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Impact of protein pre-coating on the protein corona composition and nanoparticle cellular uptake

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ABSTRACT

Nanoparticles (NPs) are functionalized with targeting ligands to enable selectively delivering drugs to desired locations in the body. When these functionalized NPs enter the blood stream, plasma proteins bind to their surfaces, forming a protein corona that affects NP uptake and targeting efficiency. To address this problem, new strategies for directing the formation of a protein corona that has targeting capabilities are emerging. Here, we have investigated the feasibility of directing corona composition to promote targeted NP uptake by specific types of cells. We used the well-characterized process of opsonin-induced phagocytosis by macrophages as a simplified model of corona-mediated NP uptake by a desired cell type. We demonstrate that pre-coating silica NPs with gamma-globulins (γ-globulins) produced a protein corona that was enriched with opsonins, such as immunoglobulins. Although immunoglobulins are ligands that bind to receptors on macrophages and elicit phagocytosis, the opsonin-rich protein corona did not increase NP uptake by macrophage RAW 264.7 cells. Immunolabeling experiments indicated that the binding of opsonins to their target cell surface receptors was impeded by other proteins in the corona. Thus, corona-mediated NP targeting strategies must optimize both the recruitment of the desired plasma proteins as well as their accessibility and orientation in the corona layer.

1. Introduction

Nanoparticles (NPs) are a promising drug delivery system that may reduce the undesired side effects of cytotoxic anticancer drugs by selectively transporting the drug payload to the desired location in the body [1,2]. For this purpose, targeting ligands (*e.g.*, aptamers or antibodies) that can bind to surface receptors on the desired cells are often grafted onto the NPs. The selective binding of these targeting ligands to receptors on the surfaces of the desired cells is expected to enhance NP uptake by the target cells [3,4]. However, NPs functionalized with targeting ligands often have low targeting efficiency *in vivo*, and accumulate in the liver and spleen rather than the intended tissue [5].

One potential cause for low targeting efficiency is that proteins and other biomolecules bind to the NP's surface when it is exposed to the biological environment [6,7]. The resulting long-lived protein coating, known as the "hard" protein corona, establishes a new biointerface that affects the biodistribution [8–10], cellular uptake [11–16], intracellular location [11], and payload release kinetics of the NPs [17]. Additionally, the targeting capabilities of functionalized NPs can be reduced by a non-biological mechanism in which the protein corona screens the targeting ligands on the NPs' surface, preventing them from interacting with their intended targets on the cell surface [18–20]. To address this obstacle, flexible

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hydrophilic polymers, such as polyethylene glycol (PEG), are often grafted onto NPs to reduce plasma protein adsorption, or used as flexible linkers that may allow the targeting ligands to reach their intended receptors on the cell surface. However, PEGylation decreases, but does not completely prevent protein binding [21,22]. Moreover, adsorbed proteins can screen the NP's targeting ligands even when they are attached to a PEG linker [19].

An emerging alternative strategy is to functionalize NPs to promote the adsorption of plasma components that are naturally targeted to the desired cells [23]. Upon contact with the blood stream, these NPs are expected to acquire a protein corona that actively targets them to the desired cells. For example, polysorbate coatings that promote the adsorption of certain apolipoproteins in the blood stream to the NPs could enhance NP transport across the blood–brain barrier [24]. Recently, protein adsorption has been directed to promote the formation of functional protein coronas that enabled active NP targeting to hepatic stellate cells [25] and cancer cells [26].

Here, we explore the feasibility of engineering NPs to direct protein corona composition for active NP targeting. Specifically, we assessed whether the targeting capabilities of protein ligands recruited directly from the plasma during corona formation would be compromised by the non-specific adsorption of undesired plasma components. Although NP uptake by immune cells is typically detrimental to targeted drug delivery, opsonin-mediated phagocytosis of NPs [27–31] provides a well-characterized testbed for assessing whether protein corona composition can be tuned to promote NP uptake by specific types of cells. The binding of immunoglobulins and activated complement factors to Fc and complement receptors, respectively, on specific types of immune cells (*i.e.*, macrophages) triggers particle phagocytosis [32–35]. Therefore, we aimed to engineer NPs to promote the adsorption of opsonins, such as immunoglobulins and complement factors, onto their surfaces, and assessed whether the resulting opsonin-rich protein corona enhanced NP uptake by immune cells through interactions with Fc and complement receptors. To promote the adsorption of opsonins from biological fluids onto the NPs [36], we pre-coated NPs with gamma-globulins (γ-globulins) from human plasma. For comparison, another set of NPs were pre-coated with human serum albumin (HSA), which is known to reduce the adsorption of opsonins onto drug carriers [37,38] and decrease their clearance from the body [39]. We demonstrate that the coronas of the NPs that were pre-coated with γ-globulins, but not HSA, were enriched with immunoglobulins and complement factors in comparison to uncoated NPs. However, the opsonin-rich corona on the γ-globulins pre-coated NPs did not enhance NP uptake by RAW 264.7 macrophages. Our findings suggest that other protein corona components shielded the immunoglobulins and complement factors in the corona from binding to their receptors on the immune cells. Thus, the targeting efficiency of proteins that were recruited from the biological environment to the corona can be compromised by non-specific protein adsorption.

2. Materials and methods

2.1. Materials

Unless otherwise indicated, all chemicals were purchased from Sigma–Aldrich. The bicinchoninic acid (BCA) protein assay kit was obtained from Pierce Biotechnology, Inc. RAW 264.7 cells were kindly provided by Prof. Ed Roy (University of Illinois at Urbana–Champaign, USA).

2.2. Synthesis of carboxylic acid-functionalized silica NPs (UC-NPs)

62.5 μL of tetraethyl orthosilicate (TEOS) was added to a well-mixed solution of methanol (1 mL), DI water (360 μL) and 80 μL concentrated ammonia (Fisher Scientific). The mixture was gently stirred for 2 h. Next, silica NPs were collected by centrifugation and washed thoroughly with ethanol and DI water. To functionalize the silica NPs with carboxylic acid moieties, 10 mg of silica NPs were dispersed in 1 mL of phosphate buffered saline (PBS, Lonza). 20 μL of carboxyethyl-silanetriol sodium salt, 25% in water (Gelest Inc.), was added to the NP solution, and the mixture was stirred for 4 h [40]. Carboxylic acid-functionalized NPs (UC-NPs) were collected by centrifugation, washed with PBS and dispersed in DI water.

2.3. Synthesis of fluorescent carboxylic acid-functionalized silica NPs

30 mg of 3-aminopropyltrimethoxysilane (APTMS) and 17 mg of rhodamine B isothiocyanate (RITC) were added to 1 mL of ethanol with triethylamine (20 μL), and stirred for 12 h at 50 °C in the dark. After removing the solvent under vacuum, the crude RITC-silane was dissolved in methanol (10 mg/mL solution). To prepare fluorescent silica NPs, tetraethyl orthosilicate (62.5 μL) was added to the well-mixed solution of methanol (1 mL), water (360 μL) and concentrated ammonia (80 μL) as described above and stirred for 2 h [41]. Next, 25 μL of RITC-silane solution was added to the mixture and the solution was gently stirred for 12 h. 20 μL of carboxyethyl-silanetriol sodium salt (25% in water) was added to the solution and it was further stirred for 12 h. Finally, fluorescent carboxylic acid-functionalized NPs were collected by centrifugation, washed with ethanol and DI water, and dispersed in DI water.

2.4. Preparation of HSA and γ-globulin pre-coated NPs (HSA-NPs and GG-NPs)

2 mg of carboxylic acid-functionalized NPs (UC-NPs) were dispersed in 1 mL of MES buffer (0.1 M, pH 6.8). 50 mg of each 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxy-succinimide (NHS) were added to the solution and the mixture was stirred for 25 min. Activated NPs were collected by centrifugation, washed with PBS, and dispersed in 1 mL of PBS. 5 mg of pre-coating proteins (albumin from human serum or γ-globulins from human blood) were immediately added to NP solution and stirred for 2 h. Protein pre-coated NPs (HSA-NPs and GG-NPs) were collected by centrifugation and washed with PBS to remove unbound or loosely bound proteins. The resulting pre-coated NPs were dispersed in PBS. The bicinchoninic acid (BCA) protein assay was used to quantify the amount of proteins on the NPs following chemical conjugation. For comparison, the BCA assay was also performed on unactivated NPs that were exposed to γ-globulin and HSA solutions under the same conditions as the activated NPs. To further assess γ-globulin conjugation to the NPs, GG-NPs were incubated with SDS-containing Laemmli sample buffer for 5 min, collected by centrifugation, and the amount of γ-globulins that remained on the NPs were quantified by a BCA assay.

2.5. Preparation of hard corona NPs (corona-UC-NPs, corona-HSA-NPs, and corona-GG-NPs)

500 μg of control (UC-NPs) and protein pre-coated NPs (HSA-NPs and GG-NPs) were exposed to 10% or 55% human plasma solutions (10% or 55% human plasma in PBS) and incubated at 37 °C for 1 h with occasional shaking. Next, NPs were collected by centrifuge and washed two times with cold PBS (4 °C) to remove