Nanoparticle–Cell Interactions: Molecular Structure of the Protein Corona and Cellular Outcomes

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CONспектus: The use of nanoparticles (NPs) in biology and medicine requires a molecular-level understanding of how NPs interact with cells in a physiological environment. A critical difference between well-controlled in vitro experiments and in vivo applications is the presence of a complex mixture of extracellular proteins. It has been established that extracellular serum proteins present in blood will adsorb onto the surface of NPs, forming a “protein corona”. Our goal was to understand how this protein layer affected cellular-level events, including NP binding, internalization, and transport. A combination of microscopy, which provides spatial resolution, and spectroscopy, which provides molecular information, is necessary to probe protein–NP–cell interactions. Initial experiments used a model system composed of polystyrene NPs functionalized with either amine or carboxylate groups to provide a cationic or anionic surface, respectively. Serum proteins adsorb onto the surface of both cationic and anionic NPs, forming a net anionic protein–NP complex. Although these protein–NP complexes have similar diameters and effective surface charges, they show the exact opposite behavior in terms of cellular binding. In the presence of bovine serum albumin (BSA), the cellular binding of BSA–NP complexes formed from cationic NPs is enhanced, whereas the cellular binding of BSA–NP complexes formed from anionic NPs is inhibited. These trends are independent of NP diameter or cell type. Similar results were obtained for anionic quantum dots and colloidal gold nanospheres. Using competition assays, we determined that BSA–NP complexes formed from anionic NPs bind to albumin receptors on the cell surface. BSA–NP complexes formed from cationic NPs are redirected to scavenger receptors. The observation that similar NPs with identical protein corona compositions bind to different cellular receptors suggested that a difference in the structure of the adsorbed protein may be responsible for the differences in cellular binding of the protein–NP complexes. Circular dichroism spectroscopy, isothermal titration calorimetry, and fluorescence spectroscopy show that the structure of BSA is altered following incubation with cationic NPs, but not anionic NPs. Single-particle-tracking fluorescence microscopy was used to follow the cellular internalization and transport of protein–NP complexes. The single particle-tracking experiments show that the protein corona remains bound to the NP throughout endocytic uptake and transport. The interaction of protein–NP complexes with cells is a challenging question, as the adsorbed protein corona controls the interaction of the NP with the cell; however, the NP itself alters the structure of the adsorbed protein. A combination of microscopy and spectroscopy is necessary to understand this complex interaction, enabling the rational design of NPs for biological and medical applications.

■ INTRODUCTION

Nanoparticles (NPs) are increasingly important for biological applications ranging from cellular imaging to drug delivery.1–11 In these applications, NPs encounter a complex mixture of cells and extracellular proteins. For example, NPs injected into the bloodstream are exposed to red and white blood cells, clotting factors, and serum proteins. Similarly, NPs used for cellular experiments are exposed to the serum proteins used as a nutrient source for cultured cells. Serum consists of hundreds of distinct proteins isolated from blood plasma following the removal of clotting factors.12–14 These extracellular serum proteins adsorb onto the NP surface, forming a protein “corona” (Figure 1).15–19 Poly(ethylene glycol) (PEG) can reduce the adsorption of serum proteins on NPs, but complete inhibition of corona formation remains a challenge.18,20,21 Understanding the protein corona is crucial for understanding how NPs interact with cells, as the corona proteins control the specific cellular receptors used by the protein–NP complex,22–24 the cellular internalization pathway,25,26 and even the immune response.27–30

■ ADSORPTION OF PROTEINS ON NP SURFACES: PROTEIN CORONA

A protein corona has been observed on a diverse range of NPs, including polymeric NPs,23,25,31–33 silica NPs,34,35 quantum dots,36,37 iron oxide NPs,38–40 silver nanoclusters,41 silver
Figure 1. Schematic of protein corona formation on a nanoparticle (NP) surface. Protein adsorption is a kinetic (k) and thermodynamic (K) function of both the individual proteins and NP properties such as surface modification, composition, and diameter. Initially, high-abundance and/or high-mobility proteins bind to the nanoparticle surface. Over time, these proteins are replaced by lower-mobility proteins with a higher binding affinity. Serum proteins commonly observed in NP coronas are shown as a representative corona: serum albumin, immunoglobulin G1 (IgG1), alpha-2 macroglobulin (A2M), and apolipoprotein A-1 (apoA1). Modified with permission from ref 19. Copyright 2013 John Wiley and Sons.

Figure 2. Formation of a protein corona on 200 nm amine-modified polystyrene NPs confirmed with SDS-PAGE. NPs (15 pM) were incubated with FBS (10% v/v) for 10 min at 4 °C. Wash steps, consisting of repeated centrifugation (16 000g, 10 min), removal of supernatant, and resuspension in water, were used to removed unbound proteins from the protein–NP complexes. After each wash step, the supernatant (S) was loaded onto the gel. S1 was diluted to 10% v/v due to the high protein concentration. After five wash steps (S5), protein is no longer visible in the supernatant. SDS was used to remove the protein from the NP surface (NP + SDS). As a control, incubation in water does not remove the protein corona (NP + H2O). SDS-PAGE was run for comparison. Adapted from ref 24. Copyright 2012 American Chemical Society.

Figure 3. Cationic and anionic NPs form similar protein–NP complexes. (a) Zeta potential of 200 nm polystyrene NPs in water and after incubation with minimum essential medium (MEM) supplemented with 10% FBS. A series of five washes consisting of centrifugation (16 000g, 10 min) and resuspension was used to remove unbound protein. Adapted from ref 24. Copyright 2012 American Chemical Society. (b) Gel electrophoresis of the washed 200 nm protein–NP complexes. SDS was used to remove the protein corona from the NPs. BSA (66 kDa) was run for comparison.

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charge, regions of positive and negative charge allow proteins to form complexes with both cationic and anionic NPs, nanorods, and planar surfaces.\textsuperscript{16,23,25,26,32,43,45,52} For example, serum albumin is net negatively charged at physiological pH with an isoelectric point at pH 4.7, but it contains 60 positively charged lysine groups.\textsuperscript{53,54}

\section*{Cellular Binding of Protein–NP Complexes}

Although the complexes formed from cationic and anionic polystyrene NPs are indistinguishable in terms of charge and protein corona following incubation with FBS (Figure 3), they have opposite trends in cellular binding (Figure 4). Fluorescence microscopy shows that in the presence of 10% FBS, the concentration of FBS typically used to culture cells, the cellular binding of cationic NPs is increased. In comparison, the cellular binding of anionic NPs is decreased in the presence of FBS. In both cases, it should be noted that the NPs form a protein–NP complex immediately following exposure to FBS. These trends were observed for multiple NP diameters (40–200 nm) and multiple cell types (monkey kidney epithelial cells (BS-C-1), human cervical cancer cells (HeLa), and Chinese hamster ovary (CHO) cells).\textsuperscript{24,55}

Using FBS, it is possible that a low-abundance protein not visible in the gel is responsible for this difference in NP binding. For example, one protein adsorbs onto cationic NPs and enhances binding and a different protein present in the mixture of FBS proteins adsorbs on anionic NPs and inhibits binding. To test this possibility, cellular binding experiments were repeated using only BSA (≥98% purity, Fisher). This removes the possibility that a low-abundance protein is responsible for the observed binding trends. Results with BSA were identical to those with FBS (Figure 5).

Competition assays were used to identify the cell surface receptor used by the BSA–NP complexes (Figure 6). Cellular binding of complexes formed from anionic NPs is inhibited by free BSA (Figure 5A), suggesting that competition for the BSA receptor is responsible for the cellular binding and internalization of albumin. This was tested using flow cytometry. Flow cytometry measures fluorescence intensity per cell in a high-throughput flow system. Although flow cytometry lacks spatial resolution, it has the advantage of measuring ∼10,000 cells/min. Using flow cytometry, we observed that increasing concentrations of BSA led to decreased binding of 93 nm carboxylate-modified NPs (Figure 6A). At a BSA concentration of 10 mg·mL\textsuperscript{−1}, similar to the concentration of protein used in cell culture, NP binding was reduced to 32% in...
comparison to a normalized value of 100% in the absence of BSA.55 This shows that BSA−NPs formed from anionic polystyrene NPs compete with free BSA for cellular receptors. In comparison, BSA−NPs formed from cationic polystyrene NPs show increased binding in the presence of free BSA (Figure 5B), indicating that a different cellular receptor is used by these complexes. A possible class of receptors for the BSA−NPs formed from cationic NPs are scavenger receptors. These cell surface receptors bind disrupted albumin and have been identified previously in the cellular binding of oligonucleotide-functionalized gold NPs.22,26−58 To determine if scavenger receptors are the cellular binding site of the BSA−NPs formed from cationic NPs, we used polyinosinic acid as a competitor. This polyanionic molecule is a competitor for scavenger receptors.22,26,56−58 If BSA−NPs bind to scavenger receptors, then we expect the addition of polyinosinic acid to compete with the BSA−NPs for binding sites on the cell surface, thereby inhibiting the cellular binding of the NPs. The approach is identical to that used for the anionic NPs, with polyinosinic acid rather than free BSA used as a competitor. Flow cytometry shows a decrease in cellular binding of 87 nm amine-modified polystyrene NPs, with 100% binding (normalized) decreased to 25% in the presence of 2.5 mg·mL−1 polyinosinic acid (Figure 6B). A control experiment with polyadenylic acid (2.5 mg·mL−1), a similar molecule that does not compete for scavenger receptors,22 showed no significant competition with the 87 nm NPs.55

The fluorescence microscopy images and flow cytometry experiments show that the same protein (BSA) adsorbed on two different NPs (cationic and anionic polystyrene) leads to binding of these protein−NP complexes to two different cellular receptors. Differences in NP−cell interactions have also been observed for cationic and anionic polymer-modified gold NPs (10−16 nm), which show different rates of cellular uptake despite the formation of identical protein coronas.51 We proposed that a difference in protein structure following adsorption on the polystyrene NP surface leads to this difference in cellular binding.

■ SECONDARY STRUCTURE OF CORONA PROTEINS DETERMINES THE CELL SURFACE RECEPTOR

Circular dichroism (CD) spectroscopy was used to probe the structure of BSA following exposure to cationic and anionic...
polystyrene NPs. CD spectroscopy utilizes a difference in the absorption of left and right circularly polarized light to probe protein secondary structure. CD spectroscopy showed that exposure to anionic NPs did not perturb the secondary structure of BSA (Figure 7A). Isolated BSA has 65% α-helix structure, calculated at 208 nm. Incubation of 60 and 200 nm anionic NPs with BSA resulted in minimal changes to the percent α-helicity, 71 and 63%, respectively.53 In comparison, incubation of BSA with 58 and 200 nm cationic NPs led to a substantial change in α-helicity, 48 and 37%, respectively.

Differences in protein–NP interactions for anionic and cationic polystyrene NPs are also observed in the thermodynamics of protein adsorption on the NP surface. Both isothermal titration calorimetry (ITC) and fluorescence spectroscopy measure a greater equilibrium association constant for the adsorption of BSA on anionic NPs (Table 1).53 Isothermal titration calorimetry also showed a greater number of BSA molecules adsorbed on the anionic NPs, with 230% coverage on anionic 60 nm NPs and 8% coverage on cationic 58 nm NPs. This value for cationic NPs is likely an underestimate because it assumes an end-on model that may not be appropriate for a denatured protein and ignores aggregation that occurs for the cationic NPs under the buffer conditions necessary for calorimetry. Like ITC, fluorescence spectroscopy showed a greater equilibrium association constant for the adsorption of BSA on anionic NPs (1.8 ± 0.1 × 10^{8} M^{-1}) compared to that of cationic NPs (7.7 ± 0.1 × 10^{8} M^{-1}) (Figure 7B).

Taken together, these results suggest that disrupted BSA on the surface of cationic polystyrene NPs causes the BSA–NP complexes to bind to scavenger receptors. It is also possible that adsorption of BSA on the NP surface could expose new peptide sequences. These epitopes could then direct the protein–NP complex to alternative receptors. However, as we observe BSA denaturation with CD spectroscopy and binding to a scavenger receptor known to bind disrupted BSA, it is likely that protein disruption, rather than altered epitope exposure, is the main reason for the binding of BSA–NP complexes formed from cationic NPs to scavenger receptors. Protein adsorption on planar surfaces is known to alter structure and lead to partial denaturation.52,59 A similar disruption of protein structure has been observed previously for NPs.62–67 In the case of albumin, disruption of secondary structure has been observed following adsorption to silver NPs,42–48 zinc oxide NPs,69 gold NPs,44,68,70,71 and gold nanorods.44 Structural changes have also been observed for lower abundance plasma proteins including fibrinogen,71 lysozyme,72 cytochrome c,73,74 and chymotrypsin.72

### CORONA PROTEINS REMAIN BOUND DURING NP INTERNALIZATION

The protein corona ultimately determines the cell surface receptors used by the protein–NP complex, as described above, and the subsequent cellular internalization of the NP. To monitor serum proteins and NPs during cellular internalization, we carried out two-color fluorescence microscopy single particle tracking experiments using fluorescently labeled cationic polystyrene NPs (green) and serum proteins (red) (Figure 8). Serum proteins and NPs bind to the cell as a single complex and remain bound for at least 18 h. Incubating cells at 4 °C allows protein–NP binding but inhibits internalization.75–77 After warming the cells to 37 °C, it is possible to track the internalization of the serum proteins and NPs simultaneously. We find that BSA–NP are internalized as a single complex and remain colocalized as they are transported through the cell.53 Transport is microtubule-dependent.
indicative of endosomes or lysosomes undergoing active transport. These experiments have two important implications. First, corona proteins determine the cellular transport of NPs, as they are not displaced during NP interactions with cells. Binding to two different cell surface receptors suggests that the BSA-NP complexes formed from cationic and anionic polystyrene NPs may use different endocytic pathways, with different rates, to reach the lysosomes. Second, proteins remain bound as the NP is internalized and transported through the cell.

**CONCLUSIONS**

The use of NPs in biology and medicine requires understanding the interactions among NPs, proteins, and cells. Our experiments show that serum proteins adsorb onto the surface of both cationic and anionic NPs. Protein structure can be altered by adsorption on a surface, including NP surfaces. For cationic polystyrene NPs, a change in the secondary structure of BSA redirects the protein-NP complex to scavenger receptors. In comparison, BSA adsorbed on anionic polystyrene NPs retains its native structure, resulting in binding of BSA-NPs to albumin receptors. In the case of anionic NPs, a similar trend was observed for carboxylate-modified quantum dots and citrate-modified colloidal gold NPs, despite the differences in NP diameter, material, and surface modification. The protein and NP remain complexed during cellular internalization and transport. These experiments illustrate the importance of serum protein structure, not just composition, for the cellular binding, internalization, and transport of NPs (Figure 9).

Moving forward, additional research is necessary to understand how the structure of other serum proteins is affected by adsorption on NPs, as each protein will vary. The number of experiments required to investigate each serum protein and NP of interest is intractable, making simulations necessary. Coarse-grained molecular dynamics (MD) simulations have recently examined the structure of corona proteins. A comparison of protein structure, CD spectra, and MD simulations can be used to predict how a specific protein will be affected by adsorption on a NP surface. In addition to computational approaches, X-ray spectroscopy and small-angle neutron scattering will provide new and complementary molecular information.

Our results have important implications for the design of NPs to target specific populations of cells or subcellular locations, a central goal for nanomedicine. Corona proteins have dedicated cell surface receptors that can be used for the binding and internalization of protein-NP complexes. For successful targeting, the targeting ligand must have a greater affinity for its receptor than the nonspecifically adsorbed serum proteins have for their receptors. It is likely that competition between targeting ligands and nonspecifically adsorbed serum proteins is responsible for the challenges associated with in vivo NP targeting. For example, transferrin-functionalized silica NPs bind to native transferrin receptors in vitro, but their targeting capabilities are masked by the adsorption of serum proteins. This highlights the importance of fundamental, molecular-level research to inform translational applications such as rationally designed NPs for drug and gene delivery.

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**Notes**

The authors declare no competing financial interest.

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**Christine K. Payne** received a B.S. (1998) in Chemistry from the University of Chicago. She obtained a Ph.D. (2003) in Chemistry from the University of California, Berkeley, under the mentorship of Professor Charles B. Harris. She spent three years in the Department of Chemistry and Chemical Biology at Harvard University as a NIH NRSA postdoctoral fellow with Professor Xiaowei Zhuang. She joined the faculty of the School of Chemistry and Biochemistry at Georgia Tech in 2007. Her interests include nanoparticle-cell interactions, conducting polymer-cell interactions, cellular biophysics, and fluorescence microscopy.

**ACKNOWLEDGMENTS**

We acknowledge the NIH Director’s New Innovator Award (DP2OD006470, C.K.P.) and a U.S. DoEd Molecular Biophysics and Biotechnology GAANN fellowship (P200A120190, C.C.F.).

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