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REVIEW

Nanostructures for protein drug delivery

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Use of nanoscale devices as carriers for drugs and imaging agents has been extensively investigated and successful examples can already be found in therapy. In parallel, recombinant DNA technology together with molecular biology has opened up numerous possibilities for the large-scale production of many proteins of pharmaceutical interest, reflecting in the exponentially growing number of drugs of biotechnological origin. When we consider protein drugs, however, there are specific criteria to take into account to select adequate nanostructured systems as drug carriers. In this review, we highlight the main features, advantages, drawbacks and recent developments of nanostructures for protein encapsulation, such as nanoemulsions, liposomes, polymersomes, single-protein nanocapsules and hydrogel nanoparticles. We also discuss the importance of nanoparticle stabilization, as well as future opportunities and challenges in nanostructures for protein drug delivery.

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1. Introduction

Research on the synthesis, properties and applications of nanoscale materials and devices has contributed to several biomedical fields such as drug delivery, imaging agents and diagnostic tools. The importance of nanotechnology can be noticed in several angles, as the scientific one; the number of

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papers in this field has risen sharply from a handful in the early 1990s to several thousand at the present time. The financial angle shows estimates for the combined market of nano-enabled medicine to overpass $100 billion in the next few years. An equally important angle, the regulatory one, also endorses nanotechnology prominence: the FDA (Food and Drug Administration) created a specific program to study the properties, risks and advantages of nanotechnology products applied to drug development, named the Center of Drug Evaluation and Research Nanotechnology Programs.[1]

Nanostructures as drug delivery systems (nanocarriers) are key to overcome challenges associated with drug therapy, including poor solubility, poor permeability, short half-life in the target organism and high toxicity.[2,3] They present at least one dimension in the nanoscale range (below 1 μm) and generally high surface to volume ratios.[4] Nanocarriers include a wide array of systems such as nanocapsules, lipid complexes, polymeric micelles, liposomes, dendrimers and others.[5–7] As any ideal delivery system, they should be non-toxic, protect their therapeutic payload and exhibit biocompatibility, biodegradation, physicochemical stability, controlled-release kinetics and improved pharmacokinetics.[8]

Therapeutic proteins, in particular, may present all challenges described above for drug therapy together with immunogenicity and inflammatory potential.[9] Proteins play a significant role in cell signaling, immune responses, cell adhesion, and the cell cycle. Native and recombinant proteins benefit major sectors of the biopharmaceutical industry and protein drugs are produced using technologically advanced microbial and mammalian cell biosystems. The biopharmaceutical industry has been performing exceedingly well in recent years and the future looks bright for protein drug development. Therefore, the use of nanotechnology to deliver protein drugs such as monoclonal antibodies, antibody fragments, peptides, replacement factors, enzymes and vaccines is increasing exponentially. Through this strategy it is possible to obtain safe/effective therapeutic protein preparations.[10] Nanodelivery systems usually stabilise protein drugs against denaturation by enzymatic digestion, increasing their biopharmaceutical applications.[11,12]

The type of nanocarrier to deliver proteins has to be carefully chosen with regards to the drug incorporation process and hydrophobic/hydrophilic loading. The process has to consider pH and thermal protein instability, whereas the carrier must be able to upload hydrophilic and large molecules, except for peptide encapsulation. Route of administration is equally important, since proteins are generally unstable in the gastrointestinal tract and present low mucosal permeability. As a consequence, oral bioavailability is usually low and erratic, making the parenteral route the first choice. Even when administered subcutaneously or intramuscularly, systemic bioavailability is often low and variable.[13] Fig. 1 summarizes the obstacles for protein drug delivery.

Considering the outstanding relevance of protein drug delivery and the potential of nanostructures to address the matter, this review presents the main features, advantages, drawbacks and recent developments of nanocarriers to this end, and also discusses the importance of nanostructure (colloidal) stabilization and its main features. We focus on systems that allow hydrophilic protein encapsulation and, therefore, protection against degradation and immunogenicity, namely nanoemulsions, liposomes, polymersomes, single protein nanocapsules and hydrogel nanoparticles.

2. Nanoemulsions (NE)

Nanoemulsions (NE) are colloidal dispersions produced with oil (O), water (W) and surfactants, considered as conventional emulsions that contain very small droplets, in the range of 50–200 nm. They can be employed for delivery of peptides and

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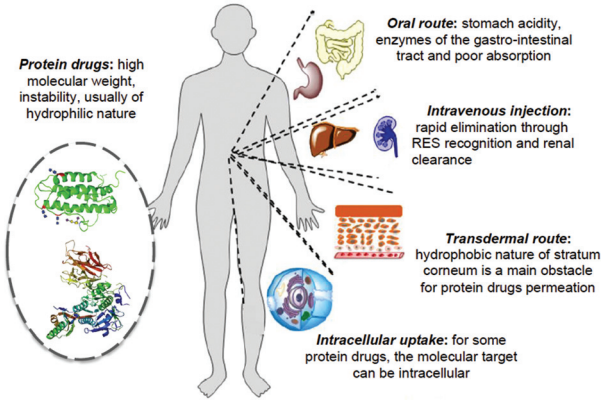

Fig. 1 Main human body challenges for protein drug delivery. RES – reticuloendothelial system.

proteins by non-parenteral routes, such as oral and transdermal delivery.[14] Despite the structural similarities between nano and microemulsions and confusing classification, these two kinds of colloidal dispersions possess some differences, as presented in Table 1.[15]

NE are formed by one liquid phase dispersed in a different immiscible continuous liquid phase. An energy input is necessary to overcome interfacial tension between the two immiscible phases, breaking larger droplets down to a smaller size; and the newly formed system is then stabilized with surfactants.[16] Droplets are formed either by a high-energy or a low-energy approach, whereas the choice will depend on the properties of the surfactant, oil and aqueous phases.[17] Fig. 2 illustrates a water-in-oil nanoemulsion and the ability of these systems to carry biomolecules.

Increasing interest in NE stems from the characteristic physicochemical properties that their small droplets size provide. It allows efficient delivery, accelerated release and rapid absorption of bioactive molecules.[11,18] Moreover, the significant surfactant film thickness relative to droplet size prevents thinning or disruption of the liquid film between the droplets.[19,20]

NE enhance the solubility, transport, dispersibility, bioavailability and bioaccessibility of proteins, and can act as excellent encapsulation systems compared to conventional emulsions.[21,22] The large surface area of NE enhances the bioavailability of peptides and proteins due to enhanced surface interaction with the absorptive epithelium.[12] In

Table 1 Main differences between microemulsions (ME) and nanoemulsions (NE)

Aspects	ME	NE
Stability	Thermodynamically stable	Thermodynamically unstable and kinetically stable
Components	Oil, water, surfactant and possibly a co-surfactant	The same components of ME. Proteins and polysaccharides can also be used as surface active agents
Surfactant/oil ratio	Large surfactant/oil ratios	Low surfactant/oil ratios
Optical properties	Transparent	Transparent to opaque
Droplet form	Sphericity depends on the optimum curvature of the surfactant monolayer and the oil content	Generally spherical due to relatively high interfacial tension
Production method	Formed spontaneously with light magnetic agitation	Requires the input of some external energy to convert the separate components into a colloidal dispersion, like ultrasound

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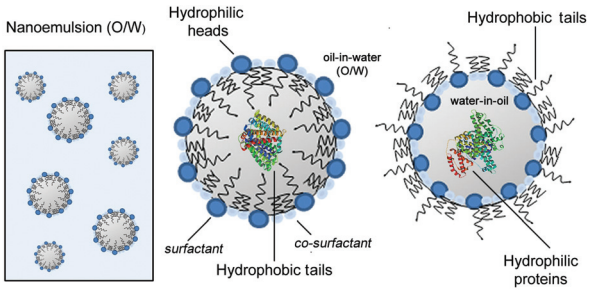

Fig. 2 Schematic diagram of nanoemulsions. The system is formed by nanodroplets with a hydrophobic core oil and surfactant tails and a hydrophobic shell of surfactant head groups.

addition, their small droplet size may enhance the transport of bioactive peptides carried within nanodroplets as droplets may pass across the intestinal wall and facilitate their absorption, bioavailability and bioaccessibility.[23]

Release of drugs from NE is generally acknowledged to be quite rapid; however, attempts toward retarded or controlled release as well as site-specific targeting have been reported as well.[24] NE support the penetration of incorporated actives into the skin and may thus promote their accumulation in the skin. In addition, the cosmetic effect of the vehicles is of further interest. In recent approaches, incorporation of hydrophilic drugs into NE systems is also being investigated.[25]

A self-nanoemulsifying drug delivery system (SNEDDS) was developed to increase oral bioavailability of fluorescent labeled-β-lactamase (FITC-BLM). This protein was loaded into the oil phase by a solid dispersion technique, forming a NE upon water addition. More specifically, the SNEDDS O/W NE-12-7 (composition: Lauroglycol FCC, Cremophor EL and Transcutol; ratio: 5 : 4 : 3) formed droplets of 22–50 nm, regardless of pH and dilution factor, with good stability (4 °C for 12 weeks) and high enzyme activity. The SNEDDS significantly increased the transport of FITC-BLM across the Madin-Darby canine kidney monolayer *in vitro*.[26] Oral absorption of FITC-BLM in rats also increased with the NE system, resulting in higher bioavailability and more than 12 h residence time; conversely, BLM loaded in the NE aqueous phase presented the same pharmacokinetics as the free one. The described observations point out SNEDDS with proteins dispersed in the oil phase as a viable alternative for oral protein delivery.[26]

As a strategy for oral delivery of insulin, NE were developed from W/O/W multiple emulsion (Tris buffer/Labrafac CC, Span™ 80, phospholipid/Cremophor EL, chitosan and alginate in water), produced by high-pressure homogenization. The hypoglycemic effect was evaluated in male Wistar rats and Goto-Kakizaki diabetic rats, with a remarkable decrease in blood glucose levels after oral administration of the NE. Additionally, sodium alginate and chitosan have mucoadhesive properties, which probably prolonged NE retention in the gastrointestinal tract and consequent drug absorption.[27]

The advantages of NE over other emulsions are derived from the smaller droplet sizes which impart distinct physicochemical properties to NE (*e.g.* bulk viscosity, optical transparency, and physical stability) compared to those of other emulsion systems.[28] Most studies conducted so far have concentrated on the use of synthetic and low molecular weight surfactants (*e.g.* the tweens and spans) due to their excellent interfacial diffusivity, compared to large biopolymers such as proteins and polysaccharides.[28–30]

NE offer distinct advantages in terms of dermal drug delivery, such as: high physical stability compared to conventional emulsions, high skin friendliness due to the low amount and the mild nature of the surfactants employed and the ease of preparation and scale-up. They avoid the limitations of other colloidal drug delivery systems, such as the limited drug loading and stability issues of liposomes and the potentially irritating compounds required for the production of nanoparticles or ME. Depending on composition and the nature of the employed drug, NE may achieve higher rates of skin penetration and drug accumulation within the skin than lipid nanoparticles.[31] In a recent study investigating the dermal delivery of lutein, a more rapid release was found with NE, which achieved higher skin permeation rates than nanostructured lipid carriers and solid lipid nanoparticles.[32]

3. Liposomes

Liposomes (LP) are composed of lipid and/or phospholipid molecules containing a hydrophilic head region and a hydrophobic tail region, which aggregate to form an enclosed lipid bilayer vesicle with an aqueous nucleus, so they are able to deliver both hydrophilic and hydrophobic protein drugs as shown in Fig. 3.[33]

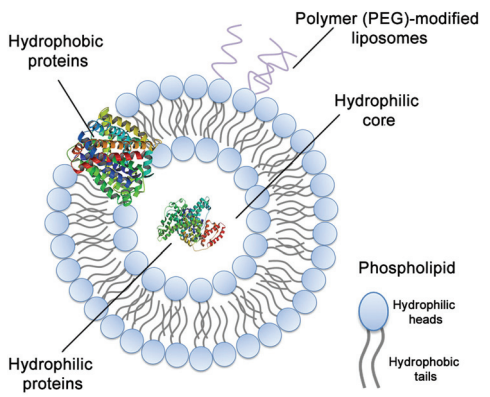

Fig. 3 Schematic representation of liposomes formed by phospholipid molecules containing a hydrophilic head region and hydrophobic tail region. Hydrophilic and hydrophobic proteins can be incorporated in its structure. By grafting polymers (PEG) on the liposome surface, dense conformational clouds can form, shielding the surface charge of liposome. The steric hindrance of polymer grafted on the liposome surface can prevent serum protein opsonization and subsequent interactions with cells of the mononuclear phagocyte system, resulting in an increased retention time of the liposomes in the blood circulation. Adapted from Mo *et al.*, 2014.[38]

Due to the aqueous core, large amounts of water-soluble protein drugs can be incorporated into LP and the native structure of proteins is usually maintained during the incorporation process.[34] LP are typically spherical self-closed structures with a size varying from 50–1000 nm[35] that can naturally occur or be artificially prepared.[36] The shape and amphiphilic nature of phospholipids (and surfactants in general) are critical to spontaneous formation of bilayers in aqueous environments and may be expressed by the critical packing parameter (s), according to eqn (1):

$$s = \frac{v}{a_0 l} \quad (1)$$

where v is the volume of the surfactant/phospholipid tail, a_0 is the effective head group cross-section area and l is the length of the extended surfactant tail. When $s \leq 1/3$ spherical micelles exist in solution. If $1/3 < s \leq 1/2$, rod-like shape or hexagonal aggregates are most likely formed. For $1/2 < s \leq 1$ the surfactant molecules may aggregate in bilayer structures (like vesicles).[37] In other words, surfactant molecular shape determines the aggregate minimal or "critical" radius of curvature R_c, and thus the shape. Note that the packing shape is not fixed or rigid; it is variable within the limits set by the condition that the length of the extended surfactant tail in the aggregate cannot exceed l. Therefore, all radii R down to R_c are granted, so there will be a distribution of structures in equilibrium with each other.

The simplest LP production process is the mechanical dispersion of dry lipids in water, resulting in concentric bilayers separated by narrow aqueous channels. These structures, known as multilamellar vesicles (MLVs), have been extensively employed to study the features of bilayer structures. The regular arrays of bilayers and relatively large size (≥400 nm diameter) makes MLVs ideally suited for X-ray studies and easier to investigate by nuclear magnetic resonance (NMR) than smaller systems. Use of MLVs in permeability studies and cell membrane fusion studies, on the other hand, is limited by the size, heterogeneity and the presence of many internal compartments.[39,40]

Usually, MLV preparations are heterogeneous and single bilayer systems are often present. The proportion of lipid exposed to the external medium, *i.e.* in the outer monolayer of the external bilayer, gives an average indication of lamellarity. For highly multilamellar systems, it corresponds to a small proportion of the total lipids, but will be approximately half of the total lipid in unilamellar systems.[39]

Several methods have been devised to produce unilamellar systems, including disruption of preformed MLVs by sonication and extrusion or modifications of the phospholipid hydration procedure to spontaneously form unilamellar systems.[39] For this last one, specific solvent evaporation procedures have been employed,[40–42] as well as dialysis in the presence of surfactants.[43]

Most part of the recent investigations with LP for drug delivery use homogeneous unilamellar vesicles in the size range of 50–150 nm. This size range is a compromise between loading efficiency (increases with increasing size), liposome stability (decreases with increasing size above an optimal 80–200 nm range) and ability to extravasate the vascular system (decreases with increasing size).[36,44] The membrane thickness is around 4 nm, and it may have a polymer coating and/or ligands with defined functions, such as specific binding or fusogenic activity.[36] To elongate the gastrointestinal tract survival of LP, the vesicles have been modified in several ways including the incorporation of bile salts and the design of multilayered or multi-vesicular carriers.[45]

Unilamellar vesicles may be small (SUV, small unilamellar vesicle) or large (LUV, large unilamellar vesicle),[42] though a clear distinction between these two types is often difficult. LUVs, which are usually more stable than SUVs and exhibit significantly larger trapped volumes, are the most popular membrane model systems, especially to investigate permeability and diffusion.

At the organizational level, defects may be present in LP bilayers having lipids with unsaturated tails.[46] This may cause an easy disruption of the lipid bilayer and subsequent leakage of the entrapped molecules into the biological system. Lipids with long hydrocarbon chains and a low degree of unsaturation and branching are used to form tightly packed lipid bilayers. Additionally, cholesterol may be introduced into the lipid bilayers to improve the packing and membrane stability.[47]

LP can improve pharmacokinetics, provide protection from degradation, mediate targeting to the pathological site and facilitate uptake by the target cells.[48,49] However, although pharmacokinetics, tissue distribution, and cellular uptake can be improved using (targeted) liposomes, the liposomal drug usually ends up in the endo- and lysosomes, where both the liposome particles as well as the encapsulated macromolecules are subject to degradation.[50,51] Also, these nanostructures are highly susceptible to enzyme degradation.[48,49]

LP have been extensively used for drug delivery due to their ability to protect and deliver hydrophilic and hydrophobic molecules, biocompatibility with cell membranes and the possibility to add specific ligands to their surface. Several types of liposomal formulations have been developed for protein drug delivery and many have been already evaluated for clinical applications.[34] Anderson *et al.*[52] evaluated a method to incorporate cytokine proteins into multilamellar LP. A variety of human cytokines including granulocyte-macrophage colony stimulating factor (GM-CSF), interleukins 1α, 2 and 6 (IL-1α, IL-2, IL-6) and interferon-γ (IFN-γ) were incorporated into LP containing a single saturated synthetic lipid, dimyristoyl phosphatidyl choline (DMPC). Sterile cytokine liposomes were produced by gamma irradiation of DMPC prior to use in cytokine liposome synthesis. Meyer *et al.*[53] studied the encapsulation of proteins within multilamellar LP. These researchers found an efficient and gentle method for granulocyte colony stimulating factor (rhG-CSF) encapsulation in LP and slow release of encapsulated material was demonstrated both *in vitro* (90% serum, 37 °C) and *in vivo* after subcutaneous injection.

Regarding transdermal delivery of liposome-encapsulated biomolecules, Guo *et al.*[54] applied insulin-loaded vesicles on the abdominal skin of mice and showed a significant drop in

the blood level of glucose. In another study, Kajimoto *et al.*[55] investigated the iontophoretic delivery of charged LP loaded with insulin through the skin of diabetic rats after administration of glucose, and reported a gradual decrease in blood glucose levels, reaching 20% of initial values at 18 h. A significant concentration of insulin was also detected in plasma 18 h after the iontophoretic application.

Although LP are typically made from natural, biodegradable, non-toxic, and non-immunogenic lipid molecules, their interaction with high-density lipoproteins in blood may lead to a premature release of entrapped drugs.[56] Several different strategies can be applied to improve molecule bioavailability from targeted and internalized liposomal nanocarriers, for example, using pH-dependent fusogenic peptides[57] or lipids[58] as light-sensitive probes enhancing endolysosomal escape.[59] Furthermore, some LP have very short blood circulation times due to the fast uptake by the reticuloendothelial system (RES).[34,60] As an alternative, long-circulating LP can be obtained by coating the LP surface with inert, biocompatible polymers, such as polyethyleneoxide (PEG or PEO) (Fig. 3). In this sense, we can define first-generation LP as simple phospholipid vesicles, while second-generation are long-circulating aggregates obtained by surface modifications, mostly PEGylation.[61] The polyethylene oxide groups attached form a protective layer over the LP surface that slows down system identification by opsonins and subsequent clearance. Besides preventing opsonization, PEGylation may improve encapsulation efficiency (formulation related).[62] Park *et al.*[63] showed that PEGylated LP presented higher encapsulation yields of insulin compared to its non-PEGylated counterpart. In addition, Kedar *et al.*[64] observed that IL-2 encapsulated in PEGylated LP showed improved interaction with cells compared to first generation LP.

Additionally to PEGylation, LP may be functionalized with surface ligands, such as antibodies and aptamers, for cellular recognition. The attachment of pH-sensitive polymers to the liposome surface is another strategy to provide longevity and promote loading release. After endocytosis of the pH sensitive LP in the intact form, they fuse with the endovascular membrane as a result of the low pH inside the endosome and release their contents into the cytoplasm.[65] Regarding the targeting ligand, a careful selection is required, concerning its selective expression or overexpression on the target cell, possible shedding of the targeting ligand and its capacity for receptor-mediated endocytosis.[66]

Several targeting ligands have been studied for development of targeted liposomes, including antibodies and their fragments, peptides, vitamins, carbohydrates, nucleic acids and charged lipids. Liposomal attachment of targeting ligands should not interfere with normal physicochemical characteristics of the liposomes and of the targeting moiety such as size, stability, molecule retention and receptor-binding affinity. In addition, it should not affect liposome pharmacokinetics, biodistribution and tumor accumulation.[67,68]

These targeting ligands can result in (cell-) specific localization and retention of the liposomal drug in tumors, for example, on tumor cells or tumor vasculature. Specific ligands can also promote active cellular uptake of the drug-containing liposome through binding to targeted internalizing receptors. By these means, the molecule is transported across the cell membrane, which normally forms a significant barrier for drug uptake.[68,69]

Successful examples of liposomal protein formulations can be found in the literature, for example, heat shock proteins (HSP) 70/Blc-2 for cancer[70] and Melan-A/MART-1_{27-35} peptides for melanoma.[71,72] Studies regarding the use of liposomes in vaccines include encapsulation of hepatitis C peptides[73] and HIV peptides.[74] Nonetheless, the majority of current liposomal protein formulations are still in preclinical research stages, with relatively little known or reported human clinical findings to date. There are still pending challenges to create commercially stable and bioactive protein formulations with lipids.[75] Additionally, poor stability of liposomal delivery systems limits their use in drug delivery and resulted in a few marketed products regardless of extensive and long research in this area. Chemical instability results from oxidation, acylation and rapid hydrolysis of phospholipids whereas physical instability is mainly due to low M_w of phospholipids that lead to a thin (typically 3–4 nm) and leaky membrane.[76]

4. Polymeric micelles and polymersomes

Similar to classical surfactants, amphiphilic block copolymers can self-assemble into a wide range of morphologies upon hydration of the copolymer, including spherical micelles, cylindrical micelles or even vesicles (Fig. 4).[77–79] The critical packing parameter (s) can also be employed to predict the resultant morphology of these amphiphilic aggregates.

For amphiphilic block copolymers, the curvature of the hydrophobic–hydrophilic surface as described by its mean curvature (H) and its Gaussian curvature (K) are related to the packing parameter (s) as shown in eqn (2), in which V is the volume of the copolymer hydrophobic branch, a_0 the interfacial area per copolymer molecule and l is the chain length of the copolymer hydrophobic branch normalized to the interface:[77,79,80]

$$s = \frac{V}{a_0 l} = 1 + Hl + \frac{Kl^2}{3} \quad (2)$$

The volume fraction of the hydrophilic block of the copolymer (f) is also employed to determine the morphology of the self-assembled system. Usually, vesicular structures (polymersomes) are favored when f is 10–40%. At $f \sim 40$–55%, cylindrical micelles tend to form and at $f \sim 50$–70%, spherical micelles are predominantly formed.[78,79]

Polymeric micelles (PM) are generally of 10–80 nm in size, with the hydrophobic core and hydrophilic surface, and are significantly more stable than surfactant-based micelles.[81] They do not dissociate immediately after extreme dilution fol-

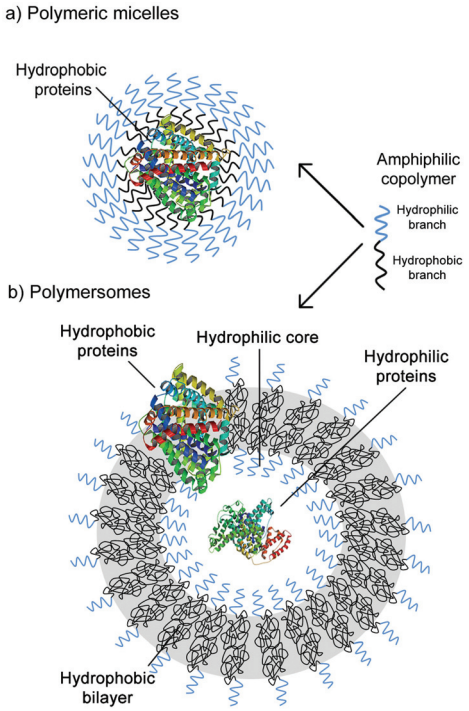

Fig. 4 (a) Polymeric micelles can be spherical or cylindrical, with the hydrophobic core and hydrophilic surface, and are significantly more stable than surfactant-based micelles. (b) Polymersomes are nanovesicles composed of synthetic copolymers.

lowing intravenous injection into the body because they have remarkably low critical aggregation concentrations (CAC = 10^{-6}–10^{-7} M) and slow kinetics of dissociation.[82,83] Due to the hydrophobic nature of the PM core and considering that most therapeutically employed proteins are hydrophilic, proteins cannot be easily encapsulated in these nanostructures. Nonetheless, there are some examples of modified proteins incorporated within the micellar structure. Weissig *et al.*[84] studied a model protein, soybean trypsin inhibitor (STI) modified with a hydrophobic residue of *N*-glutaryl-phosphatidyl-ethanolamine (NGPE) and incorporated into polyethylene oxide (M_w 5000)-distearoyl phosphatidyl ethanolamine (PEO-DSPE) micelles (<20 nm) and PEO-DSPE-modified long-circulating liposomes (*ca.* 100 nm). When injected in mice bearing subcutaneously-established Lewis lung carcinoma, higher accumulation yields were observed for STI incorporated into the polymeric micelles than in PEO-liposomes (PEGylated liposomes).

Polymeric vesicles or polymersomes (Ps) are a good alternative to liposomes, to encapsulate proteins.[85] Ps are usually in the size range of 10 nm to 5 μm and are formed by a hydrophobic shell that can incorporate hydrophobic proteins/drugs and an aqueous core that can encapsulate hydrophilic proteins/drugs.[77–80,86,87] The composition and M_w of the polymers employed can vary, which allows not only the preparation of Ps with different properties and responsiveness to stimuli but also Ps with different membrane thicknesses and permeabilities.[88] Furthermore, the constituent block copolymers might be finally excreted into the urine due to their M_w being lower than the threshold of glomerular filtration, suggesting the safety of Ps (as well as PM) with a low risk of chronic accumulation in the body.[89]

In the last few years, Ps have attracted attention as versatile carriers because of their colloidal stability, tunable membrane properties and ability to encapsulate or integrate a broad range of drugs and molecules.[78] Such as liposomes, Ps, can be prepared by the film rehydration method, direct dissolution, double emulsion in microfluidic device or electroformation. The choice of method depends on the type of molecule to be encapsulated and on the copolymer characteristics. For protein drugs, film rehydration is usually preferable since there is no contact between protein and organic solvent.

The tunability of Ps structure and properties has expanded considerably with the recent advances in block copolymer chemistry. Based on their multi drug loading capacity, membrane robustness and stealth properties, Ps are highly interesting for drug delivery applications and a lot of work has been directed to develop Ps for targeted drug delivery.[78,90,91] The development of stimuli-responsive Ps to further control the release of drugs by switching the stability and permeability of the membrane has also received a lot of attention and various block copolymers that are responsive to pH, temperature, redox conditions, magnetic field, ionic strength and glucose concentration have been synthesized and used to prepare Ps.[78]

Previous studies also show that Ps are mechanically stronger and possess superior camouflage ability than PEG-liposomes. Moreover, Ps offer more flexibility in the choice of vesicle size, bilayer thickness, and camouflaging ability *via* appropriate selection of the M_w of the hydrophilic/hydrophobic blocks.[88,92,93] While the membrane thickness of liposomes and other natural membranes are universal ($d \approx 3$–4 nm), the hydrophobic core thickness of PS can be engineered to exceed 4 nm by simply varying the copolymer hydrophobic block molecular weight.[53,94–96] Thicker membranes provide better stability and mechanical strength as well as influence the permeability of PS to small molecules.[53,97,98] According to Lee[93] and Discher,[98] giant polymersomes with 8 nm membrane thickness were found to be almost an order of magnitude more mechanically resilient and at least 10 times less permeable to water than liposomes. These features can reduce osmotic pressure gradients encountered with liposomes. Besides, it can increase the nanostructure circulation time.

The encapsulation of recombinant insulin in Ps provides a good model for the encapsulation of therapeutic proteins. Enhanced circulation kinetics and controllable release of insulin *in vivo* are desirable to increase patient compliance and to decrease the need for intravenous injections. Although insulin is a peptide of only 5.8 kDa, it was shown to aggregate to dimers, hexamers, and eventually fibrils when exposed to agitation or hydrophobic interfaces. Therefore, insulin was a challenging first model of therapeutic protein encapsulation in PEO-based Ps.[99] Ps composed of poly(styreneboroxole) (PBOx) and its block copolymers with PEO demonstrated the possibility of use as a sugar-responsive delivery vehicle for

insulin in neutral phosphate buffer (pH 7.4). Encapsulated insulin could be released from the Ps only in the presence of sugars under physiologically relevant pH conditions.[100]

Ps with large aqueous compartments as well as robust hydrophobic membranes have emerged as ideal polymeric nanocarriers for encapsulation and controlled delivery of proteins and peptides.[88] Lee *et al.*[93] proved the possibility of encapsulating proteins such as myoglobin, hemoglobin and bovine serum albumin (BSA) in Ps formed by poly(ethylene oxide)-*b*-polyethylethylene (PEO-*b*-PEE). However, the loading efficiency was low, and the mechanism of encapsulation was not fully understood. More recently, glucose oxidase was encapsulated in Ps of polystyrene$_{40}$-*b*-poly-(L-isocyanoalanine(2-thiophen-3-ylethyl)amide)$_{50}$ (Ps-PIAT) developed as enzyme nanoreactors for cascade reactions. The enzyme loading efficiency was 25%.[101] O'Neil *et al.*[102] developed an effective method for ovalbumin and BSA encapsulation. They mixed poly (ethylene oxide)-*b*-poly(propylene sulfide) (PEO-*b*-PPS) and PEO and then added a protein aqueous solution, leading to the rapid formation of vesicles and effective protein encapsulation. A formulation of PEO-*b*-PPS/PEG500 yielded a mixture of different sized Ps and encapsulated proteins at an efficiency of 15–37%.

Wang *et al.*[103] studied the encapsulation of BSA, myoglobin, immunoglobulin G and lysozyme in poly(2-(methacryloyloxy)-ethyl phosphorylcholine)-*b*-poly(2-(diisopropylamino)ethyl methacrylate) (PMPC-*b*-PDPA) Ps by electroporation. The morphology and size of the Ps remained essentially unchanged after incorporation, indicating efficient self-healing of the Ps membranes after the applied high voltage pulses. The surface charge of the proteins played a key role in electroporation and negatively charged molecules were loaded in higher amounts than positively charged molecules. Also, encapsulation efficiency, loading number (L_N), and loading efficiency (L_E) were found to increase with the number of pulses, but were little affected by the applied voltage.[104]

Liu *et al.*[105] demonstrated that biodegradable chimeric Ps based on asymmetric poly(ethylene oxide)-*b*-poly(ε-caprolactone)-*b*-poly(2-(diethylamino)ethyl methacrylate) (PEO-*b*-PCL-*b*-PDEA) triblock copolymers are multifunctional nanocarriers that efficiently deliver and release exogenous proteins into cancer cells. Cytotoxicity assays (MTT) showed that the unloaded Ps were non-toxic up to a concentration of 0.5 mg mL^{-1}. These chimeric Ps, showed remarkably high L_N, and L_E values for BSA, cytochrome C, lysozyme, ovalbumin and immunoglobulin G. Moreover, protein encapsulation did not significantly alter the Ps size distribution and zeta potential. Protein release studies showed that both BSA and cytochrome C were released in a controlled manner and confocal laser scanning microscopy showed that fluorescein isothiocyanate-cytochrome C loaded Ps efficiently delivered the protein into the cytoplasm of RAW 264.7 cells.

Zhang *et al.*[106] developed pH and reduction dual-bioresponsive nanosized Ps based on poly(ethylene oxide)-*SS*-poly-(2-(diethyl amino)ethyl methacrylate) (PEO-SS-PDEA) diblock copolymers. These copolymers exist as unimers in water under mildly acidic conditions, but readily form monodisperse nanosized Ps (54–67 nm) on adjusting solution pH to 7.4. Therefore, they are highly sensitive to intracellular pH and reductive environments, which results in fast dissociation and aggregation. Fluorescein isothiocyanate (FITC)-labeled bovine serum albumin (FITC-BSA) and cytochrome C (FITC-CC) were encapsulated into PEO-SS-PDEA Ps, as a result of electrostatic interactions between proteins and PDEA. *In vitro* release studies showed that protein release was minimal (<20% in 8 h) at pH 7.4 and 37 °C, but significantly enhanced (67.7% in 8 h) at pH 6.0 due to collapse of Ps. The fastest protein release was observed under intracellular-mimicking reductive environments. Confocal laser scanning microscopy revealed that FITC-CC-loaded PEO-SS-PDEA Ps efficiently delivered and released cytochrome c into MCF-7 cells following 6 h of incubation, demonstrating the anti-tumor potential of this nanostructured system. Cheng *et al.*[107] also developed reduction and temperature dual responsive crosslinked Ps based on two thermo-sensitive triblock copolymers, PEO$_{5k}$-PAA$_{1.7k}$-PNIPAM$_{22k}$ and PEO$_{5k}$-PAA$_{0.7k}$-PNIPAM$_{12k}$ (PEO-PAA-PNIPAM), with elevated lower critical solution temperatures. They demonstrated that both are good nanocarriers for intracellular protein release.

Another interesting application refers to the use of Ps as non-cellular hemoglobin (Hb)-based oxygen carriers. Arifin *et al.*[92] demonstrated that poly(butadiene)-poly(ethylene oxide) Ps loaded with Hb (PEH) presenting oxygen affinity comparable to that of human erythrocytes and radii larger than 50 nm could be easily obtained. PEH loading capacities were higher than PEG-liposome encapsulating hemoglobin (LEH) and PEGylated actin-containing liposomes loaded with Hb (LEAcHb).

Encapsulation of proteins within the aqueous lumen of Ps can in principle take advantage of the extended circulation kinetics and controlled release properties of Ps. Nonetheless, Meier *et al.*[108] proved that proteins could also be incorporated into hyperthick triblock copolymer membranes while maintaining their functionality as measured by membrane conductance. Incorporation of proteins in "black films" of block copolymers has been expanded for applications in sensors[109] and protein-driven energy transduction across polymeric biomembranes.[110]

Although Ps are attractive candidates for protein encapsulation, further developments are necessary to overcome the poor encapsulation efficiency usually observed. Still, these nanocarriers have opened a new avenue to intracellular delivery of proteins and peptide drugs and may further be employed as a powerful tool for understanding protein functions in cells.[105]

As a major drawback of Ps in protein drug delivery, one can cite their poor encapsulation efficiency, previously discussed in the literature.[106,111,112] Comparing to liposomes, for example, the encapsulation of proteins in polymersomes seems complex because their large membrane thickness ($d \approx 8$–21 nm) compared to liposomes ($d \approx 3$–5 nm) increases thermodynamic barriers to mobility, and thus the polymer can remain in a kinetically trapped bulk phase if water is directly added without a thin film first.[102,111] Lee[113] has found an encapsulation efficiency of 5% for BSA and 4.5% for hemoglobin by film rehydration, and Arifin and Palmer[92] describe encapsulation efficiencies of 2.7–12% for bovine haemoglobin by film rehy-

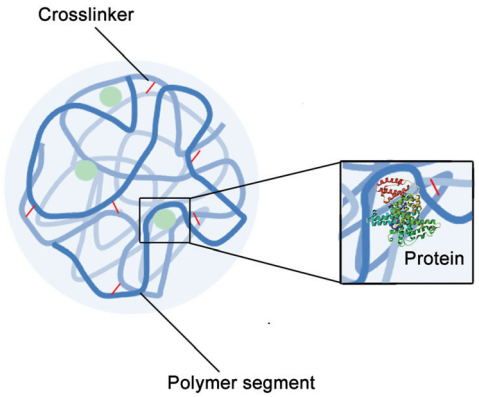

Fig. 5 Hydrogel nanoparticles (HN). Red line: crosslinker (hydrophobic interaction, hydrogen bond, electrostatic interaction, coordination, host–guest interaction). Blue lines: polymer segments.

dration employing different conditions. Preparation method can directly interfere with Ps encapsulation ability. In this sense, O'Neil[102] showed that direct hydration of polymers composed of poly(ethylene glycol)-*bl*-poly(propylene sulphide) results in Ps with encapsulation rates of 37% for ovalbumin (Ova), 19% for BSA and 15% for bovine γ-globulin (γ-globulin).

5. Hydrogel nanoparticles

Hydrogel technology has led to dramatic advances in pharmaceutical and biomedical fields.[114–116] Over the years, researchers have described many definitions for this term; the most common is that hydrogel is a water-swollen, crosslinked polymeric network produced by the simple reaction of one or more monomers (Fig. 5). Another definition states that hydrogel is a polymeric material that exhibits the ability to swell and retain a significant fraction of water within its structure, but will not dissolve in water.[117] This type of carrier can be classified based on source (natural or synthetic); polymeric composition (homopolymeric, copolymeric, multipolymer interpenetrating polymeric), configuration (amorphous, semi-crystalline, crystalline), type of crosslinking (chemical or physical nature), physical appearance (matrix, film, or microsphere) and according to network electrical charge (nonionic, ionic, amphoteric electrolyte and zwitterionic).[117]

Hydrogels have some common physical properties resembling that of the living tissues: high water content, soft and robbery consistency and low interfacial tension with water or biological fluids.[118] The polymer ability to absorb water comes from hydrophilic groups such as –OH, –CONH–, –$CONH_2$–, and –SO_3H.[119] Hydration degree will depend on aqueous environment and polymer composition (up to 90 wt%).[120,121] In accordance, polymeric networks of hydrophobic groups (*e.g.*, poly(lactic acid) (PLA) or poly(lactide-*co*-glycolide) (PLGA)) have limited water absorbing capacities (<5–10%). Despite their high water absorbing affinity, hydrogels show a swelling behavior, instead of dissolution, as a consequence of the critical crosslinks present in their structure. The crosslinks in the polymer network are provided by covalent bonds, hydrogen bonds, van der Waals interactions, or physical entanglement.[120]

Considering the source, hydrogels are divided into natural, synthetic and combinational, *i.e.*, based on semisynthetic polymers (Table 2). Among the natural polymers, chitosan and alginate are the most studied ones for HN preparation, while poly(vinyl alcohol), poly(ethylene oxide), poly(ethyleneimine), poly(vinyl pyrrolidone), and poly-*N*-isopropylacrylamide are the most employed synthetic polymers.

Natural polymers are mainly polysaccharides, generally subdivided into cationic (chitosan), anionic (hyaluronic acid, alginate, heparin, pectin, and others) and nonionic (pullulan and dextran). The use of hydrogel technology can bring many advantages, namely: stable nanostructures, biocompatibility, biodegradability and ability to load a large variety of protein

Table 2 Natural and synthetic polymers, and their derivatives used in hydrogels. Adapted from Hamidi *et al.*[120]

Natural polymers and their derivatives	
Anionic polymers	Hyaluronic acid, alginic acid, pectin, carrageenan, chondroitin sulfate, dextran
Cationic polymers	Chitosan and polylysine
Amphipathic polymers	Collagen (and gelatin), carboxymethyl chitin and fibrin
Neutral polymers	Dextran, agarose and pullulan
Synthetic polymers	
Polyesters	PEO-PLA-PEO, PEO-PLGA-PEO, PEO-PCL-PEO, PLA-PEO-PLA, PHB, P(PF-*co*-EO)$_6$ acrylate end groups and P(PEO/PBO terephthalate)
Other polymers	PEO-bis-(PLA-acrylate), PEO_6CDs, PEO-*g*-P(AAm-*co*-Vamine), PAAm, P(NIPAAm-*co*-AAc), P(NIPAAm-*co*-EMA), PVAc/PVA, PNVP, P(MMA-*co*-HEMA), P(AN-*co*-allylsulfonate), P(biscarboxy-phenoxy-phosphazene) and P(GEMA-sulfate)
Combinations of natural and synthetic polymers	
P(PEG-*co*-peptides), alginate-*g*-(PEO-PPO-PEO), P(PLGA-*co*-serine), collagen-acrylate, alginate-acrylate, P(HPMA-*g*-peptide), P(HEMA/Matrigel®) and HA-*g*-NIPAAm	

Abbreviations: PEO, poly(ethylene oxide); PLA, poly(lactic acid); PLGA, poly(lactic-*co*-glycolic acid); PCL, polycaprolactone; PHB, poly(hydroxybutyrate); PF, propylene fumarate; EO, ethylene oxide; PBO, poly(butylene oxide); CD, cyclodextrin; PAAm, polyacrylamide PNIPAAm, poly(*N*-isopropyl acrylamide); PVA, poly(vinyl alcohol); PVamine, poly(vinyl amine); PVAc, poly(vinyl acetate); PNVP, poly(*N*-vinyl pyrrolidone); PAAc, poly(acrylic acid); HEMA, hydroxyethyl methacrylate; PAN, polyacrylonitrile; PGEMA, poly(glucosylethyl methacrylate); PPO, poly(propyleneoxide); PHPMA, poly(hydroxypropyl methacrylamide); PEMA, poly(ethyl methacrylate); PAN, polyacrylonitrile; PMMA, poly(methyl methacrylate).

drugs. According to Vermonden,[122] hydrogel porous structure and water content are extremely suitable to accommodate high loads of water-soluble proteins and peptides.

Additionally, protein immobilization in the HN polymeric network provides stability and preserves protein 3D structure. Hydrogel swelling and degradability can also be tailored according to the type of polymer to achieve sustained release of both small peptides and large proteins.[123] Another advantage is that HN may surpass immune system detection; if recognition occurs, it triggers T independent immune responses that generally result in lack of immunological memory.[124] The latter feature is important for prolonging nanostructure plasma half-life.

As an example, insulin-loaded chitosan HN have been prepared by ionic gelation with tripolyphosphate.[125] Chitosan HN were obtained within a size range of 300–400 nm, a positive surface charge ranging from +54 to +25 mV, and loading efficiency up to 55%. Pan *et al.*[126] also prepared an insulin-loaded chitosan HN that enhanced *in vivo* intestinal absorption of insulin in alloxan-induced diabetic rats to a greater extent than an aqueous solution of chitosan. After oral administration of 21.1 IU kg^{-1} of insulin loaded in the chitosan HN, hypoglycemia was prolonged over 15 h. Many ongoing investigations point to the improved oral bioavailability of peptides and proteins in chitosan HN. In these studies, it is claimed that the bioadhesion property of chitosan HN further enhances the intestinal absorption of protein drugs.[127,128]

Regarding synthetic polymers, poly(vinyl alcohol) (PVA) HN were explored as protein/peptide delivery systems. The water-in-oil emulsion was produced by cyclic freezing–thawing procedure and no emulsifiers.[129] Average diameter of PVA HN obtained was 675 ± 43 nm, with BSA loading efficiency of 96.2 ± 3.8% and diffusion-controlled release trend. More recently, biodegradable polyesters consisting of short poly(lactone) chains grafted to PVA or charge-modified sulfobutyl-PVA (SB-PVA) were prepared and used as a novel class of water soluble comb-like polyesters.[120] These polymers undergo spontaneous self-assembling to produce HN, which form stable complexes with a number of proteins such as human serum albumin, tetanus toxoid and cytochrome C.[130]

Stimuli responsive (pH, temperature) hydrogels have also been prepared and found to be tissue compatible.[131] For example, pH-sensitive hydrogels with immobilized glucose oxidase were investigated for a glucose-sensitive insulin-releasing system.[61] Pattou and Palmer[132] developed a novel type of temperature-responsive HN of poly(*N*-isopropyl acrylamide) encapsulating bovine hemoglobin, which might benefit tissue hypoxia caused by decreased body temperature.

6. Single-protein nanocapsules

Recently, an innovative delivery system with a core–shell structure named single-protein nanocapsules (SPN) was proposed.[133] In SPN (Fig. 6), a protein forms the core and a polymeric shell is covalently linked to this core. The polymeric shell comprises a thin permeable layer of degradable or non-degradable polymer. To obtain SPN, polymerizable groups are covalently linked to the protein and the polymerization occurs in an aqueous solution containing monomers (ionic or neutral) and a crosslinker (degradable or not), resulting in each protein core being enfolded in a thin polymer shell. From the morphological point of view, these protein carriers are spherical, with a uniform diameter in the nanoscale range.[133]

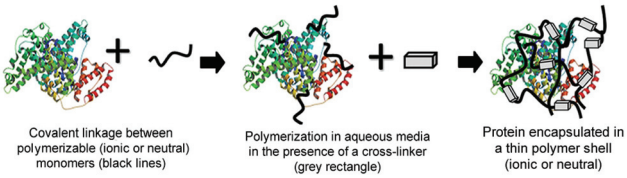

Fig. 6 Single-protein nanocapsules (SPN) preparation, using a hydrophilic protein as a model.

From the diagnostic point of view, non-degradable SPN offer an interesting alternative for the use of proteins such as green fluorescent protein (GFP), horseradish peroxidase (HRP), bovine serum albumin (BSA), superoxide dismutase (SOD) and caspase-3 (CAS). The ability of these SPN to circulate and accumulate in certain tissues, such as tumors due to an enhanced permeation and retention effect, opens a new direction for imaging and tumor tracking. Du *et al.*[134] encapsulated the enzyme horseradish peroxidase by SPN and decorated the polymeric shell with quantum dots, as a new possibility for bioluminescence imaging and therapeutics.

Despite the use of covalent linkage in the original description of this system, noncovalent encapsulation of proteins into a positively-charged polymeric shell organized by disulfide-containing crosslinkers was also reported. This non-covalent strategy resulted in SPN susceptible to cell internalization and further dissociation in the reducing cytosol to release the protein.[135] In this paper, the authors show that caspase 3 (CP-3) can be delivered as a SPN and can induce apoptosis in a variety of human cancer cell lines, including HeLa, MCF-7 and U-87 MG.

Therefore, SPN is also an interesting alternative for intracellular delivery of proteins. Proteases are commonly present in a physiological environment and may lead to protein drug degradation. In this sense, the polymeric shell of SPN protects the protein core from proteolysis. Yan *et al.*[133] showed that the fluorescence intensity of native enhanced green fluorescent protein (EGFP) exposed to proteases (trypsin and a-chymotrypsin) corresponds to 60% of its original fluorescence intensity, whereas the EGFP in SPN retained more than 90% of fluorescence intensity.

It is clear to us the similarity between SPN and the well-established PEGylation strategy to improve protein stability and pharmacokinetics. PEGylation defines the modification of a protein, peptide or non-peptide molecule by the linking of one or more poly(ethylene oxide) (PEO or PEG) chains.[136] In spite of the beneficial effects achieved with PEG coupling, this strategy is usually associated with reduced protein bioactivity.

Nonetheless, the number and location of PEG chains covalently attached to the protein as well as geometry (*e.g.* linear *versus* branched) and molecular weight of PEG can be studied to minimize activity loss.[137] Similarly to PEGylation, SPN can be deleterious for protein activity because amino acids that are fundamental for protein–target interaction may be directly involved in the coupling. Also, steric hindrance may occur if the polymer is attached close to the protein recognition surface. Nonetheless, SPN seems an intelligent and evolutionary strategy to PEGylation that allows engineering of the thin polymeric shell to either degrade or remain stable at different pHs and/or other stimuli.

7. Nanostructure stabilization – colloidal stability

Nanostructure stabilization is remarkably important for commercial drugs, vaccines and biomarkers, *i.e.*, for products where long-term stability is essential for an acceptable shelf life. Colloidal instability may arise from different physicochemical properties, such as pH, surface charge of the particle and so on. In a simplified physical view, considering the spherical approximation for colloidal particles, stability regarding aggregation depends on a balance between repulsive and attractive forces, in which gravity and buoyancy forces will dictate the particle precipitation behavior. Dominance of attraction makes particles adhere to each other leading to aggregation or fusion; and when gravity forces dominate, particles settle down. On the other hand, if buoyancy forces dominate, particles can cream up or flocculate. Moreover, in the case of a liquid dispersed phase, instability results in phase separation. Several models are available in the literature attempting to describe and/or predict these events, but there is no model able to universally describe colloidal stability regardless of nanoparticle type.[138] Nonetheless, some common features are important concerning colloidal stability. In the simplest case, considering a sphere in solution, there will be, at most, three forces acting on the particle: gravity (g), the viscous force (F_{visc}) and the buoyancy (F_b), written as:

$$F_b = \rho_{solution} \frac{4\pi}{3} R^3 g \quad (3)$$

where $\rho_{solution}$ is the solution density and R the sphere radius.

The viscous force (F_{visc}), on the other hand, can be written as:

$$F_{visc} = 6\pi\eta R v_{sed} \quad (4)$$

where η is the solution viscosity, and v_{sed} the particle sedimentation velocity, which is directly connected to the particle colloidal stability and can be written as:

$$v_{sed} = \frac{2R^2\left(\rho_{particle} - \rho_{solvent}\right)g}{9\eta} \quad (5)$$

Note that the velocity can have two different directions depending on $(\rho_{particle} - \rho_{solvent})$ value. Moreover, eqn (5) shows that v_{sed} decreases when (i) the solution dynamic viscosity (η) increases; (ii) the difference between particle and liquid densities $(\rho_{particle} - \rho_{solvent})$ decreases, and (iii) the particle size, R, is reduced.[139] Viscosity increment is performed with thickening agents like biopolymers and polyacrylic acids[140] as long as compatibility with the administration route and manufacturing process is maintained. Density alterations are mostly performed for the dispersion medium. Nonetheless, thickening agents and density modifiers may alter the electrical charge of the dispersion medium, favoring repulsion or attraction.

Since v_{sed} is proportional to the square of particle radius (eqn (5)), reduction in the particle size can effectively decrease sedimentation. However, size reduction increases the surface energy and may result in particle aggregation. If this is the case, stability will require the addition of excipients to keep the particles apart.[141]

Nanostructures dispersed in aqueous medium can acquire a surface electric charge through protonation, ionization, ion adsorption or ion dissolution. One interesting physical parameter that can be evaluated regarding protein nanoparticle charge surface is the ζ-potential, defined as the electric potential in the slipping plane relative to the potential far away from the particle.

This electrical potential is related to the colloidal stability, thus, the higher the ζ-potential the higher is the repulsion between adjacent particles in solution and, as a consequence, higher is the colloidal stability. Other forces may also act in colloidal stability, like van der Waals interactions, and must be taken into account for some specific cases.

Electrostatic stabilization of nanostructures might come from pH change, chemical reactions or ion adsorption.[142,143] As a rule, ζ-potentials above an absolute value of 30 mV provide long-term stability for electrostatically-stabilized nanostructures.[144] To decide the appropriate ζ-potential and ionic charge, nanostructure applications should be considered. For instance, positively charged nanostructures have enhanced loading of negatively-charged proteins. However, if high ζ-potential values may favor loading, excessive positive charge causes non-specific binding and consequent uptake by non-targeted cells.[145]

Steric stabilization of nanostructures relies on a variety of molecules, including: nonionic surfactants (*i.e.* tweens, triton X-100)[146] and nonionic polymers (*i.e.* polyethylene oxide, polyvinyl alcohol, polyvinylpyrrolidone).[147] These molecules may be linked to the nanostructures by adsorption or chemical conjugation (*e.g.* PEGylated liposomes). Electrostatic and steric stabilization can be combined with the use of ionic macromolecules, like negatively charged alginate or positively charged chitosan.[148,149]

8. Conclusions

Nanobiotechnology has already proven its ability to overcome barriers involved in protein drug delivery and we expect to see

more successful examples with FDA and EMA approval in the upcoming years. Also, the possibility of different formulation methods, physicochemical properties, release mechanisms and even targeting chemistries make nanocarriers very attractive to encapsulate proteins of therapeutic interest. While liposomes are well-established nanostructures, with therapeutically approved examples, chemical and physical instability have prevented significant development of commercially stable and bioactive protein formulations with lipids. We believe polymersomes are very promising and will attract more attention, with many possibilities of copolymers to be employed, low RES recognition and extended circulation time. In comparison with lipids employed in liposomes formulations, relatively stable and biodegradable copolymers can be used to produce polymersomes, owing to the progress in polymer chemistry. Hydrogel nanoparticles also deserve attention and show great potential for non-parenteral protein drug delivery. Again, the progress in polymer chemistry, has been contributing significantly to the synthesis of hydrogels with well-defined and fine-tunable degradation kinetic as well as mechanical properties. Regarding single-protein nanocapsules, we consider it an interesting alternative, especially for diagnostics. For therapeutic proteins, one should keep in mind that covalent linkage of polymers may result in loss of protein activity and degradable crosslinkers are more appropriate.

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References

1 FDA Online, http://www.fda.gov/ScienceResearch/SpecialTopics/Nanotechnology/ucm309677.htm (accessed February 2015).
2 O. Kammona and C. Kiparissides, *J. Controlled Release*, 2012, **161**, 781–794.
3 A. Bernkop-Schnurch, Nanocarrierr, *Eur. J. Pharm Sci.*, 2013, **49**, 272–227.
4 L. Brannon-Peppas and J. O. Blanchette, *Adv. Drug Delivery Rev.*, 2004, **56**, 1649–1659.
5 K. S. Sahoo and V. Labhasetwar, *Drug Discovery Today*, 2003, **8**, 1112–1120.
6 R. Lobenberg and J. Kreuter, *AIDS Res. Hum. Retroviruses*, 1996, **12**, 1709–1715.
7 M. Rawat, D. Singh, S. Saraf, *et al.*, *Biol. Pharm. Bull.*, 2006, **29**(9), 1790–1798.
8 J. H. Adair, M. P. Parette, E. I. Altınoglu, *et al.*, *ACS Nano*, 2010, **4**(9), 4967–4970.
9 S. Mao, W. Sun and T. Kissel, *Adv. Drug Delivery Rev.*, 2010, **62**, 12–27.
10 Z. Antosova, M. Mackova, V. Kral, *et al.*, *Biotechnology*, 2009, **27**(11), 628–635.
11 V. M. Balcão, C. I. Costa, C. M. Matos, *et al.*, *Food Hydrocolloids*, 2013, **32**(2), 425–431.
12 C. Prego, D. Torres, E. Fernandez-Megia, *et al.*, *J. Controlled Release*, 2006, **111**(3), 299–308.
13 J. H. Park, G. Saravanakumar, K. Kim, *et al.*, *Adv. Drug Delivery Rev.*, 2010, **62**, 28–41.
14 C. Liu and S. Yu S, *Colloids Surf., B*, 2010, **79**, 509–515.
15 D. J. McClements, *Soft Matter*, 2012, **8**, 1719–1729.
16 M. M. Fryd and T. G. Mason, *Annu. Rev. Phys. Chem.*, 2012, **63**, 493–518.
17 J. Komaiko and D. J. McClements, *J. Colloid Interface Sci.*, 2014, **425**, 59–66.
18 Y. Yang and D. J. McClements, *Food Hydrocolloids*, 2013, **30**(2), 712–720.
19 C. Qian and D. J. McClements, *Food Hydrocolloids*, 2011, **25**(5), 1000–1008.
20 S. Graves, K. Meleson, J. Wilking, *et al.*, *J. Chem. Phys.*, 2005, **122**, 134703.
21 C. Bilbao-Sáinz, R. J. Avena-Bustillos, D. F. Wood, *et al.*, *J. Agric. Food Chem.*, 2010, **58**(22), 11932–11938.
22 C. Qian, E. A. Decker, H. Xiao, *et al.*, *Food Chem.*, 2012, **135**(3), 1440–1447.
23 S. Martins, B. Sarmento, D. C. Ferreira, *et al.*, *Int. J. Nanomed.*, 2007, **2**(4), 595–607.
24 G. Eskandar, N. Simovic and C. Prestige, *Pharm. Res.*, 2009, **26**, 1764–1775.
25 J. C. Schwarz, V. Klang, S. Karall, *et al.*, *Int. J. Pharm.*, 2012, **435**(1), 69–75.
26 S. V. R. Rao, K. Yajurvedi and J. Shao, *Int. J. Pharm.*, 2008, **362**, 16–19.
27 X. Li, J. Qi, Y. Xie, *et al.*, *Int. J. Nanomed.*, 2013, **8**, 23–32.
28 F. Donsì, M. Annunziata, M. Vincensi, *et al.*, *J. Biotechnol.*, 2012, **159**(4), 342–350.
29 V. Ghosh, A. Mukherjee and N. Chandrasekaran, *Ultrason. Sonochem.*, 2013, **20**(1), 338–344.
30 S. Lee and D. McClements, *Food Hydrocolloids*, 2010, **24**(6–7), 560–569.
31 S. B. Calderilla-Fajardo, J. Cazares-Delgadillo, R. VillalobosGarcia, *et al.*, *Drug Dev. Ind. Pharm.*, 2006, **32**(1), 107–113.
32 K. Mitri, R. Shegokar, S. Gohla S, *et al.*, *Int. J. Pharm.*, 2011, **414**(1–2), 267–275.
33 D. M. Webster, P. Sundaram and M. E. Byrne, *Eur. J. Pharm. Biopharm.*, 2013, **84**, 1–20.
34 K. Y. Lee and S. H. Yuk, *Prog. Polym. Sci.*, 2007, **32**, 669–697.
35 X. Liu and G. Huang, *Am. J. Plant Sci.*, 2013, **8**, 319–328.
36 D. L. Lasic, *Trends Biotechnol.*, 1998, **16**(7), 307–321.
37 J. Israelachvili, *Colloids Surf., A*, 1994, **91**, 1–98.
38 R. Mo, T. Jiang, J. Di, *et al.*, *Chem. Soc. Rev.*, 2014, **43**, 3595–3629.

39 M. J. Hope, M. B. Bally and L. D. Mayer, *Chem. Phys. Lipids*, 1986, **40**, 89–107.
40 A. D. Bangham, M. M. Standish and J. C. J. Watkins, *Mol. Biol.*, 1965, **13**, 238–252.
41 Y. Barenholz, S. Amselm and D. A. Lichtenberg, *FEBS Lett.*, 1979, **99**(1), 210–214.
42 F. Jr-Szoka and D. Papahadjopoulos, *Biochemistry*, 1978, **75**(9), 4194–4198.
43 L. T. Mimms, G. Zampighi, Y. Nozaki, *et al.*, *Biochemistry*, 1981, **20**(4), 833–840.
44 S. C. A. Lopes, M. V. M. Novais and C. S. Texeira, *Biomed. Res. Int.*, 2013, 1–7.
45 X. Zhang, J. Qi, Y. Lu, *et al.*, *NATO Science. Peace*, 2014, **10**, 167–176.
46 Y. Barenholz, *Curr. Opin. Colloid Interface Sci.*, 2001, **6**, 66–77.
47 O. G. Mouristen and K. Jorgenson, *Pharm. Res.*, 1998, **15**, 1507–1519.
48 A. A. Gabizon, H. Shmeeda and S. Zalipsky, *J. Liposome Res.*, 2006, **16**, 175–183.
49 J. M. Metselaar, E. Mastrobattista and G. Storm, *Mini Rev. Med. Chem.*, 2002, **2**, 319–329.
50 R. M. Straubinger, K. Hong, D. S. Friend, *et al.*, *Cell*, 1983, **32**, 1069–1079.
51 J. Huwyler, J. Yang and W. M. Pardridge, *J. Pham. Exp. Ther.*, 1997, **282**, 1541–1546.
52 P. M. Anderson, D. C. Hanson, D. E. Hasz, *et al.*, *Cytokine*, 1994, **6**, 92–101.
53 J. Meyer, L. Whitcomb and D. Collins, *Biochem. Biophys. Res. Commun.*, 1994, **199**(2), 433–438.
54 J. Guo, Q. Ping and L. Zhong, *Drug Delivery*, 2000, **7**(2), 113–116.
55 K. Kajimoto, M. Yamamoto, M. Watanabe, *et al.*, *Int. J. Pharm.*, 2011, **403**, 57–65.
56 M. J. Fonseca, M. A. Alsina and F. Reig, *Biochim. Biophys. Acta, Biomembr.*, 1996, **1279**, 259–265.
57 E. Mastrobattista, G. A. Koning, L. van Bloois, *et al.*, *J. Biol. Chem.*, 2002, **277**(30), 27135–27143.
58 S. Simoes, J. N. Moreira, C. Fonseca, *et al.*, *Adv. Drug Delivery Rev.*, 2004, **56**(7), 947–965.
59 M. M. Fretz, A. Hogset, G. A. Koning, *et al.*, *Pharm. Res.*, 2007, **24**(11), 2040–2047.
60 G. Poste, C. Bucana, A. Raz, *et al.*, *Cancer Res.*, 1982, **42**, 1412–1422.
61 C. Kiparissides and O. Kammona, *Can. J. Chem. Eng.*, 2013, **91**, 638–651.
62 M. L. Immordino, F. Dosio and L. Cattel, *Int. J. Nanomed.*, 2006, **1**(3), 297–315.
63 S. Park, S. G. Choi, E. Dava, *et al.*, *Int. J. Pharm.*, 2011, **415**, 267–272.
64 E. Kedar, H. Gur, I. Babai, *et al.*, *J. Immunother.*, 2000, **23**, 131–145.
65 V. P. Torchilin, *Nature*, 2005, **4**, 145–160.
66 T. M. Allen and P. R. Cullis, *Science*, 2004, **303**(5665), 1818–1822.
67 G. A. Koning and G. C. Krijger, *Adv. Anticancer Agents Med. Chem.*, 2007, **7**(4), 425–440.
68 M. Dicheva and G. A. Koning, *Expert Opin. Drug Delivery*, 2014, **11**(1), 83–100.
69 B. Smith, I. Lyakhov, K. Lommis, *et al.*, *J. Controlled Release*, 2011, **153**(2), 187–194.
70 A. Ito, F. Matsuoka, H. Honda, *et al.*, *Cancer Immunol. Immunother.*, 2004, **53**, 26–32.
71 R. Schumacher, M. Adamina, R. Zurbriggenb, *et al.*, *Vaccine*, 2004, **22**, 714–723.
72 M. Adamina, M. Bolli, F. Albo, *et al.*, *Br. J. Cancer*, 2004, **90**, 263–269.
73 I. P. Hunziker, B. Grabscheid and R. Zurbriggen, *Int. Immunol.*, 2002, **14**(6), 615–626.
74 B. Cornet, M. Vandenbranden, J. Cogniaux, *et al.*, *Biochem. Biophys. Res. Commun.*, 1990, 167–122.
75 T. M. Allen and P. R. CulliS, *Adv. Drug Delivery Rev.*, 2013, **65**, 36–48.
76 A. D. Bangham, *Chem. Phys. Lipids*, 1993, **64**, 275–285.
77 J. Du and R. K. O'Reilly, *Soft Matter*, 2009, **5**, 35–44.
78 J. S. Lee and J. Feijen, *J. Controlled Release*, 2012, **161**, 473–483.
79 J. S. Lee, W. Zhou, F. Meng, *et al.*, *J. Controlled Release*, 2010, **146**, 400–408.
80 M. Antoniett and S. Foster, *Adv. Mater.*, 2003, **15**(16), 1323–1333.
81 R. M. Grallert, C. O. Rangel-Yagui, K. F. M. Pasqualoto, *et al.*, *Braz. J. Pharm. Sci.*, 2012, **48**(1), 1–16.
82 S. B. La, T. Okano and K. Kataoka, *J. Pharm. Sci.*, 1996, **85**(1), 85–90.
83 Y. Yamamoto, K. Yasugi, A. Harada, *et al.*, *J. Controlled Release*, 2002, **82**, 359–371.
84 V. Weissig, K. R. Whiteman and V. P. Torchilin, *Pharm. Res.*, 1998, **15**(10), 1552–1556.
85 K. Letchford and H. A. Burt, *Eur. J. Pharm. Biopharm.*, 2007, **65**(3), 259–269.
86 A. J. Pamell, N. Tzokova, P. D. Topham, *et al.*, *Faraday Discuss.*, 2009, **143**, 29–46.
87 J. P. Jain, W. Y. Ayen and N. Kumar, *Curr. Pharm. Des.*, 2011, **17**(1), 65–79.
88 F. Ahmed and D. E. Discher, *J. Controllled Release*, 2004, **96**, 37–53.
89 N. Nishiyama and K. Kataoka, *Pharmacol. Ther.*, 2006, **112**(3), 630–648.
90 F. Checot, S. Lecommandoux, H. A. Klok, *et al.*, *Eur. Phys. J. E*, 2003, **10**, 25–35.
91 O. Onaca, R. Enea, D. W. Hughes, *et al.*, *Macromol. Biosci.*, 2009, **9**, 129–139.
92 D. R. Arifin and A. F. Palmer, *Biomacromolecules*, 2005, **6**, 2172–2181.
93 J. C. M. Lee, H. Bermudez, B. M. Discher, *et al.*, *Biotechnol. Bioeng.*, 2001, **73**, 135–145.
94 P. J. Photos, L. Bacakova, B. Discher, *et al.*, *J. Controlled Release*, 2003, **90**, 323–334.
95 H. Bermudez, A. K. Brannan, D. A. Hanmer, *et al.*, *Macromolecules*, 2002, **35**, 8203–8208.
96 J. A. Opsteen, J. J. L. M. Cornelissen and J. C. M. van Hest, *Pure Appl. Chem.*, 2004, **76**, 1309–1319.

97 J. H. Eike and A. F. Palmer, *Biotechnol. Prog.*, 2004, **20**, 1543–1549.
98 B. M. Discher, Y. Y. Won, D. S. Ege, *et al.*, *Science*, 1999, **284**, 1143–1146.
99 D. A. Christian, S. Cai, D. M. Bowen, *et al.*, *Eur. J. Pharm. Biopharm.*, 2009, **71**(3), 463–474.
100 H. Kim, Y. J. Kang, S. Kang, *et al.*, *J. Am. Chem. Soc.*, 2012, **134**, 4030–4403.
101 S. F. M. van Dongen, M. Nallani, J. J. L. M. Cornelissen, *et al.*, *Chem. – Eur. J.*, 2009, **15**, 1107–1114.
102 C. P. O'Neil, T. Suzuki and D. Demurtas, *Langmuir*, 2009, **25**(16), 9025–9029.
103 L. Wang, L. Chierico, D. Little, *et al.*, *Angew. Chem. Int. Ed.*, 2012, **51**, 11122–11125.
104 H. Lomas, J. Du, I. Canton, *et al.*, *Macromol. Biosci.*, 2010, **10**, 513–530.
105 G. Liu, M. Shoubao and S. Li, *Biomaterials*, 2010, 1–11.
106 J. Zhang, L. Wu, F. Meng, *et al.*, *Langmuir*, 2012, **28**(4), 2056–2065.
107 R. Cheng, F. Meng, S. Ma, *et al.*, *J. Mater. Chem.*, 2011, **21**(47), 19013–19020.
108 W. Meier, C. Nardin and M. Winterhalter, *Angew. Chem., Int. Ed.*, 2000, **39**(24), 4599–4602.
109 D. Wong, T. Jeon and J. Schmid, *Nanotechnology*, 2006, **17**(15), 3710–3717.
110 D. Ho, B. Chu, H. Lee, *et al.*, *Nanotechnology*, 2004, **15**(8), 1084–1094.
111 Y. Lee, J.-B. Chang, H. K. Kim, *et al.*, *Macromol. Res.*, 2006, **14**(3), 359–364.
112 D. E. Discher and F. Ahmed, *Annu. Rev. Biomed. Eng.*, 2006, **8**, 323–341.
113 J. C.-M. Lee, H. Bermudez, B. M. Discher, *et al.*, *Biotechnol. Bioeng.*, 2001, **73**(2), 135–146.
114 A. S. Hoffman, *Drug Delivery Rev.*, 2002, **43**, 3–12.
115 N. Kashyap and M. N. Kumar, *Crit. Rev. Ther. Drug Carrier Syst.*, 2005, **22**, 107–149.
116 N. A. Peppas, *Hydrogels in Medicine and Pharmacy- Synthetic Polymers. Florida*, CRC Press, 1987.
117 O. E. Ahmed, M. S. Wael and A. E. Nazik, *Pharm. Res.*, 2013, **30**, 512–522.
118 R. D. Ratner and A. S. Hoffman, *ACS Symp. Ser.*, 2009, **31**, 1–36.
119 N. A. Peppas and A. R. Khare, *Adv. Drug Delivery Rev.*, 1993, **11**, 1–35.
120 M. Hamidi, A. Azadi and P. Rafiei, *Adv. Drug Delivery Rev.*, 2008, **60**, 1638–1649.
121 N. A. Peppas, *J. Bioact. Compat. Polym.*, 1991, **6**, 241–246.
122 T. Vermonden, R. Creusi and W. E. Hennink, *ACS Nano*, 2012, **112**, 2853–2888.
123 S. Pacelli, P. Paolicelli and M. A. Casadei, *Carbohydr. Polym.*, 2015, **126**, 208–214.
124 A. González-Fernández, J. Faro and C. Fernandez, *Vaccine*, 2008, **26**, 292–300.
125 R. Fernández-Urrusuno, P. Calvo, C. Remuñán-López, *et al.*, *Pharm. Res.*, 1999, **16**(10), 1581–1599.
126 Y. Pan, Y. J. Li, H. Y. Zhao, *et al.*, *Int. J. Pharm.*, 2002, **249**, 139–147.
127 V. H. L. Lee, *Crit. Rev. Ther. Drug Carrier Syst.*, 1988, **5**, 69–97.
128 H. L. Luessen, B. J. de Leeuw, M. W. Langemeÿer, *et al.*, *Pharm. Res.*, 1996, **13**, 1668–1672.
129 J. K. Li, N. Wang and X. S. Wu, *J. Controlled Release*, 1998, **56**, 117–126.
130 K. S. Soppimath, T. M. Aminabhavi, A. R. Kulkarni, *et al.*, *J. Controlled Release*, 2001, **70**, 1–20.
131 H. Weng, J. Zhou, L. Tang, *et al.*, *J. Biomater. Sci., Polym. Ed.*, 2004, **15**, 1167–1180.
132 J. N. Pattou and A. F. Palmer, *Biomacromolecules*, 2005, **6**, 2204–2212.
133 M. Yan, J. Du, Z. Gu, *et al.*, *Nat. Nanotechnol.*, 2010, **5**(1), 48–53.
134 J. Du, C. Yu, D. Pan, *et al.*, *J. Am. Chem. Soc.*, 2010, **132**(37), 12780–12781.
135 M. Zhao, A. Biswas, B. Hu, *et al.*, *Biomaterials*, 2011, **32**(22), 5223–5230.
136 F. M. Veronesse and G. Pasut, *Drug Discovery Today*, 2005, **10**(21), 1451–1458.
137 G. Pasut, M. Sergi and F. M. Veronese, *Adv. Drug Delivery Rev.*, 2008, **20**, 69–78.
138 S. Ganguly and S. Chakraborty, *Phys. Lett. A*, 2011, **375**, 2394–2399.
139 C. J. Kim, *Advanced Pharmaceutics: Physicochemical Principles*, CRC Press, Florida, 2004.
140 D. Kumar, N. Jain, N. Gulati, *et al.*, *J. Adv. Pharm. Technol. Res.*, 2013, **4**, 9–17.
141 A. M. Cerdeira, M. Mazzotti and B. Gander, *Int. J. Pharm.*, 2010, **396**, 210–218.
142 A. Ghadimi, R. Saidur and H. S. C. A. Metselaar, *Int. J. Heat Mass Transfer*, 2011, **54**, 4051–4068.
143 S. Liufu, H. Xiao and Y. Li, *J. Colloid Interface Sci.*, 2005, **281**, 155–163.
144 R. Greenwood, *Adv. Colloid Interface Sci.*, 2003, **106**, 55–81.
145 S. Honary and F. Zahir, *J. Pharm. Res.*, 2013, **12**(2), 255–264.
146 L. KvÍtek, A. Panácek, J. Soukupová, *et al.*, *J. Phys. Chem. C*, 2008, **112**, 5825–5834.
147 O. Gaspar-Ramírez, F. J. Pérez-Vázquez, L. G. Pruneda-Álvarez, *et al.*, *Immunopharmacol. Immunotoxicol.*, 2012, **34**(4), 627–632.
148 L. Chronopoulou, L. Massini, M. Giardi, *et al.*, *Colloids Surf., B*, 2013, **103**, 310–317.
149 O. Borges, M. Silva, A. de Souza, *et al.*, *Int. Immunopharmacol.*, 2008, **8**, 1773–1780.

