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Effect of anticoagulants on the protein corona-induced reduced drug carrier adhesion efficiency in human blood flow

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ABSTRACT

Plasma proteins rapidly coat the surfaces of particulate drug carriers to form a protein corona upon their injection into the bloodstream. The high presence of immunoglobulins in the corona formed on poly (lactic-co-glycolic acid) (PLGA) vascular-targeted carrier (VTC) surfaces was recently shown to negatively impact their adhesion to activated endothelial cells (aECs) *in vitro*. Here, we characterized the influence of anticoagulants, or their absence, on the binding efficiency of VTCs of various materials via modulation of their protein corona. Specifically, we evaluated the adhesion of PLGA, poly(lactic acid) (PLA), polycaprolactone (PCL), silica, and polystyrene VTCs to aECs in heparinized, citrated, and non-anticoagulated (serum and whole) blood flows relative to buffer control. Particle adhesion is substantially reduced in non-anticoagulated blood flows regardless of the material type while only moderate to minimal reduction is observed for VTCs in anticoagulant-containing blood flow depending on the anticoagulant and material type. The substantial reduction in VTC adhesion in blood flows was linked to a high presence of immunoglobulin-sized proteins in the VTC corona via SDS-PAGE analysis. Of all the materials evaluated, PLGA was the most sensitive to plasma protein effects while PCL was the most resistant, suggesting particle hydrophobicity is a critical component of the observed negative plasma protein effects. Overall, this work demonstrates that anticoagulant positively alters the effect of plasma proteins in prescribing VTC adhesion to aECs in human blood flow, which has implication in the use of *in vitro* blood flow assays for functional evaluation of VTCs for *in vivo* use.

Statement of Significance

This study addresses the impact of anticoagulant on altering the extent of the previously observed protein corona-induced adhesion reduction of vascular-targeted drug carriers in human blood flows. Specifically, serum blood flow (no anticoagulant) magnifies the negative effect of the plasma protein corona on drug carrier adhesion relative to citrated or heparinized blood flows. Overall, the results from this work suggest that serum better predicts targeted drug carrier adhesion efficiency *in vivo* compared to anticoagulant containing plasma. Furthermore, this study offers critical insight into the importance of how the choice of anticoagulant can greatly affect drug delivery-related processes *in vitro*.

1. Introduction

Vascular-targeted drug delivery has long been explored for use as an efficient and non-invasive alternative to current treatments for a variety of diseases, most notably, cancer [1,2]. Vascular targeting offers great potential to reduce side effects of non-specific cytotoxic drugs and increase overall therapeutic potency. However, several challenges still remain for this approach. Optimal VTC efficacy hinges on successful localization and adhesion of carriers to the vascular wall at the target site. Thus, VTC characteristics, including size, shape and surface characteristics that result in low VTC localization or adhesion to the vascular wall will undoubtedly hinder any potential benefit of this approach. Previously, rapid formation of a unique protein corona on poly(lactic-co-glycolic) acid (PLGA)-based VTCs was shown to drastically reduce their adhesion efficiency to inflamed endothelium in human blood flow; however, this effect was largely non-existent for polystyrene (PS),

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silica (Si), and titanium dioxide (TiO_2) particles [3–5]. This plasma protein effect was linked to a unique, large presence of immunoglobulin sized (~150 kDa) proteins in the PLGA corona [3].

Although the protein corona is known to influence many processes such as targeted nanoparticle (NP) uptake [6] and VTC adhesion to the vascular wall [3,5], very limited studies have explored the influence of suspending fluid (i.e. plasma anticoagulant or its absence) in these processes. Serum (no anticoagulant) and anticoagulated plasma are both routinely used to assess NP-protein interaction for drug delivery applications, yet these mediums have their own unique protein composition, which can influence the type of proteins that adsorb on particle surfaces [6–11]. Specifically, concentrations of chemokines, complement factors, and apolipoproteins have been reported to increase in serum relative to plasma [7,12]. On the contrary, the concentration of coagulation proteins, such as fibrinogen, is significantly reduced in serum relative to anticoagulated plasma, and overall, serum tends to have a slightly lower total protein content than plasma [13]. Likely a result of these composition differences, unique particle protein coronae have been reported between serum and plasma exposed particles, resulting in differential cellular interactions [14,15]. Similarly, the composition of proteins in plasma has been shown to significantly change with the choice of anticoagulant, e.g. citrate versus heparin [8,12]. Heparin binds to a number of proteins commonly called "heparin-binding proteins", including fibronectin, apolipoproteins, complement C3 and C4b, and antithrombin [16], and this binding process can affect the activity and plasma concentration of these proteins [17,18]. Indeed, Kim et al. reported an increased concentration of Apo-AI and α2-macroglobulin in heparin relative to citrated plasma [12]. Furthermore, Schöttler et al. reported corona differences (increased fibrinogen and decreased vitronectin) observed between citrate relative to heparin exposed particles [14]. Despite this knowledge of differences in protein composition of serum and plasma or of different anticoagulants, the role of these differences in protein corona formation and the potential role in prescribing VTC adhesion to the vascular wall remains unknown.

In this work, we evaluate how protein adsorption and, subsequently, particle adhesion is affected by the characteristics of the suspending blood fluid and particle material type. Specifically, we employed viscous buffer (VB), acid-citrate-dextrose (ACD) treated plasma, heparinized plasma, serum and anticoagulant-free whole blood as mediums for the flow adhesion of ~500 nm PLGA, poly(lactic acid) (PLA), polycaprolactone (PCL), PS and Si particles to activated human umbilical vein endothelial cells (HUVEC) in a parallel plate flow chamber (PPFC). A particle's material type has been identified to cause substantial differences in the composition of proteins present in the particle's surface adsorbed corona and thus represents a critical parameter to explore in this study [19,20]. Particles made from PLGA, PLA and PCL polymers were used in this study since they are biodegradable polyesters typically used in the construction of VTCs. PS and Si serve as control materials in our assays to allow comparison to existing literature. Both citrated (ACD) and heparinized whole bloods are explored as a flow medium, as these are commonly used anticoagulants for blood draw that also act by different mechanisms [8]. ACD chelates calcium ions, thus preventing the clotting process, whereas heparin acts by binding to antithrombin III, causing inactivation of thrombin [12]. These differences in mechanism of action between ACD and heparin can have profound impacts on the overall plasma composition [7,12,18,21]. We investigate differences in particle binding between serum and anticoagulated plasma to determine if the absence of clotting factors/proteins, as is the case for serum (anticoagulant free), impacts particle adhesion from flow based on the known significant changes in the protein corona formed onto VTCs between these two mediums [7,22]. Anticoagulant free whole blood is used here in an attempt to best resemble particle interaction *in vivo* in humans. We then probed whether any observed corona-induced adhesion reduction is mitigated by increased copies of targeting ligand on the particle surface for the different materials, as observed previously with PLGA microparticles [3]. Non-pegylated (non-PEG) particles are employed throughout this study to highlight the impact of material type in any observed corona-induced effects, which can shed light on the extent of surface modification required for VTCs of different materials to successfully reduce or alter plasma protein adsorption.

2. Materials and methods

2.1. Particle size and concentration characterization

PLGA, PLA, and PCL carboxylated 500 nm particles were obtained from Phosphorex, Inc. (Hopkinton, MA). The VTC particle concentration was obtained by manual counting on a hemacytometer (Hausser-Scientific). Non-fluorescent particles were used to limit any effect of a fluorescent dye on VTC-protein interaction. Particles were rendered fluorescent after the plasma/serum/VB incubation and prior to use in flow assays. Si and PS green fluorescent particles were purchased from Corpuscular (Cold Spring, NY) and Polysciences, Inc. (Warrington, PA), respectively. VTC size was measured by dynamic light scattering (DLS) using a Malvern Zetasizer instrument. Carboxylated stocks were dispersed in PBS++, with 1% bovine serum albumin (BSA) and then washed with 50 mM PBS prior to making the DLS measurement. Carboxylated biodegradable PLGA, PLA, and PCL particles were soaked in 50 mM MES at pH 7 (for PCL, pH ~ 5) for ⩾20 h prior to DLS measurement which corresponds to the time required for NeutrAvidin conjugation to these particles. VTC diameters ranged from ~400–700 nm as listed in Table 1. Table 2 lists the average sLe^a ligand site density of the various VTC materials.

2.2. Surface ligand conjugation

Particle stocks were first conjugated with NeutrAvidin via covalent carbodiimide chemistry followed by linkage to biotinylated sialyl-Lewis^a (sLe^a) (Glycotech Corporation). The coupling of NeutrAvidin has been described elsewhere [23]. Briefly, an approximate particle surface area of 9.1×10^9 μm^2 mL^{-1} of conjugation volume was used. Particle concentration was determined by manual count of a known volume of particle solution using a hemocytometer. The conjugation volume consisted of equal parts NeutrAvidin (5 mg mL^{-1}) and *N*-Ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) (Sigma) (75 mg mL^{-1}) dissolved in 50 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer. VTCs were first incubated with 5 mg mL^{-1} NeutrAvidin for 15 min, followed by addition of an equal volume of EDAC at 75 mg mL^{-1}. 1 M sodium hydroxide (NaOH) was added (~47 μL mL^{-1} conjugation volume) to pH the solution to ~7.4 and allowed to incubate on an end-to-end rotator for ~20 h. Following the 20 h incubation step, ~7.5 mg glycine

Table 1
VTC DLS sizing measurements. The average VTC diameter (Z-average) and polydispersity index (PDI) is listed for all materials studied. Data was obtained via a Malvern Zetasizer Nano equipped with a back scattering detector.

Material	Z-Average (nm)	PDI
PLA	635	0.25
PLGA	428	0.18
Si	712	0.12
PS	529	0.12
PCL	600	0.27