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The importance of selecting a proper biological milieu for protein corona analysis *in vitro*: Human plasma *versus* human serum

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ABSTRACT

Nanoparticle (NP) exposure to biological fluids in the body results in protein binding to the NP surface, which forms a protein coating that is called the "protein corona". To simplify studies of protein–NP interactions and protein corona formation, NPs are incubated with biological solutions, such as human serum or human plasma, and the effects of this exposure are characterized *in vitro*. Yet, how NP exposure to these two different biological milieus affects protein corona composition and cell response has not been investigated. Here, we explore the differences between the protein coronas that form when NPs are incubated in human serum *versus* human plasma. NP characterization indicated that NPs that were exposed to human plasma had higher amounts of proteins bound to their surfaces, and were slightly larger in size than those exposed to human serum. In addition, significant differences in corona composition were also detected with gel electrophoresis and liquid chromatography–mass spectrometry/mass spectrometry, where a higher fraction of coagulation proteins and complement factors were found on the plasma-exposed NPs. Flow cytometry and confocal microscopy showed that the uptake of plasma-exposed NPs was higher than that of serum-exposed NPs by RAW 264.7 macrophage immune cells, but not by NIH 3T3 fibroblast cells. This difference is likely due to the elevated amounts of opsonins, such as fibrinogen, on the surfaces of the NPs exposed to plasma, but not serum, because these components trigger NP internalization by immune cells. As the human plasma better mimics the composition of the *in vivo* environment, namely blood, *in vitro* protein corona studies should employ human plasma, and not human serum, so the biological phenomena that is observed is more similar to that occurring *in vivo*.

1. Introduction

Nanoparticles (NPs) are promising therapeutic delivery vehicles which can be loaded with pharmaceuticals and/or imaging agents (Janib et al., 2010; Xie et al., 2010) and functionalized with targeting moieties (Farokhzad et al., 2006; Salvati et al., 2013) that may enable selectively delivering their payloads to the desired location in the body (Albanese et al., 2012; Elsabahy and Wooley, 2012; Peer et al., 2007). As soon as NPs are injected into the body, proteins and other biomolecules, such as lipids, bind to their surfaces and form a layer that is called the "protein corona" (Cedervall et al., 2007b; Mahmoudi et al., 2011; Monopoli et al., 2011; Tenzer et al., 2013; Walczyk et al., 2010; Walkey and Chan, 2012). This protein corona can compromise cellular uptake and targeting efficiency by covering the targeting ligands (Mirshafiee et al., 2016; Mirshafiee et al., 2013; Salvati et al., 2013; Sobczynski et al., 2014). Protein corona formation also affects the NPs' distributions inside the body (Aggarwal et al., 2009; Furumoto et al., 2002; Lazarovits et al., 2015; Nagayama et al., 2007) and within individual cells (Lesniak et al., 2012). Thus, knowledge of blood protein adsorption onto NPs and its effects on biological phenomena are critical for the development of safer and more efficient NP-based drug delivery systems.

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An approach that is widely used to simplify studies of protein–NP interactions in the body and the effects of protein corona formation on various biological phenomena is to expose NPs to biological fluids (*e.g.*, human serum, human plasma, and fetal bovine serum) *in vitro*. Then the effects of protein adsorption on the NPs' interactions with cultured cells such as uptake or *in vitro* targeting efficiency are characterized (Dai et al., 2015; Lesniak et al., 2012; Lesniak et al., 2013; Mirshafiee et al., 2016). Although the exposure of NPs to blood proteins *in vivo* may be more comprehensive (Hadjidemetriou et al., 2015; Sakulkhu et al., 2014), such *in vitro* studies are convenient platforms for preliminary studies of protein corona formation. While human plasma was used as the protein-containing medium for corona formation in many *in vitro* studies (Cedervall et al., 2007a; Lundqvist et al., 2011; Lundqvist et al., 2008; Maiolo et al., 2014; Monopoli et al., 2011; Tenzer et al., 2013), numerous other studies used human or bovine serum to prepare NP–protein complexes (Ahmad Khanbeigi et al., 2015; Ashby et al., 2014; Borgognoni et al., 2015; Chinen et al., 2015; Cifuentes-Rius et al., 2013; Dai et al., 2015; García et al., 2015; Moyano et al., 2014; Yallapu et al., 2015; Zhang et al., 2015). While both plasma and serum are obtained from blood, their compositions are very different due to dissimilar preparation procedures. The preparation of plasma from blood involves the addition of anticoagulant reagents, followed by centrifugation to remove the blood cells. In contrast, serum preparation involves first coagulating the blood, followed by centrifugation to remove the blood cells and coagulated material. This process depletes the serum of coagulation factors, such as fibrinogen, and lowers its protein concentration (Issaq et al., 2007). This difference could significantly affect protein corona composition, and hence, the effects of this protein coating on the NPs' physical and biological characteristics. Therefore, identical NPs that have two different corona compositions, one obtained from NP incubation with human serum and the other with human plasma, may elicit very different cellular responses. Thus, the biological fluid that is used for protein corona formation must be carefully selected to ensure that the biological phenomena observed is representative of that occurring in the body.

Here, we explore the differences in the protein coronas that are obtained when NPs are incubated with human plasma and human serum. Silica NPs were exposed to either human plasma or human serum to form the protein corona. These NP-protein complexes were characterized regarding their size, surface charge, and the amount of proteins bound to their surfaces. Gel electrophoresis and liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) were used to compare the compositions of their protein coronas in terms of protein size and identity. Finally, the effects of the two different corona compositions on NP uptake was studied via incubation of serum exposed and plasma exposed NPs with macrophage (RAW 264.7) and fibroblast (NIH 3T3) cells.

2. Materials and methods

2.1. Materials

Ammonium hydroxide (28.0–30.0% as NH_3) and carboxyethylsilanetriol sodium salt (25% in water) were purchased from Macron Fine Chemicals and Gelest Inc., respectively. Phosphate buffered saline (PBS) was purchased from Lonza. The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Biotechnology, Inc. MSG-Trypsin was purchased from G-Biosciences. 2X Laemmli sample buffer, QC Colloidal Coomassie Stain, and 4–20% polyacrylamide Mini PROTEAN Precast Gels were purchased from Bio-Rad. RAW 264.7 cells were kindly provided by Prof. Ed Roy (University of Illinois at Urbana-Champaign, USA). NIH 3T3 cells were purchased from ATCC. All other chemicals were purchased from Sigma-Aldrich.

2.2. Synthesis of silica NPs

A well-mixed solution of 1 mL methanol, 360 μL deionized (DI) water, and 80 μL ammonium hydroxide was prepared, 62.5 μL tetraethyl orthosilicate (TEOS) was added, and the solution was stirred at rt for 2 h. The resulting NPs were collected by centrifuging the solution, and washed with ethanol and DI water. The NPs were functionalized with carboxylic acid moieties by adding 20 μL carboxyethyl-silanetriol sodium salt (25% in water) to silica NPs (10 mg) suspended in 1 mL PBS (Wang et al., 2007). After stirring the solution for 4 h, the carboxylic acid-functionalized NPs were collected by centrifugation, washed with PBS, and dispersed in DI water.

Fluorescent silica NPs were synthesized following a previously reported procedure (Mirshafiee et al., 2013; Tang et al., 2012) with minor modification. Briefly, RITC-silane conjugate was added to a solution of silica NPs that were prepared as described above. After stirring for 12 h, 20 μL of carboxyethyl-silanetriol sodium salt (25% in water) was added to the solution, and the mixture was stirred for another 12 h. The resulting fluorescent silica NPs were collected by centrifugation, washed with ethanol and DI water, and dispersed in DI water.

2.3. Formation of NPs with hard coronas

NPs (500 μg) were incubated with human plasma or human serum solutions that mimic the protein concentration *in vivo* (55% human plasma or human serum in PBS) for 1 h at 37 °C. The NP–protein complexes were collected via centrifugation, washed twice with cold (4 °C) PBS, and dispersed in PBS.

2.4. Characterization on NPs' physical properties

For hydrodynamic diameter and surface charge characterization, fluorescent NPs were dispersed in PBS (0.2 mg/mL), and measurements were performed using a Brookhaven ZetaPALS instrument. The hydrodynamic diameters of the pristine NPs were measured in deionized water instead of PBS due to their aggregation in PBS. To characterize NP size distribution by scanning electron microscopy (SEM), NPs were coated with Au/Pd using an Emitech K575 sputter coater, and imaged with a Hitachi S-4800 high resolution scanning electron microscope. For each type of NP, the diameters of 50 NPs were measured using Image J software.

2.5. Quantitative analysis of proteins bound to the NPs

The amounts of proteins in the hard corona-coated fluorescent NPs were measured using a BCA protein assay, which was performed following the manufacturer's instructions.

2.6. Analysis of proteins in the hard-corona by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

200 μg of each hard corona-coated non-fluorescent NPs (10 μg/μL, 20 μL) were incubated in 2X Laemmli sample buffer without reducing agent at 85 °C for 5 min, then cooled to rt and subjected to SDS-PAGE separation on 4–20% polyacrylamide Mini PROTEAN precast gels. After SDS-PAGE, the gel was fixed in acetic acid:methanol:water = 1:4:5 (v:v:v), stained with QC Colloidal Coomassie Stain overnight at rt, and then destained in water for 2–3 h. ImageQuant LAS4010 Imager (GE Healthcare) was used for imaging stained gels.