Published in final edited form as:

Bioconjug Chem. 2015 December 16; 26(12): 2419–2428. doi:10.1021/acs.bioconjchem.5b00474.

Differential impact of plasma proteins on the adhesion efficiency of vascular-targeted carriers (VTCs) in blood of common laboratory animals

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Abstract

Vascular-targeted carrier (VTC) interaction with human plasma is known to reduce targeted adhesion efficiency *in vitro*. However, the role of plasma proteins on the adhesion efficiency of VTCs in laboratory animals remains unknown. Here, *in vitro* blood flow assays are used to explore the effects of plasma from mouse, rabbit and porcine on VTC adhesion. Porcine blood exhibited a strong negative plasma effect on VTC adhesion while no significant plasma effect was found with rabbit and mouse blood. A brush density poly(ethylene)-glycol (PEG) on VTCs was effective at improving adhesion of micro-sized, but not nano-sized, VTCs in porcine blood. Overall, the results suggest that porcine models, as opposed to mouse, can serve as a better model in preclinical research for predicting the *in vivo* functionality of VTCs for use in humans. These considerations hold great importance for the design of various pharmaceutical products and development of reliable drug delivery systems.

INTRODUCTION

Vascular-targeted carriers (VTCs) are particulate systems that offer tremendous promise for use as an alternative treatment of several human diseases due to the provided benefit of non-

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The authors declare no competing financial interest

ASSOCIATED CONTENT

Supporting Information

The supporting information is available free of charge:

Figure S1: Exposure of HUVEC to animal blood does not alter affinity of sLe^a/E-selectin interaction

Figure S2: The adhesion trend with rabbit blood is more in alignment with that of mouse rather than porcine plasma

Figure S3: Example images of PS nanoparticle adhesion on HUVEC

Figure S4: The adhesion of PEGylated particles in porcine blood at lower PEG density is plotted, revealing that the adhesion recovery in porcine blood is dependent on the VTC PEG density

invasive and highly localized delivery to the diseased area^{1,2}. To date, research on the functionality of VTCs has focused on novel strategies for targeting that allow for precise drug delivery and an optimal release profile². However, these previous studies assume successful VTC margination (localization) and adhesion to the vascular wall in blood flow. Recent publications have highlighted the importance of various particle physical and surface properties, including size, shape and material characteristics, in the capacity of VTCs to efficiently bind to the vascular wall in flow models ranging in complexity from simple buffer to *in vitro* blood flow assays^{3–9}, as well as various animal models of human diseases^{10–12}. *In vivo* assays are preferred in drug delivery research due to the (1) inability of current *in vitro* systems to fully recreate the complexity of the *in vivo* environment and (2) capacity to generate models of many human diseases in these animals. Thus, to date, several animal species are used in drug delivery research, most notably rodents and pigs^{13–16}. However, critical differences in the physiology of these animals relative to humans as it relates to VTC circulation, such as blood vessel size, blood flow magnitude, blood cell properties (deformation, size and shape), and plasma protein composition, may limit extrapolation of *in vivo* results to clinical application in humans¹⁷. We have previously reported that human plasma proteins have a negative effect on the vascular wall interaction of vascular-targeted carriers (VTCs) constructed from poly(lactic-co-glycolic-acid) (PLGA) polymer, a biodegradable polymer ubiquitous in drug delivery formulations, in a donor (human) dependent manner¹⁸. Specifically, vascular-targeted PLGA nano- and microspheres exhibited minimal adhesion to inflamed endothelium in human blood or plasma flow whereas the same particles exhibited high binding when the flow medium is buffer. We provide evidence that lack of effective adhesion of PLGA in human blood was due to adsorption of certain large plasma proteins with particle surface. However, little is known about the potential differential interaction of animal plasma proteins with VTCs in their capacity to bind to the vascular wall, which is an essential component in understanding the translation of preclinical animal research to the clinic.

In this study, we evaluated the vascular wall interaction of model VTCs in flow of animal blood in a parallel plate flow chamber (PPFC) in order to elucidate any differential impact of plasma protein corona acquired from different animal bloods on VTC targeting functionality. Specifically, we characterized the adhesion of Sialyl Lewis A (sLe^a)-conjugated polystyrene (PS), PLGA, silica (Si) and titanium dioxide (Ti) spheres to inflamed human umbilical vein endothelial cells (HUVEC) from laminar flow of mouse and porcine blood. We focus on porcine and mouse blood since these animals are most commonly used for *in vivo* evaluation of VTCs. The targeting ligand sLe^a used is a variant of sialyl-Lewis^x – a tetrasaccharide carbohydrate typically expressed on leukocytes that exhibit specific binding interaction with selectins (E- and P-) upregulated by inflamed endothelial cells^{19,20}. The initial leukocyte adhesive contact to the vascular wall during inflammation response is facilitated by the sLe^x binding interaction with P/E-selectin²¹. Several works have shown that sLe^a-coated nano- and microspheres exhibit highly efficient and specific adhesion to activated (i.e. inflamed) monolayer of endothelial cells *in vitro*^{4–6,22} and *in vivo*¹¹. As such, sLe^a has previously been proposed for targeting therapeutics in many inflammatory diseases^{7,19,23–25}. Monolayers of activated endothelial cells, expressing E-selectin, were used as the adhesive substrates as these better mimic E-selectin expression pattern in inflamed vascular wall than the protein-

coated substrates typically used in flow adhesion assays. HUVECs are used for all assays, as they are readily available than human endothelial cells from other vascular beds. In addition, the high cost and low viability in culture of animal species endothelial cells limits their use for this study. Overall, this study offers the first evidence that the plasma protein corona formed on particles from common laboratory animals differentially impacts VTC binding to the vascular wall in blood flow. The presented results allows a better platform for the translation of results obtained from *in vivo* assessment of VTC functionality in common animal models to predict VTC performance in humans.

RESULTS

Effect of animal plasma on microsphere adhesion in buffer flows

To establish a baseline for the impact of different animal plasmas relative to our previous publication focused on human plasma-derived corona only¹⁸, we evaluated impact of surface-adsorbed plasma proteins on the adhesion of sLe^a-coated 5 μ m PLGA and PS microspheres to a monolayer of activated HUVECs under buffer and blood flow conditions. Figure 1 shows representative images of microspheres binding in whole blood or RBCs in viscous buffer flow, where viscous buffer (VB) refers to buffer with viscosity matching that of the particular animal plasma of interest²⁶. In the first set of analysis, PS and PLGA microspheres were incubated in either buffer or plasma from mouse or porcine and evaluated for their adhesion to an activated HUVEC monolayer in laminar flow of animal RBC+VB (Figure 2). HUVECs were activated via exposure of cells to IL1- β for 4 hr – a time point previously identified for maximum IL1- β -stimulated expression of E-selectin. On average, microsphere adhesion was slightly lower ($P < 0.05$) in mouse compared to adhesion in porcine RBC+VB flow, due to differences in RBC geometry between species causing differential microsphere distribution in flow as previously reported²⁷. Also, the adhesion of PLGA microspheres was slightly higher than that of PS microspheres in RBC+VB ($P < 0.05$ for porcine), which is in line with the slightly higher density of PLGA (1.3 g/cm³) relative to PS (1.1 g/cm³). The flow adhesion levels for both PS and PLGA microspheres incubated in mouse plasma prior to run in RBC+VB were not significantly different from the levels obtained for buffer-incubated microspheres in RBC+VB flow (control). However, both PS and PLGA microspheres incubated in porcine plasma prior to addition to buffer showed significantly lower adhesion, 42% and 55%, respectively, relative to adhesion of buffer incubated microspheres in RBC+VB flow. We confirmed with control experiments that the exposure of HUVEC to animal plasma does not alter protein expression or the affinity of sLe^a-selectin binding, where particle adhesion in buffer flow to naïve activated HUVEC was not significantly different from adhesion to HUVEC previously exposed to animal plasma (no particles) and washed prior to use (See Figure S1).

Evaluation of microsphere adhesion in whole blood

Microsphere adhesion was analyzed in whole blood flow to determine any variation in particle adhesion under physiologically relevant conditions. The EC adhesion of both PS and PLGA microspheres was essentially eliminated in porcine whole blood flow (95% reduction in PS and PLGA) (Figure 2) compared to the aforementioned partial reduction observed with porcine plasma-soaked microspheres. Similarly, a significant reduction in microsphere

adhesion is now observed in mouse (64% for PS and 98% for PLGA) whole blood flow relative to the buffer control despite the absence of reduced adhesion for microspheres pre-soaked in plasma in RBC+VB assays. Here, two possibilities exist for the enhanced reduction in microsphere adhesion under whole blood flow conditions: it is possible that (1) an enhanced plasma effect exists in whole blood that is due to the presence of the soft corona, added layer of loosely attached plasma proteins, surrounding particles in whole blood and/or (2) non-RBC blood components, i.e. WBCs and platelets, in animal bloods are interfering with particle adhesion. To determine which of the two possibilities further reduced microsphere adhesion to ECs in whole blood flow, we evaluated the adhesion of PLGA microspheres in flow of RBCs in plasma (RBC+Plasma; leukocytes and platelets removed) for mouse and porcine blood. Figure 3 shows a near complete elimination of microsphere adhesion in both porcine RBC+Plasma and whole blood flow compared to only a partial reduction with plasma-incubated particles in porcine RBC+VB flow relative to the control. This result suggests that a more robust plasma protein effect exists due to the added impact of the soft corona, rather than any effect from non-RBC cellular components. As a result, reduced microsphere adhesion is magnified in porcine whole blood flow relative to plasma-soaked particles (hard corona only) in RBC+VB flows. In contrast, there is no significant difference in PLGA microsphere adhesion levels between particles soaked in mouse plasma and perfused over EC in RBC-in-buffer (pVTC in RBC+VB) and particles perfused directly in mouse RBCs-in-plasma (RBC+Plasma) (Figure 3). Thus, the significantly lower to essentially absent adhesion of microspheres in mouse whole blood flow is likely due to the presence and interference of non-RBC cellular components in mouse blood. The negative impact of plasma on the adhesion of PLGA microspheres in porcine blood relative to RBC+VB flow observed here is similar to the trend previously reported for PLGA in human whole blood relative to human RBC+VB flow, where particle adhesion was similarly drastically reduced in human plasma or whole blood flow¹⁸. The presence of a negative plasma protein impact on particle adhesion in human and pig blood but not in mouse would suggest a large animal effect. To this end a quick analysis of particle adhesion in rabbit blood, another small animal occasionally used in laboratory research, was performed similar to Figure 2. The trend in microsphere adhesion in rabbit blood versus plasma soaked and buffer was in alignment with the trend observed for microsphere binding in the mouse system (Figure S2).

Plasma protein effect on vascular wall adhesion of nanospheres of various material types in blood flow

It is of interest to determine if the differential impact of plasma proteins in the two animal bloods would be present in nano-sized PLGA particles that are of interest for drug delivery and with nanoparticles of other material types. Thus, we evaluated the adhesion of 500 nm sized PS, PLGA, Si and Ti nanospheres to activated ECs from porcine and mouse laminar blood flows. Sample images of nanosphere adhesion is shown in Figure S3. Similar to results from assays with microspheres, the negative impact of plasma protein exposure on the flow adhesion of vascular-targeted nanoparticles was most prominent in assays with porcine plasma or porcine whole blood for all material types (Figure 4). Specifically, minimal to no adhesion was observed for PS and PLGA nanospheres perfused over activated ECs in porcine RBC+Plasma or whole blood flows. Though some low level of adhesion was

observed with Si and Ti nanospheres in the porcine RBC+Plasma and whole blood flows, these still represent a significant level of reduction in adhesion (83% and 92% for Si and Ti, respectively) compared to the adhesion level under buffer conditions.

In contrast, nanoparticle adhesion levels in assays with mouse RBC+ Plasma flow were slightly higher relative to adhesion in mouse RBC+VB assays for all material types, while a significant decrease in adhesion was observed for nanoparticles in mouse whole blood flow (Figure 4B). This result again reveals that a plasma effect is absent in the adhesion of nanospheres in mouse whole blood flow. Instead, non-RBC cellular component in mouse blood exert a negative adhesion effect similar to observation with microspheres. Overall, the impact of the non-RBC cells on nanosphere adhesion in mouse whole blood flow was not as pronounced as with microparticles (5 μ m PS and PLGA spheres), i.e. only a partial reduction in nanosphere adhesion by non-RBC cells compared to the complete reduction in microsphere adhesion observed.

Comparison of the plasma protein corona formed on nanoparticles exposed to the different animal plasma

In our prior work¹⁸, PLGA particles that exhibited negative adhesion to activated ECs in human plasma or whole blood flows show distinct proteins in their corona, i.e. unique IgG proteins ($\approx$ 150 kDa in size) as measured by SDS-PAGE, relative to microspheres that were effective at binding under plasma or blood flow conditions. Here, we seek to investigate whether similar unique features of the plasma protein corona on particles exposed to different animal blood was responsible for the stark difference in nanoparticle adhesion in plasma/blood flow conditions, particularly for porcine versus mouse. Thus, SDS-PAGE analysis was performed to compare the protein profile adsorbed on PLGA nanoparticles exposed to various media including buffer as well as rabbit, mouse, porcine and human plasma. The corona proteins were removed from the particle surface after exposure to plasma by solubilizing in 1 $\times$ lane marker non-reducing buffer from ThermoScientific (contains 1% SDS) for 5 minutes at 95°C. As shown in Figure 5, the corona formed on PLGA nanoparticles from porcine plasma shows a distinct band at the 150 kDa range similar to our prior observation with corona formed on PLGA in human plasma, while this band is less pronounced or completely absent in coronae formed on nanoparticles exposed to mouse and rabbit plasma, respectively - shown to have effective particle adhesion in plasma flow assays. In addition, the corona from porcine plasma shows a particularly heavy band in the high molecular weight range >250 kDa not seen with the corona stripped from other animals.

Effect of PEGylation on VTC adhesion in porcine blood

Polyethylene glycol chains are often grafted to the surface of VTCs to extend their circulation time *in vivo*. Specifically, the hydrophilic PEG chains create a hydration layer that interrupts adsorption of plasma proteins onto the carrier's surface, which in turn prevents WBCs from recognizing VTCs as foreign and hence increases the carrier's systemic circulation time²⁸. Here we explore whether the addition of PEG chains on particle surface would restore the adhesion of model VTCs under whole blood flow condition similar to its impact on particle circulation time. We chose to evaluate the adhesion conditions

where the most pronounced particle reductions were observed; thus, particle adhesion levels in porcine RBC+Plasma and whole blood flow were observed for 500 nm spheres conjugated with a 5.5 kDa PEG spacer at the maximum achievable site density of 38,000 PEG chains/ μm^2 , corresponding to a brush conformation as confirmed by flow cytometry. Although all particles studied here had a carboxyl-functionalized surface, PS spheres were used since a higher amount of surface carboxyl groups was available, which allows for grafting of PEG at the higher densities that are of interest here. PEGylated particles were then targeted to activated-ECs via sLe^a (~500 sites/ μm^2) and used in adhesion assays. The adhesion of both PEGylated and non-PEGylated nanospheres significantly decreased by ~90% in RBC+plasma and whole blood relative to their adhesion in RBC+VB (Figure 6A) suggesting that the PEG chains on particles were not effective at eliminating the negative plasma protein effect. In light of the results for PEGylated nanoparticles, we fabricated PEGylated PS microspheres (2 μm) at a maximum achievable site density of $35,400 \pm 1,500$ PEG chains/ μm^2 , corresponding to a brush conformation and then target to activated-ECs via sLe^a. In contrast to the nanospheres, the addition of PEG to microspheres significantly improved their adhesion in RBC+Plasma and whole blood – the reduction in particle adhesion relative to adhesion in RBC+VB flow was only 18% and 26% for PEGylated microspheres compared to the 95% and 93% reduction observed for non-PEGylated particles in RBC+Plasma and whole blood flow, respectively (Figure 6B). However, a lower PEG grafting density of $12,000 \pm 1,600$ chains/ μm^2 on PS microspheres resulted in similar reduction levels (~88% lower adhesion of PEG microspheres) as seen with the non-PEGylated particles in blood compare to adhesion in RBC+VB flow (Figure S4).

DISCUSSION

To date, several publications have highlighted the importance of the plasma protein corona in prescribing the *in vivo* functionality of targeted drug carriers²⁹. However, these studies have mainly concentrated on the impact of the corona on the recognition and clearance of drug carriers from the bloodstream by phagocytic cells^{30–32}. Only recently have a few works presented evidence that the particle protein corona characteristics can affect drug carrier ligand-receptor interaction necessary for target recognition/specificity^{33–35}. With regard to targeting/uptake, a few studies have reported that the presence of human serum proteins masks the NP surface, leading to reduced uptake efficiency and targeting³⁴. However, the corona has also been demonstrated to have positive implications that could act as a natural targeting mechanism. For example, enhanced drug delivery into the brain endothelium has been achieved *in vivo* via covalent attachment/adsorption of apolipoprotein A-I and B-100 on albumin-based nanoparticles³⁶. In addition, DOTAP/DNA lipoplexes exposed to human plasma adsorb high levels of vitronectin, resulting in enhanced cancer cell uptake relative to nanoparticles not exposed to plasma³⁷. These studies demonstrate that the presence of specific proteins (whether directly attached or adsorbed naturally) can have profound impacts on the efficiency of the targeted drug carrier. In addition to the role of material, some studies report that variability of the plasma composition across humans can lead to formation of a unique protein corona on a given particle's surface, potentially leading to a corona rich in specific proteins important for corona-directed targeting effects.^{18,38,39} Thus, the plasma “source” (e.g. differential individuals) could be a critical component for the

downstream targeting efficiency of a VTC in blood flow. Indeed, it is known that the concentration of specific proteins such as IgG varies in human and across different animal species plasma suggesting that plasma from different species will likely result in formation of unique coronas^{40–42}.

Thus, this study seeks to investigate the effect of plasma from common laboratory animals on prescribing vascular-targeted carrier adhesion efficiency to inflamed endothelial cells, as this may offer potential key insight into how *in vivo* data from animals will translate to use in humans. Specifically, the binding efficiency of VTCs in mouse and porcine blood flow was evaluated via an *in vitro* flow assay. Overall, contact with porcine plasma/blood was found to significantly reduce the adhesion of micro- and nanoparticles of various materials to ECs in flow. The observed negative impact of plasma proteins on the vascular wall binding of targeted particles in porcine blood is similar to the previously reported negative impact of plasma proteins on the vascular wall adhesion of vascular-targeted PLGA particles in human blood flow¹⁸. Specifically, PLGA nanospheres and microspheres exhibited significantly diminished vascular wall adhesion in flow upon exposure to either human plasma or whole blood. This impact of plasma protein in human on PLGA adhesion occurred regardless of the targeting ligand type, e.g. sLe^a or anti-ICAM-1, but appears to be material specific. Unlike the observation here with porcine blood, polystyrene spheres maintained a significant level of adhesion in human plasma or blood flow^{18,27}.

In contrast, particle contact with mouse plasma only revealed at most, minor reduction effects. The large reduction in particle binding in mouse whole blood is linked to the effect of non-RBC blood component-VTC interactions, most likely interactions with leukocytes. Indeed, in a previous publication, human leukocytes were reported to significantly reduce the adhesion of large microspheres in human whole blood linked to the collisions that occurs between leukocytes and particles at the wall⁶. Interestingly, nanoparticle adhesion in human blood flow was not impacted by leukocytes in the previous report as was seen with mouse whole blood here (Figure 4). It is possible that the larger representation of the smaller-sized lymphocytes relative to neutrophils in mouse blood compared to human and pig (e.g. 75% lymphocyte in mouse blood compare to 30% human blood⁴³) is responsible for this differential observation of the negative impact of leukocytes on the adhesion of nanoparticles in mouse blood. The increase in nanoparticle adhesion in mouse RBC+Plasma relative to the RBC+VB control, which is not seen with microspheres in mouse plasma (Figure 4) or in our previous work with nanoparticles in human RBC+Plasma flow¹⁸, may suggest a difference in mouse RBC aggregation in flow in response to subtle differences in plasma viscosity and protein composition²⁶, which results in enhanced nanoparticle adhesion in RBC+Plasma assays.

The SDS-PAGE analysis of the protein coronae acquired by particles in the different animal bloods is consistent with our previous results with PLGA in human blood, where a unique protein band was observed in the high molecular weight range of ~150kDa for the PLGA corona acquired from human blood – which was confirmed to be linked to the observed negative adhesion of PLGA particles to activated endothelium in human blood¹⁸. The fact that this protein band was faint or missing from protein coronae acquired from mouse plasma, which show no impact on particle adhesion, allow us to conclude that the adsorption

of large molecular weight IgG proteins is also responsible for the negative adhesion of vascular-targeted particles to the endothelium in porcine blood similar to human blood. Furthermore, it is possible that the extensive impact of porcine plasma on particle adhesion in flow observed here relative to previous report with human could be due to the contribution of the prominent proteins in the band observed at >250 kDa range in the porcine acquired corona overwhelming the targeting functionality of the ligand present on particles.

The addition of PEG on particles appears to be a promising approach to counteract the negative impact of plasma proteins on VTC targeting efficacy to the vascular wall in blood similar to previously reported positive impact of PEGylation on the circulation time of VTCs *in vivo* that is linked to reduction/delay of protein adsorption onto particle surface²⁸. However, this positive impact of PEG on adhesion is VTC size dependent, where the adhesion of microspheres, but not nanospheres, in porcine blood was restored with PEG chains on particle surfaces at the maximum density (~35,000 sites/ μm^2) achievable under the condition explored here. This differential response to PEGylation between the nanospheres and microspheres may be a result of differences in the extent/composition of the adsorbed plasma proteins on microspheres relative to nanospheres. Specifically, previous publications have reported particle size to have a significant influence on protein corona composition^{44,45}. In addition, Walkey et al. previously reported that the level of protein binding to gold nanoparticles increased as the particle size decreased, which resulted in the smaller nanoparticles requiring a higher density of PEGylation than larger particles to significantly reduce protein adsorption²⁸. Indeed, a surface density dependency of PEGylation is seen in the reverse direction with the microspheres, where the positive impact of PEG on the porcine blood flow adhesion was eliminated for these microspheres at a lower PEG density (Figure S4). Thus, it could be that more porcine proteins adsorbed onto nanoparticles relative to the microparticles studied here; therefore, restoring nanosphere adhesion in porcine blood may require a significantly higher PEG grafting density than used in this work. The limited density of reactive groups on the particles used in this work limits the maximum achievable PEG density, and thus precludes the testing of this assertion. Our preliminary attempt to evaluate differences in porcine plasma protein adsorption on the PEGylated PS microspheres versus non-PEGylated ones via SDS PAGE did not yield obvious visual differences (not shown) despite the observed slight improvement in the blood adhesion of microspheres with PEGylation. It may be that the presence of PEG on VTCs in porcine blood is only minimally effective at reducing protein adsorption in porcine blood, which would not be easily discerned with SDS PAGE. Unfortunately, there is no direct report of injection of VTCs for vascular wall binding in porcine models or evaluation of protein corona from porcine blood that we could find to help contextualize our observations in this work. We are now working to utilize the more comprehensive mass spectroscopy, LC-MS, method to obtain qualitative and quantitative comprehensive corona protein signatures for VTCs in porcine blood. In parallel, we are exploring alternate strategies for eliminating protein adsorption on VTCs, e.g. use of zwitterions that may be more effective than PEGylation and reversing the impact of plasma proteins on vascular adhesion of VTCs⁴⁶.

Overall, it is expected that the result presented here is of high relevance to design and evaluation of VTCs for human use. However, there are several limitations to the work that is worth noting. For one, the use of HUVEC as the substrate for adhesion of particles with

animal blood raises concerns for relevance to actual particle interaction *in vivo* in these animals. However, it is known that the specific sLe^a/E-selectin interaction on the vascular wall is conserved across animal species. sLe^a-targeted particles similar to one used here have been shown to effectively target the endothelium *in vivo* in mice^{5,11}. Moreover, our control assays show that sLe^a particles adhesion levels on HUVEC that were first exposed to porcine plasma, washed and then used in buffer binding assays were not significantly different from adhesion on naïve HUVEC not previously exposed to animal blood (Figure S1). It should be noted that the 500 nm particle size evaluated in this work is slightly larger than the 100 – 200 nm nanospheres typically proposed for use as VTCs. The 500 nm size is used here due to the availability of particles of different material types in this size. However, it is expected that the result obtained in this work for the 500 nm spheres would be relevant for the smaller nanospheres used in VTC design based on our extensive work showing minimal deviation in the blood flow adhesion/dynamics of 100 - 200 nm PS spheres compare to 500 nm ones^{5,6,27}. Finally, the shear rate used in this assay is typical of small veins in human, and it is likely that the peak levels of wall shear stress would be higher in the mouse compared to human, at least in the aorta⁴⁷. However, the focus here is to compare the effect of plasma on particle binding while maintaining the same flow conditions across different animal bloods. It is expected that the presence of higher shear on particles, as might be the case in mouse aortae, would further exaggerate the negative impact of plasma proteins on particle adhesion as we previously demonstrated for PLGA binding in human whole blood at different shear rates¹⁸.

CONCLUSION

This study investigates the impact of plasma source on binding efficiency of VTCs, and has important implications for the design of highly-functional drug delivery systems. In particular, the differential VTC adhesion efficiency seen in the porcine model, relative to the mouse model suggests that the choice of animal model or blood source for *in vitro* studies plays a critical role in drug carrier performance and therapeutic utility. Overall, our results suggest that porcine models, as opposed to the mouse models, better model the complete performance of VTCs in terms of their vascular wall adhesion and thus can serve as a better model for optimizing the *in vivo* functionality of drug carriers for their eventual clinician use. These considerations hold great importance for the design of various pharmaceutical products, and will likely lead to more efficient and reliable drug delivery systems.

EXPERIMENTAL PROCEDURES

Particle fabrication

The 5 µm PLGA spheres were fabricated via an oil-in-water solvent evaporation technique as previously described^{18,48,49}. Briefly, 50:50 PLGA polymer with acid (carboxyl) end groups (Evonik; Parsippany, NJ) was dissolved at 2 mg/mL in 20 ml of dichloromethane (DCM) (oil phase), and the solution was injected into 90 mL of polyvinyl alcohol (PVA)/poly(ethylene-alt-maleic anhydride) (PEMA) solutions (aqueous phase). The emulsion was stirred for 2 hr at 1800 rpm in order to evaporate DCM and form solid particles. Fabricated particles were then washed via centrifugation to diminish residual PVA on the particle

surface and limit polydispersity. Particles were dried using a lyophilizer and the resulting powder stored at -20°C until use. The 500 nm PLGA (50:50) spheres were obtained from Phosphorex, Inc. (Hopkinton, MA). Carboxylate-modified 500 nm Si spheres were purchased from Nanocomposix, Inc. (San Diego, CA), and the 500 nm Ti spheres were obtained from EPRUI Nanoparticles and Microspheres Co. Ltd. (Nanjing, China).

Preparation of vascular-targeted carriers

Carboxylate-modified PS, PLGA, Si and Ti spheres were covalently coupled with NeutrAvidin protein (Pierce Biotech Inc., Rockford, IL) via carbodiimide (EDAC) chemistry as previously described³⁻⁵. Briefly, 5 mg/mL NeutrAvidin in 50 mM MES buffer (800 μL) was incubated with avidin-coated spheres (5.6×10^8 beads) on rotor for 15 min at room temperature. Then, 75 mg/mL EDAC in 50 mM MES buffer (800 μL) was added to the avidin-particle mixture (pH to 9.0) and incubated for 20 hr. Avidin conjugated spheres were washed twice and resuspended in 1000 μL of 50 mM PBS (50mM sodium dihydrogen phosphate and 50 mM sodium phosphate dibasic, pH 7.4). Particles were kept at 4°C until use. Biotinylated multivalent sialyl Lewis A (sLe^a; GlycoTech, Gaithersburg, MD) was reacted with the avidin-coated spheres to achieve targeting as previously described^{3,4}. Sphere surface ligand densities were quantified via BD FACsCalibur calibration beads. A sLe^a site density of approximately 2500 sites/ μm^2 was used with non-PEGylated microspheres for all assays unless otherwise stated. For nanospheres, an average sLe^a site density of 5000 sites/ μm^2 was used for Si and PS while a density of 900 and 1600 sLe^a sites/ μm^2 was used for Ti and PLGA, respectively.

Preparation of PEGylated vascular-targeted particles

Carboxylated particles (2.1 μm or FITC-loaded 533.8 nm; Polysciences, Inc., Warrington, PA) were mixed with an amine-PEG-biotin solution (5 mg/mL) prepared in MES buffer (97.6 mg/mL) containing Na_2SO_4 (0.6 M) for 15 minutes at 7.49×10^{10} $\mu\text{m}^2/\text{mL}$ (total particle surface area/incubation volume) as described^{50,51}. An equal volume of EDAC (75 mg/mL) dissolved in MES buffer was then added to the solution and the pH adjusted to 9. The mixture was incubated for 20 hours at 60°C with gentle agitation. After conjugation, the PEGylated spheres were thoroughly washed and stored in 50 mM phosphate buffered saline (PBS). For ligand attachment to PEGylated spheres, biotinylated-sLe^a (10 $\mu\text{g}/\text{mL}$) was premixed with NeutrAvidin (20 $\mu\text{g}/\text{mL}$) at an equal volume ratio for 20 minutes followed by incubation with PEGylated spheres (100 μL total volume) for 45 minutes at room temperature. The particles were then washed and stored in phosphate buffer at 4°C until use in flow adhesion assay.

Characterization of PEG corona

PEG surface densities were quantified via flow cytometry (Life Technologies Attune). The 500 nm and 2 μm PEGylated spheres were stained with anti-biotin-PE or avidin-FITC, respectively, at 10 $\mu\text{g}/\text{mL}$ for 20 minutes at room temperature and washed with phosphate buffer. Fluorescent intensities were converted to surface densities via a standard calibration curve, fluorescein-to-protein ratio, and particle surface area. The conformation of the PEG corona was characterized as previously described⁵⁰. Briefly, the distance between adjacent PEG chains (S) were compared to the Flory's radius (R_f) given by the following equations⁵²:

$$R_f = aN^{0.64} \quad (2.1)$$

$$S = 2\sqrt{\frac{A}{\pi}} \quad (2.2)$$

where a is the length of one PEG monomer (0.35 nm), N is the number of PEG monomers obtained from the PEG molecular weight divided by the molecular weight of one PEG monomer, and A is the surface area occupied by one PEG chain calculated from the inverse of the PEG surface grafted density (# PEG Chains/nm²) obtained from flow cytometry. The PEG corona was classified as a brush conformation if $R_f < S < 2R_f^{53}$.

Preparation of human endothelial cells (ECs)

Human umbilical vein endothelial cells (HUVECs) are isolated from umbilical cords via the well-known collagenase perfusion method^{54,55}. Human umbilical cords were obtained from the U of M hospital under a Medical School Internal Review Board (IRB-MED) approved human tissue transfer protocol (HUM00026898). This protocol is exempt from informed consent per federal exemption category #4 of the 45 CFR 46.101.(b). Confluent HUVEC monolayers were cultured on coverslips treated with gelatin⁵⁶. Cell-seeded coverslips were then activated with IL-1 β at 1 ng/mL for a 4 hr period to upregulate E-selectin on cell surface prior to use in flow experiments.

Preparation of RBC-in-buffer and whole blood (WB)

Mouse whole blood was collected from surplus mice, generously provided by the breeding colony of Unit of Laboratory Animal Medicine (ULAM) according to a protocol. Mouse procedures were approved by ULAM and University Committee on Use and Care of Animals (UCUCA) at the University of Michigan. Briefly, mouse blood was drawn from anaesthetized mice by a cardiac puncture into a syringe containing heparin as an anticoagulant. Porcine and rabbit bloods were purchased from Lampire Biological Lab (Pipersville, PA). For whole blood experiments, mouse, porcine and rabbit whole blood with heparin were stored at 4°C before use. To prepare animal RBCs suspended in buffer, whole blood was spun down at 1000g for 30 min via centrifugation. RBCs was collected from the bottom layer, and plasma on the top layer was spun down again at 2250g for 30 min to diminish platelets and WBCs. RBCs layer was washed with PBS via centrifugation at 1000 g for 30 min to minimize the excess anticoagulant and/or plasma constituents. The RBC pellet was resuspended in viscous buffer (RBC+VB) where dextran is added in DPBS+ with 1% BSA to match the viscosity of each animal plasma or plasma (spun plasma) to achieve a 40% hematocrit (% Hct), i.e. volume fraction of RBCs to plasma⁴. Pig plasma viscosity is slightly higher, (~20%), compared to rabbit, mouse, and human which have similar plasma viscosity²⁶. Thus, rabbit and mouse VB assays used the buffer condition previously used for matching human plasma²⁷.

Flow adhesion experimental set up

A parallel plate flow chamber (PPFC) equipped with a silicon rubber gaskets forming the flow channel (GlycoTech, Gaithersburg, MD) was used for *in vitro* flow adhesion assays. Flow assays were constructed as described in previous publications⁴⁻⁶. Briefly, a single straight gasket was placed over an activated HUVEC monolayer cultured on a glass coverslip and vacuum-sealed to the flow deck to form the bottom adhesion substrate of the flow chamber. Vascular-targeted spheres suspended in buffer or blood at a fixed concentration of 5×10^5 beads/mL were introduced into the flow channel from an inlet reservoir via a programmable syringe pump (KD Scientific, Holliston, MA). Flow adhesion assays were observed on a Nikon TE 2000-S inverted microscope fitted with a digital camera (Photometrics CoolSNAP EZ with a Sony CCD sensor). Digital recording of experiments was via Metamorph analysis software.

For laminar flow assays, the wall shear rate (WSR; γ_w), was computed using the approximation.

$$\gamma_w \left(s^{-1} \right) = \frac{6Q}{wh^2}$$

using the volumetric flow rate (Q) through the channel (mL/min), where h is the channel height (254 μ m) and w is the channel width (1 cm). The wall shear stress (τ_w - dynes/cm²) can be calculated by multiplying the WSR by blood viscosity (μ), which is a function of temperature, Q , and blood hematocrit.

Data analysis

Particle binding density (#/mm²) is obtained by manual count of the number of particles bound on the cell monolayer after 5 min of flow and dividing this number by the area of the field of view (20 $\times$ magnification, $A = 0.152$ mm²). The data was collected at a constant position along the length of the chamber for all experiments. Each data point represents an average of at least three experiments and includes at least 10 fields of view per experiment. Standard error bars were plotted unless otherwise stated. Differences in adhesion levels were analyzed using a student t test and one-way ANOVA with Tukey post-test. A value of $p < 0.05$ was considered statistically significant⁶.

SDS-PAGE

Particles for gel electrophoresis were conjugated with avidin and sLe^a and then incubated in plasma for 1 hour at a particle surface area/plasma volume ratio of 2.54×10^8 μ m²/mL. Plasma soaked particles were then washed once with PBS+/+ 1% BSA, and then three times with 50 mM sodium phosphate buffer. Particles were re-suspended in 50 μ L of 1 $\times$ sample buffer (PierceTM ThermoScientificTM SDS-PAGE Prep Kit: 0.3 M Tris- HCl, 5% SDS, 50% glycerol, lane marker tracking dye, pH 6.8) and heated at 95 $^{\circ}$ C for 5 minutes. This step solubilizes the proteins, removing them from the particle surface. Reducing agents such as DTT are optional, and were not added. SDS gel electrophoresis was performed using Mini-PROTEAN[®] precast gels from BIO-RAD. 15 μ L was injected per lane for all sample lanes.

and 5 μ L was used for the molecular weight standard (Precision Plus™ Dual Color Protein Standards). Run time was approximately 25 minutes at 200 [V].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank Dr. Mariana Carrasco-Teja for helpful discussions regarding blood flow dynamics and assistance in ordering supplies needed to perform experiments

FUNDING SOURCES

This work is funded by the US National Institute of Health R01 HL115138 grant to Omolola Eniola-Adefeso and the cellular biotechnology training grant to Daniel J. Sobczynski

Abbreviations

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
VTC	Vascular-targeted carrier
PEG	Poly(ethylene)-glycol
PLGA	Poly(lactic-co-glycolic-acid)
sLe^a	Sialyl-lewis A
RBCs	Red blood cells
VB	Buffer with viscosity matched to human plasma

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Figure 1.
Sample image of the adhesion of 5 μm polystyrene spheres to activated HUVEC in laminar flow of porcine RBCs in viscous buffer (A) and whole blood (B) at 200 s^{-1} for 5 min. Particle concentration = 5×10^5 spheres/mL. Black dots (arrow) in (A) indicate bound microspheres and white dots indicate leukocytes.

Figure 2.

Particle adhesion to activated HUVEC in laminar flow of blood at 200 s^{-1} for 5 min. Adhesion of $5\text{ }\mu\text{m}$ (A) polystyrene or (B) PLGA spheres. Both particle types were evaluated in porcine and mouse blood. RBC+VB = washed RBCs in plasma-matched viscous buffer, pVTC in RBC+VB = 1 hr plasma opsonized particle in washed RBCs in plasma-matched viscous buffer, and WB = whole blood. Particle concentration = 5×10^5 spheres/mL. * = $p < 0.05$ relative to RBC+VB trial via one-way ANOVA. $n = 3$.

Figure 3.

Adhesion of 5 μm PLGA particle to activated HUVEC in laminar flow of blood at 200 s^{-1} for 5 min. (A) Porcine blood flow, and (B) Mouse blood flow. RBC+VB = washed RBCs in plasma-matched viscous buffer, pVTC in RBC+VB = 1 hr plasma opsonized particle in washed RBCs in plasma-matched viscous buffer, RBC+Plasma = RBCs in pure plasma (cells removed plasma), and WB = whole blood. Particle concentration = 5×10^5 spheres/mL. * = $p < 0.05$ relative to RBC+VB trial via one-way ANOVA. $n = 3$.

Figure 4.

Adhesion of nanoparticles (500 nm) to activated HUVEC in laminar flow of blood at 200 s^{-1} for 5 minutes for polystyrene, PLGA, silica and titanium dioxide spheres. (A) Porcine blood flow, and (B) Mouse blood flow. RBC+VB = washed RBCs in plasma-matched viscous buffer, RBC+Plasma = RBCs in pure plasma (cells removed plasma), and WB = whole blood. Particle concentration = 5×10^5 spheres/mL. * = $p < 0.05$ relative to RBC+VB trial via one-way ANOVA. $n = 3$

Figure 5. SDS-PAGE performed for proteins adsorbed onto sLe^a-coated PLGA nanospheres (500 nm) from buffer in addition to rabbit, mouse, porcine, and human plasma. Lane 1: molecular weight ladder, Lane 2: protein corona from buffer soaked particles, Lane 3: corona from rabbit plasma, Lane 4: corona from mouse plasma, Lane 5: corona from porcine plasma, and Lane 6: corona from human plasma.

Figure 6. Adhesion of (A) 500 nm and (B) 2 μ m PEGylated spheres targeted with 500 ± 180 sLe^a sites/ μ m² and 300 ± 50 sLe^a sites/ μ m², respectively, in porcine RBCs in viscous buffer (RBC +VB), porcine RBCs in porcine plasma (RBC+Plasma), and porcine whole blood (WB). * indicates significant difference in particle adhesion relative to the adhesion of the same particles in RBC+VB ($P < 0.05$).