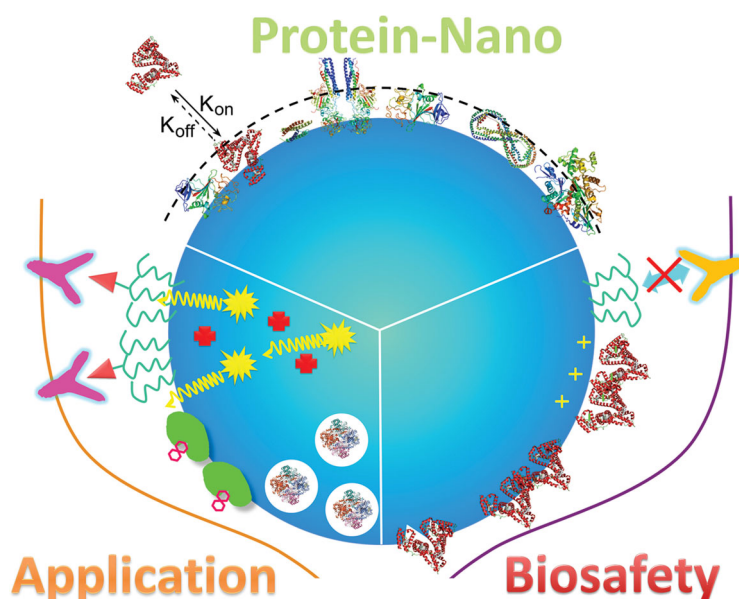


Biosafety and Bioapplication of Nanomaterials by Designing Protein–Nanoparticle Interactions

Sheng-Tao Yang, Ying Liu, Yan-Wen Wang, and Aoneng Cao*



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The protein–nanoparticle (NP) interface is a current frontier of multiple disciplines, full of challenges and opportunities. The unique behaviors of nanomaterials (NMs) bring many exciting applications, and also raise safety concerns. Beyond bioapplications, various NMs could also enter human bodies from the environment. When entering human bodies, NPs interact with various biomolecules, especially proteins, forming a protein corona. This protein–NP complex is what the biosystems 'see' and 'respond to'. Therefore, understanding how NPs interact with proteins is crucial for both bioapplications and the biosafety of NMs. In this review, the current understanding of protein–NP interactions is summarized, including the theoretical background, experimental results, and computational progresses. Guidelines for improving bioapplication performance and reducing the potential biosafety hazard of NMs by designing the protein–NP interactions are discussed, along with future directions and challenges in this exciting field.

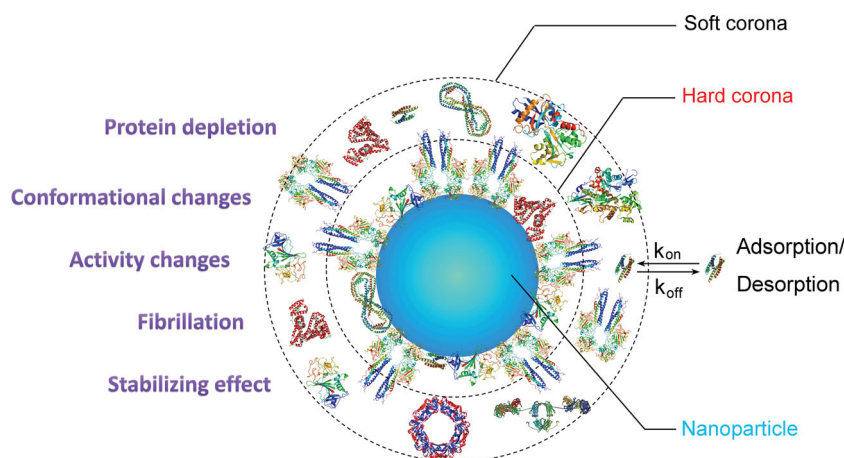
1. Introduction

Nanomaterials (NMs) have attracted tremendous interest of the scientific community for decades, due to their unique properties and potential applications in diverse areas. Various NMs have been produced and applied in many fields. Of particular interest is the recent development in nanobiotechnology, where NMs have been regarded as promising candidates in bioimaging, bioanalysis, drug delivery, therapy, etc.^[1–3] Along with the massive production and wide application of NMs, the biosafety issue of NMs has arisen in the public and academia, and impelled a large number of investigations.^[4–6] Some NMs were reported to cause unwanted toxicity to microorganisms, cells, animals, and plants. To facilitate bioapplications and biosafety evaluations of NMs, a basic knowledge of biological behaviors of NMs is indispensable.

There is a growing recognition that protein–nanoparticle (NP) interactions determine the behaviors of NMs in biosystems.^[7–12] When NMs enter biosystems, NPs will meet with biomolecules, especially proteins. Upon protein binding, a coating shell, known as protein corona, will be formed on the NP surface (Scheme 1).^[13–18] The formation of the protein corona has been observed in pure protein solution, cell culture medium, and also full serum. Structurally, the protein corona might be divided into hard and soft types.^[15,16] Proteins in soft corona are reversibly adsorbed on NPs. The composition and thickness of the protein corona depend on the physicochemical properties of the NPs and also the species of proteins in the system.^[19–22] Nevertheless, it is usually the protein–NP complex rather than the NP itself that decides the NP's fate in biosystems.

Numerous studies have indicated that adsorption on NPs influences the structure and bioactivity of proteins.^[7–10,23–27] NPs could accelerate the fibrillation of proteins and peptides, a process that is associated with several diseases, such as Alzheimer's disease.^[23] On the other hand, adsorption of proteins could also alter the properties of NPs, such as dispersibility, surface charge, and solubility.^[8] More importantly, biobehaviors of the protein–NP complex might differ from that of the protein and NP alone. For instance, graphene oxide (GO, 100–500 nm) and serum proteins are much smaller than the capillary diameter (~7 μm), thus, none of them are expected to be trapped by the pulmonary capillary.^[28] However, when they interact and form large agglomerates, the protein–GO complex is filtered by the pulmonary capillary and hence trapped in the lungs.

An understanding of the protein–NP interaction undoubtedly will facilitate the ongoing research into the biosafety and bioapplications of NMs. A number of good reviews have been dedicated to protein–NP interactions, focusing on different aspects.^[7–12] In this review, we will focus on the biosafety and bioapplication aspects of the protein–NP interaction. After thorough analysis of theoretical principles, influencing factors,



Scheme 1. Protein corona on NPs. Protein binding on the surface of the NPs will cause a series of consequences.

and the consequences of protein–NP interactions, we review the available biosafety and bioapplication data of NMs from the perspective of the protein–NP interaction, and summarize some guidelines for designing protein–NP interactions for greater biosafety and bioapplications of NMs.

2. Protein–NP Interactions

2.1. Principles of Protein–NP Interactions

2.1.1. Forces Contributing to Protein–NP Interactions

Many interactions might contribute to protein adsorption on NPs, such as van der Waals (vdw) interactions, H-bonds, electrostatic interactions, hydrophobic interactions, and π – π stacking interactions. The characteristics of these interactions are summarized in Table 1.

The vdw interaction usually is a major contribution to protein–NP interactions, if the interface topography matches well. The more close-contacted atom pairs at the interface (within distances close to the sum of the vdw radii of the two atoms), the stronger the vdw interaction (attraction) is. In principle, the vdw interaction can operate over a long range, and all atoms of the NPs and proteins have a contribution. But in solution with solvent molecules surrounding, only closely contacted atoms have a net contribution to the binding. Therefore, the vdw force is actually a short-range one for protein–NP interactions, which decreases dramatically as the contacting atoms depart (Lennard-Jones potential

Dr. S.-T. Yang, Y. Liu, Y.-W. Wang, Prof. A. Cao
Institute of Nanochemistry and Nanobiology
Shanghai University
Shanghai, 200444, China
E-mail: ancao@shu.edu.cn

Dr. S.-T. Yang
College of Chemistry and Environment
Protection Engineering
Southwest University for Nationalities
Chengdu, 610041, China

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or 6–12 potential). The vdw force is weak for a single pair of atoms. However, since both proteins and NPs contain a huge amount of atoms, the total vdw force may become dominant in some cases. The vdw interaction tends to maximize the contact interface, so shape complementarity is preferred. For NPs, the bigger particle has a larger potential interface, therefore it usually has a stronger vdw interaction with proteins. For proteins, those with more flexible conformations tend to have better contact with NPs, and might have stronger vdw interactions with them. Unfolded proteins are likely to bind with NPs more strongly than the native folded ones.

H-bonding is another important player in the intermolecular interactions. A protein surface has many hydrogen donors and acceptors, while the number of hydrogen donors and acceptors on the NP surface varies significantly. Although the H-bond is much stronger (5 to 30 kJ/mol) than the vdw interaction, the number of H-bonds between proteins and NPs is much smaller than that of vdw interactions. In aqueous solution, water molecules are also a good hydrogen donor/acceptor, therefore the net contribution of H-bonding to the protein–NP interaction is usually small. But, since H-bonding is directional, it may provide specificity for the protein–NP interaction.

Electrostatic forces between proteins and NPs is commonly observed. Both proteins and NPs need surface charges to keep them stable in an aqueous phase. The pH value of the solution decides the charge states of both proteins and NPs, and hence the electrostatic force between them. The electrostatic force is a long-range force (inversely proportional to the square of distance) that can act through media. But a high ion strength in biological fluids may shield the electrostatic force.

Hydrophobic interactions are another important contribution to the protein–NP interaction. It is actually an entropic effect, originating from excluding the ordered water molecules from a nonpolar surface. Hydrophobic interactions tend to minimize the nonpolar surface, by clustering hydrophobic groups to avoid exposure to hydrophilic media. Most globular protein folding is driven by hydrophobic interactions, forming a hydrophobic core and hydrophilic surface. If NPs have large hydrophobic surface, they not only are prone to aggregate in aqueous solutions, but also tend to bind the partially exposed nonpolar residues of proteins. The hydrophobic interaction between proteins and NPs is usually an unfolding force for



Sheng-Tao Yang received his BSc in 2006 and PhD in 2011 at Peking University majoring in chemistry. Since 2011, he has worked as a lecturer at the College of Chemistry and Environment Protection Engineering, Southwest University for Nationalities in China. His current research focuses on the preparation, application, and bioeffects of nanomaterials.



Aoneng Cao received his BS in Chemistry in 1989 and MS in Material Sciences in 1995 from Zhejiang University, and obtained his PhD in Chemistry in 1998 from Peking University. After two years of post-doc training at Cornell University, he joined the faculty of Peking University in 2000. He is currently a full professor at the Institute of Nanochemistry and Nanobiology, Shanghai University. His current interests focus on the nano–bio interface, including protein–NP interactions, nanotoxicology, bio–nanocomposites for biocatalysis, bioimaging, and drug delivery.

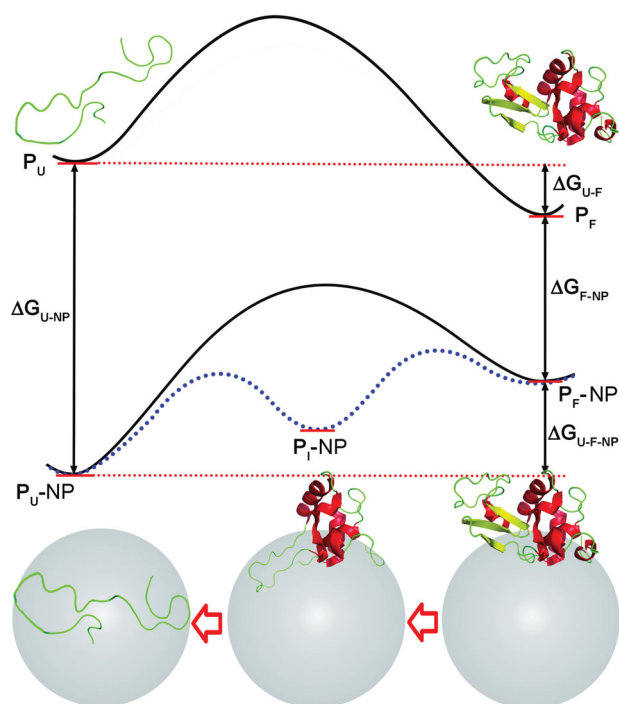
the protein's native structure, since hydrophobic NPs bind more tightly with unfolded proteins, which have more hydrophobic residues exposed than the native proteins.

π – π stacking is an attractive interaction between aromatic rings. The two most preferred conformations for aromatic ring pairs are the parallel displaced and T-shaped ones. The average π – π stacking distance is slightly larger than the vdw radius. Both experimental and computational studies show that the π – π stacking widely exists in and contributes greatly to the interaction between proteins and sp^2 carbon NMs.^[29,30] It was found that aromatic rings of amino residues on proteins are parallel to aromatic rings of a fullerene cage.^[29] The π – π stacking is also a specific interaction for protein–NP complexes, since only a few amino acids contain aromatic rings, and many of them are buried inside the hydrophobic core of the proteins.

Table 1. Forces contributing to the interactions between proteins and NMs.

Forces	Strength	Amount	Net contribution	Range [nm]*	Specificity	Main factors
vdw interaction	weak	a huge number	large	0–10	No	interface complementarity
H-bond	moderate	many	small	<0	partial	hydrogen donor/acceptor at interface
Coulombic forces	moderate	many	moderate	0–10	No	charge state, ion strength
hydrophobic interaction	strong	some	large	0–10	partial	hydrophobic surface
π – π stacking	strong	a few	large	0–5	Yes	aromatic ring orientation
salt bridge	strong	a few	moderate	<1	Yes	multiple recognition

*0 indicates direct atom contact (based on vdw radius).



Scheme 2. A schematic representation of the energy profiles of the protein–NP interaction and its influence on the folding of the protein.

The formation of a salt bridge is a combination of H-bonding and electrostatic interactions, and is commonly observed in folded protein conformations. Due to the lack of structural information about protein–NP complexes, salt bridges have not been reported in protein–NP interactions, but their existence is highly possible. Since the distance between salt bridge elements is less than 0.4 nm, multiple interactions are involved; the salt bridge could be a specific interaction for proteins and NPs. In the future, when more structural details are revealed, it is expected that salt bridges will be found in specific protein–NP interactions.

2.1.2. Thermodynamics of Protein–NP Interaction

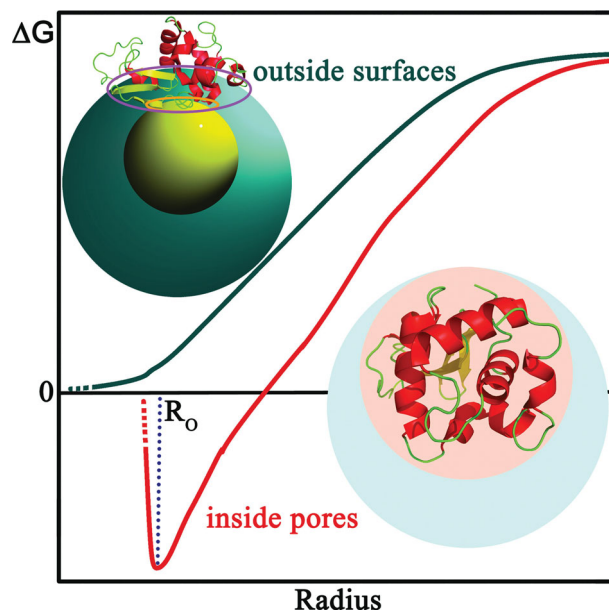
From the thermodynamics point of view, the native or folded state of protein (P_F) is only marginally more stable than the unfolded state (P_U) at physiological conditions. The protein-folding free energy change (ΔG_{U-F}) is usually equivalent to a few H-bonds. Since P_U is a flexible random coil, which possesses a much larger binding surface than P_F does, the binding energy of P_U with a NP is usually larger than that of P_F . Correspondingly, the P_U -NP complex is usually more stable than the P_F -NP complex. From **Scheme 2**, it is easy to get:

$$\Delta G_{U-NP} = \Delta G_{U-F} + \Delta G_{F-NP} + \Delta G_{U-F-NP} \quad (1)$$

At given conditions, ΔG_{U-F} and ΔG_{U-NP} are constant, so it is easy to infer from equation (1) that a larger free energy change of the binding between the native protein and the NP (ΔG_{F-NP}) means a smaller free energy change of the unfolding of the bound protein on the NP surface (ΔG_{U-F-NP}). When a protein binds to a NP, the binding force may induce conformational change or partial unfolding of the protein,

which has widely proven on many proteins and NPs by using various experimental techniques.^[9] The partially unfolded state is not stable for the free protein, but is stabilized by the interaction with NPs. High concentration of partially unfolded protein on the NP surface is the reason that NPs accelerate the fibrillation of some proteins. Partially unfolded protein can also serve as an unfolding intermediate, which substantially decreases the unfolding free energy change. However, binding with NPs might also stabilize the bound proteins and make further unfolding kinetically more difficult.

It is often observed that the larger the size or surface of a NP is, the stronger it binds and affects the stability of proteins.^[13,31] However, when proteins bind to the internal pore surface of nanoporous NMs, the situation is a little different. The binding interface inside pores may not increase upon protein unfolding, the P_U -NP complex is not necessarily more stable than the P_F -NP complex. Actually, due to space confinement, at a certain optimum pore size (R_0 in **Scheme 3**, usually close to sizes of the entrapped proteins), pore surface can significantly stabilize the entrapped proteins.^[32,33] This stabilization effect by pore confinement is attributed to the reduction of configurational entropy of the unfolded protein, which destabilizes the unfolded state of the entrapped protein and raises the energies of both the unfolded protein and its complex with the NP. If multiple covalent anchoring exists between the protein and NP pores, the stability of the entrapped protein can be further improved.^[34] The net stabilizing/destabilizing effects on the NP surface and porous surface are illustrated in Scheme 3.



Scheme 3. Size-dependent destabilizing (positive ΔG)/stabilizing (negative ΔG) effects on proteins after binding to a NP surface or porous surface. The orange and purple ellipses (above) represent the interfaces of the protein binding on small (yellow) and big (cyan) NP surfaces. R_0 represents the optimal pore (pink circle on the right) radius that has the highest stabilizing effect on the protein.

As a simple model, the binding of a protein (P) on NP can be expressed as:



$$K_d = \frac{[P] \cdot [NP]}{[P - NP]} = \frac{k_{\text{off}}}{k_{\text{on}}} \quad (3)$$

where K_d is the dissociation constant, $[P]$ is the equilibrium concentration of free protein P, $[NP]$ is the equilibrium concentration of free NP, and $[P - NP]$ is the equilibrium concentration of the protein–NP complex. k_{on} and k_{off} are the association and dissociation rate constants, respectively.

Interactions between protein and NP with a K_d larger than 10^{-4} M can be classified as weak interactions, where NP usually binds little protein nonspecifically in vivo. With a K_d between 10^{-5} and 10^{-7} M the interaction is moderate, while a K_d less than 10^{-7} M indicates a strong interaction. Taking as an example serum albumin (SA), the most abundant protein in mammal blood, with a concentration of around 10^{-4} M, a K_d larger than 10^{-4} M means that only less than half of the binding sites on the NP are occupied by SA.

It is necessary to point out that the above discussion is based on simplified binding between a single species of protein and NP. The in vivo environment is very crowded and complex. The total concentration of various proteins inside a cell reaches 10^{-2} M. Thus even for NPs with a low K_d around 10^{-4} M, NPs will be all covered by various proteins, forming a protein corona. In addition, when a NP enters a biosystem and gets into contact with different proteins, those proteins will compete and replace each other, hence, the composition of a protein corona on the NP surface will change with time. This Vroman-like effect has been well-analyzed mathematically by Vilaseca et al.^[35] Therefore, protein binding on NPs is always a dynamic process and may never reach equilibrium in vivo. The kinetics (such as diffusion and competition, association and dissociation) of such interactions are therefore particularly important and complicated in studying in vivo behaviors of NMs.

2.2. Structure of the Protein–NP Complex

Comparing this with thermodynamics, much less has been done on the exact model of the protein–NP complex, which is highly demanded for a better understanding of the binding mechanism and a more rational design of the protein–NP interaction. Although from various experimental and computational approaches, studies have revealed or inferred partial binding conformation, such as the binding specificity (orientation and binding location for both proteins and NPs), it is still a great challenge to elucidate binding models with conformational detail.

2.2.1. Experimental Approaches

Currently, to determine the all-atom structure of a protein–NP complex is almost an impossible task. Using model proteins with known 3D structures, partial binding conformational information can be obtained by combining advanced

biotechnologies with other experimental and computational techniques.

Enzymatic activity change upon binding is usually an indicator of conformational change or binding close to the active sites of an enzyme. Fullerene could inhibit activities of various enzymes,^[26,36,37] which implies that fullerene might fit into the active pockets of these enzymes.

Microscopy, such as transmission electron microscopy (TEM), scanning electron microscopy (SEM), and atomic force microscopy (AFM), may provide a direct observation of protein–NP complexes, and be used to reveal binding models of proteins on NPs with a resolution limit of about 1 nm. For example, Goldberg–Oppenheimer and Regev visualized the binding of bovine SA (BSA) on single-walled carbon nanotubes (SWCNTs) by gold NP (AuNP)-labeling and proposed a binding model of BSA on SWCNT.^[38]

Spectroscopy is another category of experimental approach to investigate the structure of the protein–NP complex. Circular dichroism (CD) is a widely used technique to characterize the conformational change of proteins in solution. CD is especially powerful to quantify partial conformational changes of proteins, which is very common upon protein binding on NP surfaces.^[26,39] Intrinsic fluorescence of proteins is a natural probe for investigating binding specificity of proteins with NPs, because only Trp (strong) and Tyr residues are fluorescent (Phe is very weak), and their fluorescence can be distinguished by exciting at both 280 nm and 295 nm.^[26] Combining Trp fluorescence spectra, enzyme activity assays and other techniques, Yang et al. concluded that fullerene specifically binds to the active pockets of lysozymes through strong π – π interactions between the fullerene cage and the Trp62 of lysozyme.^[26] This strategy has been widely used in studying protein–NP complexes.^[40,41]

Fluorescence resonance energy transfer (FRET) is a powerful technique to study interactions, and a nanoruler to measure the distance between a fluorescence donor and a fluorescence acceptor. Using Rhodamine red-labeled maltose-binding protein (MBP) coordinated to the surface of a 555 nm emitting CdSe–ZnS core–shell quantum dot (QD), Medintz et al. were able to determine the distance from each of the six unique MBP–Rhodamine red dye-acceptor locations to the center of the energy-donating QD, and found the preferred orientation of MBP on the QD surface.^[42]

Upon binding to NPs, protein residues at the binding interface will have limited solvent accessibility and slow hydrogen-deuterium (H/D) exchange rates. This property could be used to determine the binding surface of proteins with nuclear magnetic resonance (NMR).^[43,44] Since the binding of proteins on NPs is a dynamic process, this method is not suitable for weak and fast kinetic interactions.

Isothermal titration calorimetry (ITC) is a classical technique to characterize protein binding with various materials, as well as enzymatic reaction kinetics.^[45] ITC experiments can provide reliable thermodynamic parameters of protein binding. An apparent advantage of ITC is that it only measures the heat effects of the binding, which exist for almost all meaningful protein–NP interactions. Recently, using ITC, Ratnikova et al. revealed that $C_{60}(\text{OH})_{20}$ binds to tubulin at a molar ratio of 9:1 with a binding constant of $1.3 \times 10^6 \text{ M}^{-1}$.^[39]

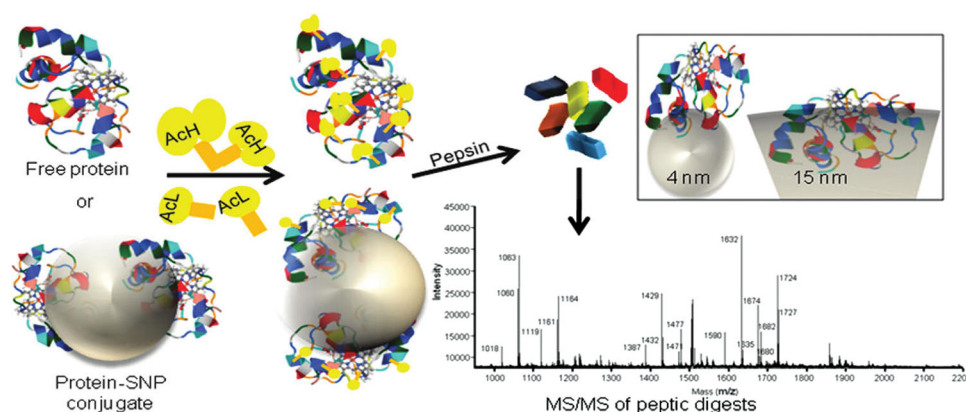


Figure 1. Determination of the orientation of proteins on silica NPs using chemical modification combined with proteolysis–mass spectrometry. Reproduced with permission.^[52] Copyright 2012, American Chemical Society.

For heme proteins, the orientation of the protein on the NP could be inferred from the heme tilt angles, which can be determined by resonance Raman spectroscopy^[46] or polarized internal reflection.^[47] It has to be pointed out, however, that the obtained heme tilt angles are averages, which may be dramatically different from the real binding modes. Near-edge X-ray absorption fine structure spectroscopy (NEXAFS) can also be used to investigate protein orientation on NPs.^[48]

Bioengineering techniques can be used to determine binding models by manipulating the binding proteins. Surface residues of the binding protein can be individually mutated to cysteine or other amino acids for site-directed labeling with probes that can be used to detect the interaction with NPs. Karlsson and Carlsson used this approach to attach fluorescent probes on the surface of human carbonic anhydrase II, and found that proteins bind to silica NPs with their N-terminal domains.^[49] Phage display technique is another choice to screen and demonstrate the specific binding residues of a protein or peptide.^[50]

A protein surface buried at the interface is protected from protease digestion. This property can be used to determine the binding region of proteins. Using H/D exchange combined with proteolysis–mass spectrometry (MS), Buijs et al. studied the conformational stability of various regions of myoglobin binding on silica NP.^[51] Shrivastava et al. determined the binding orientation of cytochrome C (cyt C), RNase A, and lysozyme on silica NPs using chemical modification combined with proteolysis–MS,^[52] and found that both the protein structure and NP size influence the protein orientation on silica NPs (**Figure 1**).

In addition, some specific chemical reactions could also be adopted. Matsui et al. found that nanoshell carbon (NSC) adsorbed cyt C through the interaction between quinones and amino groups of surface lysine of cyt C.^[53]

2.2.2. Computational Approaches

Currently, experimental approaches can only obtain partial information on binding models of protein–NP complexes. Rapid development and recent successes in both protein and material sciences have proven that a computational approach can not only provide dependable binding models, but also

simulate the association/dissociation processes. Since scientists can build reliable all-atom structure models for carbon NMs, such as fullerene, carbon nanotubes (CNTs), graphene, and their derivatives, most computational approaches have focused on the interaction between proteins (including peptides) and carbon NMs.

Many free and commercial softwares can be used for modeling protein–NP interactions. Fullerene is the best model due to its simple and symmetric structure. Yang et al. performed a computational evaluation of a lysozyme–fullerenol complex with Autodock,^[26] and found that fullerenol was located in the active pocket of lysozyme, where strong π – π interactions between the fullerene cage and Trp62 existed (**Figure 2**). This computational binding model is consistent with all known experimental results. The relatively small size of fullerene made it easy to enter the clefts or pockets of the proteins. A NP size-dependent phenomenon is clearly shown in the docking of different-sized fullerenes with RNase A (**Figure 3**), where the biggest cage (C_{82}) is located outside of the cleft.^[29] PatchDock is another high throughput method to screen the protein–fullerene interaction.^[54] NP size-dependent binding behaviors were observed using PatchDock, and could be adopted for theoretically separating C_{60} and C_{70} .^[55] However, when docking with BSA, the calculated docking site was not consistent with the experimental results.^[56,57] Therefore, to verify the reliability of

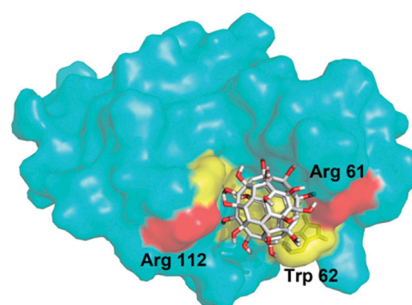


Figure 2. Binding model of the lysozyme–fullerenol complex shows good shape complementarity and π – π stacking. Reproduced with permission.^[26] Copyright 2008, Institute of Physics.

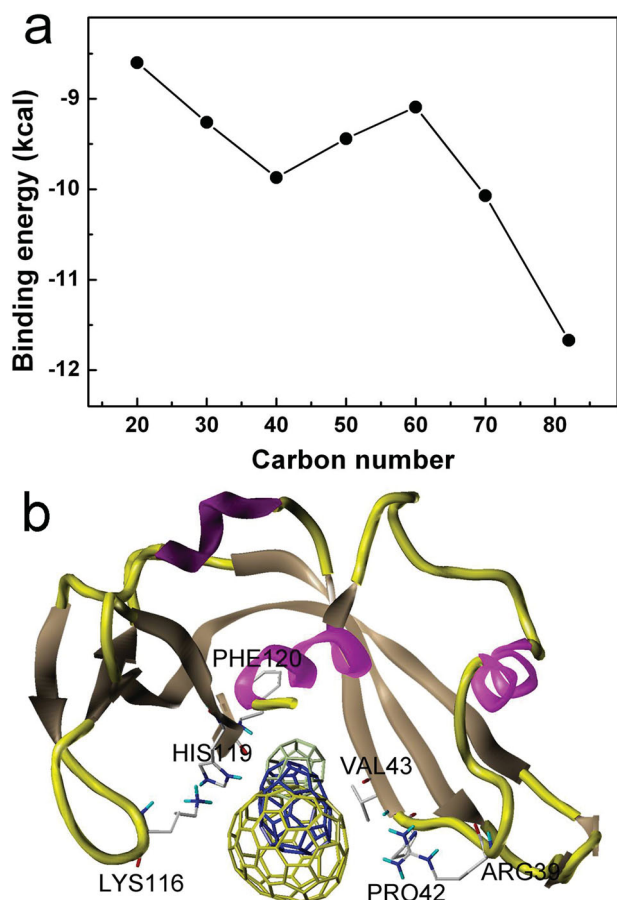


Figure 3. Binding of fullerene with RNase A. (a) influence of carbon cage size on the binding energy; (b) binding models. From inside C₂₀ (grey), C₄₀ (blue) and C₈₂ (yellow). Reproduced with permission.^[29] Copyright 2010, American Scientific Publisher.

computational results, computational binding models should be checked by experimental approaches, if possible.

For fullerene, aromatic rings and good shape complementarity benefit the binding, in which the major force might be π - π interactions. Such a phenomenon was also observed in the binding of peptides to CNTs.^[58,59]

Computational approaches can not only build binding models of protein–NP interactions, but they can also simulate the binding processes, which are usually unreachable by experimental approaches.^[60] For example, Ratnikova et al. used both molecular dynamics (MD) and docking to simulate fullerol–tubulin and fullerol–protofilament interactions.^[39] Simulations on fullerene/CNTs interacting with lipid membranes have been comprehensively reviewed by Monticelli et al.^[61]

3. Factors and Effects

3.1. Factors Regulating Protein–NP Interactions

Interaction between proteins and NPs is both protein-specific and NP-specific. Although hundreds of proteins have been identified in the protein corona of NPs in biological fluids,

it is still difficult to characterize those interactions in detail. Therefore, it is worthwhile (and only possible) to characterize protein–NP interactions in detail in vitro with model proteins that are easily available in a large amount, such as lysozyme or SA. Many factors have been found to influence protein–NP interactions. Those factors fall into three categories: protein-related (molecular weight, isoelectric point (pI), and conformational flexibility), NP-related (species, size, shape, charge, roughness, hydrophobicity, crystallization, defects, and functionalization), and medium-related (pH and ion strength).

3.1.1. Protein-Related Factors

Different proteins have different properties, which lead to different binding behaviors on NPs. Analysis has demonstrated that the protein composition of the coronae on NPs are different from that in the biosystem, indicating selective binding of proteins on NPs.^[20,22] Wang et al. showed that the adsorption capacity of immunoglobulin G (IgG) on oxidized multi-walled CNTs (O-MWCNTs) is threefold higher than that of BSA.^[62] In vitro experiments revealed that the adsorption capacity on silica NPs ranks as: RNase A < BSA < lysozyme < LPO (lactoperoxidase).^[63]

Generally, larger proteins are likely to bind NPs stronger than smaller ones. But the surface residues of a protein determine its surface properties, and thus play a more important role than its size. One surface property of a protein is its electric charge state, which depends on its pI and pH. This charge state determines the electrostatic interaction.

The conformational flexibility also has a significant influence on the protein–NP interaction. Flexible proteins usually have a larger interface and better shape complementarity with the NP surface than rigid ones.

Protein concentration is another important player, affecting not only capacity and kinetics of protein adsorption, but also corona composition. High concentrations of various proteins and other biomolecules make the in vivo environment very crowded. Such a crowded environment will generally promote protein association (favoring high adsorption on NPs) and accelerate diffusion (favoring quick binding kinetics). Monopoli et al. showed that different proteins were adsorbed by NPs at different plasma concentrations.^[20] Generally, the adsorption capacity increases with increasing protein concentration. It has been reported that adsorption of BSA on Fe₃O₄ NPs proceeds faster at higher BSA concentrations.^[64]

Most proteins in biofluids are hydrophilic proteins, with a hydrophilic surface and hydrophobic core. Some proteins may have partially exposed hydrophobic residues, which might provide binding specificity for NPs. Membrane proteins, especially their transmembrane range, are generally hydrophobic. If NPs have a hydrophobic surface, they might bind strongly with the membrane proteins while crossing a cellular membrane, bringing about a potential biosafety hazard.

3.1.2. NP-Related Factors

Different NPs have different adsorption capacities for a given protein. Casals et al. reported that in the same system Au and Ag NPs formed hard protein coronae in 2 days, while

Fe_3O_4 , CoO , and CeO_2 required 30 days.^[15] Even the same kind of NP prepared by different methods may show different adsorption behaviors. For example, NSC prepared by Fe catalysis showed a threefold higher adsorption capacity than that prepared by Co catalysis.^[53]

The surface chemistry of NPs is paramount in protein–NP interactions. Wu et al. reported that hydroxylation of fullerenes reduces the binding strength of lysozyme and RNase A on fullerene (by Autodock simulation).^[29] Surface coating with polyvinyl alcohol increased the adsorption of transferrin (Tf) on superparamagnetic iron oxide NPs.^[65] Lundqvist et al. reported that differently functionalized NPs ($-\text{NH}_2$, plain, $-\text{COOH}$) only shared about 30% similarity in the protein species adsorbed.^[22]

An important factor of surface chemistry is the surface charge of NMs, which determines the electrostatic interaction. For positively charged Al_2O_3 NPs and $\text{Al}_2\text{O}_3\text{--NH}_2$ NPs, negatively charged BSA was shown to dominate the adsorption, while positively charged lysozyme adsorbed very little.^[66] Negatively charged Al_2O_3 NPs (functionalized with $-\text{COOH}$, $-\text{SO}_3$ and $-\text{PO}_3\text{H}_2$), adsorbed much more lysozyme than BSA.

Another factor of surface chemistry is surface hydrophilicity/hydrophobicity. Cedervall et al. synthesized a series of copolymer NPs with different hydrophobicity by regulating the ratio of *N*-isopropylacrylamide (NIPAM) to *N*-tert-butylacrylamide (BAM), and demonstrated that the number of protein molecules bound to the NPs was positively related to the particle hydrophobicity.^[13] In addition, surface functionalization would increase the hydration radius of NPs, which also affects protein–NP interaction.

The physical properties of NPs, such as size, shape, and surface morphology may influence protein–NP interactions too. The size effect has been widely reported for many NPs. After incubation with human plasma, 37% of proteins identified in the protein corona formed on silica NPs was significantly affected by the size of the silica NPs.^[21] While larger NPs generally have a larger interface with bound proteins, the size effect is also NP-specific and protein-specific. Shang et al. found that RNase A was less stable on a silica NP surface than in free solution, and the larger the size of the NPs, the more unstable the bound RNase A.^[31] Perevedentseva et al. found that 100 nm nanodiamonds (NDs) adsorbed much less lysozyme than 5 nm NDs did.^[67] Moreover, they also observed that the size of NPs even affected the adsorption kinetics: the adsorption of lysozyme on 100 nm NDs reached saturation within 10–15 min, whereas 30–40 min were required for 5 nm NDs.^[67] This size-dependent adsorption was also observed in many other studies.^[22,68,69] Docking different-sized fullerenes to several proteins showed a significant size effect: that binding energy generally increased (stronger binding) along with the size of the fullerene cage, except that some fullerenes with an optimal size can fit well into the binding cleft of the protein (see Figure 3), demonstrating the importance of shape complementarity.^[29]

The shape of NPs affects the protein–NP interaction too. Lysozyme and α -chymotrypsin (ChT) were adsorbed much more on gold nanorods (AuNRs) than on gold nanospheres (AuNS).^[70] A monolayer of proteins formed on AuNS, while proteins adsorbed on AuNRs were multilayered. In addition,

the thermodynamics of adsorption of BSA on AuNPs and AuNRs were different.^[41] The binding constant for the interactions between AuNRs and BSA was about two orders of magnitude higher than that between AuNPs and BSA.

3.1.3. Medium-Related Factors

The protein–NP interaction happens mostly in aqueous media, either in a test tube or in different biofluids. The pH value is an important parameter of aqueous media, which will determine the charge states of both proteins and NPs and thus influence the protein–NP interaction. Generally, the NP's binding capacity for a certain protein reaches a maximum at a pH near the protein's pI. This is because, when the pH is close to the protein's pI, proteins are uncharged and tend to aggregate and precipitate without intermolecular repulsion. Several studies reported that adsorption of lysozyme on NPs reached a maximum at pH 11, which is close to the pI of lysozyme (pI = 11.1).^[71,72] BSA was also reported to reach a maximum adsorption on Fe_3O_4 NPs near its pI.^[64] Depending on the charge state of NPs, the optimum pH for adsorption might shift from the pI of proteins. It was reported in a study of the adsorption of BSA on *N*-methyl-D-glucamine (NMDG)-modified silica NPs that the maximum adsorption occurred at pH 5.5, slightly higher than the pI of BSA (pI ~5).^[73] As pH changes, the protein–NP interaction changes accordingly. Bharti et al. reported a pH-dependent lysozyme–silica NP interaction.^[74] At pH 3, silica NPs were uncharged and few proteins were adsorbed. At pH 4–6 lysozyme adsorption bridged silica NPs, thus resulting in compact aggregates; at pH 7–9 only a few proteins bridged NPs, leading to loose aggregation; at pH 10 lysozymes stabilized the silica NPs by forming monomers and small aggregates.

The ionic strength of the medium is another important parameter affecting the protein–NP interaction. High ionic strength can shield electrostatic interactions, and hence enhance the hydrophobic interaction. Wang et al. reported that adsorption of lysozyme and horseradish peroxidase (HRP) on silicon nanowires was inhibited at high NaCl concentrations.^[75] High concentrations of NaCl have also been reported to inhibit adsorption of BSA on Fe_3O_4 NPs.^[64] The dual effects of NaCl on the adsorption of BSA to NMDG-modified silica NPs was reported: at concentrations lower than 0.1 M, an increase in the NaCl concentration leads to an increase of the adsorption; however, further increases in concentration would inhibit adsorption.^[73]

When multivalent ions are present, the situation will be more complicated than that of monovalent salts. Multivalent metal ions usually have strong interactions with both proteins and NPs, and are conducive to strong protein–NP interactions.

3.2. Effects and Consequences

Protein adsorption on NPs will change the physicochemical properties of both the proteins and the NMs, leading to an alteration of functions. Since proteins are generally more flexible than NPs, usually the alteration is more remarkable for the proteins upon protein–NP interaction.

3.2.1. Effect on Proteins

Upon binding on NPs, the most common alteration of proteins is conformational change. Varied degrees of conformational change, including both the secondary and tertiary structures, have been widely reported.^[26,40] Conformational changes of proteins are dependent on many factors, such as concentration, size, and shape of NPs.^[25,41,65,67,68,70,76] For example, MWCNTs with larger diameters induce more serious conformational changes of BSA and carbonic anhydrase.^[76] AuNR induces more helix loss than AuNP.^[41] It is noteworthy that, even after release from NPs, the conformational changes might be irreversible.^[64,65] The conformational changes, especially the irreversible ones, might lead to the loss of protein function (e.g. enzyme activity) and consequently unwanted toxicity.

The effects of NPs on activities of bound enzymes could be inhibiting or promoting, depending on the nature of enzymes and NPs.^[24–27] Similar to structural changes, enzyme activity is regulated by the surface coverage, size, and shape of the NPs.^[29,41,68–71,77] The orientation and binding sites are more crucial for enzyme activity. Usually, NPs binding to or close to the active sites of enzymes will inhibit their activity.^[26] But in some cases, adsorption of an enzyme onto a NP might make its active site more accessible to a substrate and consequently increase the enzyme activity. For example, poly-DL-lactic acid NPs could convert lipase into an open form, thus increasing the enzyme activity.^[24] From the point of view of safety, the enzyme activity change, whether inhibiting or promoting, might lead to toxicity, since many diseases are associated with enzyme activity. For in vitro applications, loss of enzyme activity is a cost when the enzyme is immobilized on NMs. Designing proper protein–NP interactions to keep or increase enzyme activity is worth investigating in the future.

Interacting with NPs could result in stabilizing or destabilizing proteins, depending on the many factors discussed above. RNase A is reportedly destabilized upon binding on the surface of silica NPs.^[31] and is stabilized when encapsulated in mesoporous silica NPs.^[32] The encapsulation of proteins in NPs not only stabilizes the proteins, but also protects them from protease digestion.^[33,34,78] Undoubtedly, the stabilizing effect is important for biocatalysis, where immobilized enzymes can be cycled and applied in more extreme conditions.

Recently, NPs were reported to accelerate protein fibrillation by shortening the nucleation lag phase.^[23] In contrast, Cabaleiro-Lago et al. reported that NIPAM/BAM NPs inhibited amyloid β protein A β (1–40) fibrillation.^[79] The NP-mediated fibrillation was affected by the concentration, size, and surface chemistry of the NPs.^[80–87] Skaat et al. found that F- γ -Fe₂O₃–HSA-PEG (polyethylene glycol)-A β (1–40) accelerated the fibrillation of A β (1–40), while F- γ -Fe₂O₃ did not.^[88] The underlying mechanism of NP-mediated protein/peptide fibrillation needs further investigation.

3.2.2. Effect on NMs

Effects on NMs, such as energy transfer from NPs to proteins, defect passivation by proteins, and protein-affected electron configurations, have been widely reported and utilized. Here, we focus on the biological relevant effects on NMs.

Upon protein adsorption, the first change to the NMs is in their surface chemistry. For hydrophobic NPs, protein adsorption makes the surface hydrophilic and compatible with an aqueous environment. After coating with proteins, the hydrophobic pristine carbon NMs, such as fullerene, CNT, and ND, become more hydrophilic and water-dispersible.^[89,90] Adsorption of charged proteins alters the surface charge of NMs accordingly.^[63,65–67] In addition, surface chemistry determines the colloidal behavior of NPs. Surface chemistry changes occurring upon protein adsorption should be carefully considered when studying the environmental fate and toxicity of NMs.

Another effect of protein adsorption is that it alters the dispersing/agglomerating state of the NMs.^[41,70,74] As mentioned before, increasing the hydrophilicity and surface charge enables the hydrophobic NPs to disperse in water. Many NMs are dispersed following this strategy. Alternatively, if one protein molecule interacts with multiple NPs simultaneously, this can induce agglomeration of the NPs.^[74] Overall, dispersing/agglomerating of NMs has significant implications for their bioapplication as well as the toxicity of the NMs.

Spectroscopic changes of NMs upon protein adsorption are well-documented in the literature, and have been used to characterize interactions and related properties. Yang et al. observed specific binding of fullerenol with lysozyme, leading to the symmetry break of fullerenol and thus inducing strong near-UV CD signals from the non-chiral fullerenol.^[26] When green fluorescent protein (GFP) is encapsulated in silica NPs, its fluorescence is remarkably enhanced due to the nanoconfinement effect and isolation of the fluorophore from the surrounding medium.^[33] Binding of BSA affected the surface plasmon resonance (SPR) absorption band of AuNPs and AuNRs, which could be taken as an indicator of the state of dispersion of the AuNPs.^[41] In addition, when the fluorescent NPs are bound with proteins, there might be energy transfer between the two, which can be utilized to design biosensors.^[91]

4. Biosafety by Design

When entering the body, NPs will eventually be coated by protein corona. This protein–NP complex is what the biosystems really deal with, and it also determines the absorption, distribution, metabolism, excretion, and toxicity (ADME/T) of NMs. Accumulated knowledge has shown clear clues as to how to reduce the potential toxicity of NMs by designing protein–NP interactions.

Designing measures including minimization of the hydrophobic surface and charges of NPs could be taken to reduce strong binding of proteins, especially for in vivo bioapplications. Such measures may reduce adverse impacts on proteins, such as strong irreversible binding, depletion of critical low-concentration proteins, bioactivity loss, conformational changes, and protein fibrillation.

Other designing measures, such as agglomeration of NPs in environmental media, could be taken to reduce the exposure to biosystems, and NPs could also be functionalized to reduce their uptake in tissues and promote the elimination of NMs from biosystems.

4.1. Reducing Exposure to Biosystems by Promoting Agglomeration of NPs

Exposure is the first issue to consider when evaluating the biosafety of NMs. Agglomeration/aggregation of NPs would lead to the sedimentation of NPs in environmental media. Thus, a possible route to mitigate toxicity is to reduce exposure levels of NMs to a biosystem by facilitating the agglomeration of NPs in the environmental media. For example, Lankoff et al. found that agglomerated Ag and TiO₂ NPs induce less toxicity to cells, because cells took up fewer agglomerated NPs.^[92] Proteins can agglomerate NPs in some circumstances.^[28,93,94] Therefore, it is possible to agglomerate NPs on purpose in a biological environment by designing the protein–NP interaction.

Serum proteins, particularly SA, can agglomerate certain NPs. Wang et al. have shown that GO agglomerates in the presence of serum proteins in both cell culture medium and pure serum.^[28,93] Orts-Gil et al. showed that 28 nm silica NPs agglomerate in the presence of culture medium DMEM (with 10% fetal bovine serum (FBS)).^[94]

Lysozyme is a readily available, cheap protein, and also a good candidate to agglomerate NPs.^[74] Because not all NPs can be agglomerated/aggregated by serum or lysozyme, testing is always needed to verify and optimize the efficient agglomeration of NPs.

4.2. Reducing Cellular Uptake of NMs

Cellular uptake of NMs has a significant influence on their bioeffects. Many NMs induce cytotoxicity only after they are ingested by cells. Cellular uptake is closely correlated with protein binding and recognition, which could be regulated by designing the protein–NP interaction (**Scheme 4**).

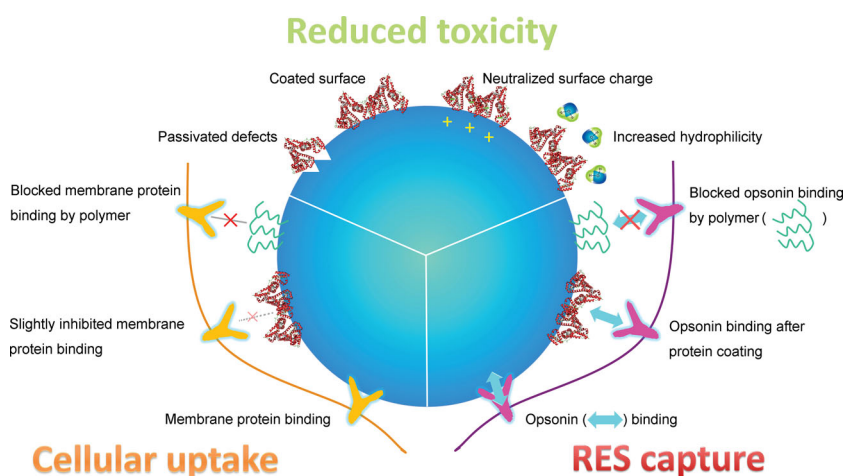
Some NPs can bind to membrane proteins and consequently be internalized. Therefore precoating with proteins or formation of a protein corona on the surface could shield those NPs from binding with membrane proteins and therefore reduce cellular uptake of NPs. Baier et al. reported

that adsorption of SA always leads to a slight inhibition of the cellular uptake of NPs, and the presence of FBS inhibits the uptake significantly.^[95] Similar uptake inhibition of MWCNTs, carbon black, and PS NPs was reported by Zhu et al.^[96] and Smith et al.^[97] Patel et al. proposed a possible mechanism of the protein-inhibited cellular uptake of NPs.^[98] DNA–AuNPs were internalized by HeLa cells through the scavenger receptor-mediated pathway. Binding of BSA on DNA–AuNPs inhibited the binding of these NPs to scavenger receptors, hence the cellular uptake was reduced. Jiang et al. found that the formation of a protein corona inhibits the uptake of FePt NPs by HeLa cells.^[19] Lesniak et al. compared the effects of heat inactivation on protein corona compositions and NPs uptake, and found that a lower cellular uptake correlates with more proteins in the hard corona.^[99]

For phagocytes, decrease of the protein affinity of NPs suppresses their cellular uptake, because protein binding is important for phagocyte recognition. Safi et al. found that, upon exposure to cells, citrate ion-coated γ -Fe₂O₃ NPs aggregate and interact with the membrane, and then are internalized as aggregates by lymphoblastoid cells.^[100] In contrast, after coating with poly(acrylic acid), γ -Fe₂O₃ NPs are dispersible and protein-resistant, and only internalized by cells as individual particles. Lartigue et al. found that cellular uptake of γ -Fe₂O₃ NPs is enhanced by protein adsorption. After coating with amino alcohol derivatives of glucose, NPs become protein-resistant and are seldom captured by macrophages.^[101]

No matter the cell type, one can always reduce cellular uptake of NMs by making them 'invisible' to cells by PEGylation (polyethylene glycol functionalization). Many studies have demonstrated that the PEG-functionalized NPs adsorb fewer proteins and are seldom ingested by cells.^[102–105] Walkey et al. showed that increase of PEG density decreases the protein adsorption on AuNPs, resulting in reduced capture by J774A.1 macrophage-like cells.^[106]

As discussed above, a major issue to consider is the recognition of NPs via membrane protein binding when designing protein–NP interactions to reduce the cellular uptake of NPs. Protein coating will slightly shield NPs from membrane protein binding for non-phagocytic cells. However, NPs with a high protein affinity could promote binding of opsonins, leading to the recognition by phagocytes and the increase of cellular uptake. To achieve a lower uptake by both non-phagocytic and phagocytic cells, PEGylation should be a good choice. After PEGylation, binding of membrane proteins and opsonins is suppressed, due to the protein-resisting character of the PEG coating. This might be important not only for the biosafety of NMs but also for designing protein-resistant NMs for bioapplications.



Scheme 4. Strategies to improve the biosafety of NMs by designing the protein–NP interaction.

4.3. Optimizing Biodistribution of NMs

Since in vivo systems are much more complicated than the in vitro models, the

effects of protein–NP interactions on in vivo biosafety are rarely tackled directly. Fortunately, studies have proven that ADME of NMs, which influences the toxicity of NMs, can be regulated by protein binding.

When NPs enter the blood stream, the first barrier to overcome is the pulmonary capillary. Particulates larger than 2 μm will be filtered out by pulmonary capillaries: so called 'pulmonary capillary filtration'.^[107] There are many reports showing that NPs form larger agglomerates upon strong interaction with proteins in blood. Such agglomerates are readily trapped in the lungs by pulmonary capillary filtration and induce pulmonary damage. For example, Liu et al. have shown that dominating accumulation of GO in lungs was due to the agglomeration of GO with serum proteins.^[28]

Being aware of this mechanism, we can reduce the pulmonary capillary filtration of NMs in blood by regulating the protein–NP interaction. Taking GO as an example, using smaller and less concentrated GO, smaller agglomerates are formed and the pulmonary uptake of GO is dramatically reduced.^[28] Beyond that, a more direct and effective approach is to coat the GO surface with protein-resistant polymer, such as PEG, which can eliminate pulmonary capillary filtration of GO.^[108]

After escaping pulmonary capillary filtration, the next issue is reticuloendothelial system (RES) capture. NMs can be captured by RES to a greater or lesser extent and accumulate in RES-rich organs, such as the liver, spleen, and lungs.^[109,110] Opsonization (binding of opsonins to xenobiotics) is regarded as the trigger for phagocytic recognition and RES capture.^[111] 'Opsonins' commonly refer to complement proteins and IgG. However, generally, any blood serum component which aids the process of phagocytic recognition could be regarded as an opsonin. Obviously, the protein–NP interaction decides the opsonization of NMs. Previous studies have indicated that complement proteins and immunoglobulins are important components of the protein corona after NPs are introduced into serum.^[18,20–22]

Consequently, suppressing opsonin binding will allow us to reduce NM uptake by RES. Like other proteins, the adsorption of opsonins on NPs could be regulated by the particle size, surface chemistry, and serum concentration.^[22] Tenzer et al. observed that 125 nm silica NPs adsorb more immunoglobulins, while 8 and 20 nm silica NPs adsorb more complement proteins in human blood plasma.^[21] Monopoli et al. found that NPs adsorb less complement proteins from plasma at higher plasma concentrations.^[20] To reduce RES capture, designing weaker opsonin–NP interactions or creating a protein-resistant surface for the NPs has also been proven effective.^[112–114]

Clearly, for dispersible NPs, designing a weaker non-specific protein-binding property is always preferred in order to optimize the ADME properties of NPs in body. Although optimizing particle size does affect the opsonin binding, such improvement is generally small. Surface chemistry plays a more powerful role in reducing opsonin binding. In particular, PEGylation has been well established as a general approach to reduce protein–NP interaction, which can inhibit agglomeration of NPs in the blood stream and also the binding of opsonins to NPs. After reducing RES capture,

the accumulation of NMs in organs can be minimized, thus restricting their potential toxicity.

4.4. Mitigating Toxicity of NMs

4.4.1. Reducing Harm to Proteins

When a NM enters the body, it will bind various proteins, causing many possible adverse effects via changes to the proteins, such as depleting proteins, unfolding proteins, inhibiting function, promoting protein fibrillation, and even transferring proteins to the wrong locations. All these direct potential hazards could be reduced by reducing the binding potential of NMs.

As discussed above, designing the surface chemistry of NPs is the most effective approach to manipulate the protein–NP interaction. Just to emphasize that, the hydrophobic surface of NPs should be generally regarded as a potential safety hazard. The best way to reduce the hydrophobicity of NPs is to functionalize them with hydrophilic groups. Carefully choosing functional groups can reduce protein–NP interactions and increase the biocompatibility of NMs. Considering that biosystems contain differently charged proteins, a safe choice is to functionalize NPs with nonionic groups, such as hydroxyl groups.

Although protein binding on NPs is generally size-dependent, unfortunately, there is no general rule for designing particle size to minimize protein–NP interaction. Case-by-case studies are required to optimize the size as well as the surface morphology of NMs.

Accelerated protein fibrillation by NPs raises a new alert on the biosafety of NMs. Basically, this acceleration is due to a high concentration of partially unfolded proteins stabilized on the NP surfaces. Those partially unfolded protein species are unstable in natural conditions, and it is very difficult for them to reach a critical concentration to form fibril nuclei. This is why all known protein fibrillation-associated diseases are age-related, requiring long times to nucleate. NMs can shorten the nucleation time, thus inducing a potential safety hazard. Given that proteins not associated with any known diseases could also be turned into fibrils,^[115] and that non-natural dialysis-related amyloidosis is only found in patients on long-term hemodialysis,^[116] precautions should be taken for NMs which are intended for long-term in vivo applications.

4.4.2. Mitigating Cytotoxicity

There is growing evidence that protein coatings could mitigate the cytotoxicity of NMs. In this strategy, the effective coating and high coverage of proteins on NPs are crucial. Ge et al. reported that several serum proteins adsorb on SWCNTs and reduce the cytotoxicity dramatically (**Figure 4**), following the order: Bovine fibrinogen (BFG) > gamma globulin > Tf > BSA > ferritin.^[117] BFG shows the best detoxification effect by rearranging itself to cover SWCNTs compactly. Hu et al. reported that adsorption of FBS on GO reduces the cytotoxicity of GO, while the uncoated GO destroys the cell membrane and enters the cytoplasm.^[118] The presence of serum can also reduce the toxicity of many other NMs, such

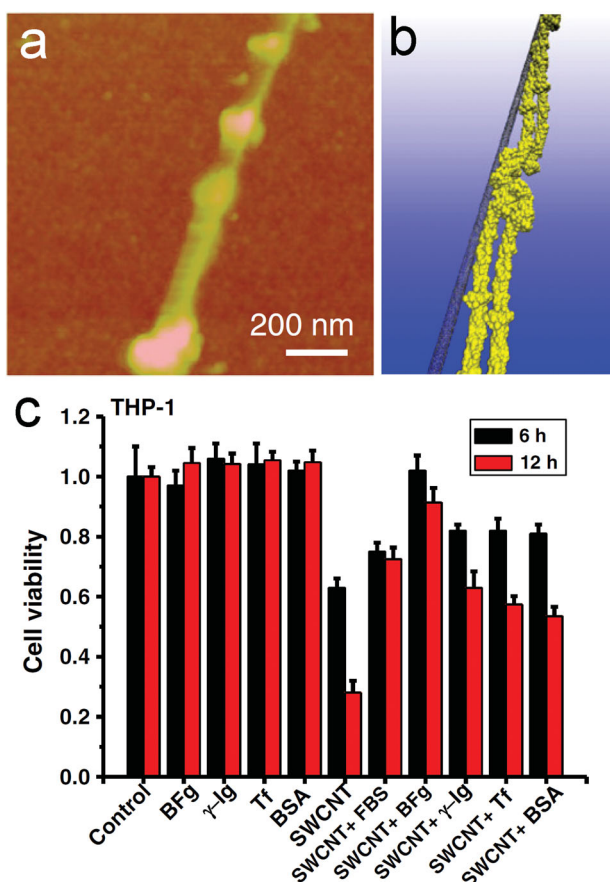


Figure 4. Reduction of the cytotoxicity by protein binding. (a) AFM image of a SWCNT coated by BFG; (b) binding model of BFG on SWCNT; (c) BFG-reduced cytotoxicity of SWCNTs to THP-1 cells. Reproduced with permission.^[117] Copyright 2011, National Academy Science.

as polysaccharide cationic NPs, MWCNTs, carbon black NPs, silica NPs, and Ag NPs.^[92,96,119]

Pre-coating with excess proteins can also mitigate the cytotoxicity of NMs caused by nutrient depletion. Horie et al. found that while NMs could deplete proteins and calcium in the culture medium, pre-treatment of NMs with FBS reduced the depletion, thus, reducing the cytotoxicity of NMs.^[120]

Even though protein coating reduces the cytotoxicity of NMs in many cases, protein–NP interactions should be carefully designed, because binding with proteins does not always result in detoxification. For example, Clift et al. observed that FBS enhances the toxicity of 20 nm PS beads.^[121]

Available nanotoxicity data also infer other possible strategies to mitigate the toxicity of NMs by designing the protein–NP interaction. First, reactive oxygen species (ROS) are involved in a widely reported toxicological mechanism of NMs.^[122] It is believed that surface defects of NPs may cause ROS generation.^[123] Protein binding might passivate the surface defects, hence reducing ROS generation and therefore the cytotoxicity of NMs. Second, over-charged NMs are usually more toxic, especially positively charged ones. We can select proteins with opposite charges to neutralize the NMs. Third, hydrophobic NMs might interrupt cell membranes,

because the inner lipid bilayer is hydrophobic. Protein binding can make NMs hydrophilic and therefore increase their biocompatibility.

4.4.3. Mitigating *In vivo* Toxicity

Cytotoxicity of NMs is already complex, and the situation at an animal level becomes much more complicated. Although information on how protein binding affects *in vivo* toxicity of NMs is scarce, from *in vitro* studies we can speculate that proper surface coatings of proteins might help to reduce the *in vivo* toxicity of NMs. In addition to dispersing NPs, protein coatings could make NPs more biocompatible, such as softening the NP surface and relieving mechanical damage to tissues caused by rigid NPs. Also, proteins might passivate defects of NPs, where ROS are usually generated. However, strong protein–NP interactions should be avoided to prevent the possible adverse effects discussed above. For NPs applied *in vivo*, it is preferable to design weak non-specific protein-binding surfaces for NPs, thus NPs will bind those abundant proteins *in vivo*, such as SA, avoiding depletion of important rare proteins *in vivo*. In addition, even though biofluids in the lungs or the gastrointestinal tract contain proteins, formation of protein coronae on NP surfaces requires time, therefore, pre-coating NPs with proteins might give better performance.

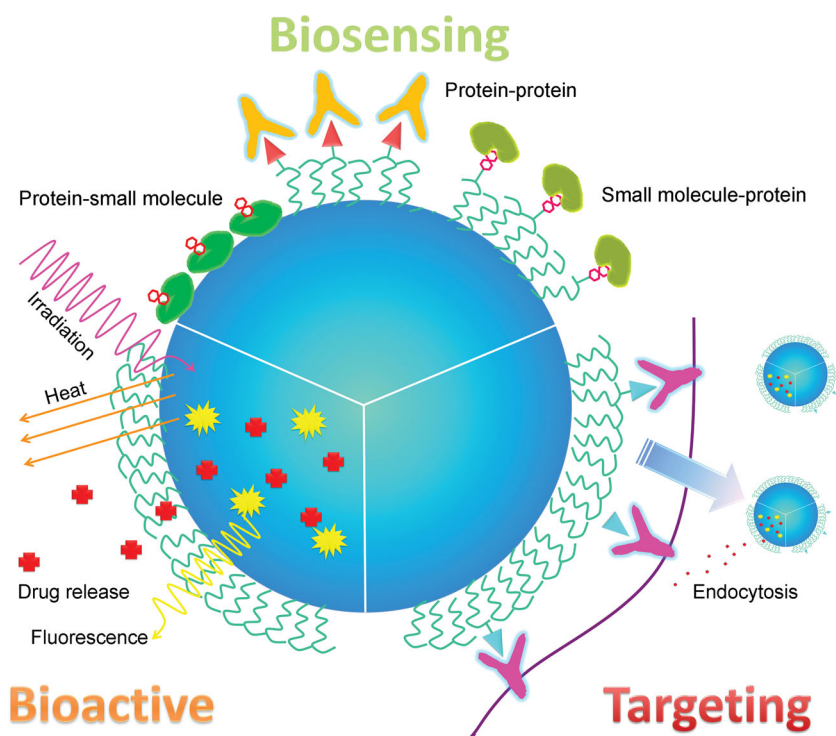
5. Bioapplications by Design

Adsorption of proteins on NPs is not always a hazard. The interactions between proteins and NPs and their effects can be explored for various beneficial bioapplications, including biosensors, biocatalysis, and drug delivery. For those bioapplications, binding sites of the protein–NP complex should be well controlled by a rational design.

5.1. Biosensors

Biosensors are an important bioapplication of NMs. NM-based biosensors have many advantages over traditional ones, such as high sensitivity and specificity, which are crucial for early diagnosis of diseases. Nano-biosensors are usually based on specific interactions between functionalized NPs with biomarker molecules (including proteins; see **Scheme 5**).

The common design for biosensors has NPs bound with proteins, which further recognize substrates. Medintz et al. designed an elegant QD–MBP FRET sensor for maltose detection (**Figure 5**).^[124] Fluorescence of QDs was quenched due to FRET when QSY-9 bound to MBP. When maltose was present in the system, fluorescence was recovered, because maltose replaced QSY-9 to bind specifically on MBP. If the substrate is a protein, design measures should be taken to avoid nonspecific adsorption of a protein substrate on the biosensor. Taking reduced GO (RGO)-based field-effect transistors (FETs) for IgG detection as an example,^[125] anti-IgG was anchored on RGO via AuNPs to provide a specific interaction with IgG. Nonspecific interactions could be



Scheme 5. Bioapplications made possible by designing protein–NP interactions.

blocked by pre-treating the FET device with blocking buffer containing BSA. In another strategy, NPs were functionalized with small molecule moieties that can be recognized by proteins. For instance, sugar-functionalized CNTs could be used for detecting glycoproteins, where the sugar moieties specifically interact with the glycoprotein.^[126]

It is noteworthy that the specific interaction is a key factor to consider for building a biosensor. Rational design of the proper orientation of proteins on NMs is crucial to guarantee a specific interaction as well as correct sensing.

which is over-expressed on the membrane of cancer cells. Wang et al. linked FA on silica NPs to achieve selective targeting of HeLa cells.^[127] Peptides work well in ligand–receptor based targeting, for example, arginine–glycine–aspartic acid (RGD) peptide is among the most used. Kumar et al. linked RGD to AuNPs to target neuropilin-1 (Nrp-1) receptors.^[128] The RGD–AuNPs showed a 5-fold higher uptake in MDA-MB-321 cells than AuNPs alone. Proteins could be adopted, too. For example, Tf-coupled carbon dots could target HeLa cells efficiently.^[129]

5.2. Protein-Guided Targeting and Stealth Characteristics

NMs provide a powerful platform to carry multiple functional agents, such as imaging probes and therapeutic drugs. NM-based theranostic nanomedicine can be used for targeted drug delivery and to monitor the therapeutic response simultaneously, and it is expected to play an important role in future personalized medicine. Nano drug-delivery systems could increase the bioavailability of drugs and reduce their toxicity to healthy tissues, mainly by providing guided targeting and stealth characteristics.

5.2.1. Protein-Guided Targeting

For bioapplications such as imaging, drug delivery, and bioanalysis, the design of specific interactions for targeting is required. Linking targeting moieties onto NPs is the most used strategy.

Protein-guided targeting can be based on ligand–receptor interactions. For example, folic acid (FA) is one of the most used moieties, targeting the FA receptor,

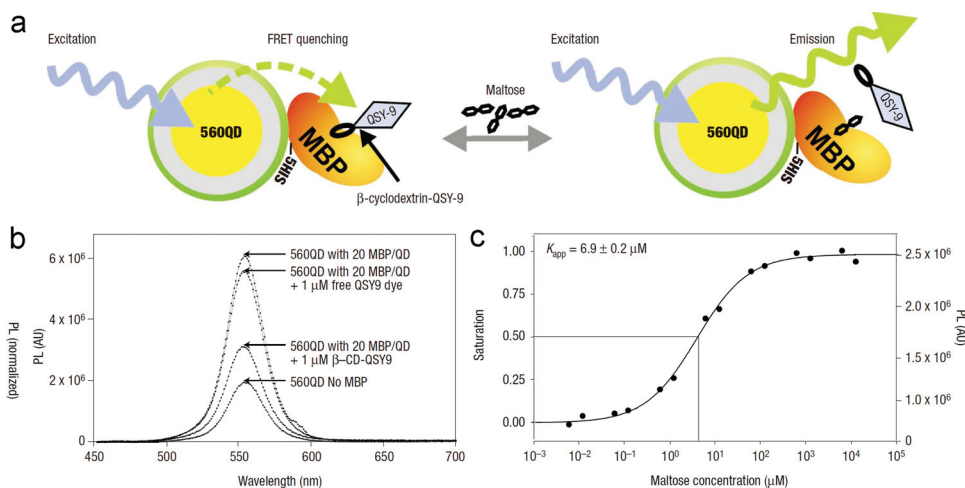


Figure 5. QD–MBP FRET sensor. (a) Function schematic; (b) determination of QD–MBP FRET quenching; (c) transformation of titration data. Reproduced with permission.^[124] Copyright 2003, Nature Publishing Group.

Another kind of targeting moiety is based on antibody–antigen interactions. The human single-chain variable-fragment (scFv) antibodies that can specifically bind c-Met proteins can be linked to QDs and liposomes to target tumors specifically, as aberrant expression of c-Met protein is higher in tumors.^[130]

Aptamer is an alternative to antibodies. Farokhzad et al. prepared NPs functionalized with A10 2'-fluoropyrimidine RNA aptamers that recognized the extracellular domain of the prostate-specific membrane antigen (PSMA).^[131] Since PSMA is over-expressed on the surfaces of prostate cancer cells, the aptamer-functionalized NPs accumulate more in the LNCaP prostate epithelial cells and the xenograft model.

Interestingly, protein coronae provide a new pathway. Prapainop et al. functionalized PEGylated QDs with 5,6-seco-sterol atheronal-B, and these QDs induced aggregation of apo-B100, forming a hard corona on the QDs.^[132] The formation of the apo-B100 corona facilitated the cellular uptake of QDs through an SR-A receptor-mediated pathway and finally the QDs located in the endoplasmic reticulum.

5.2.2. Stealth Character

To achieve specific targeting, being resistant to nonspecific protein adsorption is essential. When this issue comes to an in vivo level, a protein-resistance character would allow NPs to escape recognition by opsonins and capture of RES (so called 'stealth' properties). By careful design of the protein–NP interaction, stealthy NMs could be prepared and applied.

PEGylation is believed to be the most effective way to make NMs stealthy.^[111,113,133] Other surface functionalizations are also useful in preventing protein adsorption, such as poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO), poloxamers, and polaxamines.^[111]

Longer or even branched PEG chains and higher PEG densities could achieve better a stealth character.^[134,135] However, when PEG chains have a MW higher than 5000, protein adsorption on NPs did not change any more with further increasing MW, likely due to PEG chain folding. As for PEG density, it was found that protein adsorption on NPs containing 5% PEG was threefold lower than those containing less PEG.^[133] In addition, PEGylation methods regulate the stealth character of NMs, for which a covalent PEGylation is better than a noncovalent one. For example, to achieve similar pharmacokinetic improvement of SWCNTs, a much longer PEG chain was needed for noncovalent PEGylation than covalent PEGylation.^[113]

5.2.3. Targeting and Stealth NMs for Bioapplications

By designing the protein–NP interaction and incorporating multiple functions, NMs with both targeting and stealth characteristics could be applied in many biological areas (Scheme 5). The following examples show how it works.

Kievit et al. designed a complex nanosystem for cancer diagnostics.^[136] An iron oxide core was first coated with chitosan–PEG, where Alexa Fluor 647 was buried. Then, neu antibody was linked to the NPs by a PEG linker, and the resulted neu–NPs selectively accumulated in the MMC cells in vitro and in tumor. The tumor showed very good contrast in T2 maps in vivo and in fluorescence images ex vivo at 48 h

postexposure. In contrast, the control IgG–NPs did not work. Similarly, Ren et al. built an O-MWCNT-based drug delivery system for brain tumor therapy.^[137] O-MWCNTs were suspended using DSPE-PEG and a targeting moiety (angiopep-2) was linked at the end of PEG. Then doxorubicin was loaded onto the CNT surface via hydrophobic and π - π interactions. The CNT vehicle reached a brain tumor and prolonged the median survival time by 13 days compared to saline and by 10 days compared to free doxorubicin.

5.3. Protein Immobilization and Biocatalysis

Proteins immobilized on NP surfaces or encapsulated inside NP pores, either noncovalently or covalently, could facilitate biocatalysis applications of the proteins. When enzymes are immobilized or encapsulated, NPs increase the enzyme stability and enable them to be used more widely. Wang et al. immobilized enzymes on gold nanoporous structures and found that lipase and catalase retain about 80% activity after 20 cycles in use, and HRP retained 60%.^[138] In an organic phase, more than 40% of activity still retained after 20 cycles, compared to only 30% in the first run for the free enzyme. In addition, the thermal stability of the enzyme increased upon immobilization. Lipase retained 40% activity at 70°C after 20 cycles.

Immobilization of enzymes combined with other techniques may provide new applications. When HRP is immobilized on AuNPs, the photothermal effect of AuNPs enables easy control of HRP activity.^[139] Under 532 nm laser irradiation, the enzyme could be deactivated in a laser power-dependent manner. Immobilization of enzymes might be combined with cellular delivery for intracellular catalysis, for example, by immobilizing luciferase on silica NPs. Sun et al. delivered luciferase into HeLa cells where luciferase converted luciferin and to oxyluciferin in cytoplasm.^[140]

A suitable immobilization or encapsulation method is highly demanded for certain systems and applications. Wu et al. reported a new strategy to stabilize lipase and lyase by NPs.^[141] A typical Pickering emulsion containing enzymes was stabilized by silica NPs, where NPs (140 nm) form a large cage (5–15 μ m) encapsulating the emulsion. The stabilized enzyme could be used for catalysis in the organic phase, and its specific activity increased significantly due to the improved mass transfer and accessibility of the enzyme catalyst in the reaction system. Due to electronic repulsion, negatively charged proteins and peptides are difficult to noncovalently encapsulate in negatively charged silica NPs. To overcome this problem, we designed a new histidine tag (His tag) method. With the simple addition of a small amount of calcium ions in a common reversible emulsion, the negatively charged His-tagged proteins or peptides could be effectively encapsulated in silica NPs by the interaction between calcium ions incorporated in the silica matrix and His tags of proteins or peptides, which overcame the electronic repulsion (**Figure 6**).^[133]

5.4. Protein Assisted Synthesis of NMs

Living organisms can produce highly ordered nanostructured biominerals, such as shells and bones. Proteins play an important role in this biomineralization process by providing the

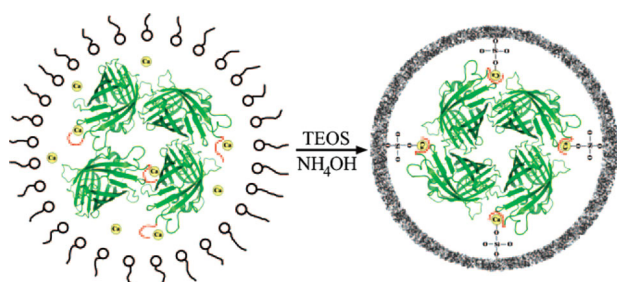


Figure 6. Schematic illustration of the His tag immobilization technique. The addition of Ca^{2+} provides anchoring positions for His-tagged proteins, overcoming the electronic repulsion between proteins and silica NPs. Red ribbons indicate the His tags. Reproduced with permission.^[131] Copyright 2010, Wiley-VCH Verlag GmbH & Co. KGaA.

template and/or confined reaction environment, and controlling the shape, hierarchical organization, and morphology of inorganic particles. Many efforts have been exerted to mimic this bioprocess, and many NMs have been synthesized under the control of proteins.

Proteins can provide residues to chelate metal ions and the cavity of proteins can be used as the template.^[142–144] Xie et al. synthesized fluorescent gold nanoclusters (AuNCs) with the help of BSA (**Figure 7**).^[142] Upon adding Au (III) ions to aqueous BSA solution, Au ions were sequestered and entrapped by BSA. Then BSA reduced Au (III) at pH 12 and AuNCs were generated within BSA as BSA–AuNC bioconjugates. In Fan et al.'s study, AuCl_4^- was used for preparation of AuNPs inside unmodified horse spleen apoferritin.^[143] AuCl_4^- was adsorbed into apoferritin and desalted, and then reduced by NaBH_4 to form Au clusters acting as the seeds for growing AuNPs.

Protein–NP interaction could also be used for the selective fabrication of NM composites. One main strategy uses proteins to bind multiple NPs, where the proteins serve as a glue as well as a template. Han et al. prepared porous magnetite fibers with the assistance of glutin.^[145] Mechanically, Fe_3O_4 NPs bound to glutin through Coulombic attraction. Since the glutin template is a fibrous structure, Fe_3O_4 NPs are fibrous assemblies. Then, Fe_3O_4 NPs were reoriented and assembled effectively along the [111] direction under a

magnetic field. Liu et al. adopted a protein–NP interaction to fabricate a NM–NM composite.^[146] Firstly, BSA was adsorbed on GO, then Au, Pt, Ag, Pd or Latex NPs could stick on GO by the interaction between BSA and the NPs. When GO was reduced by BSA under 55–90 °C at pH 12, RGO–BSA–NPs composites were prepared.

5.5. Miscellaneous Applications

The separation of proteins is a basic and important process in various biological studies and in biomedical production. Taking advantage of protein–NP interactions, efficient and selective separation of proteins can be achieved. Shao et al. prepared a $\text{Fe}_3\text{O}_4@\text{SiO}_2$ @layered double hydroxide (LDH) sphere for the separation of His-tagged GFP.^[147] An NiAl-LDH layer was grown in situ on $\text{Fe}_3\text{O}_4@\text{SiO}_2$ NPs. The separation induced very limited changes to His-tagged GFP, and 90% fluorescence could be recovered. $\text{Fe}_3\text{O}_4@\text{SiO}_2$ @LDH showed good stability and specificity to His-tagged proteins. Similarly, $\text{Fe}_3\text{O}_4@\text{NiSiO}_3$ and Au– Fe_3O_4 NPs were prepared for selective separation of His-tagged GFP.^[148,149]

Separation of proteins can also be achieved using NMs by filtration. El-Safty et al. prepared building-block-based mosaic cage silica nanotubes (NTs) for protein separation.^[150] Synthesis of silica NTs was performed on an anodic alumina membrane. The resulting nanofilter membrane was used for the size-exclusive separation of proteins, such as cyt C, myoglobin, hemoglobin, lysozyme, and β -lactoglobulin.

As discussed above, dispersing NMs could be achieved via protein–NP interactions too. Many applications require well-dispersed NMs with good biocompatibility. For example, hydrophobin could disperse MWCNTs well and be applied in the controlled immobilization of IgG.^[151] Shimkunas et al. found that NDs could be well dispersed by insulin.^[152] The well-dispersed NDs could be taken up by cells and release insulin there. Thus, this dispersing system became applicable for insulin delivery. Yang et al. found that lysozyme dispersed GO well and also protected the colloid during the reduction reaction, resulting in well-dispersed RGO.^[153]

Computational results indicate that proteins could sort carbon NMs of different sizes.^[29] Accordingly, Nie et al. utilized lysozyme for diameter separation of double-walled CNTs (DWCNTs). Lysozyme preferred to bind DWCNTs of larger diameter. By carefully controlling the binding conditions, lysozyme could selectively suspend DWCNTs of larger diameters or separate DWCNTs from the mixture of SWCNTs and DWCNTs.^[90] Since diameter has a significant influence on the bioactivity of CNTs, protein-assisted size separation of CNTs might find important application.

Not only do NMs protect proteins, but the surface coating of proteins could also protect some NMs. Ishii et al. reported that CdS QDs could be encapsulated in the cave of chaperonin proteins GroEL or

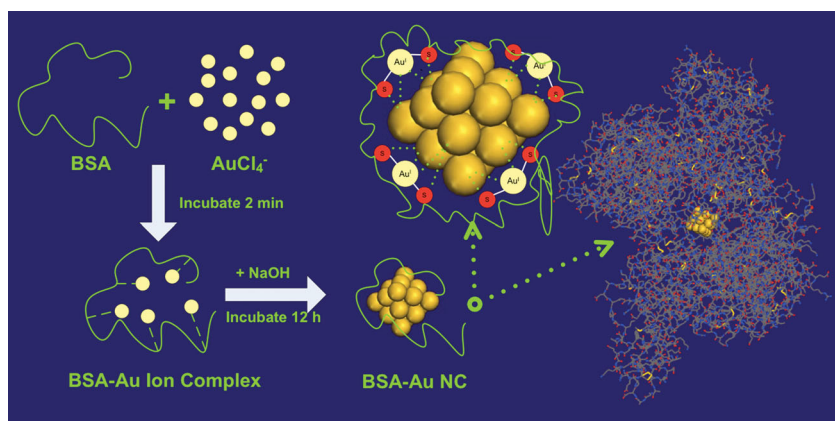


Figure 7. BSA-directed synthesis of fluorescent AuNCs. Reproduced with permission.^[142] Copyright 2009, American Chemical Society.

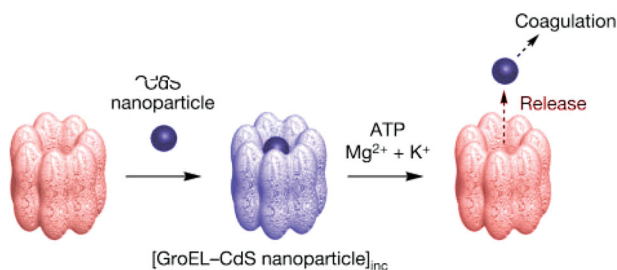


Figure 8. CdS NP encapsulation into and ATP-triggered release from the cylindrical cavity of GroEL. Reproduced with permission.^[154] Copyright 2003, Nature Publishing Group.

T.th cpn, and released upon addition of ATP (**Figure 8**).^[154] Encapsulation of CdS QDs in proteins protected the QDs from methyl viologen, which could quench the fluorescence of QDs. The high-temperature stability of chaperonins enables the encapsulated QDs good thermal stability under 80 °C. Encapsulation in protein coronae could protect NMs, too. Sahoo et al. found that protein coronae protect the fluorescence of QDs against incubation at 37 °C at different pHs.^[155] This protection effect might find application in bioimaging, for example, obtaining NMs with more stable fluorescence.

6. Summary and Outlook

Due to the complexities of both proteins and NMs, the protein–NP interface is only poorly understood even in the simplest in vitro conditions. The lack of a thorough understanding of protein–NP interactions impedes biosafety assessment and the bioapplication of NMs. Joint efforts from multiple disciplines are needed to explore this exciting and challenging field.

Current results have demonstrated that the physicochemical properties of both proteins and NMs affect the protein–NP interaction. However, there are still no systematic and consistent rules. For example, some studies suggest that smaller NPs bind proteins more tightly, while others show converse results. Such fundamental issues should be worked out under in vitro conditions using model proteins and standardized NMs by systematic investigations.

Once they have entered a biosystem, NPs will be covered by protein coronae (likely including other biomolecules, too). The protein corona will confer a biological identity to NPs, which determines the fate and effects of the NM in vivo. Therefore, it is necessary to establish NM–Bio profiles in different biological fluids for both safety evaluation and bioapplication of NMs. Such profiles should include both thermodynamic and kinetic information. Because NMs in vivo may be transported to different parts of the body, and may translocate into different subcellular compartments, such in-depth profiles necessitate particular future research endeavors.

One step forward in the understanding of protein–NP interactions is to control the formation of protein coronae on NPs by rational design. Current studies have shown clues as to how to manipulate the protein–NP interaction, however, this knowledge is still at an infant stage. For future studies, designing the synthesis and functionalization of NMs to control

the protein coronae for both biosafety and bioapplication considerations is encouraged.

To push knowledge and application of the protein–NP interface one more step forward, we could merge proteins and NPs: protein–NP hybrids or artificial nanoproteins. Some efforts have already been made to design artificial proteins. But most such studies are based on grafting a large number of functional groups on NP surfaces, and they have relied on multivalency to promote the original function of the grafted groups. This is not how the natural protein works at all. Natural proteins are the most delicate and precise nanomachines evolved over millions of years by nature, and their functions come from the collaboration of a set of amino acids that do not have individual functions.

The advance of nanotechnology promises better designs and better NMs. One ultimate goal of nanotechnology would be to design NMs with precisely controlled atomic structures and functions that are comparable to natural proteins. Recent developments in protein science suggest that usually only a few key residues on protein surfaces carry out their major functions, while all other residues mainly function as the skeleton to hold those key residues in the right positions.^[156] We believe that NPs could also serve as such skeletons, and in the future it will be possible to precisely functionalize a set of functional groups with well-defined positions on the surfaces of NPs and thus generate a ‘natural’ nanoprotein with the advantages of both proteins and NPs. To achieve this goal, it is crucial to locate a set of functional groups on the surface of the NPs with well-defined geometry at the sub-nanoscale level, which remains a huge challenge. A possible route to achieve this goal is to functionalize a set of groups with the right geometry on a big rigid molecular template first, and then transfer them to the surface of the NPs. This is one of the almost unexplored fantastic frontiers at the protein–NP interface, showing “plenty of room at the bottom” and it is full of opportunities and possibilities in future.

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