4
Proteins and Nanoparticles: Covalent and Noncovalent Conjugates

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4.1
Overview

Nanoparticles (NPs) provide a versatile tool for the integration of biological and materials systems. These organic–inorganic hybrid materials feature an inorganic core surrounded by a monolayer conjugated with organic and/or biomolecular ligands. Materials used for the cores include metals (e.g., Au, Pt, Ag, Co, FePt), semiconductors (e.g., CdSe, CdS, ZnSe, InP, PbSe), and core/shell hybrids (e.g., CdSe/ZnS, FePt/Fe_2O_3) [1]. With these systems, the composition of the core material dictates the primary physical and chemical properties of the NP, providing unique and useful intrinsic properties. Gold NPs (AuNPs), for example, are optically dense and have useful electronic and plasmonic properties, while CdSe NPs (quantum dots, QDs), are highly stable and efficient fluorophores for use as optical probes. The magnetic characteristics of iron oxide NPs make them functional probes for techniques such as magnetic resonance imaging (MRI). Taken together, the diversity of available core materials and properties make NPs pragmatic tools for numerous applications [1].

The organic monolayer of the NP is likewise important, providing the interface between the core and the surrounding environment. At the simplest level, the monolayer acts as a barrier between the NP core and the environment, effectively protecting and stabilizing the core. On a more functional level, the reactivity, solubility and interfacial interactions of NPs are dictated by the chemical nature of the monolayer periphery.

For the biological applications featured in this volume, solubility in aqueous environments can be provided by charged or polar groups at the monolayer periphery. The four general monolayer designs used to achieve water solubility are: amphiphilic ligands [2–6], silica shells [7], lipids [8], and polymers [9]. Building upon the water-soluble scaffold, the monolayer can be further tailored with more complex headgroups to modulate intermolecular interactions of the particle. These headgroups can range from simple relatively nonspecific ligands to biologically active components including peptides, proteins, and DNA.

Two fundamentally different approaches can be used to conjugate proteins to NPs. The first approach uses noncovalent interactions between the particle and protein, while the second method uses direct covalent linkage of the protein to the particle surface. Both approaches have their strengths and limitations, and hence their place in the bionanotechnology ''tool kit''.

4.1.1 Covalent Protein-Nanoparticle Conjugates

The unique optical [10, 11], photophysical [12] and electronic [13] properties of metal and semiconductor NPs makes them ideal for biorecognition and biosensing processes. Conjugating proteins covalently to the surface of NPs allows for greater control of protein reactivity and aggregation of biomolecule-functionalized NPs. Covalent attachment is generally achieved by either coupling with active amino acid residues (e.g., $-NH_2$, $-SH$ and $-COOH$) on the surface of a protein, or by solid-phase synthesis of peptides, terminated with functional residue that can be further conjugated onto the NP surface.

Optical biosensing is one of the technologies enabled by covalent particle–protein conjugation. Two ways of transducing binding events using NP-biomolecule conjugates have been recently reported. The first method is simply to use metallic NPs as local quencher of a fluorophore, allowing the interaction between the NP and the fluorescent protein or fluorescent-tagged protein to be determined quantitatively [14]. The second method is through spectroscopic shifts generated by aggregated or conjugated metal NPs through plasmonic coupling [15, 16].

Similar to DNA systems, the specificity of enzyme–substrate interactions can be exploited for the creation of optical biosensors. Recently, Simonian et al. [17] reported a system for the detection of paraoxon, an organophosphate neurotoxin. In their study, these authors functionalized Au NPs with the enzyme organophosphate hydrolase (OPH), and the conjugates were then incubated with a fluorescent enzyme inhibitor. The fluorescence intensity of the inhibitor is sensitive to the proximity of the Au NP (Figure 4.1); when paraoxon was introduced to the OPH–NP–inhibitor conjugate mixtures, an increase in fluorescence was observed via displacement of the inhibitor by paraoxon.

Semiconductor NPs (i.e., QDs, in particular CdSe) are very useful as fluorescence labels due to their favorable intrinsic properties, including high fluorescence quantum yield, photostability, and size-dependent tunable fluorescence bands [7, 18, 19]. In a model system, effective fluorescent resonance energy transfer (FRET) was observed by the specific interaction of biotin-labeled CdSe NPs with Texas red-labeled streptavidin. The extent of the energy transfer was in proportion to the concentration of the dye-labeled protein, and provided proof of concept for an effective biosensor.

Controlled aggregation of NPs causes a shift in optical absorption in the surface plasmon resonance peak and broadening of the absorption spectrum of the NPs, reflecting the extent of aggregation. Otsuka et al. reported a lactose-

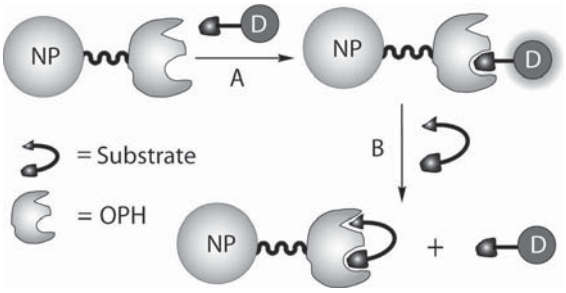

Fig. 4.1 (A) Schematic representation of Decoy **D**-Enzyme interaction for enhancement of fluorescence due to the proximity of nanogold in the absence of substrate. (B) Substrate displacement of decoy from the OPH–gold complex, leading to a decrease in the fluorescence signal from the decoy.

conjugated gold NP to target agglutinin, a bivalent lectin with D-galactose specificity [20]. As shown in Figure 4.2, the lactose on gold NPs promotes lectin-induced aggregation, leading to distinct changes in the absorption spectrum. Also, the change of color from red to purple was observed due to the aggregation. The aggregation is reversible in nature, which can be released by addition of excess galactose. Significantly, since the degree of aggregation is proportional to the

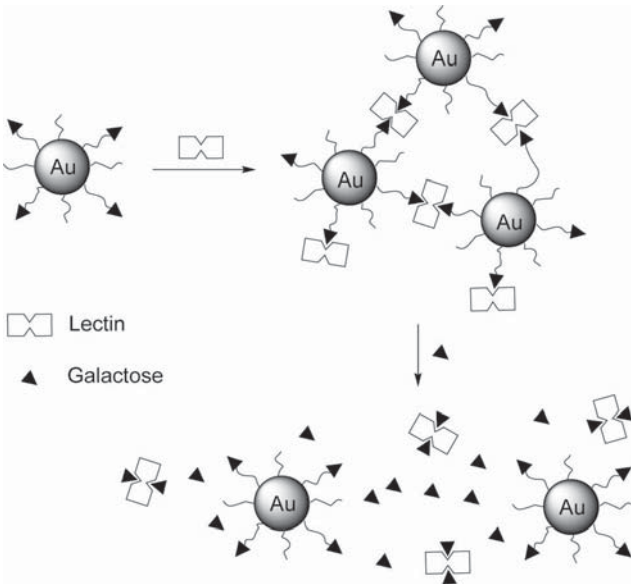

Fig. 4.2 Schematic representation of reversible lectin-induced association of gold nanoparticles modified with lactose.

lectin concentration, the target molecule could be detected quantitatively at high sensitivity. By taking advantage of the specific biotin–streptavidin interaction, Perez-Luna et al. fabricated biotinylated gold NPs and investigated their aggregation by optical properties in the presence of streptavidin [21].

The intrinsic catalytic and photoelectrochemical properties of NPs can be utilized to fabricate electronic biosensors [22, 23]. Redox enzyme functionalized NPs have been used extensively for these bioelectroanalytical systems [24]. In this system, enzyme functionalized NPs are linked through electrodes, and the concentration of substrate or specific proteins is measured by the development of electrolytic current [13, 25, 26]. The catalytic deposition of metals on biomolecule–NP hybrid labels has also been used to generate conductive domains and surfaces. This unique property of these systems allows them to be used as an electronic biosensor, as the conductivity can be measured quantitatively [27].

Electronic biosensors and biofuel cells [28, 29] can be driven by the transfer of electrons generated via biological reactions to the surface of the NP. This transfer changes the surface plasmon resonance spectra of the NP, yielding a bioelectronic/biosensing system. Based on this concept, Willner et al. reported several systems using a NP–enzyme hybrid as a sensor. In one representative example [25], the redox enzyme glucose oxidase (GOx) was connected through a single Au NP by reconstitution of the apo-flavoenzyme, apo-glucose oxidase (apo-GOx), on a 1.4 nm Au NP that was functionalized with N^6-(2-aminoethyl) flavin adenine (FAD). This enzyme–NP hybrid system was connected to the electrode by a suitable dithiol ligand. Alternatively, the FAD-functionalized NP can be assembled on the electrode first, after which the apo-GOx can be introduced (Figure 4.3A). The rate of electron transfer from the enzyme depends on dithiol linkers. Using a dithiol linker (Figure 4.3C), this system exhibits a highly efficient electrical communication with the electron-transfer turnover rate at ~50 000 s^{-1}. As

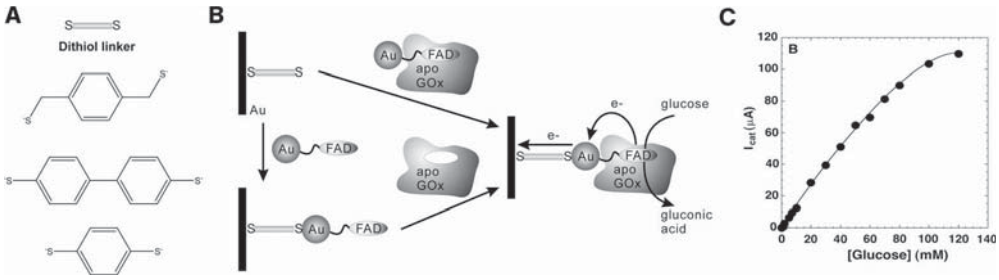

Fig. 4.3 (A) Structure of different dithiol linkers. (B) Assembly of glucose oxidase (GOx) electrode by the reconstitution of apo-enzyme on a FAD-functionalized gold nanoparticle. (C) Calibration plot of the electrocatalytic current developed by the reconstituted GOx electrode in the presence of different concentrations of glucose.

shown in Figure 4.3B, the amount of glucose present is directly proportional to the amount current produced, providing a highly efficient glucose biosensor.

This idea of "charging" NPs can be used for other catalytic application, such as the recently reported hydrogen evolution demonstrated by Yeni Astuti et al. [30]. In these studies, zinc-substituted cytochrome c (ZnCyt-c) was immobilized on metal oxide NPs. The efficient electron injection from the triplet state of ZnCyt-c to the TiO_2 NP electrode then served to generate H_2. Another example recently reported by Ipe and Niemeyer uses electrostatically attached cytochrome $450_{BS\beta}$ to cadmium sulfite (CdS) QDs via a histidine linker [31]. In the presence of hydrogen peroxide, the enzyme is able to catalyze the hydroxylation of myristic acid to hydroxymyristic acid. Since superoxide (O_2^-) and OH is generated when CdS absorbs light, this allows the enzyme to activate its catalytic machinery through scavenging the free radicals. By combining the unique properties of CdS QDs and cytochrome $450_{BS\beta}$, the authors were able to create a nanohybrid that acts as a light-switchable photocatalyst.

4.1.2
Noncovalent Protein–NP Conjugation

The simplest way to produce noncovalent NP–protein conjugates is through complementary electrostatic interactions between the NP and the protein. This can be done either on the surface of a "naked" NP (e.g., citrate-stabilized gold) or by the use of a functionalized monolayer. The use of functionalized monolayers allows the facile generation of either positively or negatively charged NPs that can bind oppositely charged proteins. Perhaps the most significant useful attribute of noncovalent protein–particle conjugation is the reversibility of appropriately chosen systems, facilitating applications in sensing and delivery. For example, Ag_2S NPs conjugated with bovine serum albumin (BSA) can be assembled and disassembled, with the change of pH causing association and dissociation of the protein [32]. Noncovalent interactions with peptides are also useful for templation of peptide assemblies to yield *de-novo* proteins [33–35]. The reversible inhibition and activation of enzymatic activity provides a further use of noncovalent electrostatic conjugation [36]. An important issue for all of these applications is the retention of native protein structure in the particle–protein conjugate. Recent studies have demonstrated that oligo (ethylene glycol) monolayer-protected NPs minimize nonspecific binding of proteins (Figure 4.5A), making these systems quite useful [36, 37].

The controlled interactions of NPs with proteins is a potential tool for both fundamental and applied biomedical investigations. In a fundamental study by Fischer et al., the use of α-chymotrypsin (ChT) with NP **1** (Figure 4.4A) revealed effective inhibition of the enzyme via a two-step process: fast reversible initial inhibition followed by a kinetically irreversible conformational change in secondary structure as determined by CD and fluorescence spectroscopy [5]. The initial binding in this system is electrostatic; the anionic monolayer of the NP is complementary to the cationic side chains surrounding the active site of ChT. Upon

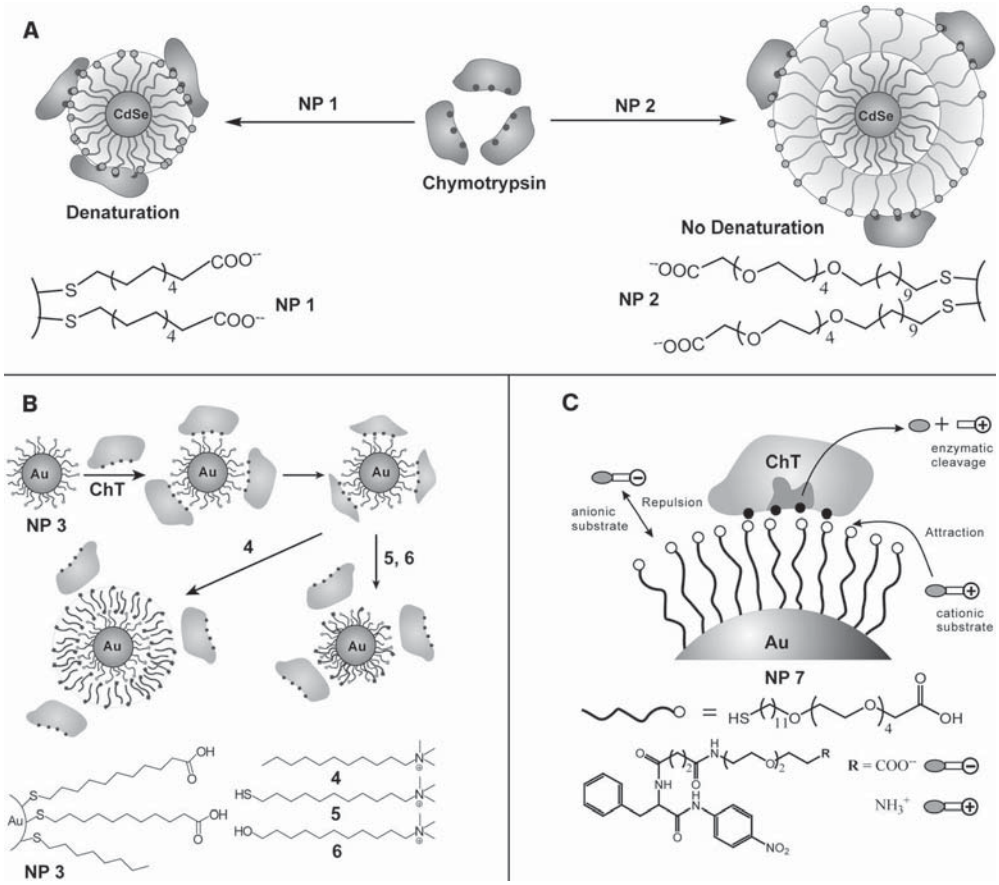

Fig. 4.4 Nanoparticle–protein complexation through complementary electrostatic interactions. (A) Structure and interaction of nanoparticles (NP) **1** and **2** with ChT. (B) ChT inhibition by NP **3** and its release mechanism from the surface of NP by addition of different surfactant (**4**–**6**). (C) Enzyme selectivity induced through substrate–monolayer interaction. Cationic substrates are attracted and hydrolyzed by the enzyme, but anionic substrates are repulsed by the monolayer.

binding with the NP, the active site of ChT is sterically hindered, resulting in inhibition of the protein. The second irreversible step was proposed to be the result of ChT burying its hydrophobic residues into the surface of the monolayer; this hypothesis is supported by the fact that binding without denaturation was observed for NP **2** [6].

As the nature of binding of the above NP–protein system is electrostatic in nature, surfactants can be used to restore enzyme activity. Subsequent studies conducted by Fischer et al. demonstrated that the addition of positively charged surfactants attenuated the monolayer charge (Figure 4.4B), releasing and reactivating

the protein [36]. Dynamic light scattering (DLS) measurements demonstrated that NP **3** had the same hydrodynamic radius before and after surfactant addition of thiol **5** and alcohol **6**. Upon incubation with alkyl surfactant **4**, however, a bilayer is formed. Taken together, these results indicate that NP–protein interactions can be tailored to facilitate applications such as protein refolding and intracellular protein release.

Substrate-selective behavior of the NP–protein complex was observed in further investigations with NP **7** and ChT. To elucidate the role of substrate charge on the selectivity, three SPNA derivatives, each with different charges, were synthesized and analyzed [38]. Enhanced chemoselectivity of ChT activity was observed when bound to the surface of NP **7** (Figure 4.4C). The NP–ChT complex showed very low activity towards negatively charged SPNA substrate, but ~50% and almost 100% relative activities of bound ChT to free ChT were observed towards the neutral SPNA substrate and the positively charged SPNA substrate, respectively. Considering the substrate charge together with the anionic nature of the NP monolayer, this chemoselectivity can be explained by a combination of steric hindrance and electrostatics.

The introduction of functionality onto the surface of NPs can enhance the affinity and specificity of this type of binding [39]. A report on diverse L-amino acid-terminated NPs demonstrates varying affinity towards ChT, supporting this hypothesis [39]. In addition to modulating the binding affinity to ChT, it was found that the hydrophilic side chains destabilize the structure of ChT through either competitive hydrogen-bonding or breakage of salt bridges, with denaturation much slower with hydrophobic amino acid side chains. Significantly, correlation between the hydrophobicity index of amino acid side chains and the binding affinity and denaturation rates was observed once again.

The above examples demonstrate effective binding with little or no specificity outside of electrostatic complementarity. Specificity of binding can be imparted via conjugation of the particle with biomolecular ligands. Zheng and Huang fabricated a biotin group or glutathione capped onto the surface of gold NPs protected by tri(ethylene glycol) thiols [40]. These authors were able to show specific binding of either streptavidin or glutathione-*S*-transferase to their respective capped NPs. In another example, Lin et al. fabricated a series of carbohydrate-capped gold NPs to explore their interaction with concanavalin A (Con A) [41]. It was shown that gold NPs functionalized with a mannose linker had a high affinity towards Con A, although the size of the NP and the mannose linker affected the interaction. By extending their investigation using mannose-conjugated gold NPs, the same authors were able to demonstrate mannose-specific adhesion FimH of type 1 pili in *Escherichia coli* [42].

Gold NPs have also been successfully tagged with covalent DNA–streptavidin conjugates. Building upon their earlier findings [27], Niemeyer et al. functionalized citrate-stabilized gold NPs with two different thiolated oligomer strands forming a difunctional DNA–gold NP [43] scheme allowing for the detection of protein antigens with enhanced sensitivity. One of the sequences on the NP is used to immobilize antibodies, while the other is used for signal amplification

by means of DNA-directed assembly of multiple layers of NPs. It is suggested that this system will allow for the rapid detection of proteins, at a low cost.

4.2 Methods

The coupling and functionalization of NPs with proteins is carried out using a variety of methods, including electrostatics, ligand recognition, metal-mediated complexation, chemisorption, and covalent binding through bifunctional linkers [44–46]. These methods are summarized in the following sections.

4.2.1 General Methods for Noncovalent Protein–NP Conjugation

The simplest way to form protein–NP conjugation is through electrostatic interactions. In this case, the proteins are electrostatically attracted to oppositely charged NPs, allowing absorption. The affinity and stability of these interactions

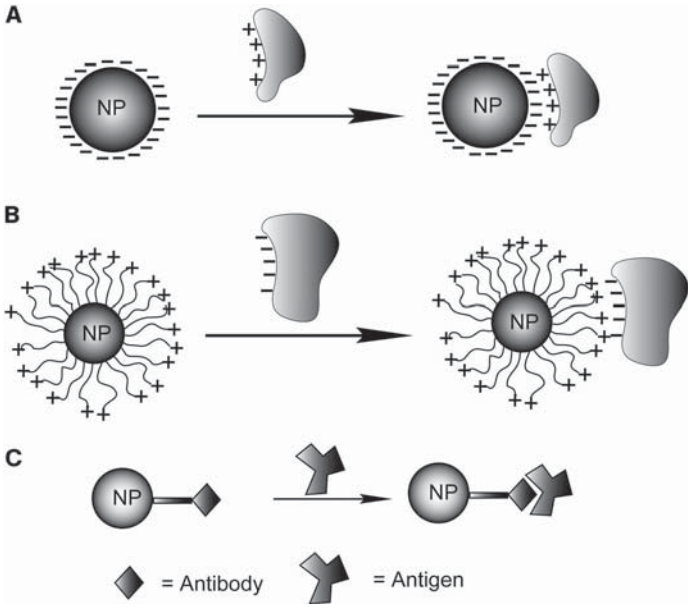

Fig. 4.5 Formation of the noncovalent biomolecule–nanoparticle (NP) conjugates. (A) Electrostatic interactions via direct absorption of the protein onto the NPs surface. (B) NP–protein conjugates formed by absorption of the protein onto the NPs monolayer. (C) Biomolecule–nanoparticle conjugates can also be assembled by antibody–antigen associations.

can be measured using various techniques such as isothermal titration calorimetry (ITC) and enzymatic activity assays. For examples of this interaction, see Section 4.1.2.

Nanoparticle–protein conjugates can also be formed through specific protein–ligand interactions. This recognition is achieved by functionalizing NPs with groups that provide specific affinity to certain proteins or oligonucleotides. For example, streptavidin-functionalized gold NPs have been used for the binding with biotinylated proteins [7], while NPs functionalized with antibodies have been used for affinity binding with their respective antigen [47]. It has been observed that the antigen–antibody binding constant on NPs is higher than in free systems [48].

Metal-mediated complexation provides a versatile method for the creation of noncovalent protein–NP conjugates. Nickel and cobalt nitrilotriacetic acid (NTA) complexes have a high affinity to histidine-tagged proteins, without nonspecific binding via metal chelation [49]. In one study, Xu et al. affixed a nickel–NTA complex onto the iron oxide shell of magnetic NPs with a dopamine anchor, and further used them to target the histidine-tagged proteins [49]. The protein–NP complex can be separated magnetically and then released by the addition of EDTA (Figure 4.6).

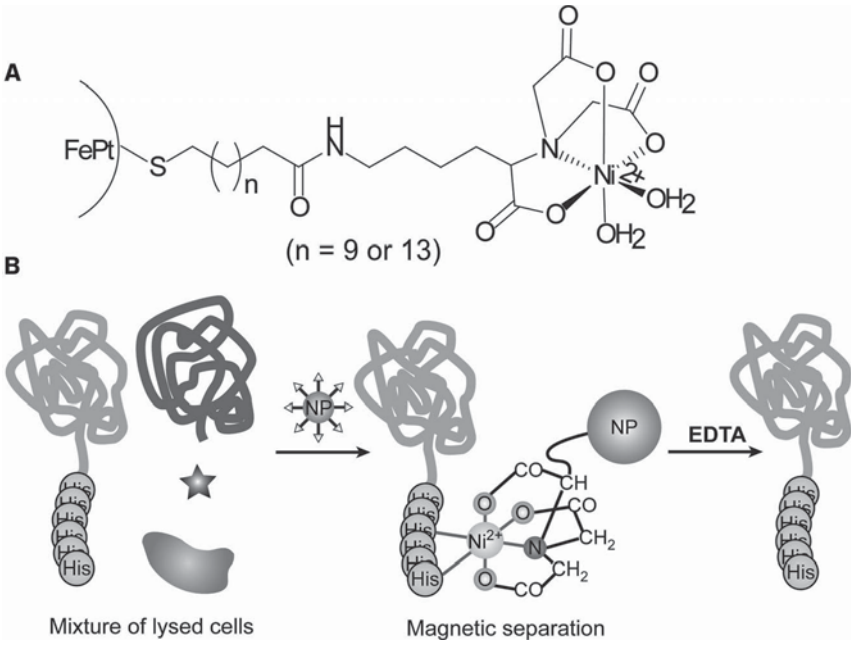

Fig. 4.6 (A) Structure of nanoparticles targeting histidine-tagged proteins. (B) Selective binding to histidine-tagged proteins and purification by magnetic separation.

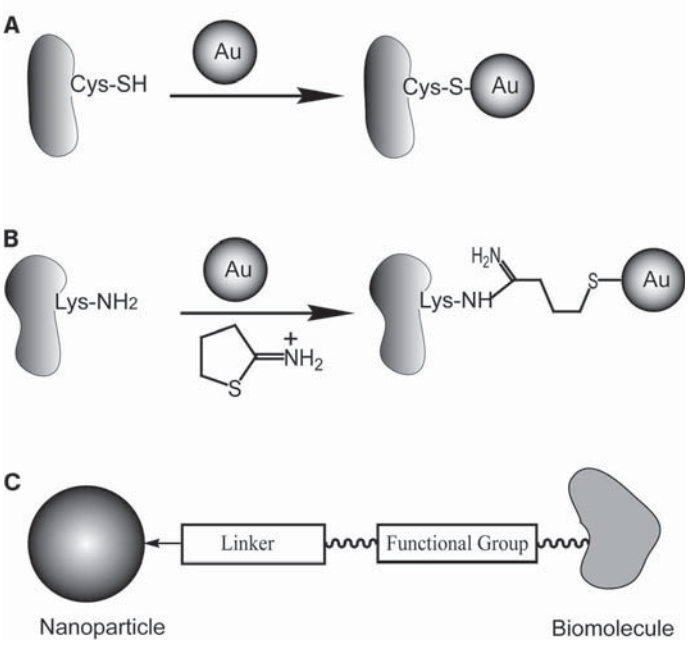

Fig. 4.7 Formation of covalent biomolecule–nanoparticle (NP) conjugates. (A) NP–protein conjugates formed by adsorption of NPs onto native thiol groups of the protein. (B) Conjugation using proteins chemically functionalized with an external thiol residue. (C) Schematic representation of covalent binding of protein through a bifunctional linker.

4.2.2 General Methods for Covalent Protein–NP Conjugation

The problems of instability and inactivation of proteins can be overcome when they are covalently conjugated to the surface of metal NPs [50]. This has been achieved in two ways: (i) chemisorption via thiol derivatives; and (ii) through the use of bifunctional linkers. The chemisorption of proteins onto the surface of NPs (which usually contain a core of Au, ZnS, CdS and CdSe/ZnS) can proceed through cysteine residues that are present in the protein (e.g., oligopeptide, serum albumin) [51], or chemically in the presence of 2-iminothiolane (Traut's reagent) [51, 52].

Bifunctional linkers are highly diverse and offer versatile covalent conjugation of proteins onto NPs. Thiols, disulfides or phosphine ligands are often used as anchor groups to bind Au, Ag, CdS, and CdSe NPs for these bifunctional linkers. Weakly absorbed molecules are displaced by the above anchor groups to further stabilize the NPs, or they are added during NP synthesis. Biological compounds

are often covalently linked to NPs using bifunctional linkers via carbodiimide-mediated amidation and esterfication coupling with thiol groups [24].

4.3 Outlook

During the past few years, the development and application of protein–NP conjugates has burgeoned, mainly because the useful characteristics of NPs – such as ease of functionalization, tunable core size, and variety of core materials available – make these systems excellent scaffolds for the conjugation of proteins. There are numerous opportunities for expanding the utility of these systems. For example, in the area of noncovalent protein–particle interactions there are many unanswered questions regarding the nature of the interface, and a better understanding of this issue will provide access to a range of new diagnostics and therapeutics.

In another potential direction, proteins and NPs can self-assemble into highly organized superstructures, offering opportunities for the creation of novel materials and devices [53, 54]. Thus, an understanding of protein–NP conjugates is significant both for fundamental research and for biotechnological and nanotechnological approaches. In turn such knowledge should lead to the creation of advanced materials that can be applied to sensing, catalysis, signal transduction, transport, and other applications associated with the biomedical and/or biomaterial sciences.

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