Vascular-targeted particle binding efficacy in the presence of rigid red blood cells: Implications for performance in diseased blood

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The field of drug delivery has taken an interest in combating numerous blood and heart diseases via the use of injectable vascular-targeted carriers (VTCs). However, VTC technology has encountered limited efficacy due to a variety of challenges associated with the immense complexity of the *in vivo* blood flow environment, including the hemodynamic interactions of blood cells, which impact their margination and adhesion to the vascular wall. Red blood cell (RBC) physiology, i.e., size, shape, and deformability, drive cellular distribution in blood flow and has been shown to impact VTC margination to the vessel wall significantly. The RBC shape and deformability are known to be altered in certain human diseases, yet little experimental work has been conducted towards understanding the effect of these alterations, specifically RBC rigidity, on VTC dynamics in physiological blood flow. In this work, we investigate the impact of RBCs of varying stiffnesses on the adhesion efficacy of particles of various sizes, moduli, and shapes onto an inflamed endothelial layer in a human vasculature-inspired, *in vitro* blood flow model. The blood rigid RBC compositions and degrees of RBC stiffness evaluated are analogous to conditions in diseases such as sickle cell disease. We find that particles of different sizes, moduli, and shapes yield drastically different adhesion patterns in blood flow in the presence of rigid RBCs when compared to 100% healthy RBCs. Specifically, up to 50% reduction in the localization and adhesion of non-deformable 2 μm particles to the vessel wall was observed in the presence of rigid RBCs. Interestingly, deformable 2 μm particles showed enhanced vessel wall localization and adhesion, by up to 85%, depending on the rigidity of RBCs evaluated. Ultimately, this work experimentally clarifies the importance of considering RBC rigidity in the intelligent design of particle therapeutics and highlights possible implications for a wide range of diseases relating to RBC deformability.

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I. INTRODUCTION

Particle therapeutics have received much attention for use in disease treatment due to their high potential for achieving localized delivery of drugs and imaging agents and, in some cases, for the added benefit of controlled release of therapeutics.¹ The administration of therapeutic particles via intravenous (IV) injection is attractive due to the (1) non-invasive nature and (2) extensive reach of the vascular system to target tissues throughout the body. Often, disease-specific proteins expressed by the endothelium—the monolayer of cells lining the lumen of most blood vessels—are employed for target recognition by the particle therapeutics, i.e., vascular-targeting.

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For example, in certain diseases, such as atherosclerosis, the endothelium produces an inflammatory response,^{2,3} which upregulates inflammatory surface markers not found on the endothelium in healthy tissues and, thus, is attractive for use as targets to disease.³ Therefore, targeted-drug delivery systems employ the conjugation of surface moieties, such as peptides and antibodies, with high affinity for these differentially expressed inflammatory disease markers. In theory, particulate carriers fitted with the disease-specific moieties will be captured to the blood vessel wall expressing the targeted protein in the diseased tissue. However, to target the diseased vascular wall effectively, particularly in mid-to-large arterioles and postcapillary venules, particles must first localize, i.e., marginate, to the vessel walls.^{4–6} Researchers have shown that particle margination is highly dependent on the biophysical, multi-component nature of blood.^{6–14}

Blood is a highly concentrated non-Newtonian suspension of red blood cells (RBCs), white blood cells (WBCs), platelets, and plasma.¹⁵ The high deformability of RBCs is a unique characteristic that significantly influences particle margination in blood flow.^{7–10} In particular, the deformable RBCs are vastly susceptible to experiencing a lift force, which causes them to migrate away from the vessel wall and localize in the radial center of the vessel as blood flows.^{8,11–13} Particle-RBC collisions in the core of the vessel then promote the migration of particles towards the vessel wall.^{7,13} It is known that particle size is a critical factor which determines the margination efficacy of particle therapeutics.^{16–21} In particular, work done by Charoenphol *et al.* indicates that spherical microparticles (MPs) best marginate to the vascular wall and nanometer-sized vascular-targeted carriers (VTCs) exhibit less efficient margination.^{22,23} Namdee *et al.* attributed this reduced margination to spherical nanoparticles (NPs) getting trapped in the RBC core.²⁰ Once out of the RBC core, the near-absolute absence of RBCs near the vessel wall, i.e., cell-free layer (CFL), allows the proper binding of particles to the vessel wall.^{7,13} Given the importance of RBC deformability in the margination of particles and hence their adhesion to the vascular, any alteration in RBC flexibility, as found in certain human diseases,^{24,25} would undoubtedly affect particle margination and adhesion.

RBC deformability is known to be drastically altered not only in certain diseases of genetic origin, such as sickle cell disease (SCD) and hereditary spherocytosis,^{24,25} but also in others that developed upon a viral or parasitic invasion, such as human immunodeficiency virus (HIV) and malaria.^{25,26} The RBCs in patients with Type 2 Diabetes (T2D) have also been reported to be significantly less deformable than healthy cells.²⁷ Metabolic disorders such as obesity and lifestyle habits such as smoking can also reduce the deformability of RBCs.^{28–30} Additionally, studies have shown that RBC populations become more *stiff* and less deformable, on average in natural aging.^{30,31} Since particle therapeutics aim to treat diseases that are most common in older age groups, such as coronary artery disease, understanding particle dynamics and localization functionality in the presence of rigid RBCs could hold significant implications for designing particle therapeutics with high efficacy in disorders with altered RBCs.^{32–34} To date, despite a vast number of computational and experimental studies that have examined RBC and particle margination in blood flow, there is no investigation into how changes in RBC deformability impact particle margination and, hence, binding efficacy. Thus, this work experimentally probes the direct impact of rigidified RBCs on the adhesion dynamics of particles in blood flows via *in vitro* assays in a parallel plate flow chamber (PPFC). We report that particle adhesion in human blood flow can be significantly reduced or improved depending on the particle modulus, shape, and size where being deformable can lead to a positive impact of rigid RBCs on particle adhesion. We believe that this proposed work is the first comprehensive, experimental investigation into how rigidified RBCs affect the ability of VTCs to marginate and adhere to the vascular wall. The knowledge presented is of importance as it can provide opportunities for developing new therapeutics with high efficacy in human diseases.

II. MATERIALS AND METHODS

A. Study approvals and preparation of human blood

Human blood donors signed a written informed consent for the collection of their blood via venipuncture. All blood flow assays were done with fresh blood, which was collected into a syringe

containing citric acid-sodium citrate-dextrose (ACD) as an anticoagulant and stored at 37 °C until used, as described in the study by Onyskiw *et al.*³⁵ The blood drawing protocol was approved by the University of Michigan Internal Review Board (IRB-MED). 6% Dextran was added to the anti-coagulated blood to assist with the isolation of RBCs from WBC-rich plasma. Once separated, RBCs were washed by suspending in phosphate-buffered saline (PBS) (–/–) and centrifugation. WBC-rich plasma was stored independently from the RBCs in a water bath at 37 °C.

B. Red blood cell rigidification and characterization

Washed RBCs were treated with a 2% Hct in PBS (–/–) with varying concentrations of tert-butyl hydroperoxide (TBHP) for 30 min as described by Gutierrez *et al.*³⁶ Treatment with TBHP induces oxidative stress on the cellular membrane by lipid peroxidation, and this causes the RBC membrane to become rigid.^{37,38} Different concentrations of TBHP generate distinct degrees of rigidity. Gutierrez *et al.* previously characterized the rigidities of three different TBHP concentrations, 1.0 mM, 0.75 mM, and 0.5 mM, using an ektacytometer (LORRCA; Mechatronics, Hoorn, The Netherlands), which showed significant alteration in RBC deformation.^{36,39} Cell deformation is expressed as a dimensionless elongation index (EI), Eq. (1), where the major and minor axes of a RBC are represented as A and B, respectively.³⁶ The level of RBC rigidity was found to scale with the concentration of TBHP, where 1.0 mM was the least deformable, while the 0.75 mM and 0.5 mM treatments displaced an intermediate and low rigidity, respectively, relative to the untreated RBC.³⁶ Elongation indices were generated for a range of shear stresses for each rigidity condition. At a shear stress of ~4 Pa, analogous to a shear rate of 1000 s^{–1}, the TBHP treatments of 1.0, 0.75, and 0.5 mM generated RBCs with EIs that were 9, 4, and 2 times smaller, and thus more rigid, than those of healthy RBCs, respectively. In this work, these rigidity treatments are represented as *highly*, *moderately*, and *slightly* rigid RBCs, respectively. The elongation index versus shear stress curves for the various treatments are shown in Fig. S1

$$EI = \frac{A - B}{A + B}. \quad (1)$$

C. Preparation of the endothelial cell monolayer

Healthy human umbilical cords were obtained from Mott Children's Hospital (Ann Arbor, MI). Using a collagenase perfusion method described by Onyskiw *et al.*, human umbilical vein endothelial cells (HUVECs) were isolated and briefly cultured into T75 flasks.^{34,36} HUVECs were seeded onto 30 mm glass coverslips coated with 1% gelatin and incubated with standard media at 37 °C and 5% CO₂ until confluent density was reached.^{19,23} Confluent cells on coverslips were activated with 1-ng/ml interleukin-1 β (IL-1 β) for 4 h, to induce inflammation and the expression of cell-adhesion molecules for particle binding, before use in blood flow experiments. Previous reports show that E-Selectin receptor expression on HUVECs is maximal (~20-fold increase compared to unactivated HUVECs) 4–8 h after addition of IL-1 β .⁴⁰ No HUVECs beyond passage 4 were used in this investigation.

D. Deformable hydrogel particle fabrication and characterization

Deformable hydrogel particles of 2 μ m diameter were fabricated by UV-initiated polymerization of polyethylene (glycol) diacrylate (PEGDA) and 2-carboxyethyl acrylate (CEA) as described by Fish *et al.*⁴¹ PEGDA, CEA, acryloyloxyethyl thiocarbonyl Rhodamine B (Rhodamine), and lithium phenyl-2,4,6-trimethylbenzoylphosphine photoinitiator (PI) were combined into methanol (MeOH) at 20% solids (80% MeOH). The MeOH-PEGDA solution was then emulsified into silicone oil by probe sonication and subsequently exposed to a 365 nm UV light for 10 min to initiate polymerization. All materials are obtained commercially, except the PI which is synthesized internally as previously published.^{42,43} Hydrogel particles of 2 μ m

diameter were captured by a series of centrifugation and filtration steps as previously described by Fish *et al.*⁴¹ The deformability of the particles can easily be controlled by modulating the hydrogel cross-linking density. The Young's moduli (~ 113 kPa) of the hydrogel particles (20% solids) closely match a value reported for WBCs previously.⁴¹

E. Ellipsoidal shaped particle fabrication and characterization

Polystyrene (PS) rods are produced by using polyvinyl alcohol films to stretch $2\text{ }\mu\text{m}$ Fluoresbrite YG Carboxylate Microspheres (Polysciences, Inc). The spherical $2\text{ }\mu\text{m}$ particles were suspended in 100 ml aqueous solution of 7% w/v, 13 000–23 000 MW polyvinyl alcohol (PVA) (Sigma Aldrich). The vials were placed on a rotator for 1 h to be mixed in thoroughly. The solution was then poured onto a single-well OmniTray (Thermo Fisher) and put on a flat shelf in a convection oven at 50°C overnight. The resulting films were cut into $2\text{ in.} \times 1\text{ in.}$ strips and attached to a single direction stretching apparatus within a convection oven that holds the strips with two parallel clamps—one stationary and the other moved along a pipe in one direction. The oven was preheated to 160°C before a pump pulled the moving clamp down the pipe and stretched the films to produce rod particles. The un-stretched ends of the films were cut off. The remaining films were dissolved with 30% v/v isopropanol (Sigma Aldrich). The rod particles were centrifuged and washed several times to remove the remaining PVA. The recovered particles were imaged using a Philips XL30 FEG scanning electron microscope (courtesy of the University of Michigan EMAL). The particle dimensions were measured using SEM images via Metamorph Software (Molecular Devices, LLC). The rods produced have a ratio of the major axis length to the minor axis length (aspect ratio) of 4 (AR4).

F. Particle conjugation with targeting ligands

FluoresbriteTM YG Carboxylated polystyrene rods and spheres were conjugated with a NeutrAvidin Biotin-Binding Protein (Thermo Scientific) via carbodiimide coupling chemistry as previously described.²⁰ Subsequently, avidin-coated particles were conjugated with biotinylated sialyl-Lewis A (sLe^a) (Glycotek, Inc.) and stored in PBS (–/–) with 1% wt. bovine serum albumin (BSA) at 4°C until ready for use in blood flow experiments. To estimate the particle ligand density, particles with NeutrAvidin bonded on their surface were reacted with a range of Sialyl Lewis A (sLe^a) concentrations up to $10\text{ }\mu\text{g/ml}$. Particles with reacted sLe^a were then stained with fluorescently labeled anti-CLA (allophycocyanin; APC) and subsequently examined via flow cytometry (Attune; Applied Biosystems). The particle populations of interest were gated, and the mean fluorescent intensity (MFI), in the APC regime $\sim 660\text{ nm}$, of each subset of particles was recorded as demonstrated in Fig. S2 and Table S1. Using calibration beads (Bangs Laboratories, Inc.), we created a calibration curve to convert MFI values into molecules of equivalent soluble fluorochrome (MESF) values. With the MESF and surface area of a single particle, we can approximate the number of sLe^a sites on a single particle. The ligand site density is determined by normalizing the sLe^a sites by the particle surface area. We interpolated, in the linear regime, in between the range of concentrations tested to determine which sLe^a concentration can achieve the desired number of sites on the different types of particles used, as demonstrated in Fig. S3. A fixed sLe^a site density of $\sim 1000\text{ sLe}^a\text{ site}/\mu\text{m}^2$ was used in all assays unless otherwise stated.

G. Parallel plate flow chamber laminar blood flow assays

Laminar blood flow assays were conducted with a parallel plate flow chamber (PPFC, Glycotek, Inc.) in combination with HUVEC confluent coverslips as previously described by Gutierrez *et al.*³⁶ All adhesion experiments were performed at 37°C . The PPFC was fitted with a rectangular silicone gasket ($0.25\text{ cm} \times 2\text{ cm} \times 127\text{ }\mu\text{m}$) and the coverslip with a confluent HUVEC monolayer (IL-1 β -activated) held with vacuum to the PPFC. The gasket thickness ($127\text{ }\mu\text{m}$) dictated the channel height. RBCs isolated from whole blood were first treated with TBHP solutions independently from other blood components, thoroughly washed, and reconstituted into the whole

blood at the desired concentrations with the final hematocrit (Hct) fixed at 40%. Blood samples were prepared to have different rigid RBC concentrations, e.g., 20% of the total RBC volume was treated and 80% were healthy cells as previously described.³⁶ The rigid RBC volume percentages examined were 20%, 30%, 50%, 70%, 90%, and 100%; all labels in figures represent the percent rigidified RBCs present. In the 100% rigid RBC volume conditions, all of the RBCs (at 40% Hct) were treated with the specified TBHP concentration, i.e., rigidity treatment. Healthy blood conditions were used as an experimental control, i.e., reconstituted to 40% Hct with healthy (no TBHP treatment) RBCs. Subsequently, individual blood samples were perfused through the open-loop, straight PPFC in a laminar flow profile for 5 min at a wall shear rate (WSR; γ_w) of 1000 s^{-1} , as determined by Eq. (2), which is physiologically relevant in certain arterioles and potentially some venules.^{44–46} h is the channel height (cm), w the channel width (cm), and Q the volumetric flow rate (ml/s), which was controlled via a syringe pump (Harvard Apparatus)

$$\gamma_w = \frac{6Q}{h^2w}; \text{ s}^{-1}. \quad (2)$$

The PPFC was carefully flushed with PBS+/+ buffer (PBS, containing 1% BSA crystals, Ca^{2+} , and Mg^{2+} ; $\text{pH} \sim 7.4$) prior to the perfusion of the blood to clear out HUVEC surface debris and bubbles. Particles were added to whole blood samples at a concentration of $\sim 10^6$ particles/ml of blood and immediately perfused over the HUVEC layer in the PPFC. After blood perfusion (5 min), PBS+/+ buffer was continuously added to the PPFC to maintain the fully developed flow profile and to wash out any unbound particles still in flow. Previous publications have shown that E-selectin expression, endothelial cell layer integrity (detachment), and the flow profile are maintained within the experimental time frame explored in this work.²² Fluorescent particle adhesion to the activated HUVEC layer was observed on a Nikon TE2000-S inverted microscope fitted with a Photometrics CoolSNAP EZ digital camera with a Sony CCD sensor. The particle adhesion density was quantified using ImageJ. To avoid the potential variation in fluxes along the chamber length, adhesion data were collected at a constant position of $\sim 1 \text{ cm}$ (center) away from the channel entrance and exit. Entrance length estimations predict that the flow profile is fully developed $\sim 20 \mu\text{m}$ from the channel entry, well before the center of the channel where most of the data acquisition occurred. All adhesion results were quantified as the number of bound particles/ mm^2 HUVEC. All particle adhesion data were expressed as the percent reduction relative to the healthy control of the same blood donor, thus normalizing for variability across donors.

H. Particle localization in blood flow studies leveraging a confocal microscope

Blood samples were perfused through a $\sim 100 \mu\text{m}$ ibidi channel, similar to the *in vitro* PPFC setup, as previously described.²⁰ Images were taken of the entire height of the channel in increments of $2 \mu\text{m}$ using a confocal microscope with 488-nm (to image FITC polystyrene particles) and 543-nm (to image rhodamine hydrogel particles) lasers at 80% power. The microscope ran in a round-trip 0.488 ms/line using a $2\times$ digital zoom and a $20\times$ water immersive objective. Fluorescent images of particles were counted using ImageJ by setting a constant threshold and the “Analyze Particle” function. Laser, photo-multiplier tube (PMT), and threshold settings were optimized for blank samples (no fluorescent particles) to contain less than 1% count of fluorescent particle-positive trials. The shutter speed was set to allow for visualization of individual fluorescent particles in the flow. The inside of the channel was marked with a red fluorescent protein (RFP) marker to facilitate the calibration of the starting height (inside-top of the channel) for the image scan to begin. The results presented represent the number of detected fluorescent particles in the scanned focal plane, not simply the mean fluorescent intensity. All experiments were normalized to the corresponding healthy control.

I. Statistical analysis

Particle adhesion data collected from the *in vitro* PPFC model are an average of ten pictures from each trial, with $n=3$ blood donors for each data point presented. Similar to the

work done by Gutierrez *et al.*,³⁶ the ten brightfield images obtained for each data point ($n = 1$) were average, and the standard error was calculated and represented as error bars shown in all the figures. The average values obtained for each “n” were averaged over multiple donors ($n = 3$), and the standard error was obtained by error propagation calculations. A 2-way ANOVA integrated package on GraphPad Prism was used to analyze any statistical difference between rigid and healthy conditions. Asterisks indicate p values of $* < 0.05$, $** < 0.01$, and $*** < 0.001$, and lack of asterisks indicates not significant (n.s.).

III. RESULTS

A. Effect of rigid RBCs on 2 μm stiff polystyrene particles as a function of RBC rigidity

We began by examining how the presence of rigidified RBCs affects the adhesion of 2 μm “rigid” polystyrene (PS) microspheres (MPs) to an activated HUVEC layer in whole blood flow. We tested a range of RBC rigidities ($9\times$, $4\times$, and $2\times$ times more rigid than healthy RBCs) and at different concentrations (20%, 30%, 50%, 70%, 90%, and 100%) of rigid RBCs in blood. Polystyrene spheres were used as a proxy for biodegradable, polymeric particle carriers. As previously described, the 2 μm PS spheres were functionalized with biotinylated sLe^a at ~ 1000 sLe^a site/ μm^2 and introduced into whole blood samples at a concentration of $\sim 10^6$ particles/ml of blood.^{22,35} RBCs that were $9\times$ more rigid than healthy RBCs are presented as the *highly rigid* condition, ones that were $4\times$ more rigid presented as *moderately rigid*, and RBCs that were $2\times$ more rigid are labeled as the *slightly rigid*. The EIs produced by the 0.75 mM and 0.5 mM TBHP treatments are of particular interest due to their pathological relevance; the rigidities of RBCs in SCD patients in stable conditions and SCD patients in crisis closely resemble the RBC rigidities generated with 0.5 mM and 0.75 mM TBHP, respectively.^{36,47} Also, it is known that patients can have different fractions (percentages) of rigidified RBCs present in their whole blood.^{36,47}

Figure 1 shows the impact of RBC rigidity on particle adhesion to the vessel wall as a function of RBC stiffness in a 127 μm height channel. The data are represented as the fraction reduction in particle adhesion in rigid RBC conditions relative to the adhesion of the same particles in a healthy condition, i.e., with no rigid RBCs present, to eliminate donor-to-donor variations. The raw adhesion density for the healthy condition represented in Fig. 1 is shown in Fig. S4. We show in Fig. 1 that the presence of rigid RBCs in the blood flow produced a reduction in the adhesion of 2 μm PS MPs relative to the binding found in the healthy control regardless

FIG. 1. Adhesion of 2 μm polystyrene particles to inflamed endothelium *in vitro* by RBC rigidity: Quantified 2 μm polystyrene particle binding to an IL-1 β inflamed endothelial layer at 10^6 particles/ml of whole blood as a function of rigid RBCs present, i.e., % volume rigid RBC. Each figure utilized rigid RBCs with different degrees of rigidity: (a) 1.0 mM treated RBCs (*highly rigid*), (b) 0.75 mM treated RBCs (*moderately rigid*), and (c) 0.5 mM treated RBCs (*slightly rigid*). A WSR of 1000 s^{-1} was used for all the shown conditions. Polystyrene particles are conjugated with NeutrAvidin and functionalized with a sLe^a binding ligand, ~ 1000 sites/ μm^2 . All trials are plotted as reduction compared to respective healthy controls, i.e., with no rigid RBCs present, thus eliminating the donor variation. Error bars represent the standard error. The statistical analysis of adherent density was performed using two-way ANOVA to test all particle adhesion versus healthy particle adhesion control. (*) indicates $p < 0.05$, (**) indicates $p < 0.01$, and (***) indicates $p < 0.001$.

of the RBC stiffness and percentage of rigid RBCs present. For assays with *highly rigid* RBCs, a maximum reduction ($\sim 50\%$, $p < 0.01$) in the adhesion of the PS MPs occurred when 20% of the total RBCs present were rigidified [Fig. 1(a)]. As we increased the percentage of the rigid RBCs in the blood sample, there was a slight decrease in the adhesion reduction to $\sim 25\%$ lower than adhesion in the healthy control. However, the reduction in MP adhesion beyond the 30% rigid RBC in flow mark was not significant, that is, PS MP adhesion in blood with *highly rigid* RBCs was not statistically different from adhesion in healthy conditions. Figure 1(b) shows a plot of the trend in $2\ \mu\text{m}$ PS particle adhesion reduction for the case where *moderately rigid* RBCs were present in the blood flow. Here, we observed a non-linear relationship between adhesion reduction and the percent of rigid RBCs present in the blood. Initially, the level of decrease in MP adhesion increased as the percentage of rigid RBCs in blood is increased up to the 50% mark, corresponding to the highest adhesion reduction ($\sim 52\%$, $p < 0.01$). As the percentage of rigid RBCs in blood increased beyond the 50%, the decrease in MP adhesion was reduced. At 100% of *moderately rigid* RBCs present in the flow, no difference in particle adhesion was observed relative to the binding in the healthy condition. Interestingly, there was no particular trend in the impact of the *slightly rigid* RBCs on the adhesion of the $2\ \mu\text{m}$ PS MP as the percentage of rigid RBCs is increased [Fig. 1(c)]. Instead, we observed the highest reduction in adhesion ($\sim 58\%$, $p < 0.001$) when 20%, 50%, and 90% of total RBCs present were slightly rigid. Conversely, there was a negligible reduction in MP adhesion when 30% of RBCs present were slightly rigid. At 70% and 100% of the *slightly rigid* RBCs present, the adhesion of the $2\ \mu\text{m}$ PS MPs was reduced by $\sim 25\%$, which was not significant from the healthy condition.

B. Effect of rigid RBCs on $2\ \mu\text{m}$ deformable hydrogel particles as a function of RBC rigidity

To evaluate the impact of particle deformability on the dynamics of adhesion in the presence of rigid RBCs, we fabricated $2\ \mu\text{m}$ hydrogel (PEG-based) particles (PEG-MP) and modulated the cross-linking density to achieve particle deformability similar to the deformability reported for WBCs ($\sim 113\ \text{kPa}$, Young's modulus).⁴¹ The PEG-MPs were conjugated with sLe^a at $\sim 1000\ \text{sLe}^a\ \text{site}/\mu\text{m}^2$ similar to polystyrene MPs. Figure 2(a) shows the percent difference in the adhesion of the $2\ \mu\text{m}$ PEG-MP in the presence of the *highly rigid* RBCs relative to the healthy condition. At the lowest concentration of *highly rigid* RBCs, e.g., 20%, we observed a

FIG. 2. Adhesion of $2\ \mu\text{m}$ hydrogel particles to inflamed endothelium *in vitro* by RBC rigidity: Quantified $2\ \mu\text{m}$ hydrogel particle binding to an IL-1 β inflamed endothelial layer at 10^6 particles/ml of whole blood as a function of rigid RBCs present, i.e., % volume rigid RBC. Each figure utilized rigid RBCs with different degrees of rigidity: (a) 1.0 mM treated RBCs (*highly rigid*), (b) 0.75 mM treated RBCs (*moderately rigid*), and (c) 0.5 mM treated RBCs (*slightly rigid*). A WSR of $1000\ \text{s}^{-1}$ was used for all the shown conditions. Hydrogel particles are conjugated with NeutrAvidin and functionalized with a sLe^a binding ligand, $\sim 1000\ \text{sites}/\mu\text{m}^2$. All trials are plotted as reduction compared to respective healthy controls, i.e., with no rigid RBCs present, thus eliminating the donor variation. Error bars represent the standard error. The statistical analysis of adherent density was performed using two-way ANOVA to test all particle adhesion versus healthy particle adhesion control. (*) indicates $p < 0.05$, (**) indicates $p < 0.01$, and (***) indicates $p < 0.001$.

slight reduction in the adhesion of $2\text{ }\mu\text{m}$ PEG-MP, which represents the maximum adhesion reduction seen for this particle type across all rigid RBC concentrations similar to the observation with the non-deformable polystyrene MPs. Interestingly, as the level of rigid RBCs present in blood increased, the reduction in hydrogel particle adhesion relative to the healthy condition decreased linearly. A negative percent reduction in PEG-MP adhesion was observed beyond 50% *highly rigid* RBCs in blood, which translates to a raw particle adhesion that is higher than the level achieved in a healthy blood flow condition. Surprisingly, when 100% of the RBCs present were rigid, we observed an adhesion reduction of -85% ($p < 0.001$), i.e., nearly twice the adhesion density of PEG-MPs which was found for the healthy condition. A visual representation of the enhanced $2\text{ }\mu\text{m}$ PEG-MP adhesion to the endothelial layer is presented in Fig. S5. When examining the impact of the *moderately rigid* RBCs, we observed a similar trend to the one seen for the *highly rigid ones*. At low percentages of the *moderately rigid* RBCs present in blood flow, a slight reduction in adhesion was observed for the PEG MPs relative to the healthy condition. As the percentage of rigid RBCs present increased, the particle adhesion improved towards similar values as obtained for the healthy condition. However, unlike the *highly rigid* RBC case, an enhanced PEG-MP adhesion relative to the healthy condition was only observed for the 100% *moderately rigid* RBCs present in flow, $\sim -78\%$ ($p < 0.001$). Finally, when we examined the adhesion of the PEG-MP in the presence of the *slightly rigid* RBCs, we saw a similar oscillate-trend as was observed for polystyrene MPs in Fig. 1(c). A reduction in particle adhesion occurred when 20%, 50%, and 90% of the RBCs present were rigid. When 30%, 70%, and 100% of the RBCs present were rigid, no difference in $2\text{ }\mu\text{m}$ hydrogel particle adhesion was observed compared to particle adhesion in the healthy condition.

C. Effect of rigid RBCs on rigid 500 nm polystyrene particles as a function of RBC rigidity

Given the significant impact of rigid RBCs on adhesion patterns observed for MPs, we investigated the adhesion performance of rigid polystyrene NPs, which are typically proposed for drug delivery applications. Here, we used 500 nm sized polystyrene particles and functionalized them with sLe^a as described with MPs. We have previously demonstrated that spherical nanoparticles between 100 and 500 nm show the same level of margination and adhesion in human blood flow.^{22,23} Again, the adhesion of the 500 nm polystyrene NPs in blood flow with rigidified RBCs is presented relative to adhesion in a healthy blood flow condition. Figure S4 shows the raw particle adhesion density for 500 nm polystyrene particles at a WSR of 1000 s^{-1} ($\sim 900\text{ \#/mm}^2$), which was lower than the adhesion observed for polystyrene MPs in line with previous works.²² As shown in Fig. 3, rigidified RBCs in blood did not have any significant impact on the adhesion of PS NPs regardless of the level of RBC rigidity. Also, no specific particle adhesion reduction trend was visible when the percentage of rigid RBCs was varied.

D. Effect of rigid RBCs on rigid ellipsoidal polystyrene particles as a function of RBC rigidity

We also investigated the adhesion performance of non-spherical particles in whole blood containing rigidified RBCs due to previous interest in the use of such particles in cellular targeting and drug delivery. Previous reports have shown that non-spherical, specifically rods, particles exhibit better adhesion capabilities when compared to their spherical counterparts.^{16,17,21,48,49} We used polystyrene rod particles that were generated from heat-stretching of $2\text{ }\mu\text{m}$ spherical particles to an aspect ratio of 4 (AR4). Figure 4 shows the adhesion reduction trends for sLe^a-coated rods in the presence of rigid RBCs across the different levels of rigidity evaluated. The raw rod particle adhesion density in normal blood at a WSR of 1000 s^{-1} is shown in Fig. S1. Interestingly, negligible differences in adhesion were observed for most blood flow conditions with the exception being rods in blood with 100% *highly rigid* RBCs [Fig. 4(a)]. Here, the level of rod particle adhesion reduction was $\sim -50\%$ ($p < 0.05$), which translates to a 50% greater rod adhesion density compared to their adhesion when no rigid RBCs

FIG. 3. Adhesion of 500 nm polystyrene particles to inflamed endothelium *in vitro* by RBC rigidity: Quantified 500 nm polystyrene particle binding to an IL-1 β inflamed endothelial layer at 10^6 particles/ml of whole blood as a function of rigid RBCs present, i.e., % volume rigid RBC. Each figure utilized rigid RBCs with different degrees of rigidity: (a) 1.0 mM treated RBCs (*highly rigid*), (b) 0.75 mM treated RBCs (*moderately rigid*), and (c) 0.5 mM treated RBCs (*slightly rigid*). A WSR of 1000 s^{-1} was used for all the shown conditions. Polystyrene particles are conjugated with NeutrAvidin and functionalized with a sLe^a binding ligand, $\sim 1000 \text{ sites}/\mu\text{m}^2$. All trials are plotted as reduction compared to respective healthy controls, i.e., with no rigid RBCs present, thus eliminating the donor variation. Error bars represent the standard error. The statistical analysis of adherent density was performed using two-way ANOVA to test all particle adhesion versus healthy particle adhesion control. (*) indicates $p < 0.05$, (**) indicates $p < 0.01$, and (***) indicates $p < 0.001$.

were present. While the rod particles exhibited negative reductions for other conditions with the *highly rigid* RBCs, these were not significant relative to the healthy condition.

E. Effect of targeting ligand density on particle adhesion trends

With the understanding that particle adhesion in flow is a function of the receptor-ligand kinetics, which can vary with the targeting ligand density, we evaluated the impact of rigid RBCs on the adhesion of particles at a higher targeting ligand density. The experimental conditions in Figs. 1(a) and 2(a) were repeated but with MPs at three times the sLe^a density using the most rigid RBCs (1.0 mM TBHP). We focus on only the $2 \mu\text{m}$ spheres for this evaluation, given that minimal differences in adhesion were observed with the presence of rigid RBCs for

FIG. 4. The adhesion of $2 \mu\text{m}$ volume polystyrene rod (AR4) particles to an inflamed endothelium *in vitro* by RBC rigidity: quantified AR4 rod polystyrene particle binding to an IL-1 β inflamed endothelial layer at 10^6 particles/ml of whole blood as a function of rigid RBCs present, i.e., % volume rigid RBC. Each figure utilized rigid RBCs with different degrees of rigidity: (a) 1.0 mM treated RBCs (*highly rigid*), (b) 0.75 mM treated RBCs (*moderately rigid*), and (c) 0.5 mM treated RBCs (*slightly rigid*). A WSR of 1000 s^{-1} was used for all the shown conditions. AR4 polystyrene rod particles are conjugated with NeutrAvidin and functionalized with a sLe^a binding ligand, $\sim 1000 \text{ sites}/\mu\text{m}^2$. All trials are plotted as reduction compared to respective healthy controls, i.e., with no rigid RBCs present, thus eliminating the donor variation. Error bars represent the standard error. The statistical analysis of adherent density was performed using two-way ANOVA to test all particle adhesion versus healthy particle adhesion control. (*) indicates $p < 0.05$.

FIG. 5. Adhesion of $2\ \mu\text{m}$ particles at $\sim 3000\ \text{sites}/\mu\text{m}^2$ ligand densities to inflamed endothelium *in vitro* by RBC rigidity. quantified $2\ \mu\text{m}$ (a) polystyrene and (b) hydrogel particle binding to an IL-1 β inflamed endothelial layer at 10^6 particles/ml of whole blood as a function of rigid RBCs present, i.e., % volume rigid RBC. Each figure utilized rigid RBCs $9\times$ more rigid than healthy RBCs, i.e., treated with 1.0 mM TBHP. A WSR of $1000\ \text{s}^{-1}$ was used for all the shown conditions. Polystyrene and hydrogel particles are conjugated with NeutrAvidin and functionalized with sLe^a at $\sim 3000\ \text{sites}/\mu\text{m}^2$. All trials are plotted as reduction compared to respective healthy controls, i.e., with no rigid RBCs present, thus eliminating the donor variation. Error bars represent the standard error. The statistical analysis of adherent density was performed using two-way ANOVA to test all particle adhesion between both sLe^a ligand densities, ~ 3000 and $\sim 1000\ \text{sites}/\mu\text{m}^2$. (*) indicates $p < 0.05$ and (**) indicates $p < 0.01$.

the smaller spheres and rods. The raw adhesion data for particles in the healthy blood (0% rigid RBCs) are shown in Fig. S7. The particle adhesion reduction trends for both polystyrene and hydrogel $2\ \mu\text{m}$ MPs at the higher density of $3000\ \text{sites}/\mu\text{m}^2$ were found to be similar to the adhesion trends observed with particles at the lower site density of $1000\ \text{sites}/\mu\text{m}^2$ for all but one of the tested conditions (Fig. 5). The higher site density only significantly altered the particle adhesion difference for the hydrogel MPs at the 70% rigid RBC composition, where the adhesion level at the $3000\ \text{sites}/\mu\text{m}^2$ density did not change with or without rigid RBCs present, contrary to the positive response observed with rigid RBCs at $1000\ \text{sites}/\mu\text{m}^2$. Also, the raw particle adhesion levels were only slightly higher with $3000\ \text{sites}/\mu\text{m}^2$ relative to the $1000\ \text{sites}/\mu\text{m}^2$ sLe^a density for the hydrogel and not the polystyrene, MPs (Fig. S7), suggesting that the adhesion kinetics was in a transport-limited regime for both ligand site densities evaluated.²²

F. Effect of rigid RBCs on particle localization

To better understand how the particle adhesion trends presented above develop in the presence of rigid RBCs, we investigated particle localization to the cell-free layer in whole blood flows. We examined the localization of $2\ \mu\text{m}$ PS, $2\ \mu\text{m}$ PEG-MP, and $500\ \text{nm}$ PS particles by first mixing non-functionalized particles into reconstituted ($\sim 40\%$ Hct) whole blood with varying rigid RBC conditions at a concentration of $\sim 10^6$ particles/ml of whole blood. The blood sample was perfused through a $\sim 100\ \mu\text{m}$ channel (with no HUVECs present) at a WSR of $\sim 1000\ \text{s}^{-1}$, and a confocal microscope was used to image particle density in the CFL at about $2\ \mu\text{m}$ from the vessel wall. The CFL has been reported to range from 2 to $7\ \mu\text{m}$ in humans depending on variables such as hematocrit, vessel geometry, and flow rates.^{50–53} Given the particle sizes examined in this study, $500\ \text{nm}$ – $2\ \mu\text{m}$ diameter, focusing on the plane $2\ \mu\text{m}$ from the vessel wall should provide a good indication of whether the particles of interest were able to exit the bulk flow and localize near the wall, i.e., successfully marginate. A full scan of the channel height is represented as a distribution in Fig. S6. We normalized the particle localization density in all conditions where rigid RBCs were present to the particle localization density for the healthy condition. Figure 6(a) shows the raw particle localization density for all particles in healthy conditions. Figure 6(b) shows the percent change in localization density of $2\ \mu\text{m}$ polystyrene in the presence of rigid RBCs. For most conditions where rigid RBCs were present,

FIG. 6. **Particle localization to CFL in the presence of rigid RBCs:** quantified particle localization densities $2\ \mu\text{m}$ away from the vessel wall in a channel of height $\sim 100\ \mu\text{m}$ at $\sim 1000\ \text{s}^{-1}$ and $\sim 10^6$ particles/ml of whole blood as a function of rigid RBCs present, i.e., % volume rigid RBC, and rigid RBCs with different degrees of rigidity, 1.0 mM (*highly rigid*), 0.75 mM (*moderately rigid*), and 0.5 mM treated RBCs (*slightly rigid*). (a) The raw particle localization density in a healthy control, i.e., with no rigid RBCs present. The normalized percent change in particle localization for varying rigid RBC conditions for the (b) $2\ \mu\text{m}$ stiff polystyrene spheres and (c) $2\ \mu\text{m}$ deformable hydrogel spheres is shown. All particles are not functionalized with a targeting ligand. Error bars represent the standard error. The statistical analysis of localization density was performed using two-way ANOVA to test particle localization density in rigid RBC conditions compared to healthy control, i.e., with no rigid RBCs present. (*) indicates $p < 0.05$ and (**) indicates $p < 0.01$.

we observed a significant decrease in the localization of $2\ \mu\text{m}$ PS particles to the CFL in the presence of the *highly* and *moderately* rigid RBCs. Conversely, Fig. 6(c) shows that the percent change in the localization of $2\ \mu\text{m}$ PEG MPs dramatically increased compared to localization in a healthy blood condition when a high amount of the *highly* and *moderately* rigid RBCs were present (70% and 100%). For the 500 nm PS particles, particle localization density significantly decreased relative to the healthy condition for all rigid RBC conditions (Fig. S7). Previous work has shown that nanosized particles do not marginate to the vessel wall as efficiently as MPs, and the results shown in Fig. 6(a) are in agreement.^{16–21} The presence of RBC rigidity in blood seemed to exacerbate this poor NP margination effect.

IV. DISCUSSION

Several human conditions can be characterized by the loss of RBC deformability,^{24–31} with SCD being the most described²⁴ but also includes, HIV, T2D, and natural aging.^{26–31} The presence of altered RBC membrane deformability in blood is not without consequences; increased RBC membrane rigidity has been linked to pulmonary vascular resistance, crisis pain in SCD, and lowered cardiovascular health.⁵⁴ Gutierrez *et al.* recently reported that less deformable RBCs in blood severely reduced the adhesion of WBCs to an inflamed vessel wall in blood flow.³⁶ However, to date, no experimental evidence has been put forth in the literature which evaluates vascular-targeted carriers (VTC) adhesion performance in a diseased (rigid RBC) blood model. The margination and adhesion efficacy of a polymeric drug carrier highly depend on their interaction with cellular components of blood; thus, it is likely that the simultaneous presence of VTCs and rigid RBCs in the bloodstream could significantly alter the margination and adhesion dynamics of VTCs to the vascular wall. In this work, we evaluated the impact of artificially rigidified RBCs on the margination and adhesion of rigid polystyrene (PS) and deformable hydrogel particles, as models for polymeric VTCs, *in vitro* via human blood flow assays. We examined microparticles (MPs) along with nanoparticles (NPs) precisely because, under normal conditions, MPs exhibit a higher margination capability over NPs as shown by previous investigators.^{22,23,55} In the previous work by Gutierrez *et al.*, the disruption to WBC adhesion by rigid RBCs was most pronounced at the highest wall shear rate of $1000\ \text{s}^{-1}$ evaluated, which inspired the examination of the impact of rigid RBCs on particle adhesion at the same shear condition in this work.³⁶ Our findings demonstrate that many different levels of rigidified RBCs mixed in with healthy RBCs result in a decrease in the margination and adhesion of these PS particles to the wall, but in some cases, they result in a dramatic increase in the margination and adhesion of deformable MPs. Overall, this work highlights the significance of factoring the impact of RBC rigidity into designing translatable particle technology into

practical therapeutics. The previously noted shift towards stiffer RBCs with natural aging further highlights the relevance of the presented work as the bulk of the proposed use of VTCs is focused on the elderly, including cardiovascular diseases that continue to be the primary cause of mortality globally.⁵⁶

In line with the previous work with WBCs, our results show that the presence of rigid RBCs of varying rigidities reduces the blood flow adhesion of polystyrene microspheres by up to $\sim 50\%$, depending on the level of RBC rigidity and the fraction composition of the rigid RBCs in the blood. Notably, the maximum impact of these altered RBCs is seen when they co-exist in the blood flow with healthy RBCs. Gutierrez *et al.* previously attributed the rigid RBC-induced reduction in WBC adhesion to heterogeneous collisions in blood flow,³⁶ which Graham and coworkers describe as the physical interaction between rigid (*stiff*) and deformable (*soft*) cells.^{6,14} First, the heterogeneous collisions between stiff and soft RBCs lead to an expanded RBC core, i.e., rigid RBCs are found closer to the vessel wall (in the CFL), which occur because the rigid RBCs experience a substantial displacement upon collision with the deformable RBCs. Second, the increased presence of RBCs in the CFL causes an increase in heterogeneous collisions between RBCs and WBCs.³⁶ We suspect that the same two-step effect of heterogeneous collisions is responsible for the reduced adhesion of the PS microspheres in blood flow with rigidified RBCs. Although a direct comparison between WBC and PS particle behaviors in rigid RBC blood flow conditions may not be entirely relevant due to size and modulus differences, the general similarity of the observed trend between the two suggests a likely scenario of rigid RBCs present in the CFL, preventing MPs from entering the CFL in large numbers. Essentially, rigid RBCs displaced to the wall by collisions with their softer counterparts take up spots in the CFL which otherwise would be occupied by MPs. Indeed, we observed a decrease in the localization of polystyrene MPs at a $2\text{ }\mu\text{m}$ distance from the vessel wall [Fig. 6(b)] as compared to the localization of the same particles in healthy blood flow. While it is possible that collisions between rigid RBCs and polystyrene MPs could also disrupt adhesion, the results presented in Fig. 5 showing similar levels of adhesion reduction with the presence of rigid RBCs for MP with $3\times$ more ligand density suggest that altered adhesion kinetics is not a major factor in dictating the observed particle adhesion trends. Thus, this leads us to speculate that particle margination, or alteration in particle motion in the cell-free layer, plays a more significant role in influencing how particles adhere in the presence of rigid RBCs.

We also probed how particle size can affect the particle adhesion trends in blood flow with the presence of rigid RBCs; we compared the adhesion of polystyrene MPs and NPs ($2\text{ }\mu\text{m}$ and 500 nm). We find that the adhesion reductions observed for NPs are not as significant as the reductions seen for MPs despite a similar adhesion trend for both particle sizes in the case where *highly* and *moderately* rigid RBCs were present. We speculate that the primary source of this different response to rigid RBCs between microspheres and nanospheres is the higher propensity that MPs have to marginate towards the vessel wall than NPs. With more microspheres at the cell-free layer, there is a greater opportunity for heterogeneous collisions with rigid RBCs and hence a more significant adverse effect on the final particle adhesion at the wall. Of note, even with a more substantial reduction due to rigid RBCs, the microsphere adhesion density is still higher than that for nanospheres (Fig. S4). However, it is possible that the physical size of particles itself also contributes to the difference in magnitude of the negative impact of rigid RBCs on microspheres versus nanospheres. With other things being equal, microspheres will experience a more significant displacement upon collision due to having a more substantial momentum, which then likely translates to the larger spheres needing to overcome a higher force to adhere firmly.⁵⁷ Along this line, we find that the levels of the adhesion reductions experienced by the $2\text{ }\mu\text{m}$ PS evaluated here, while significantly higher than for NPs, are generally lower than previously reported for WBCs, with an average diameter of $7\text{--}12\text{ }\mu\text{m}$, under the same rigid RBC flow conditions.³⁶

The case where RBCs were (*slightly rigid*) is interesting because an oscillate-type trend is observed for the adhesion of PS microspheres but not for nanospheres. Here, RBCs are not as stiff as in previous conditions. However, specific percentages (20%, 50%, and 90%) of the *slightly rigid* RBCs present produced a reduction in microsphere adhesion, while others had no

effect (30%, 70%, and 100%) [Fig. 1(c)]. This oscillate-type behavior in microsphere adhesion at different percentages of rigid RBCs present is also observed in the adhesion of hydrogel microspheres [Fig. 2(c)]. Thus, this behavior seems to be particle-size dependent. Possible variations in the localization of *slightly* rigid RBCs are likely responsible for these adhesion trends. However, the motion of individual RBCs is not readily visualized experimentally in high shear blood flows with the hematocrit at physiological levels as evaluated here. A theoretical simulation could potentially help clarify this oscillate-type trend. Theoretical models have been utilized to investigate how the cellular shape and deformability impact how objects behave in flow.^{6,58–60} Some computational models have also examined how MPs and NPs marginate and affect drug delivery.⁵⁵ Work by Müller *et al.* simulates the margination of MPs and NPs, concluding like many others that MPs have a higher probability of localizing closer to the vessel wall compared to NPs.⁵⁵ Other interesting theoretical investigations have been able to modulate cellular rigidity in their simulations to investigate heterogeneous collision effects on margination.⁶ Such models have served as adequate approximations that highlight some of the governing physics, such as lift force and heterogeneous collisions, which aid in giving rise to the observed blood flow phenomena. However, these simulations cannot fully recapitulate the complex behavior of blood flow due to limitations in computing power, which is often overcome by oversimplifications. To date, no theoretical approach has been established which investigates how increases in RBC membrane rigidity impact the ability of MPs and NPs to localize and ultimately bind to the vessel wall. Thus, future work, experimental and computational, is still needed to understand this phenomenon further.

Perhaps the most intriguing result from this study is the adhesion trend of the 2 μm deformable PEG-based hydrogel particles. Recent works in particle drug delivery have looked into modulating particle elasticity as a method to increase the therapeutic efficacy.^{41,61–65} Deformable particle drug carriers have become of high interest due to their capability for reduced cellular internalization and increased circulation time *in vivo*.^{65,66} Previous investigations have developed deformable particle carriers with a wide range of sizes (22 nm–9 μm) and moduli (200 Pa–3 MPa),^{41,61,67–69} with the modulus tuned to closely resemble RBCs and even platelets in some cases.^{62,63} Here, we examined deformable PEG-based MPs with a modulus similar to what is observed for WBCs.⁴¹ When small percentages of *highly* and *moderately rigid* (1.0 mM and 0.75 mM TBHP treated) RBCs are present, a slight reduction in hydrogel particle binding is observed. However, as the percentage of rigid RBCs is increased, particle adhesion to the vessel wall began to improve. We hypothesize that a combination of distinct phenomena, including increased heterogeneous collisions with rigid RBCs, affects the localization capability of hydrogel particles in the presence of rigid RBCs. Previous theoretical works suggest that deformable particles do not marginate towards the vessel wall as compared to rigid particles.^{14,70} Here, we see a substantial increase, up to 136%, in particle localization in the CFL for the 2 μm hydrogels with the presence of rigid RBCs [Fig. 6(c)]. These results are counterintuitive since it would be expected that rigid particles experience greater displacement and thus would experience enhanced localization in the presence of rigid RBCs. Previous work has shown that the presence of WBCs in whole blood enhanced hydrogel MP adhesion to the vascular wall.⁴¹ The presence of WBCs improves deformable MP (modulus similar to the WBC modulus) adhesion as compared to adhesion in an RBC-plasma solution, showing that the WBCs in the CFL enhanced deformable MP adhesion.⁴¹ We suspect that mismatched, i.e., heterogeneous, collisions with rigid RBCs in addition to WBCs in the CFL promote deformable particle margination to the vessel wall. Still, this does not offer a complete explanation as to why having a small percentage of rigid RBCs in blood produces a slight reduction in hydrogel MP adhesion. Additionally, although PEG-MPs had a modulus closely resembling the modulus of WBCs, they do not behave like WBCs in the presence of rigid RBCs as was reported by Gutierrez *et al.*, suggesting that the size is a key factor in predicting particle/cell behavior in the presence of rigid RBCs.³⁶ Another possible explanation for the differences in adhesion patterns between stiff and deformable MPs is the ability of deformable particles to spread onto the surface once they are adherent to the wall. Unlike WBCs and deformable particles, stiff polystyrene particles do not spread on the surface and thus would experience a higher disruptive

force from flow and, potentially, from colliding rigid RBCs localized near the vessel wall. Also, although the same equivalent diameter, stiff particles would have a smaller contact area with the wall, which impacts adhesion strength. However, the fact that the impact of rigid RBCs on polystyrene microspheres did not change significantly with the significant increase in particle ligand density [Fig. 5(a)] would suggest that particle spreading, and hence improved adhesion kinetics, is not a significant contributor to the differences in the adhesion pattern between polystyrene and hydrogel microspheres.

The particle shape has been proposed as a major factor influencing particle localization and binding efficacy. Previous results have shown that ellipsoidal shaped particles exhibit enhanced adhesion compared to spherical counterparts.^{17,21,49} In this study, we probe how the particle shape alters adhesion in the presence of rigid RBCs. Our obtained results show that the adhesion of rigid, rod-shaped particles is not reduced in the presence of rigid RBCs unlike the spherical PS particle types tested, thus hinting that the particle shape is a major factor in particle-RBC interactions. The relatively unaffected, or in some cases enhanced, adhesion of rod particles in the presence of rigid RBCs is likely due to rod particles having (1) a larger contact area with the endothelial layer and (2) a streamlined shape with a small surface area exposed to shear flow. Localization of rigid RBCs near the vessel wall results in increased collisions with particles in the CFL, preventing them from firmly adhering or even knocking them off the vascular wall completely. Having a streamlined shape while bound to the endothelial layer would then explain why rod particle adhesion is not as drastically affected by the presence of rigid RBCs. In addition to this, the typical tumbling, rolling dynamics, and lateral drifting velocity observed in rod particles in the flow could be enhanced by heterogeneous collisions with rigid RBCs.⁷¹ Future experimental and computational analysis is needed to verify particle adhesion in the presence of rigid RBC data.

V. CONCLUSIONS

This work shows that the presence of rigid RBCs in whole blood can have a significant impact on the localization and adhesion capabilities of vascular-targeted carriers. We utilize an artificial rigidification method to rigidify healthy RBCs aiming to mimic various hematologic diseases that directly affect RBC membrane deformability. Then, we reconstitute rigid RBCs, with varying degrees of rigidity, into whole blood at distinct fractions and perfuse through a microfluidic model at physiologically relevant conditions. We aim to probe how changing particle deformability, size, and shape in the presence of rigid RBCs could affect particle adhesion capabilities. First, we find that the adhesion capability of micro-sized, rigid polymeric spheres is highly reduced in the presence of rigid RBCs regardless of the degree of rigidity or concentration of the rigid RBCs. Interestingly, deformable micro-sized sphere adhesion is reduced in the presence of small amounts of rigid RBCs. However, when a high percentage of rigid RBCs are present, the adhesion of deformable micro-sized particles is enhanced by up to 85%. We observe that the adhesion capability of nanosized particles is only slightly reduced in the presence of rigid RBCs. Moreover, we find that ellipsoidal particle adhesion is either enhanced or not affected by the presence of rigid RBCs. Overall, this work offers compelling evidence that the presence of rigid RBCs in whole blood can either significantly impact the localization and adhesion functionality of targeted drug carriers in whole blood, which can hold critical implications for a wide range of diseases affecting RBC deformability. The variable adhesion performance of particles observed under distinct percentages of rigid RBCs and RBC stiffness suggests the particle therapeutics efficacy can vastly vary between patients and disease severity. This unique information presented in this work can be highly valuable for the future development of personalized particle therapeutics with high adhesion efficacy in diseased blood.

SUPPLEMENTARY MATERIAL

See [supplementary material](#) for ektacytometry elongation curves, sample flow cytometry analysis, particle localization, images of fluorescent hydrogel 2 μm particles on the HUVEC layer, and particle adhesion reduction trends using particles of higher adhesion ligand density.

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