CHAPTER 3

Protein Corona: The Challenge at the Nanobiointerfaces

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3.1 INTRODUCTION

There is rising public interest in the field of nanomedicine. The most significant area of nanotechnology has been nanoparticles (NPs) in recent years [1–12]. Currently, it is widely known that the surface of NPs is covered with multiple kinds of biomolecules when they enter biological fluids [13–17]. Compared with manufactured products in laboratories, the biomolecule-coated NPs (e.g., protein "corona"-coated particles) can thus be regarded as completely new materials. Recent investigations uncovered that our insufficient understanding of the interactions between NPs and living organisms is largely responsible for the broad gap between bench discoveries and clinical translation [18]. When NPs enter the body, their surface is quickly covered by proteins to create a dynamic corona (the so-called "soft" corona) [17]; minutes later, a long-lived (hard) corona layer surrounds the NPs and continues to be tightly adsorbed to their surfaces [19].

The interactions between NPs and proteins depend on various parameters, including the sizes, shapes, charges, and chemical functionalities of these moieties. Noncovalent interactions are managed by these parameters, including van der Waals interactions, hydrogen bonding (H-bond), hydrophobic interactions, electrostatic interactions, and π-π stacking interactions that cause NP-protein assembly [20]. Table 3.1 overviews the features of these interactions.

The relative significance of each of these forces is dependent on multiple parameters, such as the size and the chemical properties of the NP surface [21]. One type of force is VDW, which are weak and short-range electrostatic attractions between dipoles. Another is H-bonding that forms between

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Table 3.1 Characteristics of the various chemical interactions between NPs and proteins [20]

Forces	Strength	Range (nm)[a]	Specificity	Main factors
VDW forces	Weak	0–10	No	Interface complementarity
H-bond	Moderate	<0	Partial	Hydrogen donor/ acceptor at interface
Electrostatic forces	Moderate	0–10	No	Charge state, ionic strength
Hydrophobic interaction	Strong	0–10	Partial	Hydrophobic surface
π-π stacking	Strong	0–5	Yes	Aromatic ring orientation
Salt bridge	Strong	<1	Yes	Multiple recognition

[a]0 indicates direct atom contact (based on VDW radius).

uncharged hydrophilic regions of the proteins (e.g., serine, threonine, asparagine, and glutamine residues) and polar residues on the surface of the NP (e.g., hydroxyl on oxidized metals). H-bonding interactions are individually stronger than VDW but typically less plentiful in aqueous physiological environments [22]. Materials with charged surface groups (e.g., amines or carboxylic acid) can create cooperative electrostatic interactions with ionized proteins as well. Despite the presence of many ions in biological fluids, these interactions occur because surface charges are frequently better neutralized by polyionic macromolecules. Electrostatic interactions are thought to have a critical function in bringing proteins close to the surface of NPs. The surface of certain materials may also have hydrophobic interactions. Rigid dehydrated regions in proteins with secondary and tertiary structures are often formed by hydrophobic residues, which are buried inside the core to minimize contacts with surrounding water. The protein conformation can be altered and create strong interactions with the NP due to weak forces bringing these apolar regions in the vicinity of hydrophobic surfaces [23].

The composition of the corona has a strong influence on the effect of any NP on biological components such as cells (and vice versa), and it is in turn influenced by the physicochemical properties (e.g., surface chemistry and size) of the particle [23–25]. Thus, although NPs can be synthesized with specific physicochemical properties, much is unknown regarding the effect of the protein corona formation on the hypothetical therapeutic efficacy of NPs and induction of undesirable mistargeting and side effects [26,27].

The biological recognition properties of NPs depend on the way they interact with biomolecules and their surroundings. Thus, their use requires

a general knowledge of the biological behavior of NPs. The engineering of protein interactions is facilitated by the tunability of the core size and tailor ability of the NP surface. While proteins have catalytic activity, recognition, and inhibition, NPs can exhibit characteristics such as optoelectronic and magnetic properties [28].

With respect to the physicochemical properties of biological fluid that they come in contact with, only a specific group of proteins that have high affinity for the NP surface will attach and remain tightly bound for an extended period of time [13,14,29]. Since the protein corona layer remains strongly bound to the NP surface for some hours, the living organisms "feel" the NP-protein corona rather than the original surface of the NPs [30–38].

Because of their multifunctional biomedical capabilities (e.g., targeted imaging [39], targeted delivery of drugs [40], hyperthermia [41], transfections [42,43], and stem cell tracking [44]), superparamagnetic iron oxide NPs (SPIONs) are known as some of the most promising materials among many kinds of NPs used in biomedical applications. To completely understand their toxicities and biological fate, in depth information regarding the protein corona composition at the surface of different types of SPIONs is needed. While the biological responses (e.g., the cellular uptakes, the cellular signaling pathways, and the biodistribution) of SPIONs with various physicochemical properties have been examined in detail [45–54], few accounts on the compositions of the protein corona at their surface exist [55].

Numerous techniques have emerged to explore the protein corona on the NP surface [56]. Of these techniques, centrifugation has been acknowledged as the most dependable method for separating protein-NP complexes [33]. However, due to inadequate washing, high-abundance proteins may be identified as being attached to the NP surface. The centrifugation force, the washing duration, the washing solution, and the solution volumes on the detachment of the loosely attached proteins all influence the outcome of the centrifugation method [57]. To overcome these obstacles, a magnetic separation technique and the magnetic properties of SPIONs are helpful. Furthermore, the precise gradient removal of the NP-attached protein coronas can be achieved using this method [58,59].

3.2 THE PROTEIN CORONA

As previously mentioned in the introduction, a major driver of therapeutic delivery involves the protein-particle interactions that produce the protein corona. In the bloodstream, plasma proteins have a pivotal role and exist at high concentrations. The protein corona is the layer of proteins that

immediately surrounds NP surfaces upon NP entrance into biological fluids (e.g., blood, plasma, cell culture media, and intracellular environment). Even NPs functionalized with vector proteins (not considered as a corona) would obtain an extra layer of corona proteins when entering a biological fluid. The biokinetics and in vivo fate of NPs can be notably influenced by protein coronas. NP surfaces gain a new bioidentity due to the adsorption of proteins that affect their biological interactions. The nature of proteins adsorbed in the protein corona is affected by the NP size [19], NP shape [60], and various surface characteristics, such as the surface functional groups [61], topography of the surface [62], and surface hydrophobicity/hydrophilicity [13,63,64]. In addition, the corona composition can be affected by temperature [61,65], NP dispersal environment [56], source of protein [55], incubation time [66], concentration [16,66,67], and gradient concentration [68]. Furthermore, NP biokinetics and fate will vary with respect to the nature and amount of proteins adsorbed. The overall entropy increase of the surface proteins, along with the nonspecific interactions between the proteins and NP surface, is largely responsible for the adsorption of proteins to the surfaces [69]. Proteins with isoelectric points (pI) > 5.5 bind to NPs with acidic surfaces, and those with pI < 5.5 primarily bind to NPs with basic surfaces [70]. However, important to note is that protein adsorption to certain surfaces may be specific as well. In spherical nucleic acids, for instance, different sequences cause coronas having different compositions to form, which in turn influences the level of cellular uptake [71]. The vast protein complexity and protein interactions on NP surfaces are especially challenging to corona composition characterization and its evolution in biological systems [17].

The NP-protein corona concept is an essential component for designing the surface properties, surface charges, and hydrodynamic size of NPs (Fig. 3.1). Specifically, the interactive NP surface may be prebound to chemical species that retain their previous history and may affect its protein adsorption kinetics. These preexisting surface substances may arise from (i) manufacturing process remnants or ambient gas exposure, (ii) industrial chemicals and stabilizers in preparing dispersions, or (iii) organic and inorganic constituents of biologically applicable buffers used to produce laboratory stock solutions [22].

A preliminary nanobio interface comprising particles bound to proteins in biological fluids experiences active alterations as the particles then move onto or into cells. The particle's interactions with biological surfaces and receptors, and thus its fate, are strongly dictated by the kinetics of

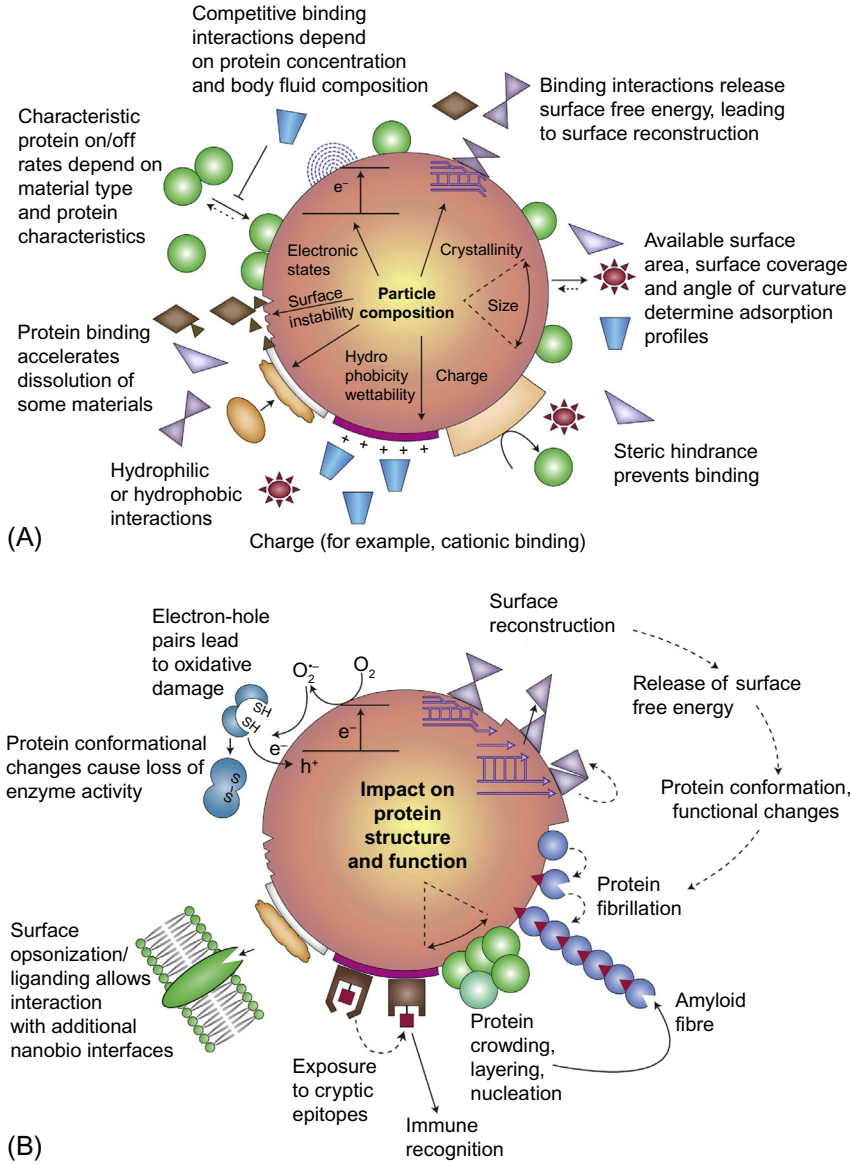

Fig. 3.1 Effects of protein corona surrounding a nanoparticle. The corona forms a primary nanobio interface that decides the fate of the nanoparticle and can lead to deleterious effects on the interactive proteins. (A) Preexisting or initial material characteristics contribute to corona formation in a biological environment. Dynamic changes in the corona are caused by characteristic protein attachment/detachment rates, competitive binding interactions, steric hindrance by detergents and adsorbed polymers, and protein profile of the body fluid. The corona can be altered when particles move from one biological compartment to another. (B) Potential alterations in protein structure and function because of interactions with the nanoparticle surface can give rise to possible molecular mechanisms of injury that may contribute to disease pathogenesis. The colored symbols denote many kinds of proteins, such as charged, lipophilic, conformationally flexible proteins, catalytic enzymes with sensitive thiol groups, and proteins that gather together or interact to form fibrils [22].

NP-protein association and dissociation and simultaneous exchange with free proteins in the media. The lifetimes of particle-ligand complexes span from microseconds to days. Multiple techniques are available for the quantitative analysis of these complexes.

Since cellular interactions with nanomaterials can be influenced by the protein corona, an important question is whether particle composition affects these protein interactions (Fig. 3.1A). The biomolecules that interact with the particles are determined by the nature of the particle surface (e.g., its hydrophobicity, size, radius of curvature, charge, and coatings that exert steric or electrosteric effects; Fig. 3.1A). Therefore, their access to cells is mediated by the particle surface [14]. There are several known proteins that form transient complexes with NPs. Certain particle types and the biological fluids that they are suspended in engage in favorable binding interactions due to sizable variations in their dissociation rates. Furthermore, binding is affected by protein concentrations close to the particles. For the dynamic NP corona in the blood, the particle surface may be dominated by human serum albumin and fibrinogen for brief periods of time, while they may eventually be supplanted by lower abundance proteins with higher affinities and slower kinetics [14]. On the contrary, in a lower protein environment (e.g., bronchial or ocular fluid), the particle surface may be dominated by lower affinity binding proteins (e.g., albumin). Albumin, immunoglobulins, complement, fibrinogen, and apolipoproteins are proteins that bind most tightly to carbon nanotubes (CNTs), iron oxide particles, liposomes, and polymeric NPs. Particle opsonization, which encourages receptor-mediated phagocytosis, results from complement and immunoglobulin binding [72]. Because of immune activation, such behavior may be useful in creating new vaccines or minimizing unfavorable health effects [72]. While endocytic uptake can be increased by deliberately attaching protein ligands, less understood is how the spontaneous formation of a protein corona may contribute to tissue uptake in vivo. The prevailing notion is that plasma protein binding plays a significant role in controlling the in vivo organ distribution and removal of carrier particles from the circulation. For example, longer circulation times and modified biodistribution caused by decreased protein absorption of injected polyethylene glycol-coated particles may be accounted for by this behavior [72].

At least two mechanisms involving proteins and organic substances increase the dissolution rates of particles of ZnO, CdSe, iron oxides, aluminum oxides, and oxyhydroxides [73]. These mechanisms are aqueous complexation and ligand-enhanced dissolution. The latter mechanism has been

tested for iron and aluminum oxides and oxyhydroxides and can likely occur for ZnO as well.

Crucial for understanding possible biological injury due to alterations such as fibrillation, exposure of new antigenic epitopes, and loss of function like enzymatic activity is examining the reverse effects of particles on proteins. In one of the most well-investigated experimental models, NPs serve as catalysts that expose protein interaction domains to bring about aggregation through hydrogen bonding and the formation of disease-promoting fibrils [32]. Human β2-microglobulin fibrillation, for instance, takes place on the surface of cerium oxide and copolymer NPs and CNTs [32]. The process of amyloid fibril formation is quantifiable. But whether these experimental conditions can be duplicated in vivo needs to be ascertained, because the nucleation surface may be screened by competitive binding in complex biological fluids. Currently, no evidence exists to indicate that protein fibrillation induced by NPs is implicated in any disease pathogenesis. However, repeating the same process in the brain can theoretically contribute to neurodegenerative processes (e.g., Alzheimer's disease). While the precise trigger of protein unfolding at the particle surface has not been discovered, contact forces, including free surface energy release through surface reconstruction, may be involved. Relaxation of the particle crystal structure through protein binding is one possibility [74] (Fig. 3.1B). Likewise, electron confinement or the formation of electron-hole pairs at the material surface may cause structural bond cleavage or covalent cross-linking of protein SH domains.

For instance, when chicken egg lysozyme binds to SiO_2 NP surfaces, a critical α-helix unfolds, thereby disrupting the catalytic activity of the enzyme [75]. Furthermore, a loss of function may result when critical thiol groups are cross-linked in glycerol aldehyde phosphate dehydrogenase and may be quantified experimentally [73]. Similarly, after the denaturation of a protein comprising cryptic epitopes on a particle surface, an immune response may be initiated if new antigenic sites are exposed. If a self-protein is targeted against, the response may lead to autoimmune disease (Fig. 3.1B).

3.3 "HARD" VERSUS "SOFT" CORONA

The protein layer on the surface of the NPs, known as the protein corona, can be categorized into "soft" and "hard" varieties [17]. "Soft coronas" have a dynamic structure due to the exchange of biomacromolecules from the surrounding medium and the NP surface. For "hard coronas,"

macromolecules are fixed to NP surfaces in a static manner and typically involve large-scale protein denaturation. Complicating comprehensive characterization of the proteins in the "soft corona" is their volatility. Therefore, more investigations have concentrated on materials with hard coronas [17]. Lundquist and colleagues [33] discovered that for a fixed material type, the size and the zeta potential of the NPs strongly influence the biological activity of the proteins in the corona. Moreover, the adsorption of blood serum proteins onto NPs shows time dependence [76]. First, the most motile proteins are attached to the surface, and then, they are replaced by proteins with lower motility, which have a greater affinity for the surface. It is thought, and Casals et al. [66] confirmed, that this process can take a few hours. Over time, a "soft corona" loosely bound to the NP surface becomes an irreversibly attached "hard corona." However, according to very recent studies, "hard coronas" can form in less than a minute without undergoing further compositional change [19]. Important to note is that conflicting reports may be explained by the newly discovered "personalized protein corona" effect [77]. In particular, alterations in plasma composition due to health issues and diseases may give rise to the formation of different protein coronas on identical NPs in different individuals. In the hard protein coronas of identical NPs in different diseases, certain proteins have been demonstrated to specifically appear or disappear [77]. Proteome composition differences should thus be considered when analyzing results as well.

3.4 REVERSIBLE VERSUS IRREVERSIBLE INTERACTIONS BETWEEN NPs AND PROTEINS

Proteins may either reversibly or irreversibly bind to NPs. Although both types of interactions can change protein structure and behavior, irreversible binding is more difficult in terms of understanding the functional utility of nanomaterials. Proteins must maintain their structures and activities to be functional. Protein denaturation on particle surfaces leads to a "hard" protein corona that can extensively change particle behavior in vivo.

In early investigations, the enzymatic activity of proteins like α-chymotrypsin (ChT) was inhibited using anionic NPs [78]. At the active site of this protein, these NPs engaged in electrostatic interactions through cationic patches. Enzymes were first inhibited, and then, the secondary structure of the protein slowly and irreversibly denatured. The interaction between the nonpolar interior of the NP and hydrophobic residues of

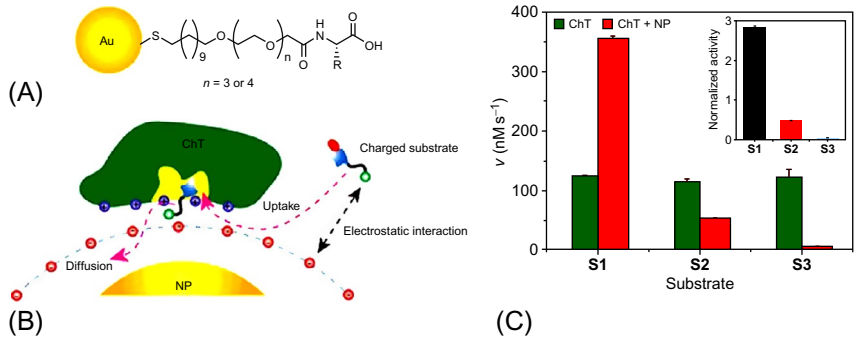

Fig. 3.2 (A) The chemical structure of anionic amino-acid-functionalized gold nanoparticles. (B) Schematic depiction of the interaction between negatively charged NPs and ChT, showing the charge affecting not only the binding but also the regulation of the enzymatic activity. (C) Generation rates for each substrate (S1 cationic, S2 neutral, and S3 anionic) to support that the binding selectivity is caused by electrostatic interactions [26].

ChT led to the structural denaturation of this protein. Most applications of protein-NP conjugates necessitate retention of protein activity, so structural retention is a major priority. The NP surfaces can be properly tailored to prevent the proteins from structural denaturation. For example, introducing oligo (ethylene glycol) (OEG) [79] functionality to the NP surface substantially decreases the rate of denaturation of ChT.

The generation of ensembles, in which the collective properties of the conjugate structure and the properties of the individual precursor components differ, has become an interesting area of research in bioconjugate chemistry. Enzymatic inhibition of ChT using anionic NPs not only results from ChT active site blockage by the NPs but also is determined by the charge state of the substrate (Fig. 3.2). The interaction between ChT and anionic NPs gives rise to a threefold increase [78] in the catalytic activity of ChT for cationic substrates and decreases its activity by 50% and 95% for neutral and anionic substrates, respectively. This substrate selectivity phenomenon is due to the distinctive electrostatic environment provided by the protein-NP conjugate.

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