R E V I E W

Overview of the main methods used to combine proteins with nanosystems: absorption, bioconjugation, and encapsulation

Mariagrazia Di Marco[1]
Shaharum Shamsuddin[2]
Khairunisak Abdul Razak[3]
Azlan Abdul Aziz[4]
Corinne Devaux[1]
Elsa Borghi[1]
Laurent Levy[1]
Claudia Sadun[5]

[1]Nanobiotix, Paris, France; [2]School of Health Sciences, Health Campus Universiti Sains Malaysia, Kelantan, Malaysia; [3]School of Materials and Mineral Resources Engineering, Engineering Campus, [4]School of Physics, Universiti Sains Malaysia, Penang, Malaysia; [5]Department of Chemistry, Sapienza, University of Rome, Rome, Italy

Abstract: The latest development of protein engineering allows the production of proteins having desired properties and large potential markets, but the clinical advances of therapeutical proteins are still limited by their fragility. Nanotechnology could provide optimal vectors able to protect from degradation therapeutical biomolecules such as proteins, enzymes or specific polypeptides. On the other hand, some proteins can be also used as active ligands to help nanoparticles loaded with chemotherapeutic or other drugs to reach particular sites in the body. The aim of this review is to provide an overall picture of the general aspects of the most successful approaches used to combine proteins with nanosystems. This combination is mainly achieved by absorption, bioconjugation and encapsulation. Interactions of nanoparticles with biomolecules and caveats related to protein denaturation are also pointed out. A clear understanding of nanoparticle-protein interactions could make possible the design of precise and versatile hybrid nanosystems. This could further allow control of their pharmacokinetics as well as activity, and safety.

Keywords: nanoparticles, drug delivery, proteins, polypeptides, absorption, bioconjugation, encapsulation

Introduction and background

Nanotechnology has the potential to create new materials and devices with wide-ranging applications in medicine,[1–3] agriculture,[4] and energy or electronic production.[5,6]

The size-dependent optical, electrical, and magnetic properties of nanoparticles make nanotechnology a promising candidate for bioapplications such as in vivo imaging, sensing, catalysis, therapeutics, and cell targeting.[7–9]

Based on different approaches, physicians, physicists, chemists, biologists as well as bioengineers share a common interest to treat severe diseases through nanotechnology. Theoretically, nanoparticles can be tailored to reach the right target at the right time. Pathogenic agents such as viruses or bacteria, and cancer cells could be precisely targeted and affected without disturbing healthy tissues. This crucial task has been one of the highest priorities for the past 10 years.

Among several medical applications, nanoparticles could be largely employed as carriers of therapeutical biomolecules.[10] The combination of nanoparticles with biomolecules such as proteins or specific polypeptides offers opportunities for the design of very precise and versatile hybrid systems mostly useful in helping to fight cancer and immunological diseases.[11–13]

There are more than 50,000 different proteins in the human body.[14] Proteins are present in complex biological processes such as muscle contraction, immune protection and transmission of nerve impulses. All enzymes and most hormones are proteins;

Correspondence: Maria Di Marco
Nanobiotix, 60 rue de Wattignies, 75012 Paris, France
Tel +33 I 40 26 06 26
Fax +33 I 40 26 04 44
Email maria.dimarco@nanobiotix.com

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hence, proteins are vital sources for the body's metabolism and their lack can result in several diseases (eg, lack of insulin in type 1 diabetes).

The latest development of protein engineering allows the production of proteins having desired properties and great potential market;[15] however, protein fragility is one of the major drawbacks for their utilization. Consequently, the discovery and development of new therapeutic proteins have also created new opportunities for drug-delivery systems involving the design of appropriate nanocarriers such as liposomes, micro-, and nanoparticles.[16–19]

The oral route is a comfortable way for drug administration especially when repeated or routine dosing is necessary.[20] Nevertheless, the development of oral carriers for many proteins remains a challenge due to the fact that bioavailability of these molecules is limited.[21] Indeed, most polypeptides and proteins are quickly degraded in the gastrointestinal (GI) tract by proteolytic enzymes.[22,23] Moreover, the intestinal epithelium is a major barrier to the absorption of hydrophilic drugs that cannot easily diffuse across the cells through the lipid-bilayer cell membranes.

Numerous investigations have shown that nanocarriers can improve the stability of therapeutic agents against enzymatic degradation and achieve desired therapeutic levels in target tissues for the required duration. Nanoparticle drug-delivery systems (nano-DDS) could permit an optimal pharmacokinetic profile and meet specific needs. For example, nanoparticles as oral protein carriers could protect the active ingredient in the GI tract and/or prolong the residence time of its contents on the mucous membrane. After administration, nano-DDS can be taken up and transported across the intestinal mucosa by enterocytes or M cells in the Peyer's patches because of their small size.[24]

Several articles and reviews on the use of nanoparticles or microparticles for oral drug delivery are dedicated to insulin.[25–28] In 1980, Couvreur and colleagues performed the first study on hypoglycemic effects after oral and parenteral administration of insulin-loaded nanoparticles to diabetic rats.[26]

Proteins are also difficult to be delivered via topical or transdermal routes and therefore their parenteral administration is still largely applied.

Besides the general complications of the parenteral route (such as local infections, thrombophlebitis, rarely tissue necrosis), small proteins (<30 kD) are quickly filtered out by the kidneys. Without an appropriate drug carrier, proteins can also cause unwanted allergic reactions, can be targeted by the immune system and be rapidly degraded.

For example, rapid clearance from the circulation can be an explanation of the modest in vivo antitumor effects of the antiangiogenic RGD (Arg–Gly–Asp) peptides.[29]

Bone morphogenetic proteins (BMPs) induce bone formation after implantation; their orthopedic application in repair of bone fractures and defects is focused in local device and spinal fusion procedures. The problem of BMP is its rapid diffusion from the administration site when applied without a carrier. Currently, one of the most effective and biocompatible carriers for BMP delivery is the type I bovine absorbable collagen sponge (ACS). However the BMP release rate is difficult to control and to maintain constant for long term because of a high initial burst release of this device. The use of new nanotechnologies could maintain BMPs at the treatment site preventing extraneous bond formation and optimizing the drug release.[30]

Synthetic antigenic peptides are specific sections or a variant sequence of viral/bacterial proteins able to induce an immune response in the host. These small peptides are very useful in the vaccine development compared to the use of the whole protein/antigen. To date, several antigenic peptides have been identified but delivery problems still limit their application. Even in this case, the design of an effective delivery system is an important challenge in nanotechnology field.

An efficient protein carrier should solve different problems allowing the access to the target sites, at the right time and for the proper duration. In order to choose the best nanosystem, five factors must be considered: nature of the protein, route of administration, pattern of drug release, method of delivery and formulation.[31,32]

Proteins such as albumin, antibody, growth factors, transferrin, cytokines and low-density lipoprotein can be also used as active ligands to help nanoparticles loaded with chemotherapeutic or other drugs to reach particular sites in the body.[33–35] Abraxane® (Abraxis Bioscience, Los Angeles, CA; AstraZeneca, Wilmington, DE), albumin–bound nanoparticle of paclitaxel, is an example of US Food and Drug Administration (FDA)-approved protein-based active ligand for the treatment of metastatic breast cancer.[35]

Monoclonal antibodies (mAb) has been widely used as bioprobes in diagnostics as well as delivery drug to specific tumors.[36,37] OX26 mAb can help nanoparticles to cross the blood–brain barrier and diffuse in the brain tissue in order to transport drugs (eg, the anticaptase peptide, Z-DEVD-FMK) for the treatment of neurological and psychiatric disorders.[38] Nanoparticles can be also coated with mAb for cell surface antigen and used as a bait for detection or isolation of various kind of cells including lymphocyte and tumor cells.[39,40]

Despite many potential applications, the interaction of nanoparticles with biomolecules and living systems is still not fully understood.[41–50] Continuous study on this subject contributes to the current knowledge and stimulates the development of novel therapies such as nonviral vectors for gene therapies or as precise anticancer molecules.[37,51–53] Furthermore, by clarifying these aspects, specific protein-based nanovectors with optimized functions could be developed. This review aims to provide an overall picture on current progress and general aspect of the most successful approaches used to combine proteins with nanosystems. This combination is mainly achieved by absorption, bioconjugation or strong binding via avidin–biotin technology and encapsulation. These methods and the correlated problems of protein denaturation are discussed in turn in this review.

Nanoparticle–protein absorption, bioconjugation, and encapsulation
Absorption of proteins on nanoparticles surface

The interaction between biological and synthetic materials impacts on a vast range of medical issues from implants to pharmacokinetic aspects. The study of the materials biocompatibility starts, therefore, with the analysis of protein absorption on surfaces.[54] Synthetic materials for biomedical applications are immediately covered by proteins when put in contact with a biological environment.[55,56] After protein binding, nanoparticles are quickly cleared by the mononuclear phagocytic system (MPS), also known as the reticuloendothelial system (RES).[57,58] These macrophages, which are typically Kupffer cells of the liver, cannot directly identify the nanoparticles themselves, but rather recognize specific opsonin proteins bound to the surface of the particles.[59]

The interaction between proteins and nanoparticles surface leads to the formation of proteins "corona" around nanoparticles that largely defines their biological identity as well their potential toxicity.[49,50,58,60–64] Recently, Lynch and Dawson postulated the importance of the "protein corona" as the vehicle and the biological identity of a nanoparticle for its transport through cell membranes.[60]

The nanoparticle surface is immediately occupied by proteins with high concentrations and high association rate constants and successively by proteins having lower concentrations but a higher affinity.[47] Competitive absorption of proteins is influenced by several factors such as electrostatic interactions, protein stability, and kinetic parameters.[65]

As the protein corona could affect the nanoparticle behavior, including its biological effect, the nanoparticle could also have an effect on the protein behavior. Some nanoparticles seem able to promote the protein assembly into amyloid fibrils in vitro by assisting the nucleation process.[66] Bellezza and colleagues found that nanoparticles affect the morphology of the myoglobin absorbed onto phosphate-grafted zirconia nanoparticles, inducing prefibrillar-like aggregates.[67] This phenomenon could have important implications for medical application of nanoparticles because the self-assembly of a variety of proteins and peptides is known to be the cause of human amyloid diseases where fibrous protein aggregates are formed, resulting in amyloid plaque deposition in the extracellular tissues.[68–74] Moreover, fibrillar structure seems to be related to heavy human disorders such as Alzheimer's disease, Parkinson's disease, and spongiform encephalopathies.

However this action seems strictly related to the type of nanosystems chosen. For instance, there are nanosystems such as C60 hydrated fullerenes that can relax fibrillar structures.[60,68] Certainly, the control of the protein absorption on nanoparticle surfaces is an important issue to control their fate in biological systems.[75]

In order to prevent or control the opsonization, several methods of disguising nanoparticles have been developed. In these methods, generally, nanoparticles are coated with biocompatible polymers that have the double function of preventing their aggregation and retarding the protein absorption.[57,76,77]

A common strategy to improve blood compatibility and to increase the blood circulation half-life of the nanoparticles is the construction of a protein-coated surface resistant to the absorption of the other opsonines.[78] A thin layer of protein appears to minimize adhesion and aggregation of nanoparticles, avoiding subsequent macrophage recognition or, in the worst case, a thrombus formation. Moreover, it is possible to properly tune the cells uptake of the nanoparticles using specific proteins.[35]

Proteins are mainly amphiphatic molecules that typically adhere to the surface of a biomaterial in a nonspecific way. In various cases, this nonspecific adhesion is sufficient to artificially immobilize proteins on the nanoparticles surface, and no surface modification is necessary.

Despite of the large number of studies, the absorption of a protein on whatever the solid surface is still a complex and not well understood process.[60,79–85] In the case of nanoparticles, size and radius of curvature become significant when compared to the protein size resulting in new interactions not shown with the bulk materials.[47]

The high hydrophobicity of many proteins seems to play an important role in their absorption on the nanoparticles surface.[86] Several models of protein absorption on surfaces identify two main steps in the process. The first step could involve the arrival of the protein at the interface, through a diffusion process following the Brownian law of motion, and its further collision with the solid surface. Depending on the balance of the energetic interaction, proteins can remain on the solid surface or return to solution. If the protein has been absorbed, the second step could lead to conformational changes (because of van der Waals interactions), surface charge, protein dipole moment, and protein size or solution ionic strength.[84,85,87–90] This second step often involves irreversible changes in the protein structure up to denaturation.[91–93]

Proteins can be divided in two groups: hard and soft proteins. The first group includes proteins with high internal stability, while proteins in the second group have a low internal stability. Soft proteins seem to be able to change their conformation better than the hard ones. This characteristic results in a gain in conformational entropy when absorbed on solid surfaces, improving the efficacy of the absorption process when compared to the hard proteins. On the other hand, it seems that some degree of denaturation upon absorption is more probable for soft proteins than for the hard ones, especially on hydrophobic surfaces.[84,94–97]

During the artificial absorption of protein to nanoparticles surface, the use of a large excess of the target material could allow the retention of sufficient biological activity and native epitopes, even if some proteins are denatured. However, problems associated with denaturation of the protein over time, or its exchange with other proteins in solution, could make this strategy satisfactory only for short-term uses.

The success of an absorption strategy to deliver drug or therapeutic proteins using protein-based nanoparticles as a carrier can be influenced by several factors such as the type of nanoparticles, delivery route and the nature of proteins to be absorbed. For this reason, nanoparticle–protein affinity needs to be intensely examined case-by-case.

The knowledge of how the protein-based nanoparticles interact with other proteins present in the blood is fundamental for the understanding of their biological and toxicological properties.[77] Many methods based on established techniques could be applied such as size-exclusion chromatography, isothermal titration calorimetry, surface plasmon resonance, atomic force microscopy, differential scanning calorimeter, and circular dichroisim (CD) spectroscopy.[47,67]

Even if several existing characterization methods for measuring the nature and the amount of absorbed protein on solid surfaces could be applied to nanoparticle systems,[96] the development of new physical and biophysical methods may be necessary to fully understand the relationship between proteins and nanomaterials.

Bioconjugation of proteins on nanoparticle surfaces

Conjugation of biomolecules on nanoparticle surfaces has attracted widespread interest in biotechnology and medicine.[7,98–100] The conjugation of specific proteins with nanoparticles has introduced a new advancement in molecular and cellular biology which has further led to a vast improvement of in vivo gene delivery, clinical diagnosis, medical/cancer imaging, receptor-targeted delivery.[40,101–105]

A preferred method used in many areas of biochemistry to couple specific protein to solid surface is the bioconjugation by covalent binding. While protein absorption on solid surfaces such as nanoparticles can be reversible depending on pH, salt concentration, temperature or other environment physicochemical characteristics, protein covalent bounds are highly stable. To fulfill the purpose of stable covalent binding, a large number of reactions have been proposed and many protein modifications using new techniques have been developed.[7,106–111]

The choice of the bioconjugation procedure depends strictly on physicochemical and biochemical properties of nanomaterials and proteins. Protein made by various side chains and residues can interact by multiple coating ligands with the same nanoparticles or even with more nanoparticles. Moreover, nanoparticles can be more or less polydispersed and have different physicochemical surface properties such as area, porosity, and charge. These aspects are very important since the hydrophobicity, charge and site affinity could affect the interaction and thus jeopardize the stability of final covalent-coupled products.

The most popular approach for coupling covalently nanoparticle to protein is based on the existence on proteins of specific and reactive functional groups such as amino–NH_2 (lysine), carboxylic acid–COOH (aspartic, glutamic), hydroxyl–OH (serine, tyrosine) and –SH (cysteine).[112]

Proteins can be chemically coupled to different kinds of nanoparticles using established reagents such bifunctional cross-linker molecules. In this case, nanoparticles need to be functionalized with functional groups such as carboxylic acid, hydroxyl, sulfhydryl and amino groups.

Proteins, including antibodies, generally have several primary amines in the side chain of lysine residues and the N-terminus of each polypeptide that are available as targets

for N-hydroxysuccinimide-ester and carbodiimide reagents. Cysteine residues on proteins can react with maleimides and iodoacetamides reagents to give thioether-coupled products.[113] These reagents react rapidly at physiological pH and can be usually coupled with thiol groups selectively in the presence of amine groups. Maleimides and iodoacetamides have the same application but the first reagent seems to have better selectivity than the second one, not apparently reacting with histidine or methionine.

Cross-linking reagents contain reactive ends to specific functional groups (such as primary amines, sulfhydryls) on proteins or other molecules. They can be divided into homobifunctional (same reactive groups) and heterobifunctional (different reactive groups) which chemical cross-links may or may not be reversed.[114] Homobifunctional cross-linkers have a disadvantage of potentially connecting two neighboring groups, either on the nanoparticle surface or on the protein inducing undesired cross-linking. Heterobifunctional crosslinkers allow sequential conjugations, minimizing polymerization. For example, sulfosuccinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC) can be used to couple thiol-containing biomolecules with amine–coated nanoparticles, or vice versa. Whereas the hetero-bifunctional cross-linker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is commonly used to link $-NH_2$ and $-COOH$ groups (Table 1).[114–118]

Many cross-linkers are available in the market and they can be chosen for specific needs (such as chemical specificity, spacer arm length, cleavability). Among several cross-linkers, the zero-length ones such as carbodiimides are widely used allowing covalent bonds between nanoparticles and proteins without insertion of an exogenous spacer. Nevertheless, the direct attachment of a protein to a surface without a spacer can cause steric

constraint modifying the protein reactivity compared to the protein in solution. In addition, without a spacer, multiple contacts between protein and nanoparticle surface are more probable favoring total or partial protein denaturation and thus decreasing protein activity.[119]

When protein does not have the suitable residue necessary for the specific conjugation, the most common way to get it is the chemical introduction of sulfhydryl groups. This process (Figures 1a and 1b) can be mainly made by the following four methods: 1) reduction of protein disulfide bonds using reductive agents such as dithiotreitol (DTT = Clelands reagent). 2) Coupling of protein primary amino groups with 2-iminothiolane (Trauts reagent). 3) Quenching of reactive protein aldehyde residues with cystaminiumdichloride reagents or 4) coupling of cystaminiumdichloride to carboxyl groups via 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC); both cases followed by the disulfide bonds reduction with DTT as outlined above.[112,120–123]

The avidin/streptavidin–biotin bound is the strongest noncovalent biological interaction known; for this reason this technology is commonly used in biological labs.[124,125]

Biotinylated proteins/antibodies/enzymes can be efficiently coupled on amino nanoparticle surfaces by streptavidin-biotin technology accomplished by streptavidin activation through carbodiimide (EDC) chemistry. Biotin binds strongly to this biochemically modified surface in the most specific and sensitive way. Furthermore, streptavidin through carbodiimide (EDC) chemistry can be covalently coupled with different ligands such as mAb and enzymes which make the biotin–streptavidin system widely used in a variety of biotinylated nanoparticles.[38,126–128]

Proteins having cysteine residues can be directly attached to some metal nanoparticle surfaces such as gold and silver by stable metal–sulfur bonds.[129,130] In the other cases, the

Table 1 The most popular cross-linker reagents for coupling protein to nanoparticle based on their respective functions

Reactive groups	Eg of functional cross-linker	Functional groups on nanoparticles/proteins	Functional groups on proteins/nanoparticles
–NHS ester Maleimide or Iodoacetamides	SIAB, SMCC, SPDP, SPMB, MBS	$-NH_2$	–SH
Carbodiimide	EDC or EDAC + sulfo-NHS stabilizer	–COOH	$-NH_2$
–NHS ester	EGS, DSP, DSS, BS3	$-NH_2$	$-NH_2$
Maleimide	BMME	–SH	–SH

Abbreviations: SIAB, N-succinimidyl(4-iodoacetyl)aminobenzoate; SMCC, succinimidyl-4-(N-maleimidomethyl)cyclohexana-1-carboxylate; MBS, m-maleimidobenzoyl-N-hydroxysuccimide ester; SPDP, succinimidyl 3-(2-pyridyldithio)propionate; SPMB, succinimidyl (4-p-maleimidophenyl)butyrate; EDC, 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride; EGS, ethylene glycolbis(succinimidylsuccinate); BS3, bis-(sulfosuccinimidyl) suberate; BMME, bis(maleimido methyl) ether; DSS, disuccinimidyl suberate; DSP, dithiobis (succinimidyl propionate).
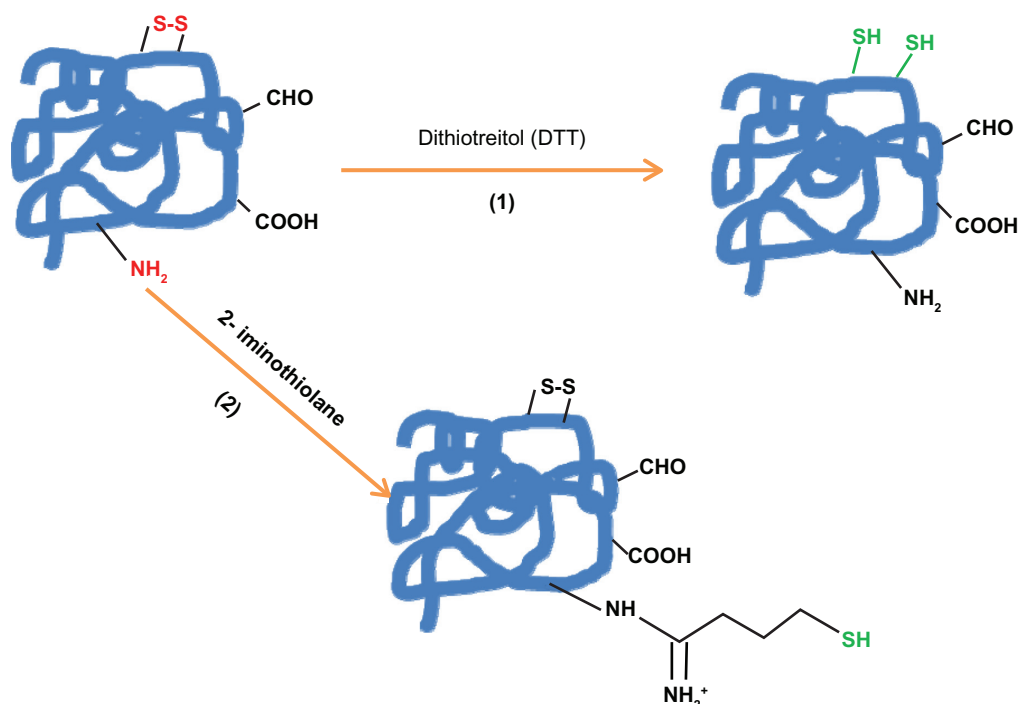
Figure 1a The introduction of sulfhydryl groups by: **1**) the reduction of protein disulfide bonds using reductive agents such as dithiotreitol (DTT = Cleland's reagent). **2**) Coupling protein primary amino groups with 2-iminothiolane (Traut; s reagent).

covalent coupling of proteins on nanoparticle surfaces is always a long experimental procedure.

Covalent bioconjugation procedure can be summarized in: 1) Coating of nanoparticles with the selected active functional groups. 2) Chemical activation of thiol groups on the protein side with specific reductive agents, if necessary. 3) Total removal of the reduction agent in excess; this step can create

unplanned reactions and spoil the whole coupling process. 4) Post conjugation procedures such as removal of unbound protein/remnant excess.

In addition to the disadvantage of the long experimental procedure, covalent bioconjugation can affect the protein structure and function resulting in its partial denaturation (Figure 2). Moreover, modification of enzymes under strong
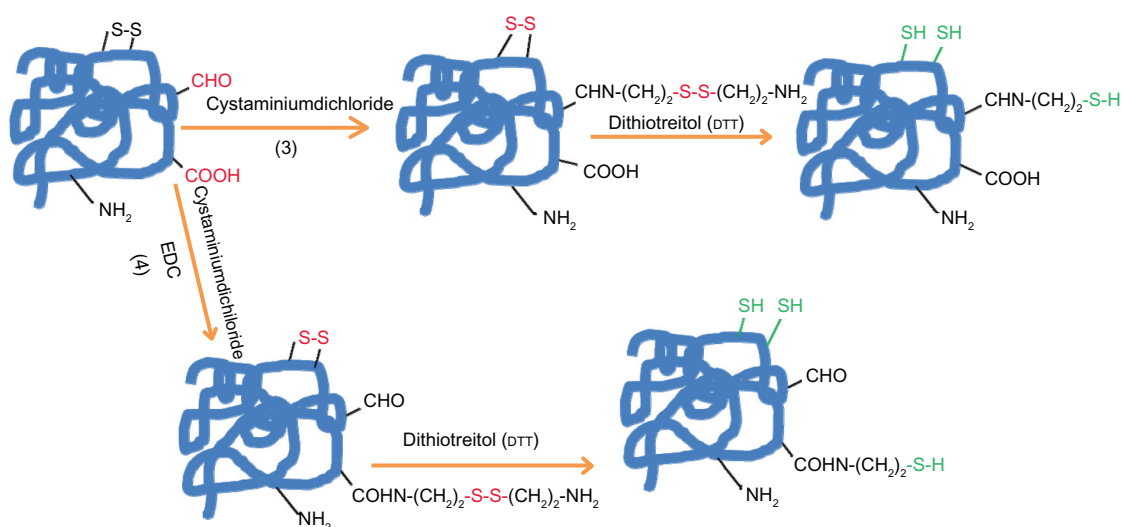

Figure 1b The introduction of sulfhydryl groups by: **3**) Quenching of reactive protein aldehyde residues with cystaminiumdichloride reagents or **4**) coupling of cystaminiumdichloride to carboxyl groups via 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC); both cases followed by the disulfide bonds reduction with DTT.
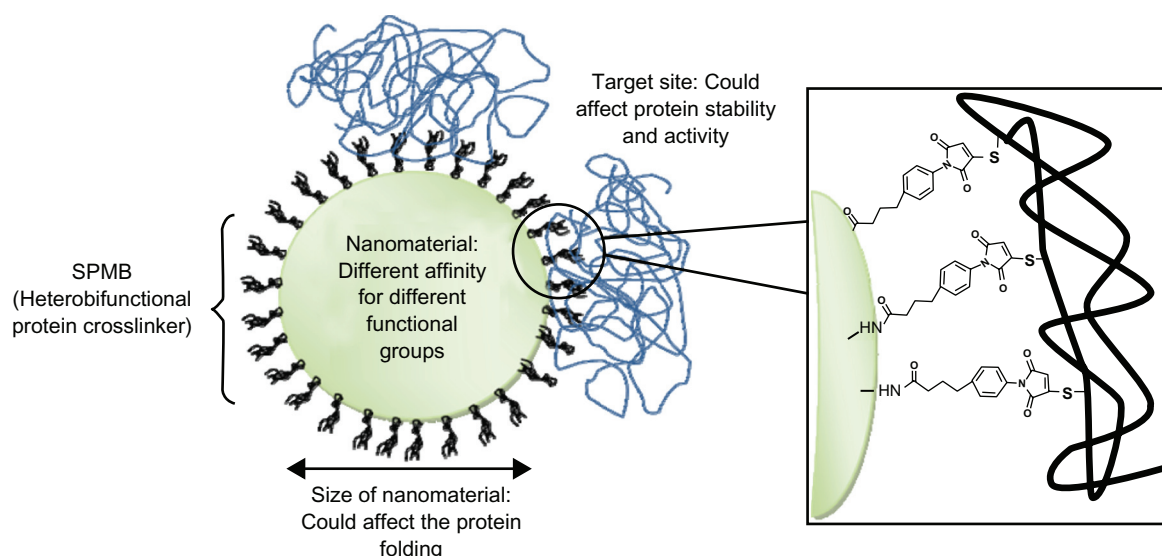
Figure 2 Protein–nanoparticle interactions: main factors that can affect proteins resulting in their denaturation. In this example, proteins are conjugated on amino functionalized nanoparticles using the cross-linker SPMB.

denaturing conditions can result in their complete loss of activity.

Proteins can be denaturated during manipulations or formulations mainly by two mechanisms: conformational denaturation (eg, reversible unfolding and irreversible aggregation via noncovalent interactions) and chemical denaturation (covalent bonds such as deamidation, hydrolysis, oxidation, β-elimination, incorrect disulfide formation, Maillard reaction, and transamidation).

Even if the first denaturation mechanism can happen during the nanoparticles bioconjugation process, the second one is often necessary to obtain high efficacy of the coupling. For example, the –SH or –S-groups in cysteine –SH or disulfide –S–S– bridges are important in maintaining the conformation of the proteins. As a result, the engineering introduction of sulfhydryl groups in the protein changes its natural disulfide bonds resulting in partial conformational and chemical denaturation.

The DTT reagent, widely used to reduce disulfide bonds in biochemical systems, can alter protein function not only by thiol-disulfide exchanging but also by interacting with protein domains in the absence of cysteine residues.[131]

While carboxyl groups seem to play an important role in enzymes catalytic activity,[132] their modification likely results in a change of protein secondary and tertiary structure.

The ε-amino groups of lysine are often specifically targeted because of their high reactivity and their modification seems to have fewer effects on protein properties. Unfortunately, the high abundance of these groups in many proteins can lead to increased heterogeneity and restricted conformational flexibility owing to multipoint attachment on a nanoparticles surface.

It is also possible that other reagents used during the coupling chemical process can contribute to the protein denaturation and to its activity loss. Therefore biological function checking as well as close monitoring of the quality and quantity of conjugated protein are extremely important to be assessed before being used.[111]

Gold or silver nanoparticles too, due to the similar strength bond between Au/Ag–S and S–S, can potentially break up protein disulfide –S–S– bridges leading to denaturation.

Specific ELISA kits can be used to explore the activity of the proteins coupled to nanosystems. However there are nanoparticles such as quantum dots (QD) that can have an overlap in the absorption spectra and the ELISA essay end product. In this case the proper specific activity of the protein needs to be assessed directly by in vitro testing.[133]

Protein encapsulation

Therapeutic biomolecules based on peptides, proteins or enzymes can be extremely fragile and easily aggressed by external agent such as proteases. Encapsulation of these fragile drugs in nanocarriers is a possible strategy for preventing their aggression and denaturation. This process can also improve the drug pharmacokinetic pathway and reduce immunological reactions.[19]

An optimal drug delivery system should be biocompatible, biodegradable and should not cause any immunological
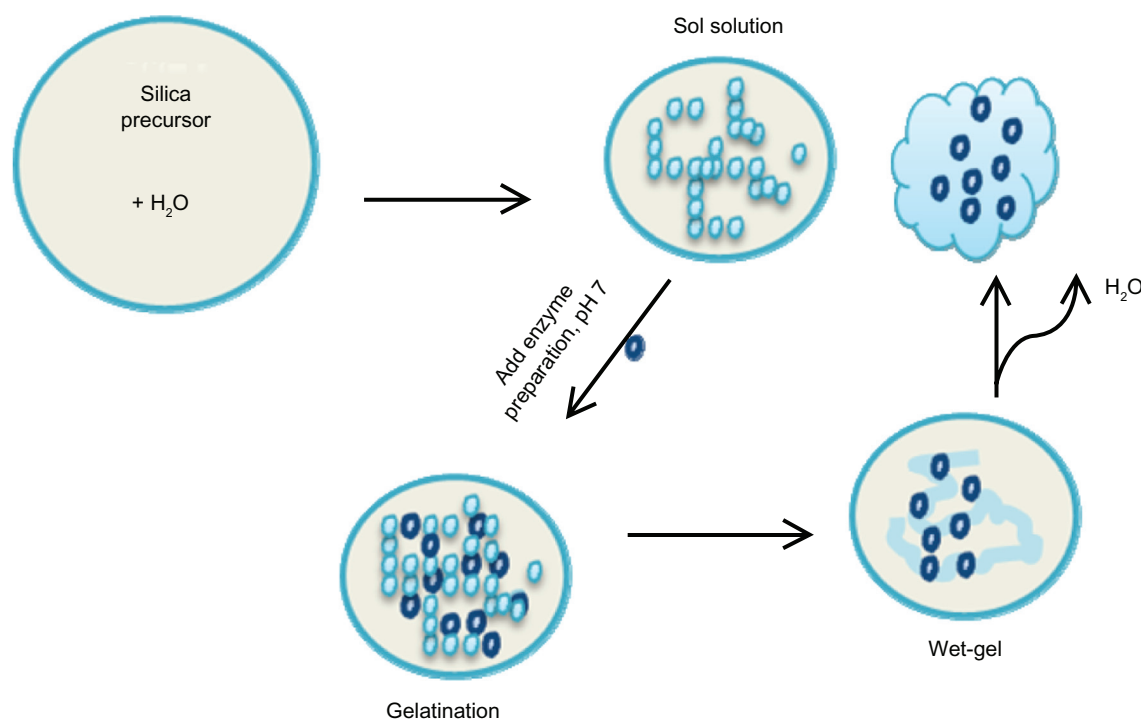
Figure 3 Entrapment of enzymes using sol-gel chemistry: A schematic overview of the sol-gel process. Several silicate precursors can be used to modify the surface chemistry of the sol-gels such as TMSO, APTES, MTMOS, and ETMOS.
Abbreviations: TMSO, tetramethyl orthosilicate; APTES, 3-aminopropyltriethoxysilane; MTMOS, methyltrimethoxysilane; ETMOS, ethyltrimethoxysilane.

adverse reaction in the human body. Among several candidates, liposomes are considered as the most promising vectors for proteins delivery due to their biocompatibility and their capacity to improve the drug pharmacokinetic. Liposomes, also known as lipid-based vesicles, are generally composed of concentric amphiphilic lipids, such as phospholipids, containing a water compartment. These carriers are versatile and their physico–chemical characteristics can be properly tuned.[19] Liposomes synthesized from dehydrated–rehydrated vesicles are widely used due to the ease of this preparation process and the low amount of stress applied to the proteins.[134]

Liposome formulations are most frequently considered for parental administration of the drug, but may also be a potential formulation principle for alternative routes such as topical and nasal administration.

Several liposomes have immunoadjuvant properties and their application in vaccines based on recombinant protein subunits and synthetic–peptide antigens is attractive. The first liposome based vaccine (against hepatitis A) that has been licensed for human use is commercially known as Epaxal Berna® vaccine.[135]

The main drawback of liposomes is their instability in biological media as well as their sensitivity to many external parameters such as temperature or osmotic pressure. Theoretically, it could be possible to increase their stability following several strategies such as the polymerization of a two–dimensional network in the hydrophobic core of the membrane, coating the liposome with a polyelectrolyte shell or adding surface active polymers to form mixed vesicular structures.[136–138] However, poor loading and partial protein/enzyme denaturation during the entrapment process can occur.

Another well established technique to encapsulate biological species such as enzymes, antibodies and other proteins in a functional state is based on the sol–gel chemistry method.[139] Silica is indeed considered a very appealing material for drug delivery systems because it is relatively inexpensive, chemically inert, thermally stable, and biocompatible. Amorphous silica, used for decades as a food additive and for specific applications, is generally regarded as safe. Up until now, the FDA has not established if existing silica safety data can be applied to nanoscale forms of the material. In this approach, polypeptides, especially enzymes, could be entrapped inside silica matrix allowing the retention of enzymatic activity.[139–141]

On the other hand, process difficulties such as uncontrolled release, denaturation and the hardness control of the protein

orientation can be found.[142,143] The control of the drug release of such silica nanoparticles is the most important and difficult parameter that needs to be properly tuned. The encapsulation efficacy of insoluble protein is greatly different compared to the soluble one and the existence of soluble and insoluble part of polypeptides in the same therapeutic protein subunit complicates the synthesis process. Additionally, in the crowded environment of a silica matrix, the physical and chemical properties of the silica can directly influence protein structure and activity. Furthermore, functional activity of proteins entrapped into the sol-gel matrix needs to be accurately analyzed case-by-case using several techniques such as CD spectropolarimetry.[144,145]

The drug release and the capability of the carrier to be metabolized can be important factors to be considered when chronic or repeated treatments are necessary. The disadvantage associated with inorganic and synthetic carriers are the poor or slow biodegradability and possible inflammatory responses.[146]

Biodegradable polymers nanosystems are an attractive alternative to liposomes since they have the advantages of longer circulation in the blood stream and generally higher drug carrying capacity.[147] Polymers such as poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA) have been extensively investigated for their biocompatibility and potential capability of releasing therapeutically proteins in a controlled way even over a prolonged period of time.[148–154] These polymers are degradable by bulk erosion through hydrolysis of the ester bonds. The hydrolysis rate depends on several nanoparticles physicochemical parameters and can be tailored according to the desired release pattern of the protein to be incorporated.

PLA and PLGA are FDA-approved as excipients to achieve sustained release of the active ingredient. However, their application in protein delivery systems is often characterized by low entrapment efficiency, burst release, instability of encapsulated hydrophilic protein and partial protein release.[155–158] To improve the performance of these polymer nanoparticles, polysaccharides such as alginate (ALG) and chitosan (CS) could be applied.[151,159] CS and its derivatives have been intensively studied as carriers for proteins and drugs. More specifically these nanoparticles can be totally made by CS or used in several copolymer combinations.[25,160]

Copolymers made by the combination of CS/ALG are able to generate a more "friendly" environment which protects peptides and proteins from stressing conditions and allows their stabilization during encapsulation, storage and release.[161–166]

Glycol chitosan nanoparticles modified with hydrophobic bile acid analogs self-assemble into polymeric nanoparticles with hydrophilic shells of glycol chitosan and hydrophobic cores of bile acid derivatives have been reported as possible vehicle for RGD (Arg–Gly–Asp) peptide.[29,30]

Regardless of the nanomaterial chosen for protein encapsulation, an important issue that needs to be considered is the understanding of protein–protein interactions. There are large numbers of transient protein–protein interactions that occur in the cell, which in turn control a large number of cellular processes. These transient interactions of protein complexes can cause several effects such as activation/inactivation of certain proteins, resulting in the formation of a new binding site.[167,168] Kinetics properties of enzymes can be also altered by denaturation during the entrapment process allowing potential change of the protein specificity to its substrate.[169–171]

Gaining a clear picture of these basics knowledge will definitely lead to a change of object design to increase the protein load, to control the protein release and to retain the protein integrity and efficacy.

Conclusion

Although new proteins are available for medical purposes, their administration as therapeutics still remains difficult. Nanosystems seem to be the optimal solution to improve protein bioavailability, biodistribution and safety. Moreover, the combination of nanoparticles with proteins could also be a valid system to achieve the design of efficient nanovectors for drug delivery. Indeed, nanoparticles can be properly tuned for specific applications and could be precisely designed to meet biological needs. However, to completely fulfill this purpose, it is necessary to better clarify the nature of interaction between nanoparticles and biomolecules. The control of the protein denaturation is another important parameter that needs a deeper understanding. Further investigations should help to manage these hybrid nanosystems, opening new therapeutic and diagnostic perspectives as well as new challenges in the near future.

Disclosures

The authors report no conflicts of interest in this work.

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