisition. Blood was collected from healthy human donors after obtaining informed consent in accordance with the University of Melbourne Human ethics approval 1443420 and the Australian National Health and Medical Research Council Statement on Ethical Conduct in Human Research. For WBC studies, blood was drawn by venipuncture into Vacuette collection tubes (Greiner Bio-One) containing sodium heparin and gently inverted 5 times. For studies involving platelets/RBCs, the tourniquet was applied loosely and the first 2 mL of blood was discarded before collecting into ACD-B Vacutainer collection tubes (BD Biosciences).

2.4. Cell Line Association Studies. C1R cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U mL$^{-1}$ penicillin, and 100 μg mL$^{-1}$ streptomycin. Cells were seeded at 9.0 × 10$^4$ cells per well in 96-well plates on the day of the experiment. Five microliters of particles (300 μg mL$^{-1}$) was added to cells in 145 μL of media to give a final concentration of 10 μg mL$^{-1}$ of particles in 150 μL. Cells were incubated with particles for between 10 and 120 min at either 4 or 37 °C and then washed four times in 1% BSA/PBS.
Samples were resuspended in PBS containing propidium iodide (PI; 0.5 μg mL⁻¹) for analysis by flow cytometry (Stratagene S100EXi). All experiments were performed in triplicate. PBS: fetal bovine serum.

2.5. Association with WBC Populations. Freshly drawn blood (100 μL) was added to 5 mL polystyrene round-bottom tubes (“FACS tubes; Falcon”) before incubating on ice or at 37 °C for 10 min. NPs (diluted in PBS) were added directly into whole blood at a final concentration of 10 μg mL⁻¹ and incubated for 1 h, before placing all tubes on ice. RBCs were lysed with Pharm Lyse buffer (BD Biosciences) at 20× blood volume and topped up to 4 mL using 1× PBS. WBCs were pelleted (500 g, 5 min) and washed twice more with PBS. Cells phenotyped on ice (1 h) by using titrated concentrations of antibodies against CD3 (AF700, SP34-2), CD14 (APC-H7, MoP9), CD20 (BV421, 2H7), CD45 (V500, HI30), CD56 (PE, B159). Lineage-1 cocktail (FITC), and HLA-DR (PerCP-Cy5.5, G46-6) (all antibodies from BD Biosciences, except CD20 (BioLegend). Cells washed twice in cold (4 °C) PBS wash buffer (FWB; WB; 1× PBS containing 5% w/v bovine serum albumin (Sigma-Aldrich) and 2 mM EDTA pH 8 (Ambion)), fixed (1% formaldehyde) and analyzed by flow cytometry (LSRFortessa, BD Biosciences). Data was analyzed on FlowJo V10 and graphed using GraphPad Prism 6.

For high-dimensional viSNE analysis, NP cell association data from five donors was concatenated using FlowJo V10, before exporting to Cytobank for viSNE analysis.30,31 viSNE analysis allows 2D projection using FlowJo V10 and graphed using GraphPad Prism 6. All experiments were performed in triplicate.

2.6. Confocal Microscopy. The NP-blood incubation was repeated by the method described in section 2.5 using a single blood donor. However, star polymers were added at 100 μg mL⁻¹, and a modified antibody panel was used: CD3 (AF488, SP34-2), CD14 (PE-Cy7, M5E2), CD19 (PE, HIB19), and CD66b (BV421, 2H70), all purchased from BD Biosciences. Cells were sorted into FWB on an BD FACS ARIA III (100 μm nozzle). Coverslips (#1.5, Menzel-Glaser) treated with poly-L-lysine (Sigma-Aldrich) for 2 h, room temperature (RT), washed thrice with 1× PBS before sorted cells were added to coverslips and allowed to set for 1 h, RT. Cells were fixed with 1% formaldehyde, rinsed twice in PBS, and mounted onto glass slides using ProLong Diamond Antifade with DAPI (Thermo Fisher). Cells were imaged by confocal microscopy using 20× and 63× oil objectives (Zeiss LSM710 and ZEN 2012 Black software).

2.7. Association with RBC and Platelets. Freshly drawn blood (5 μL) was added to FACS tubes, containing approximately 25 × 10⁶ RBC and 1 × 10⁵ platelets. In the tubes, the volume was made 100 μL through the addition of 1× PBS, before preincubating on ice or 37 °C for 10 min. NPs (1–10 μg in PBS) were added to blood and incubated for a further 1 h, before placing all tubes on ice. Cells phenotyped on ice using titrated concentrations of antibodies (BD Biosciences) against CD42b (PE, HP1), CD45 (V500, HI30), and CD235a (FITC, GA-R2). Cells were washed four times with 4 mL of PBS (800 g, 15 min), fixed (1% formaldehyde), and analyzed by flow cytometry (LSRFortessa).

2.8. Human Platelet Activation. NPs (0.1, 1, 10 μg) or equal volume of PBS were added to FACS tubes, before carefully transferring blood (100 μL) immediately after collection and swirling gently. Adenosine diphosphate (ADP, 1 μM final concn, Sigma-Aldrich) was used as a positive control for platelet activation.32,33 Blood was incubated for 10 min at room temp, before adding 400 μL of cold (4 °C) BSA-Tyrode’s buffer as per ref 34 (1× PBS containing 130 mM NaCl, 2.6 mM KCl, 0.42 mM NaH₂PO₄, 5.5 mM d-glucose, 10 mM HEPES and 0.3% w/v bovine serum albumin). Platelets were phenotyped by transferring 100 μL into FACS tubes containing titrated concentrations of antibodies (BD Biosciences) against CD42b (PE, HP1), CD45 (V500, HI30), CD62P (BV650, AK-4), CD235a (FITC, GA-R2), and using the gating tree in Figure 9A. Samples were diluted 20-fold further using BSA-Tyrode’s buffer, and samples were analyzed immediately by flow cytometry (BD LSRFortessa).

2.9. Statistical Analyses. Results were analyzed using Wilcoxon nonparametric two-tailed matched pairs signed rank test (GraphPad Prism 7).

3. RESULTS

3.1. Synthesis and Characterization. Functionalizing the surface of NPs with nonantibody molecules that can facilitate interactions with cell subsets is a potential avenue toward targeted use of NPs. To this end, we synthesized star polymers having pyridyl disulfide (PDS)-terminated arms as an exemplar NP with a thiol reactive group in the particle corona. The stars were prepared by using RAFT polymerization to first synthesize the arms, followed by star formation via chain extension with a difunctional cross-linker (i.e., an “arm-first” approach) (Figure 1A). In order to provide PDS moieties on the distal end of the star arms, we employed a RAFT agent with a PDS group conjugated to the “R” group of the RAFT agent (2-(pyridine-2-ylsulfonyl)ethyl 2-(((dodecylthio)carbonothioylthio)-propanoate, PDSI), thus ensuring that the PDS group was located away from the cross-linked core of the star. An antifouling corona of mPEG brushes was provided by employing oligo-ethylenyl glycol methyl ether acrylate (OEGA, M₉ = 480 g mol⁻¹) as the monomer for the arm synthesis. The resulting poly(OEGA) (POEGA) arms were

Figure 2. PDS nanoparticles display enhanced uptake by human C1R cell line. C1R cells were seeded at 9.0 × 10⁴ cells per well in 96-well plates and incubated with star polymers at a final concentration of 10 μg mL⁻¹ in DMEM supplemented with 10% FBS. Cells were incubated with particles for between 10 and 120 min at either (a) 37 °C (left) or (b) 4 °C (right). Cells were washed four times in 1% BSA/PBS and resuspended in PBS containing the viability dye propidium iodide (PI; 0.5 μg mL⁻¹) for analysis by flow cytometry (Stratagene S100EXi). All experiments were performed in triplicate. PBS: fetal bovine serum.
characterized using $^1$H NMR, with the number-average molecular weight determined to be approximately 9800 g mol$^{-1}$. Moreover, further analysis by gel permeation chromatography (GPC) revealed that the POEGA exhibited unimodal molecular weight distribution ($M_n = 12,000$ g mol$^{-1}$ vs polystyrene standards, PDI = 1.20) with little evidence of high molecular weight coupled material or low molecular weight tailing (Table S1). Subsequent chain extension using N,N′-methylenebis(acrylamide) in the presence of the functional monomer 2-vinyl-4,4-dimethyl-5-oxazolone provided star polymers with $M_n = 123,000$ g mol$^{-1}$ and PDI = 1.18 (Figure S1, Table S1).

The stars were successfully isolated from the residual arm by precipitating into a mixture of diethyl ether and chloroform (90:10 v/v) and were fluorescently labeled by reacting the 2-vinyl-4,4-dimethyl-5-oxazolone units in the core with Cyanine5 (Cy5) amine. After exhaustive dialysis against methanol (MWCO = 3000 g mol$^{-1}$), successful fluorescent labeling was confirmed using fluorescence spectroscopy and by GPC with dual UV–vis and RI detection (Figure S2). Control particles without thiol-reactive moieties were prepared by replacing the

![Figure 3. PDS nanoparticles display enhanced association with primary phagocytic cells in whole human blood. PDS and BSPA control nanoparticles were incubated with freshly drawn, heparin-treated human blood at 10 μg mL$^{-1}$ for 1 h, 4 or 37 °C. Free nanoparticles were washed and RBCs lysed, before phenotyping cells on ice. Cell association with Cy5-labeled nanoparticles was determined by flow cytometry and represented by a median fluorescence index (MFI). (a) Representative gating tree from a single donor, demonstrating identification of granulocytes (gran), monocytes (mono), and dendritic cells (DC) at 37 °C. Red histograms represent cell association with PDS nanoparticles, blue with control nanoparticles. (b) Summary of cell association profiles from 7 healthy donors across independent experiments. Cell association was analyzed at 37 °C (top) and 4 °C (bottom). * p < 0.05, ** p < 0.01.](image-url)
PDSD with the nonfunctional RAFT agent 3-((benzylsulfanylthiocarbonylsulfanyl)-propionic acid (BSPA), as shown in Figure 1A,B. We estimate from the molecular weight analysis of the arms and stars that each star has approximately 10 arms attached. By dynamic light scattering (DLS), the hydrodynamic diameter of PDSD\(^{-}\)POEGA Star Cy5 (PDS stars) was 17 \(\pm\) 0.9 nm and control BSPA-POEGA Star Cy5 (BSPA stars) were 14 \(\pm\) 0.6 nm (Table S1). By transmission electron microscopy (Figure 1C), the stars measured <10 nm.

3.2. PDS Functionality Enhances Association with Cell Lines. It has previously been shown that various immortalized cancer cells lines express substantial levels of free thiols. To first confirm that the PDS NPs interact with cell-surface thiols, we incubated PDS and BSPA NPs with the CIR human B lymphoblast cell line (Figure 2). An enhanced association of PDS NPs was observed as early as 10 min at 37 °C (Figure 2A). At 4 °C, PDS NPs demonstrated enhanced cell association at 120 min (Figure 2B). Very little cell association with control star polymers not presenting thiol-reactive groups was observed at both temperatures.

3.3. Phagocytic WBC Association Is Enhanced by PDS Functionality. Many NP applications will involve intravenous injection of NPs, where they will first encounter a wide range of circulating cells in a process that is not well modeled by

**Figure 4.** PDS nanoparticles display enhanced association with B cells but not other nonphagocytic cells in whole human blood. PDS and BSPA control nanoparticles were incubated with freshly drawn, heparin-treated human blood at 10 \(\mu\)g mL\(^{-}\)\(^{-}\) for 1 h, at 4 or 37 °C. Free nanoparticles were washed and RBCs lysed, before phenotyping on ice. Cell association with Cy5-labeled nanoparticles was determined by flow cytometry and represented by a median fluorescence index (MFI). (a) Representative gating tree from a single donor, demonstrating cell identification at 37 °C. Red histograms represent cell association with PDS nanoparticles, blue with control nanoparticles. (b) Summary of cell association profiles from 7 healthy donors across independent experiments. Cell association was analyzed at 37 °C (top) and 4 °C (bottom). ** \(p < 0.01\).
studies the intensity of association with Cy5-labeled nanoparticles. Individual cells are colored based on their Cy5 fluorescence. PBS only demonstrates different autofluorescence profiles based on cell type, while changes in the intensity of association with Cy5-labeled nanoparticles reveals global association changes between PDS and control stars.

Figure 5. Global analysis of star nanoparticle association with white blood cells. To examine the global change in nanoparticle association with all white blood cell subsets, we performed a viSNE analysis (Cytobank) on the concatenated flow cytometry data of 5 donors at 37 °C. Each dot represents a single cell and spatially distinct clusters represent cell subsets based on the combination of cell-surface markers they express. Clustering is based on the t-Distributed Stochastic Neighbor Embedding (t-SNE) algorithm. (a) Reference plot indicating the cellular identity of viSNE clusters. Colors represent the identity of cell subsets based on conventional manual gating strategies. (b) Global cell association with Cy5-labeled nanoparticles. Individual cells are colored based on their Cy5 fluorescence. PBS only demonstrates different autofluorescence profiles based on cell type, while changes in the intensity of association with Cy5-labeled nanoparticles reveals global association changes between PDS and control stars.

Figure 6. Imaging cell interactions of star nanoparticles with primary blood cells. PDS and BSPA control nanoparticles were incubated with freshly drawn, heparin-treated human blood at 100 μg mL⁻¹ for 1 h, at 4 or 37 °C. Free nanoparticles were washed and RBCs lysed, before phenotyping cells on ice and sorting individual populations by fluorescence activated cell sorting. Sorted (a) monocytes, (b) granulocytes, (c) B cells, and (d) T cells were mounted onto polylysine coverslips and imaged by confocal microscopy. Middle slice from representative cell z-stacks is displayed. Merged images false-colored with Cy5 nanoparticles (red), DAPI (blue). Scale bar = 2 μm.

immunology. Targeting these cells to modulate immunity is of growing interest, with several monoclonal antibody products marketed to suppress aberrant immune responses. We therefore assessed whether thiol-reactive PDS star polymer NPs could interact with lymphocyte populations within fresh human blood (Figure 4). The gating strategy is shown in Figure 4A. Notably, B cells demonstrated a clear association preference for PDS NPs at 37 °C, with a 3.2-fold greater mean association than control NPs (Figure 4B). In contrast, T and NK cells displayed little association with either NP at both temperatures. Taking this data altogether, we concatenated the flow cytometry data of 5 different donors at 37 °C and performed high-dimensional viSNE analysis (Figure 5). Each dot represents a single cell that is clustered in 2D space based on its expression of cell-surface markers. This enabled the visualization of global changes in NP association across all studied WBC subsets. We used the manual gating strategies of...
Figure 3A and Figure 4A to identify the clustered cell populations (Figure 5A). As the clusters consist largely of one color, viSNE clustering confirmed the accuracy of our manual gating strategies. These plots also demonstrate the relative abundance of each cell subset that NPs are exposed to in blood. For example, granulocytes comprise a large proportion of WBCs while dendritic cells are very rare. When cell clusters are colored based on their association with Cy5-labeled NPs, we observed clear global changes in NP association due to the presence of PDS groups (Figure 5B).

**3.5. Nanoparticle Cellular Localization Using Confocal Microscopy.** The above analyses demonstrated enhanced association of PDS NPs to various blood WBCs compared with BSPA control NPs. Binding of PDS NPs to exofacial thiols may subsequently lead to enhanced NP uptake, particularly among phagocytic cells. The flow cytometry studies above however do not provide spatial information on whether NPs have been internalized or remain surface bound. To study NP uptake, we performed the fresh blood association assays as above, before obtaining purified individual cell populations using fluorescence-activated cell sorting and then imaged the cells by confocal microscopy (Figure 6). We found that PDS NPs were internalized by monocytes, with Cy5-NPs localized within the cytoplasm and frequently observed in cytoplasmic and surface clusters (Figure 6A). In most cells, PDS NPs filled the cytoplasm but did not enter the nucleus, as determined by DAPI staining. BSPA control NPs did not associate with monocytes. Similarly, granulocytes internalized PDS NPs into clusters, while also displaying very strong, diffuse intracellular signals suggesting possible endosomal escape (Figure 6B). Even though the star polymers were small (<10 nm by TEM), the PDS NPs were largely restricted from the nucleus. BSPA control NPs were rarely observed associating with granulocytes. In contrast, B-cell-localized NPs were restricted to the cell surface (Figure 6C), consistent with their nonphagocytic nature. T cells very rarely associated with any NPs, although

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**Figure 7.** PDS nanoparticles display minimally enhanced binding to red blood cells at the maximum concentration tested. PDS and BSPA control nanoparticles were incubated with freshly drawn, ACD-B treated human blood for 1 h, at 4 or 37 °C. Free nanoparticles were removed by washing before phenotyping on ice. Red blood cell association with Cy5-labeled nanoparticles was determined by flow cytometry and represented by a median fluorescence index (MFI). (a) Representative gating tree from a single donor, demonstrating association at 10 μg mL⁻¹, 37 °C. Red histograms represent association with PDS nanoparticles, blue with control nanoparticles. (b) Summary of association profiles at 37 °C (top) and 4 °C (bottom) from 5 healthy donors across independent experiments.
when NPs did associate with T cells (Figure 6D), they remained on the cell surface.

3.6. RBC Association Is Marginally Altered by PDS Functionality. RBCs are the most common cell type in blood, outnumbering WBCs by ∼1000-fold. These cells are impaired in a number of genetic diseases such as sickle-cell disease and targeted by certain infections, such as malaria parasites. Although RBCs do not leave the circulation, many studies have examined the utility of RBCs for cellular hitchhiking of NPs to intravascular targets due to their long circulating lifetimes of approximately 120 days. We therefore assessed the association of star polymer NPs with this important cell subset (Figure 7). Gating on RBC (Figure 7A) demonstrated association at 10 μg mL⁻¹, 37 °C. Red histograms represent platelet association with PDS nanoparticles, blue with control nanoparticles. (b) Summary of association profiles at 37 °C (top) and 4 °C (bottom) from 5 healthy donors across independent experiments. * p < 0.05.

3.7. PDS Functionality Enhances Association with Platelets. Platelets adhere to damaged endothelium, where they become activated and recruit additional platelets and clotting factors to form a platelet plug. Platelets are an interesting component of blood, which could be used to direct therapeutics to sites of vascular damage and were therefore assessed for NP association (Figure 8). After gating on platelets (Figure 8A), we assessed NP-platelet association in 5 healthy donors (Figure 8B). At 37 °C, we observed a large association preference for PDS NPs at 10 μg mL⁻¹ and 100 μg mL⁻¹ (5.0- and 7.5-fold, respectively). At 4 °C, there was still a trend for increased platelet association with PDS NPs, suggesting a biological process (e.g., uptake) is not solely responsible for the enhanced association.

3.8. Platelets Are Not Activated by Star Polymers. Aberrant platelet activation is associated with cardiovascular disease and NPs that lead to platelet activation could potentially have long-term undesirable side effects. Since the PDS NPs had enhanced platelet association, we examined how these NPs might affect platelet activation status. We studied the expression of the surface activation marker CD62P (P-selectin) immediately after incubating particles in fresh ACD-anti-coagulated blood and identified single platelets by flow cytometry (Figure 9A). This assay is traditionally performed at room temperature, where higher platelet activation responses are observed. Incubation of the blood with 10 μM ADP, a known positive control of platelet activation, increased the number of CD62P-expressing platelets ∼2-fold compared to PBS incubations across 6 separate blood donors (Figure 9B). Incubating with either NP at 1–100 μg mL⁻¹ did not increase CD62P expression above baseline levels.
The level of exofacial thiols expressed by hematopoietic cells is graded, with granulocytes > monocytes > lymphocytes (B cells > T cells) > RBCs, although less is known about the relative exofacial thiol levels on dendritic cells and platelets. However, platelets express protein disulfide isomerase on their surface, which catalyzes thiol–disulfide interchange and may facilitate enhanced association with PDS star polymers. The multicellular nature of our human blood model captures competition between cell types, and the association of the PDS star polymers studied mirrors the ranking of exofacial thiols in blood cells. In contrast, BSPA control NPs displayed stealth properties, and we observed relatively low association with phagocytic WBC, along with nonphagocytic WBC, RBC, and platelets. Altering the arm number and size of the PDS star polymers reported herein would likely alter their interactions with blood cell components and is currently under investigation. We note that blood proteins can also contain free thiol groups (such as albumin) and may influence the availability of PDS star polymers to interact with blood cell components.

Many tumors express surface thiol groups and targeting tumors with NPs displaying PDS groups may allow for the efficient delivery of cytotoxic drugs or imaging agents. We speculate that incorporating a PDS group may also be beneficial for nanovaccinology applications. PDS star polymers displayed enhanced association with dendritic cells—professional antigen presenting cells essential in the immune response to vaccination. Moreover, B cell association was enhanced upon PDS incorporation. This may be beneficial in the treatment of B cell diseases, such as lymphomas, or manipulating humoral immunity for improved antibody responses to vaccination.

The utility of blood cells as natural NP carriers has been extensively explored. This concept, termed “cellular hitchhiking,” has been studied in cell lines and rodent models also with some success in avoiding immune clearance and improving tissue targeting. RBCs have been investigated for their ability to improve the pharmacokinetics (circulation) and biodistribution (enhanced lung targeting) of NPs. RBCs have low levels of free exofacial thiols, which may explain the relatively low association we observed with PDS NPs. In contrast, monocytes display levels of exofacial thiols that far exceed RBCs and lymphocytes, which is consistent with the high association of monocytes with PDS star polymers that we observed. Monocytes have a half-life of 20 h, after which they extravasate from the circulation and become tissue macrophages. Following extravasation, these can migrate to inflamed tissues or deep into hypoxic regions of tumors. Monocytes have therefore served as Trojan horses to increase the delivery of therapeutics to sites of inflammation and even...
deliver these across the blood brain barrier. The PDS NPs described herein also demonstrated enhanced association with platelets, which have a circulating lifespan of 7–9 days. The engineering of NPs to mimic platelets has been investigated for the treatment of vascular injuries, and hitchhiking NPs onto platelets represents another strategy to harness their innate ability to target vascular damage and/or alter hemostasis. We adapted our previously published whole blood assay to facilitate the study of platelet association and their activation state through examining the cell-surface molecule CD62P (P-selectin). The PDS NPs were nonactivating under the conditions studied; however, further safety studies are warranted.

5. CONCLUSIONS

Engineering PDS groups onto small star polymer NPs resulted in significant changes in NP association with a variety of human blood cell components and will likely alter intravenous biodistribution profiles. There are several potential applications in both tumor targeting and immune cell targeting for this technology that warrant further investigation.

■ ASSOCIATED CONTENT

1 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.6b15942.

Physical characterization summary of POEGA arms and POEGA star polymers, GPC analysis of arms and stars, fluorescence spectra and GPC chromatograms of Cy5-labeled stars (PDF)

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