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Engineering the Nanoparticle-Protein Interface: Applications and Possibilities

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Abstract

Covalent and non-covalent conjugation of proteins to nanoparticles provides access to functional hybrid systems with applications in biotechnology, medicine, and catalysis. The creation of effective conjugates requires the retention of protein structure and function, a challenging task. In this review we discuss successes, challenges and opportunities in the area of protein-nanoparticle bioconjugation.

Introduction

Nanoparticles (NPs) provide a platform for integrating biology and synthetic materials. A wide variety of core materials can be used for fabricating NPs, including metals, metal oxides, semiconductors, and core-shell hybrids [1]. With these systems, the composition of the core material dictates the physical properties of the nanoparticle, including optical and magnetic behavior. Taken together, the diversity of available core materials and properties make nanoparticles pragmatic tools for numerous applications [2••].

The organic monolayers used to passivate nanoparticles play a key role in their biological applications. Functionally, the solubility and stability of the nanoparticles are dictated by the surface properties of the particle. More importantly, the biointerfacial interactions of nanoparticles are dictated by the chemical and topological nature of the NP coverage. A common motif used for NP stabilization/solublization and biocompatiblization (Figure 1) features a ligand shell incorporating a hydrophobic interior (for micelle-like stabilization of the shell) and an oligo(ethylene glycol) spacer to minimize protein denaturation.

Two fundamentally different approaches can be used to conjugate proteins to nanoparticles. The first approach uses direct covalent linkage of the protein to the particle surface. The second method uses non-covalent interactions between the particle and protein to generate supramolecular assemblies. Both of these approaches have strengths and limitations, and hence have a place in the bionanotechnology “toolkit”. This review covers only a small subset of the recent literature in both covalent and non-covalent conjugation of proteins to nanoparticles, highlighting both advances and challenges in the area.

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Covalent Protein-Nanoparticle Conjugates

Covalent attachment of proteins to NPs provides conjugates that are stable toward dissociation, an important issue for many applications. In particular, the stability and irreversibility of covalent protein-NP conjugates makes this strategy useful for applications in complex biological media with other interfering species. Covalently conjugating proteins to the NP surface also provides control over protein reactivity and NP aggregation.

One strategy for attachment relies on direct interactions between multiple protein sidechains, e.g. lysine and glutamate, and the NP core. This approach has been used by Rotello *et al.* to generate Fe_3O_4 particles that were stable in serum for applications in hyperthermic therapy [3]. A common issue with this approach, however, is the loss of protein structure due to multivalent interactions between numerous residues of protein and the NP surface [4]. Conditions that minimize non-specific adsorption and optimize protein structure and activity are desirable for efficient direct conjugation onto NPs. Efforts have been directed to reduce this effect through appropriate NP co-functionalization and through NP loading [5•]. A potentially less disruptive approach is to use a monovalent attachment, such as a single cysteine. However, substantial denaturation can likewise occur with this approach [6••].

The use of engineered ligands for conjugating proteins to NPs has met with much greater success in terms of retaining protein structure (Figure 2). This type of attachment has traditionally been accomplished using standard amine-carboxylate coupling methods [7]. More recently, the ubiquitous alkyne-azide Huisgen “click” reaction has been used to attach proteins to NPs. In the presence of a catalyst, “click” reactions can proceed with high yield and can also allow the site-specific conjugation of the azide or alkyne tagged proteins to the NP partners. A variety of proteins have been attached in functional form using this method, including lipase [8••], horseradish peroxidase [9], and luciferase [10]. Ligand design is crucial in this strategy, with each of these studies employing biocompatible oligo(ethylene glycol) (OEG) spacers between protein and NP. The OEG chains reduce nonspecific interactions as well as provide additional degrees of freedom for the terminal functional groups.

Obtaining both site specificity and protein activity remains a challenge in NP conjugation. One particularly clever approach to this end was developed by Rao *et al.*, who conjugated quantum dots (QDs) to “HaloTag” proteins based on a modified dehalogenase that reacts specifically and irreversibly with appropriate halogenated substrates (Figure 3a). The HaloTag ligand was designed with an amino ethylene glycol group to orient the ligand away from the surface of the QD and also reduce the steric hindrance between the HaloTag and QD. The versatility of the approach was provided through the use of fusion proteins, allowing the facile attachment of luciferase functionality to the particle for biolouminescence resonance energy transfer (BRET) applications [11•].

Another attractive approach is intein-mediated site specific conjugation of nanomaterials to proteins. This approach allows the covalent conjugation of any nanostructure to proteins with retained structure and activity, and the reaction is specific in the cellular environment as well. Skourides *et al*. genetically tagged EGFP to pleckstrin-homology (PH) domain with the N-terminal half of a split intein and conjugated the C-terminal half to QDs (Figure 3b). *In vivo* intein-splicing in *Xenopus* embryos showed a fully functional QD-PH conjugate that could be tracked in real time [12].

Non-Covalent Protein-Nanoparticle Conjugation

On a fundamental level, understanding the supramolecular interactions between proteins and nanoparticles is central to the applications of NPs *in vivo*. A number of studies have

explored the kinetics and thermodynamics of binding between individual proteins and NPs [13], demonstrating that properly functionalized NPs interact with proteins in analogous fashion to protein-protein interactions [14••]. The biophysical properties, such as binding affinity, residence time, binding cooperativity of NP and the common serum proteins, have been quantitatively characterized in buffer medium to gain fundamental understanding of the behavior of proteins on the NP surfaces [15,16]. More recently, efforts have been directed towards understanding the interactions of NPs with complex protein mixtures, e.g. serum [17••,18], an important issue for drug delivery and sensing (*vide infra*). An evolution from a loosely bound to irreversibly attached protein "corona" around the NPs over time has been observed in cell culture media containing serum, a process that potentially correlates the cellular uptake and other biological processes with NP-protein complexes [19,20].

Pragmatically, non-covalent conjugation of proteins to NPs provides a complementary strategy to covalent attachment, enabling applications in sensing and delivery. As with covalent conjugation, denaturation of the protein on the surface is quite rapid [21] unless appropriately engineered ligands (almost universally OEG-based) are used for particle functionalization [22,23]. The simplest way to produce non-covalent NP-protein conjugates is through complementary electrostatic interactions between the nanoparticle and the protein, a particularly useful approach when specificity is not required [22]. To overcome the inherent non-specificity of this approach, the NP surface has been strategically modified by varying its charge and hydrophobicity, enabling regiospecific interaction [24]. Metal-mediated interactions provide a useful alternative that imparts selectivity to the NP-protein conjugation process. A particularly facile method is through nickel-mediated interactions with His-tagged proteins [25,26], a technique that also provides effective control of NP-protein stoichiometry [27]. In a particularly elegant implementation of His-tag binding, Mattoussi, Medintz *et al*. used the affinity of hexa-His peptides to directly conjugate fluorescent proteins (FPs) to QD surfaces to create caspase sensors (Figure 4) [28]. In this system the His-tag and the peptide linker were long enough to minimize the denaturation observed above with cysteine-based immobilization.

Specificity of binding can be imparted via conjugation of the particle with biomolecular ligands, exploiting the ease of functionalization inherent to many NP materials. The biotin-(strept)avidin interaction is widely used for the conjugation of NP systems [29]; Zheng and Huang fabricated a biotin group or glutathione capped onto the surface of gold nanoparticles protected by tri(ethylene glycol) thiols [30]. They were able to show specific binding of either streptavidin or glutathione-*S*-transferase to their respective capped nanoparticles. The glutathione approach has since been applied to the creation of glutathione-*S*-tranferase fusion proteins [31]. Another popular conjugation strategy is through carbohydrate-lectin interactions [32], with a particularly efficient method being that of Yan *et al*. using the photochemical attachment of unmodified sugars to the NP surface [33]. The conjugation of carbohydrate-coated magnetic particles to proteins by means of the carbohydrate-lectin specificity provides a powerful tool for rapid detection of pathogens along with utility in separation of different bacterial strains [34].

While most NP-protein conjugates are intended for applications in solution, nanocomposite assemblies provide access to systems with interesting and potentially useful properties. Initial studies of protein-NP composite assemblies focused on the use of proteins as building blocks for control of interparticle spacing and morphology [35,36], exploiting the size, charge and shape of the protein to control composite structure [37•,38]. Building upon the structural control provided by these composites, electronically-active multilayer systems have been developed using biotin-streptavidin assembly of gold nanoparticles [39]. In recent studies the magnetic properties of a protein (ferritin) have been integrated with those of FePt nanoparticles through electrostatic assembly to generate 3-dimensional assemblies featuring

novel magnetic behavior [40]. NP-protein assembly has also been explored at 2-D oil-water interfaces. Through appropriate choice of OEG-containing ligand, β-galactosidase (-gal, pI 4.6) has been assembled with cationic nanoparticles on oil-in-water emulsion interfaces, with high retention (>70%) of -gal activity [41].

The reversible nature of non-covalent NP-protein assemblies enables their application in areas including sensing and delivery. On the sensing front, non-covalent gold NP-green fluorescent protein (GFP) complexes have been employed in an array-based "chemical nose" fashion to identify five of the more abundant human serum proteins (i.e. human serum albumin, immunoglobulin G, transferrin, fibrinogen and – antitrypsin, Figure 5a) in human serum (overall protein concentration *~1 mM*), obtaining a limit of detection of 500 nM. [42••]. In more recent studies an analogous approach has been used for cell surface sensing, with NP-GFP complexes able to differentiate between healthy and cancerous isogenic cells [43]. An array-based approach using magnetic NPs with magnetic resonance imaging (MRI) transduction has been likewise used to identify cell types and states (Figure 5c) based on carbohydrate-lectin selectivity [44•].

Protein delivery presents a second area where reversible interactions would be desirable, ultimately providing the delivery of free native protein to the cell. Recently, the intracellular delivery of the large protein β-gal (465 kD) was demonstrated using cationic nanoparticles having a short peptide conjugated to a tetra(ethylene glycol) unit (Figure 6). Significantly, the enzymatic activity of the β-gal was retained in the cell, and the β-gal escaped from the endosome and into the cytosol [45•]. Non-covalent conjugation has been employed in delivering proteins by transmucosal and oral pathways. Amino acid capped gold NPs were used to carry insulin in active form with sustained release profile [46]. There are certain advantages of increased selectivity of non-covalent approaches in delivery applications as well. Specific carbohydrate-lectin conjugation enables uptake via receptor-mediated endocytosis. The preferential uptake of sugar-capped PEGylated QDs by specific asialo-gycoprotein has been demonstrated to be effective for *in vivo* targeting and imaging [47].

Summary and Outlook

Substantial progress has been made in recent years on the formation of both covalent and non-covalent protein-NP conjugates. Perhaps the most important lesson learned is that the protein-NP interface is crucial, and that the most straightforward way to provide stability is through OEG-containing ligands. Efforts to fine-tune these interfaces are ongoing, as there is still no completely reliable means of conjugating proteins to NPs. An additional challenge is provided by systems where intimate contact between protein and NP is desirable, e.g. optical and electronic systems. As seen above, direct conjugation of NPs to proteins remains an open challenge. Finally, as these issues become resolved, the potential for synergistic engineering of NPs and proteins presents the possibility for new bionanoconjugates featuring behavior unique from those of either component.

Acknowledgments

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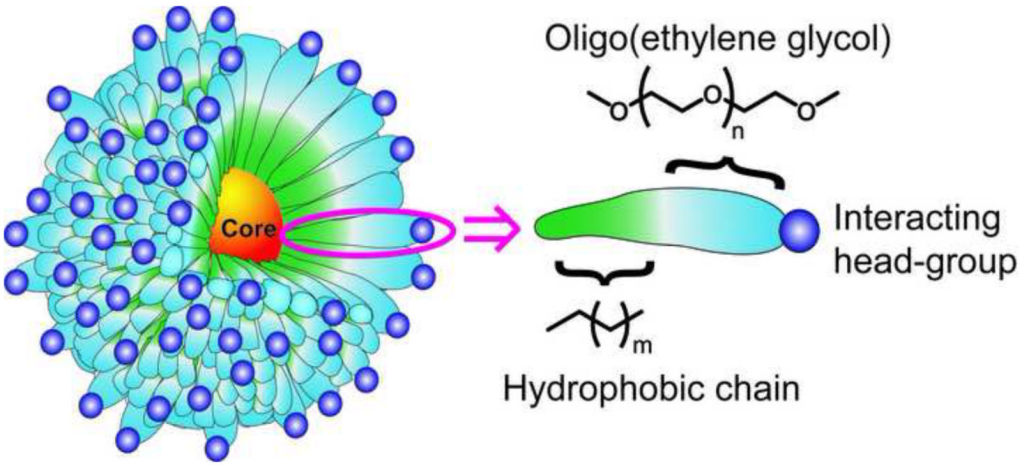

Figure 1. Structure of common nanoparticles

A general schematic of surface ligands on most of the common NPs used for interaction with biological systems. The oligo(ethylene glycol) linker helps to retain the structure and function of proteins after conjugation.

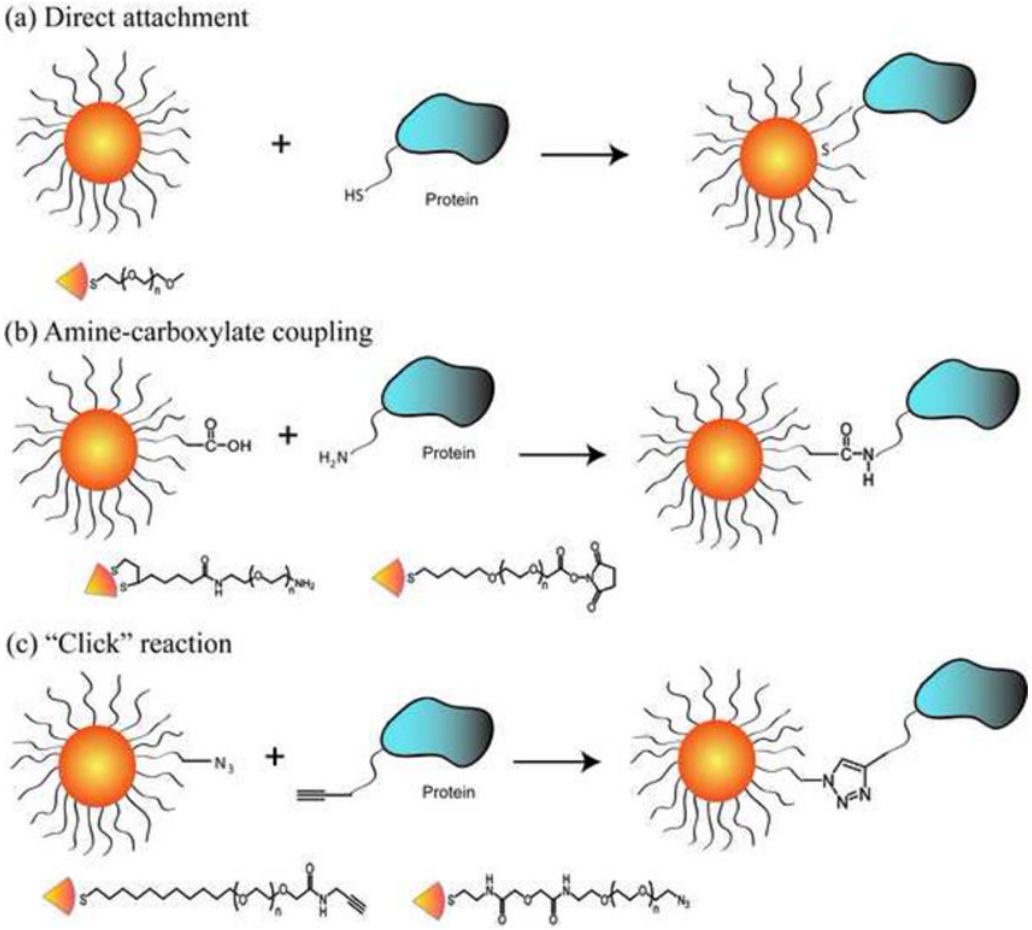

Figure 2. Nanoparticle-protein complexation through covalent conjugation

(a) The direct attachment of proteins to NPs using a thiol group on the protein surface. (b) Amine-carboxylate coupling using carboxylate-presenting NPs and amine groups on protein surface. (c) "Click" reaction using azide tagged NP and alkyne tagged protein. Representative ligands used to modify the NP surface are presented under each strategy.

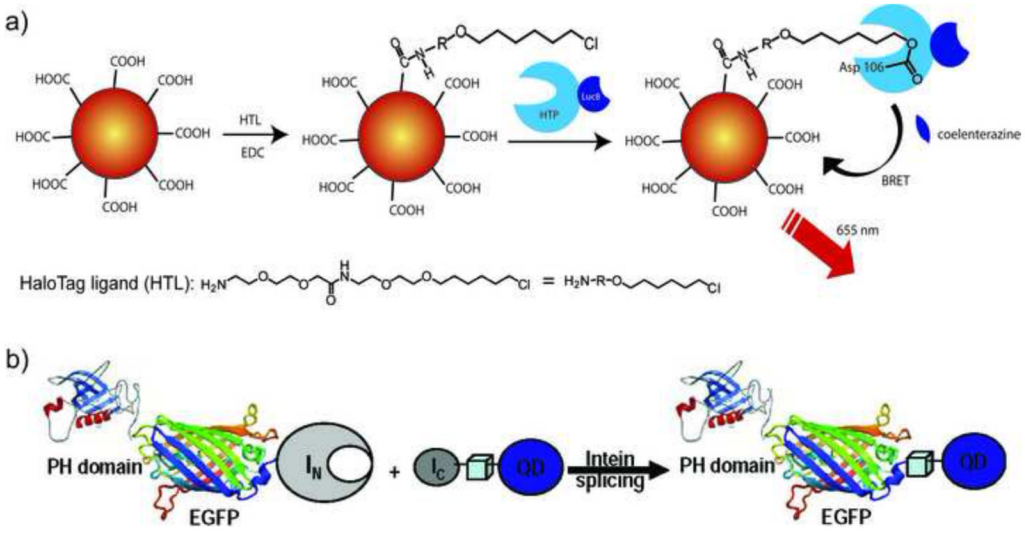

Figure 3. Site-specific conjugation of active proteins

a) Conjugation of a HaloTag protein to QDs mediated by the appropriate alkyl chloride ligand. b) Schematic representation of site-specific intein-mediated conjugation of QD to target protein (reproduced with permission from ref [12]).

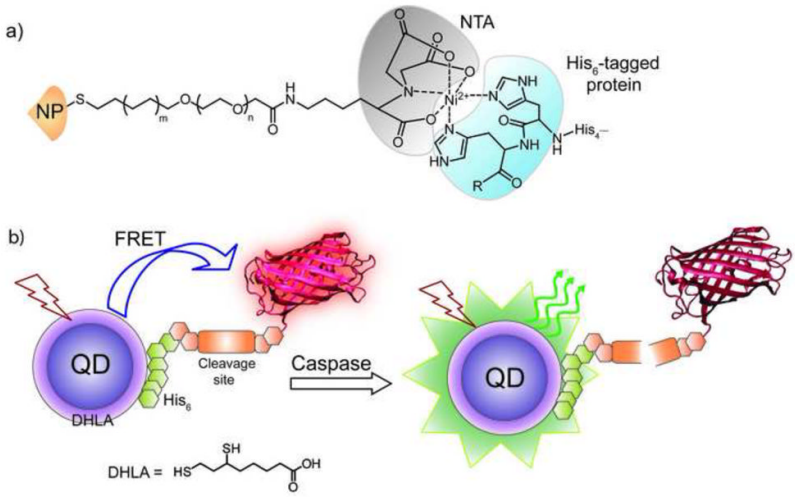

Figure 4. Metal-mediated non-covalent conjugation and its application

a) Depiction of metal-mediated high affinity conjugation of hexa-His tagged protein to nitrilotriacetic acid (NTA) functionalized NP. b) Schematic of the QD-fluorescent protein sensor constructed through non-covalent conjugation between DHLA coated QD and mCherry protein with an N-terminal linker expressing the caspase 3 cleavage site and a His6 sequence. The specific cleavage of the linker by caspase 3 removes FRET to enable protein sensing.

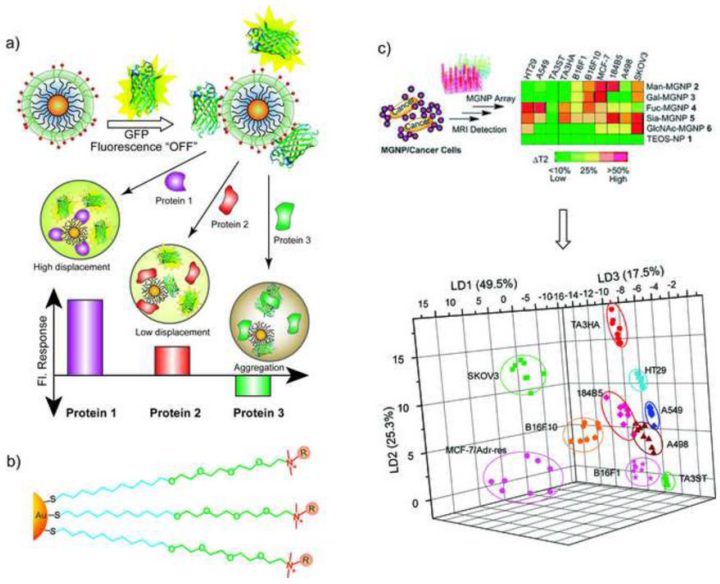

Figure 5. GFP-NP "chemical nose" sensor

a) Schematic illustration showing the competitive binding between proteins and quenched gold NP-GFP complexes, whose aggregation leads to fluorescence "turn on" or further quenching using a library of cationic nanoparticles. b) Chemical structure of cationic gold NPs. c) Percentage changes of *T*2 relaxation time (% *T*2) obtained upon incubating MGNPs with 10 cell lines (10^5 cells/mL) and the LDA plots for the first three LDs of *T*2 patterns (reproduced with permission from [44•].

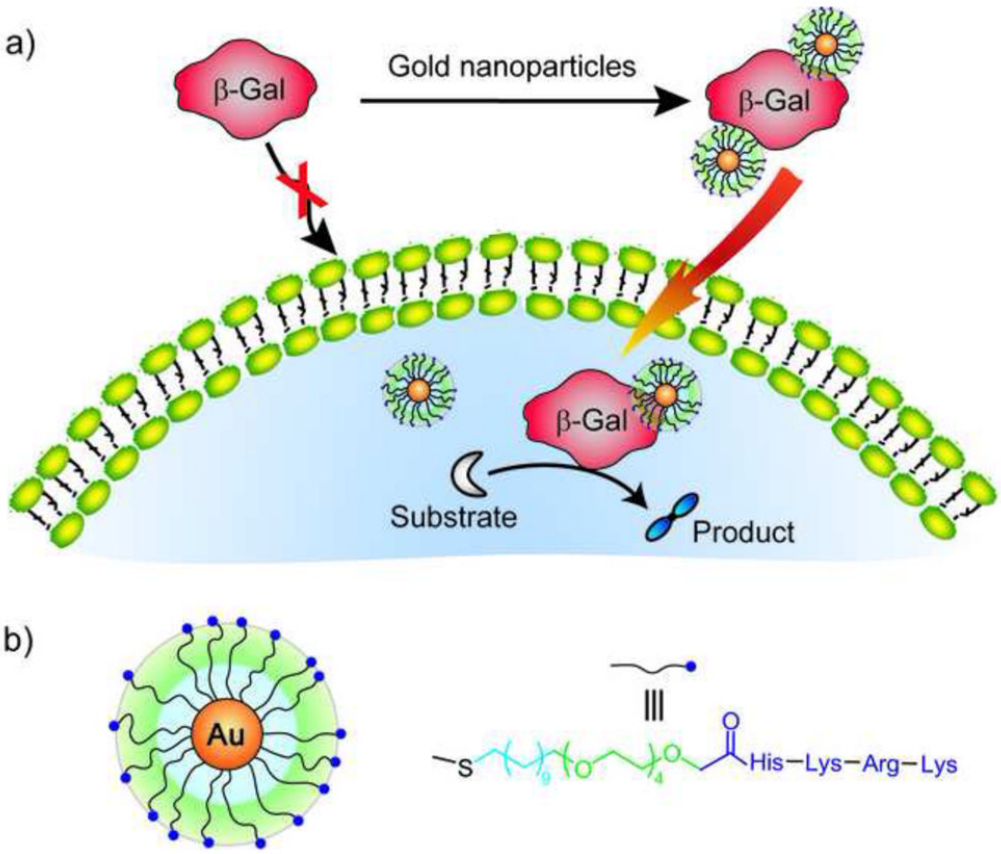

Figure 6. Delivery of protein cargo via non-covalent conjugation

a) Schematic representation of the complexation between cationic NP and anionic protein (β-Gal), leading to efficient permeation through the cell membrane compared to uncomplexed proteins. b) Structure of the cationic nanoparticle and the surface ligands.