CellPress

Review

Protein Formulations for Emulsions and Solid-in-Oil Dispersions

Madalena Martins,[1] Ana Loureiro,[1] Nuno G. Azoia,[1] Carla Silva,[1] and Artur Cavaco-Paulo[1,*]

Needs from medical and cosmetic areas have led to the design of novel nanosized emulsions and solid-in-oil dispersions of proteins. Here, we describe the production of those emulsions and dispersions using high-energy methodologies such as high-pressure homogenization or ultrasound. Recent work has resulted in new mechanistic insights related to the formation of protein emulsions and dispersions. The production method and composition of these formulations can determine major parameters such as size, stability, and functionality, and therefore their final application. Aqueous nanoemulsions of proteins can be used for drug delivery, while solid-in-oil dispersions are often used in transdermal applications.

Trends in the Production of Protein Emulsions and Dispersions

Protein formulations based on emulsions and dispersions are one of the most researched areas in biomedical and pharmaceutical/cosmetic agents. The production technology of such formulations can be found in applications ranging from targeted drug delivery to transdermal perfusion patches and cosmetics [1–3]. In this context, various methods have been used to formulate proteins, such as **coacervation/desolvation** (see Glossary), **thermal gelation**, **emulsification**, **self-assembly**, and **solid-in-oil dispersions** [4–7].

Nanoemulsions are emulsions with uniform and extremely small droplet size, which have attracted growing interest as colloidal drug carriers for pharmaceutical applications [8,9]. High-energy emulsification methods, such as sonication and high-pressure homogenization, are widely reported for preparing nanoemulsions [10,11]. The formation and stabilization of the droplets determines the preparation of a finely dispersed emulsion [12], where the high-energy methods are important through the promotion of efficient mass transfer. This phenomenon is mainly due to the formation of high turbulence that creates molecular agitation. The stabilization of the newly formed droplets against coalescence is the second step; **emulsifiers** are added to the system for this purpose [11,13]. The emulsifiers can lower the interfacial tension and prevent the agglomeration and coalescence of the droplets by increasing repulsion forces between droplets [13–15]. A wide variety of synthetic and natural emulsifiers can be used, such as surfactants, phospholipids, proteins, and polysaccharides [15]. Surfactants play a greater role in selecting emulsifiers due to their amphiphilic molecules, which can be applied in oil/water interface emulsions, resulting in the dispersion of one phase into another immiscible phase.

Solid-in-oil (S/O) dispersions consist of nanosized protein–surfactant complexes dispersed in an oil vehicle. S/O dispersions are attractive formulations for improving the dispersibility (or solubilization) of **hydrophilic** biomolecules such as **globular proteins** and peptides into the oil phase [16,17]. The method used to produce S/O dispersion is based on a combined high-energy

Trends

The design of protein-based templates of emulsions and solid-in-oil dispersions for drug delivery applications constitutes an immense platform for the delivery of active components intravenously or by skin permeation.

High-energy methodologies such as high-pressure homogenization and ultrasound play important roles in the formation and stabilization of emulsions and dispersions.

Emulsification and dispersibility methods are the most promising technologies to achieve controlled transport and delivery of active compounds.

[1]Centre of Biological Engineering (CEB), University of Minho, Campus of Gualtar, 4710-057 Braga, Portugal

*Correspondence: artur@deb.uminho.pt (A. Cavaco-Paulo).

 http://dx.doi.org/10.1016/j.tibtech.2016.03.001

approach. This method involves the use of equipment, such as high-pressure homogenizer and ultrasonic probe, which are capable to generate huge amounts of energy for emulsification and dispersion of the protein in an appropriate oil. This technique is an effective alternative method to formulate poorly water-soluble compounds [18]. S/O dispersion is a type of colloid with a material in a solid state dispersed in a media in liquid state, and this is generally designated as sol or suspension [6,18–20]. Nanodispersions are biphasic submicron colloidal dispersion formulations [17].

Here, we focus on the emulsification and dispersibility methods that are the most widely used approaches to obtain stable microscale and/or nanoscale protein formulations (Figure 1) as promising systems enabling controlled transport and delivery. We review recent works related to the production of both emulsions and S/O dispersions using high-energy methodologies. These protein formulations can be used for delivering active components intravenously or in skin permeation.

The Influence of Emulsifiers in Producing Emulsions and Dispersions

Protein microemulsions and nanoemulsions have been used to stabilize, protect, and deliver active components in different formulations. Several factors are known to influence the behavior of these formulations, including particle size and distribution, emulsifier type and concentration, aqueous solubility of the dispersed phase, temperature, surface tension, and ionic strength [21]. The nature of the emulsifier is crucial in the formation of an emulsion. The most effective emulsifiers are nonionic or mixtures (e.g., ionic and nonionic, or mixtures of nonionic surfactants). They can be more effective in emulsification for lowering the interfacial tension and stabilizing the emulsion against flocculation and coalescence. The emulsifier concentration has a direct effect on emulsion production, especially for obtaining homogeneous and small emulsions.

Protein molecules demonstrate greater mobilities at emulsion interfaces with the microsphere walls having spherical and regular surfaces, as shown in the schematic illustrations in Figure 2.

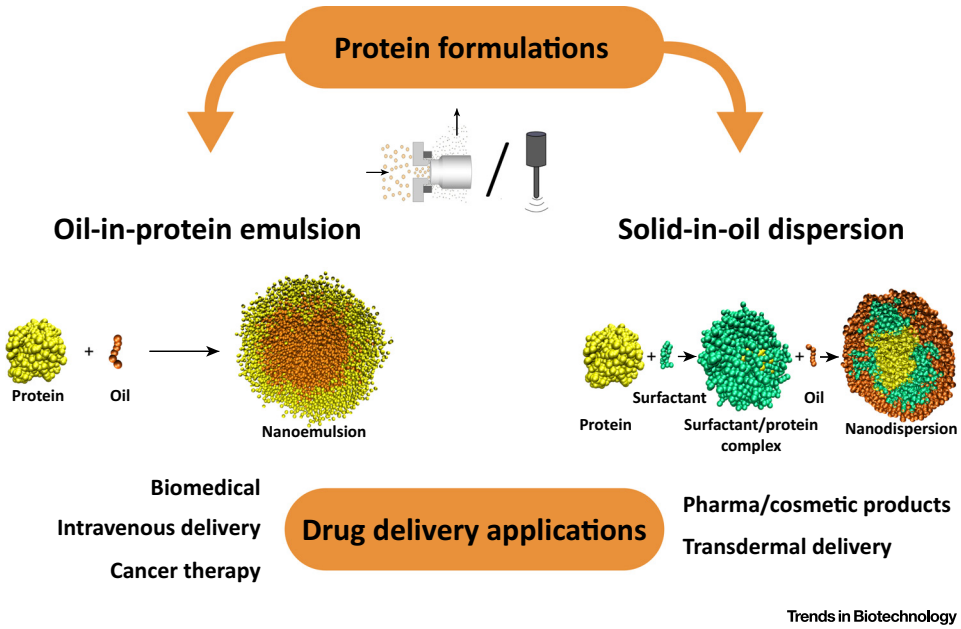

Figure 1. Schematic Illustration of Protein Formulations Based on Emulsification and Dispersibility Methods to obtain Microemulsions and/or Nanoemulsions and Dispersions, under Mechanical High-Energy Methodology for Drug Delivery Applications.

Glossary

Coacervation/desolvation: a thermodynamically driven self-assembly process for nanoparticle preparation, where a desolvating agent (acetone or ethanol) is added dropwise continuously to an aqueous protein solution under continuous stirring. In some cases, the addition of crosslinking agents is necessary to promote the nanoparticle stabilization.

Emulsification: a method widely used for preparing nanoparticles, in which an aqueous protein solution is emulsified in a nonaqueous medium, such as oil. There are different emulsification processes, including high-energy methods, which use mechanical devices such as high-pressure homogenizers or ultrasound generators; and low-energy methods, which use the stored chemical energy of the system. For stabilizing the nanoparticles, chemical or physical (thermal) crosslinking is used.

Emulsifier: an agent that helps emulsions become more stable. The chemical structures of emulsifiers contain a hydrophilic and hydrophobic part, and they act by reducing the interfacial tension between the oil and water phases.

Globular proteins: the most water-soluble proteins, which have polypeptide chains coiled into a compact shape and a tightly packed core of hydrophobic amino acids.

Hydrophilic: molecules that tend to interact with water (either polar or charged). Hydrophilic side chains tend to associate with water molecules or with other hydrophilic side chains.

Hydrophobic: molecules that tend to avoid water (nonpolar and uncharged). Hydrophobic side chains interact with each other due to their tendency to minimize their contact with water or polar side chains.

Nanocarriers: nanoscale drug delivery systems that transport drugs or biomolecules; an important objective is to improve their longevity in the blood, allowing their accumulation in pathological areas.

Self-assembly: a nanoparticle preparation method involving increasing the hydrophobicity of a protein; a protein can be made to self-assemble by breaking disulfide bonds or decreasing primary amine

groups on the protein, or by adding lipophilic compounds.

Solid-in-oil dispersion: the dispersion of a nonliquid (i.e., solid-state) material in a liquid dispersion media (oil dispersion).

Thermal gelation: a process for nanoparticle preparation that involves heat-induced conformational changes in the protein with unfolding of some polypeptide segments followed by protein–protein interactions.

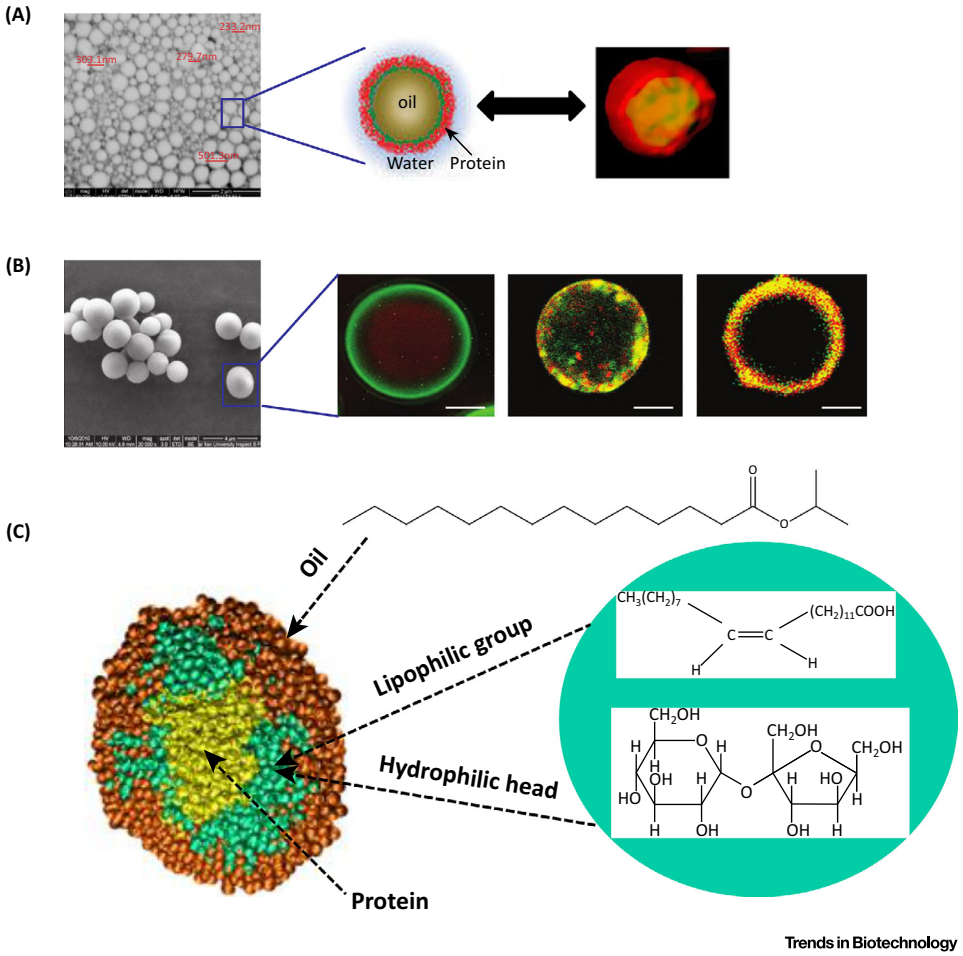

Figure 2. Schematic Illustration of the Protein Arrangement at the Interface of the Microspheres. (A) Left: scanning electron microscopy (SEM) image of bovine serum albumin (BSA) microspheres; right: BSA at the interface of an aqueous/organic phase, showing the hydrophilic residues (red color) interacting with water and the hydrophobic residues (green color) interacting with the organic phase (adapted from [1,24]). (B) Left: SEM image of RNA-loaded BSA microspheres; right: confocal microscopy images of RNA (red color) encapsulated within BSA (green color). Scale bar = 0.5 μm (adapted from [1]). (C) Solid-in-oil nanodispersion, using sucrose ester as a surfactant.

Their penetration into the nonaqueous phase helps to solvate their **hydrophobic** residues, which adsorb differently at fluid interfaces [22,23]. The adsorption of globular proteins to oil/water interfaces promotes clear changes in the conformation of the secondary and tertiary structures, when compared with the protein in solution. These alterations contribute to greater stable structures compared with their native structures in solution [10]. Figure 2A shows microspheres of bovine serum albumin (BSA) produced by high shear forces (i.e., the sonication method) applied to a biphasic water/hydrophobic solvent system. These high shear forces induce conformational changes in protein and force the protein to be disposed at the water/solvent interface, stabilizing the microspheres [24]. Figure 2B shows that the environment in the inner part of the BSA spheres changes slightly when RNA is loaded within BSA and becomes more hydrophilic [25].

Sucrose fatty acid esters have been explored as mild nonionic surfactants in the production of nanodispersion technology, assuming the formation of an external layer of a hydrophobic moiety (Figure 2C). Sucrose esters comprise mixtures with various degrees of esterification. A higher

monoester content results in a hydrophilic sucrose ester, whereas a higher esterification degree results in a lipophilic sucrose ester [26]. Their potential in the fields of pharmaceutical and cosmetic formulations have been investigated more thoroughly because of their low toxicity and excellent biodegradability [26,27]. In these fields of application, sucrose esters with low hydrophilic–lipophilic balance (HLB) values are particularly valuable because they present higher hydrophobic character. The HLB varies with the degree of esterification of the sucrose molecule, ranging from 0 to 20, where a lower HLB value indicates a more hydrophobic molecule.

Formation Mechanisms and Applications of Protein Emulsions

Emulsification Methodology Based on Proteins

Proteins are a logical choice as emulsifiers/stabilizers of emulsions due to their hydrophilic and hydrophobic amino acids and high surface activity [28,29]. The adsorption of proteins at oil/water interfaces of emulsions is an important process in the production of emulsion-based products in the pharmaceutical industry [28]. Proteins impart emulsion stability by a combination of steric and electrostatic mechanisms [29]. Upon adsorption, proteins adopt conformations that are different from their native structures in solution because the adsorption process needs conformational rearrangement to enable hydrophobic amino acids within the core to interact with the oil phase [28]. In a study using BSA and *n*-dodecane as an organic phase, albumin microspheres were produced. This biphasic system was subjected to high-intensity sonication, resulting in small and stable microspheres [24]. However, these particles produced by sonication did not present sizes smaller than 250 nm. Particle size is an important feature for intravenous therapeutic applications, and nanoparticles smaller than 100 nm can be intravenously administered and are taken up by cells more efficiently [30].

A recent study describes the production of albumin-based nanoemulsions through the high-pressure homogenization of a biphasic system [2]. The objective of this study was to develop stable protein-based nanoemulsions as drug delivery systems for intravenous therapeutic application. Albumin presents several advantageous characteristics that make it an ideal candidate for drug delivery, such as its availability, biocompatibility, biodegradability, lack of toxicity, and lack of immunogenicity [31–33]. In this study, an aqueous phase containing albumin and a PEGylated surfactant (Poloxamer 407) was emulsified with vegetable oil, as organic phase, by high-pressure homogenization (Figure 3A) [2].

Poloxamer 407 is a nonionic surfactant, whose structure is a triblock that contains a central hydrophobic block of polyoxypropylene (POP) or poly(propylene oxide) (PPO) and two identical lateral hydrophilic chains of polyoxyethylene (POE) or polyethylene glycol (PEG) [34–36]. This compound has attracted significant pharmacological interest and has been characterized as an 'inactive' ingredient for different types of preparations (e.g., intravenous, oral solution, and topical formulations) [36]. One study introduced Poloxamer 407 in the initial formulation to obtain small nanoemulsions with the ability to evade the immune system, two important characteristics required for intravenous administration. The presence of Poloxamer 407 resulted in smaller albumin-based nanoemulsions (Figure 3A) with improved stealth and decreased macrophage clearance [2,37]. Because experimentally it is very difficult to clarify the interactions between Poloxamer 407 and albumin with the oil phase, molecular dynamics simulations were performed. Results demonstrated that the hydrophobic part of Poloxamer 407 (POP block) interacts preferably with the oil, decreasing hydrophobic interactions between protein and oil, which can be responsible for reducing the size of nanoemulsions (Figure 3A). PEG chains interact preferentially with the protein, whereas neutral and hydrophilic surfaces were obtained, resulting in stealth nanoemulsions [2,37]. These protein-based nanoemulsions present suitable characteristics that improve their blood circulation time and biodistribution, being good candidates for application as drug delivery systems for intravenous application.

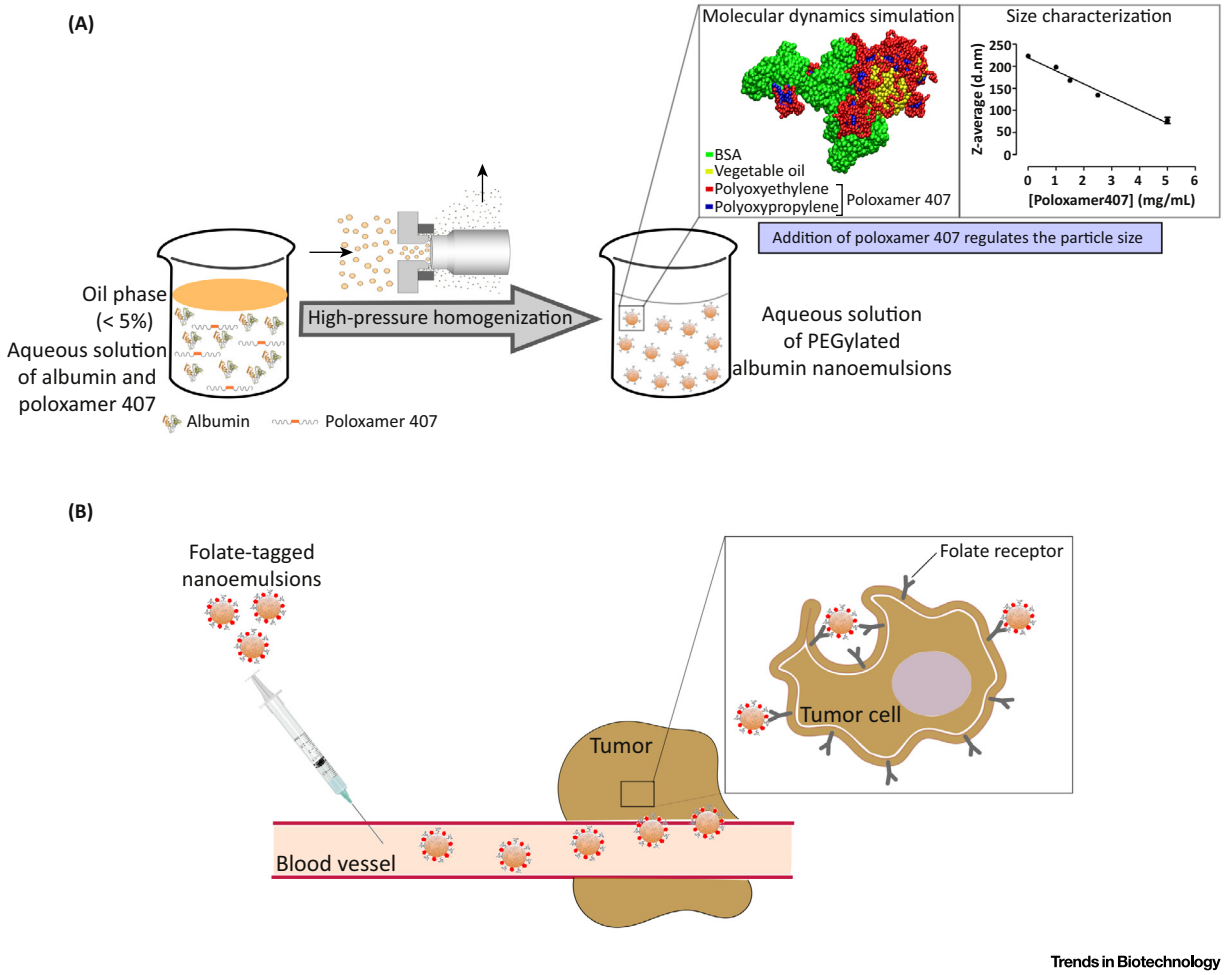

Figure 3. Mechanism of Production and Intravenous Application of Protein Nanoemulsions. (A) Schematic representation of PEGylated albumin nanoemulsion production using the high-pressure homogenization method, highlighting the results obtained by a molecular dynamics simulation of the bovine serum albumin (BSA)/vegetable oil/Poloxamer 407 system and by size characterization of developed nanoemulsions. The image of the molecular dynamics simulation was created using visual molecular dynamics (VMD) [79], and rendered with POV-Ray 3.6. (B) Illustration of an application of nanoemulsions functionalized with a targeting agent, folate. These nanoemulsions as drug delivery systems can be administered intravenously and active targeting for tumor tissues can occur through recognition of the targets (folate receptor). Abbreviation: PEG, polyethylene glycol.

Protein-Based Emulsions as Drug Delivery Systems

Nanoemulsions are used in the pharmaceutical field as drug delivery systems for parenteral, oral, ocular, and dermal administration, being designed to deliver and target drugs by these various routes of administration [38]. The production of targeted delivery systems has inspired researchers to use environmentally friendly coupling methods including tailored liposomes [39], specific functionalized nanocapsules [40,41], enzymatic synthesis of conjugates (e.g., antibody–human serum albumin) [42] and polysaccharide hydrogel systems [43], among others.

The intravenous route, a commonly used route for parenteral administration, allows quick and complete distribution across the body via the systemic circulation [44]. After intravenous administration, the drugs in circulation still have to overcome several physical and physiological barriers to reach their targets [44,45]. **Nanocarriers**, such as nanoemulsions, with suitable characteristics for intravenous administration, small size, and desirable surface properties (Figure 4A), can protect the drugs during systemic circulation, improving their blood circulation

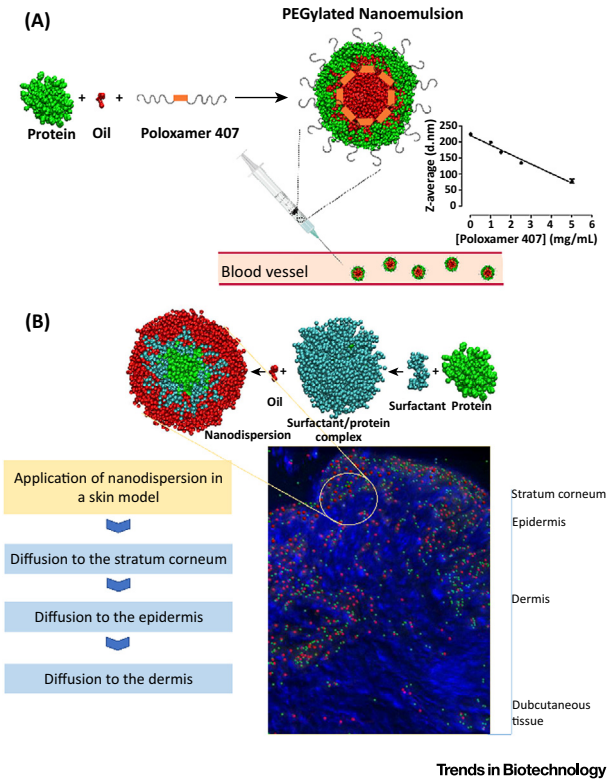

Figure 4. Applications of Nanoemulsions and Solid-in-Oil Nanodispersions. Illustration of (A) intravenous therapeutic applications and (B) transdermal perfusion, showing that the size and surface of emulsions and solid-in-oil (S/O) dispersions are crucial properties to achieve successful applications.

time and biodistribution [46]. The functionalization of the nanocarriers with targeting agents promotes the specific recognition and binding to the targeted therapeutic sites [47].

Albumin nanoparticles carry reactive groups on their surface (amino, thiol, and carboxylic groups) that can be used for drug conjugation and/or other surface modification [5,48]. There are several studies that describe the production and functionalization of albumin nanoparticles through the conjugation of the carboxylic group of the targeting agent, for example, folic acid (FA), to the amino groups present on the surface of albumin-based nanoparticles [49,50]. Our group developed functionalized albumin-based nanoemulsions composed of albumin–drug/targeting agent conjugates [51]. Those functionalized albumin nanoemulsions demonstrated capacity to release the drugs efficiently *in vitro*, which is a very important characteristic for drug delivery [51]. These FA-tagged albumin nanoemulsions loaded with a compound that presents antiproliferative capacity against cancer cells, carbon monoxide releasing molecule-2 (CORM-2), revealed suitable characteristics for targeted drug delivery in cancer therapy [2,52].

Formation Mechanisms and Applications of Protein S/O Dispersions

S/O Dispersion Technology

S/O dispersion has been developed as a novel oil-based dispersion for innovative drug delivery systems. The method of S/O dispersion was originally established in 1997 as a tool for enzymatic catalysis in organic media, and various studies were carried out using S/O nanodispersions [53]. The process in which water-in-oil (W/O) emulsion is lyophilized has many advantages in creating S/O dispersion containing proteins. The complex surfactant–protein is easily dispersed into the oil until a homogeneous and transparent oil-based protein dispersion is obtained, without loss of yield. Before this procedure was proposed, the penetration yield of proteins was very low (under 30%), except for

lipophilic proteins such as lipase [6]. The delivery of globular proteins is difficult because of their hydrophilic nature and their high molecular weight. This poor penetration into the skin is mainly due to the hydrophobic character of the stratum corneum (SC), the outermost layer of the skin [54]. S/O dispersion allows protein penetration through the intrinsic barrier of the skin assisted by surfactants and an oil vehicle (Figure 4B). *In vitro* permeation profiles through pig skin were investigated and confirmed the penetration of nanodispersions. The permeation results were represented with confocal laser microscopy by 3D imaging (Figure 4B). This 3D image represents the reconstruction of z-stack confocal images. Reconstructions were made using 'Imaris' image analysis software.

S/O methodology to incorporate a large protein of 66 kDa (BSA) was based on the methods of Tahara *et al*. and was further optimized [3,55]. This method consists of a complex between sucrose ester surfactant and the protein formed by high-pressure homogenization as a first step. Then, a lyophilization step is necessary to eliminate the solvent used for surfactant solubilization, attaining a solid surfactant–protein complex. This complex is then dispersed in an appropriate oil, such as isopropyl myristate (IPM), by ultrasonication (ultrasound probe, 20 KHz) (Figure 5A).

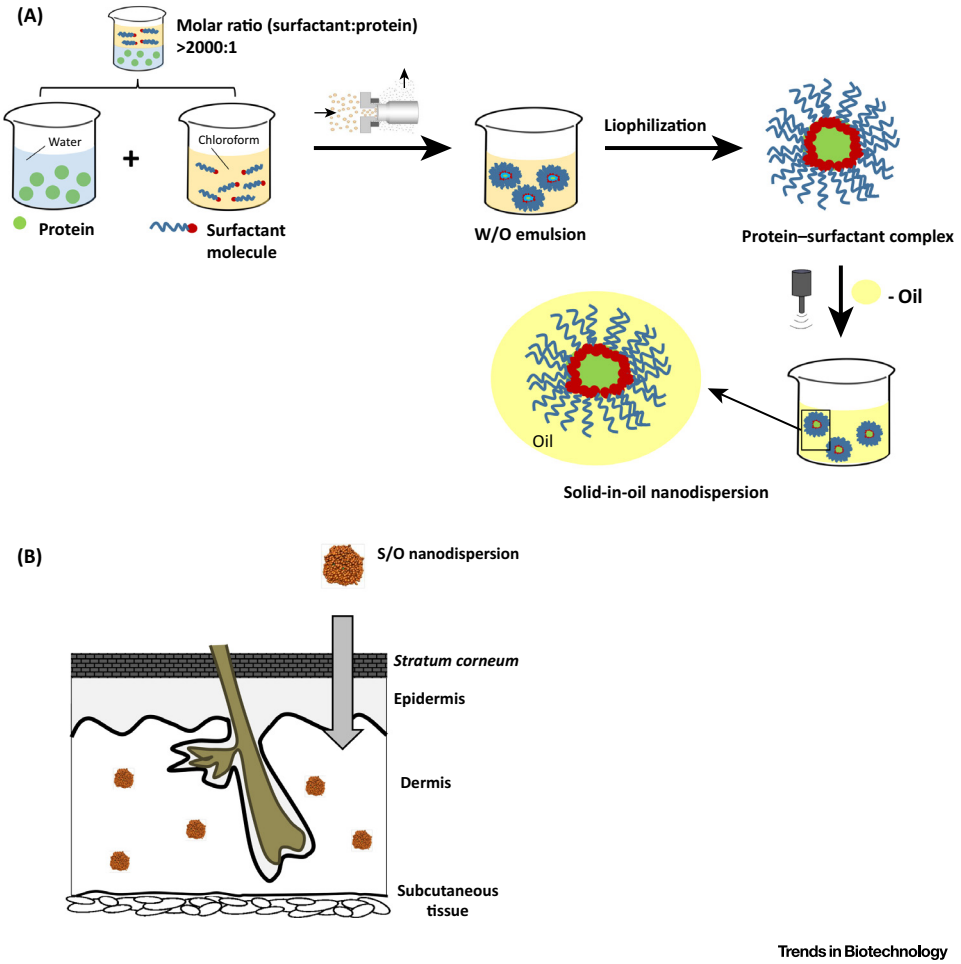

Figure 5. Mechanism of Production and Transdermal Application of Solid-in-Oil Nanodispersions. (A) Schematic representation of solid-in-oil (S/O) nanodispersion production, which comprises three stages: (i) addition of sucrose ester (surfactant) to bovine serum albumin (BSA) solution to form a surfactant–protein complex by high-pressure homogenization; (ii) lyophilization of the complex to remove water and chloroform; and (iii) dispersion of the solid complex in oil (isopropyl myristate) by ultrasonication to form the final S/O nanodispersion. (B) Illustration of transdermal perfusion application of the S/O nanodispersions.

In the S/O protein nanodispersions, complexes are formed by coating the protein with hydrophobic surfactants. Sucrose esters, which are nonionic with low HLB, determined their dispersion within the oil phase. The oil used in the dispersion phase, IPM, enhanced the skin penetration properties and increased the diffusion of the nanodispersion in the SC and/or the partition coefficient between the SC and the oil vehicle [56]. This oil acts upon the SC by liquefying the lamellar gel phase of the SC lipids and eventually partially dissolving them [57]. IPM is also able to dissolve considerable amounts of cholesterol, which may act as a membrane stabilizer [58]. The molecular dynamics simulations results, using the GROMACS [59] package and using the coarse grained force field MARTINI [60], revealed the significant importance of each component in the formulation. IPM strongly influences the interaction between the dispersion and lipids, while the surfactant molecules solubilize the hydrophilic protein within the oil [3]. With regard to stability, S/O nanodispersion showed good storage stability over approximately 2 months.

Outstanding Questions

Will emulsification and dispersibility methodologies be able to produce a desirable formulation for drug delivery and transdermal applications?

Is the use of high-energy approaches necessary for the production of protein emulsions and dispersions?

How will the delivery of active components intravenously or by skin permeation benefit with better understanding of protein formulations based on emulsions and dispersions?

Protein-Based S/O Dispersions as Transdermal Delivery Systems

Proteins have created huge challenges to creating formulations for transdermal delivery systems due to their unfriendly physicochemical properties. The design of an approach to protect the protein from enzymatic degradation enhancing its delivery without altering its biological activity has been the basis of several studies. Many attempts have been made to formulate an appropriate delivery system for proteins, with major emphasis on noninvasive routes such as transdermal patches [61]. Researchers have developed formulations including solid lipid nanoparticles, biodegradable polymers, chitosan nanoparticles, microspheres, liposomes, hydrogels, microemulsions, and/or nanoemulsions, among others, for protein delivery [62–68].

The transdermal delivery system represents an attractive alternative to other administration routes, especially oral and intravenous, which are the most common routes. The most important advantages of the transdermal transport system are noninvasiveness, prolonged delivery with the transdermal drug delivery system, avoidance of liver or gastrointestinal metabolism, and good acceptance by patients [69]. Over the past few decades, there has been a concerted effort to develop new and practical methods to enhance transdermal delivery, since several lipids can be found in the SC of human skin, the most important being ceramides, free fatty acids, cholesterol, and cholesterol sulfate. The vast majority of transdermal drug formulations are based on the passive diffusion of compounds with low molecular weights and on lipophilic drugs that permeate the skin.

However, for many hydrophilic molecules with a molecular weight greater than 500 Da [61], skin penetration is generally poor. The most therapeutic and pharmaceutical valuable proteins are typically greater than 500 Da, which greatly decreases their skin penetration [70–72]. It has been reported that larger proteins of 27 and 40 kDa gave limited perfusion results [16,55,72,73]. Another study demonstrated successful transdermal delivery of large proteins for skin vaccination using an external photodermal effect [74]. The S/O nanodispersion formulation produced by Martins *et al.* demonstrated successful skin penetration of a large protein (66 kDa) for a range of medical and pharma/cosmetic applications (Figure 5B) [3]. Another study demonstrated a nanodispersion of hyaluronic acid (HA) conjugated with BSA that successfully penetrated the skin layers into the dermis [75]. The S/O nanodispersion technique has also been developed to achieve transdermal immunization responses through the effective transcutaneous delivery of other hydrophilic molecules such as some antigens [76–78].

Concluding Remarks

The functionality of emulsions and S/O dispersions of proteins greatly depends on the formulation used. The addition of a PEGylated surfactant, Poloxamer 407, induced the production of nanoemulsions with suitable characteristics for intravenous therapeutic applications: small size (around 100 nm) and stealth behavior (PEGylated surface) (Figure 4A). The design of S/O

nanodispersions, however, assumes the formation of an external layer of a hydrophobic moiety that can cover the hydrophilic surface of the protein. The resultant S/O dispersion, with sizes around 150 nm, allows the perfusion of large hydrophilic molecules through the SC barrier of the skin (Figure 4B); this method can overcome the challenge of the 500 Da rule for skin permeation of hydrophilic molecules.

Strategies for the design of novel emulsions and S/O dispersions will depend on the specific needs of the required applications. The delivery of active components intravenously or by skin permeation will benefit greatly from the platform discussed in this review (see Outstanding Questions). Future strategies to produce novel emulsions and dispersions for hydrophilic active components are expected to have broader final target applications. We hope that drug delivery applications can benefit from continued advances in emulsions and dispersions under mechanical high-energy methodologies.

Acknowledgments

This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit and COMPETE 2020 (POCI-01-0145-FEDER-006684).

References

1. Shimanovich, U. *et al.* (2014) Protein micro- and nano-capsules for biomedical applications. *Chem. Soc. Rev.* 43, 1361–1371
2. Loureiro, A. *et al.* (2015) Size controlled protein nanoemulsions for active targeting of folate receptor positive cells. *Colloids Surf. B Biointerfaces* 135, 90–98
3. Martins, M. *et al.* (2013) In vitro and computational studies of transdermal perfusion of nanoformulations containing a large molecular weight protein. *Colloids Surf. B Biointerfaces* 108, 271–278
4. Nitta, S.K. and Numata, K. (2013) Biopolymer-based nanoparticles for drug/gene delivery and tissue engineering. *Int. J. Mol. Sci.* 14, 1629–1654
5. Lohcharoenkal, W. *et al.* (2014) Protein nanoparticles as drug delivery carriers for cancer therapy. *Biomed. Res. Int.* 2014, 180549
6. Tahara, Y. *et al.* (2012) Solid-in-oil dispersion: a novel core technology for drug delivery systems. *Int. J. Pharm.* 438, 249–257
7. Jun, J.Y. *et al.* (2011) Preparation of size-controlled bovine serum albumin (BSA) nanoparticles by a modified desolvation method. *Food Chem.* 127, 1892–1898
8. Saduní, N. *et al.* (2005) Studies on the formation of O/W nanoemulsions, by low-energy emulsification methods, suitable for pharmaceutical applications. *Eur. J. Pharm. Sci.* 26, 438–445
9. Gutiérrez, J.M. *et al.* (2008) Nano-emulsions: new applications and optimization of their preparation. *Curr. Opin. Colloid Interface Sci.* 13, 245–251
10. Day, L. *et al.* (2014) Conformational changes of globular proteins adsorbed at oil-in-water emulsion interfaces examined by synchrotron radiation circular dichroism. *Food Hydrocolloids* 34, 78–87
11. Loureiro, A. *et al.* (2016) Albumin-based nanodevices as drug carriers. *Curr. Pharm. Des.* Published online January 24, 2016. http://dx.doi.org/10.2174/1381612822666160125114900
12. Tesch, S. *et al.* (2003) Production of emulsions in high-pressure homogenizers – part I: disruption and stabilization of droplets. *Chem. Eng. Technol.* 26, 569–573
13. Emulsion formation, stability, and rheology. Tadros, T.F., ed.), 2013.pp. 1–75, Wiley-VCH Verlag
14. Malaki Nik, A. *et al.* (2010) Interfacial design of protein-stabilized emulsions for optimal delivery of nutrients. *Food Funct.* 1, 141–148
15. Qian, C. and McClements, D.J. (2011) Formation of nanoemulsions stabilized by model food-grade emulsifiers using high-pressure homogenization: factors affecting particle size. *Food Hydrocolloids* 25, 1000–1008
16. Piao, H. *et al.* (2007) A novel solid-in-oil nanosuspension for transdermal delivery of diclofenac sodium. *Pharm. Res.* 25, 896–901
17. Kolaib, E. and Sharma, R.K. (2013) Nanodispersions platform for solubility improvement. *Int. J. Res. Pharm. Biomed. Sci.* 4, 636–643
18. Rabinow, B.E. (2004) Nanosuspensions in drug delivery. *Nat. Rev. Drug Discov.* 3, 785–796
19. Yadav, G.V. and Singh, S.R. (2012) Nanosuspension: a promising drug delivery system. *Pharmacophore* 3, 217–243
20. Xiaohui, P. *et al.* (2009) Formulation of nanosuspensions as a new approach for the delivery of poorly soluble drugs. *Curr. Nanosci.* 5, 417–427
21. Fontenot, K. and Schork, F.J. (1993) Sensitivities of droplet size and stability in monomeric emulsions. *Ind. Eng. Chem. Res.* 32, 373–385
22. Iyer, P.V. and Ananthanarayan, L. (2008) Enzyme stability and stabilization – aqueous and non-aqueous environment. *Proc. Biochem.* 43, 1019–1032
23. Dimitrova, T.D. *et al.* (2004) Surface forces in model oil-in-water emulsions stabilized by proteins. *Adv. Colloid Interface Sci.* 108-109, 73–86
24. Silva, R. *et al.* (2012) Insights on the mechanism of formation of protein microspheres in a biphasic system. *Mol. Pharm.* 9, 3079–3088
25. Shimanovich, U. *et al.* (2011) Encapsulation of RNA molecules in BSA microspheres and internalization into *Trypanosoma brucei* parasites and human U2OS cancer cells. *Adv. Funct. Mater.* 21, 3659–3666
26. Szűts, A. and Szabó-Révész, P. (2012) Sucrose esters as natural surfactants in drug delivery systems – a mini-review. *Int. J. Pharm.* 433, 1–9
27. Ullrich, S. *et al.* (2008) Sucrose ester nanodispersions: microviscosity and viscoelastic properties. *Eur. J. Pharm. Biopharm.* 70, 550–555
28. Zhai, J.L. *et al.* (2013) Protein folding at emulsion oil/water interfaces. *Curr. Opin. Colloid Interface Sci.* 18, 257–271
29. Ye, A. *et al.* (2013) Oil-in-water emulsion system stabilized by protein-coated nanoemulsion droplets. *Langmuir* 29, 14403–14410
30. Suri, S. *et al.* (2007) Nanotechnology-based drug delivery systems. *J. Occup. Med. Toxicol.* 2, 16
31. Sailaja, A.K. *et al.* (2011) Different techniques used for the preparation of nanoparticles using natural polymers and their application. *Int. J. Pharm. Pharm. Sci.* 3, 45–50
32. Kratz, F. (2008) Albumin as a drug carrier: design of prodrugs, drug conjugates and nanoparticles. *J. Control. Release* 132, 171–183

33. Elzoghby, A.O. *et al.* (2012) Albumin-based nanoparticles as potential controlled release drug delivery systems. *J. Control. Release* 157, 168–182
34. Moghimi, S.M. and Hunter, A.C. (2000) Poloxamers and poloxamines in nanoparticle engineering and experimental medicine. *Trends Biotechnol.* 18, 412–420
35. Moghimi, S.M. *et al.* (2001) Long-circulating and target-specific nanoparticles: theory to practice. *Pharmacol. Rev.* 53, 283–318
36. Dumortier, G. *et al.* (2006) A review of Poloxamer 407 pharmaceutical and pharmacological characteristics. *Pharm. Res.* 23, 2709–2728
37. Nogueira, E. *et al.* (2013) Liposome and protein based stealth nanoparticles. *Faraday Discuss.* 166, 417–429
38. Figen, T. *et al.* (2010) Nanoemulsions as drug delivery systems. In *Colloids in Drug Delivery* (Fanun, M., ed.), pp. 221–244, CRC Press
39. Nogueira, E. *et al.* (2015) Peptide anchor for folate-targeted liposomal delivery. *Biomacromolecules* 16, 2904–2910
40. Rollett, A. *et al.* (2013) HSA nanocapsules functionalized with monoclonal antibodies for targeted drug delivery. *Int. J. Pharm.* 458, 1–8
41. Rollett, A. *et al.* (2012) Folic acid-functionalized human serum albumin nanocapsules for targeted drug delivery to chronically activated macrophages. *Int. J. Pharm.* 427, 460–466
42. Rollett, A. *et al.* (2013) Enzymatic synthesis of antibody–human serum albumin conjugate for targeted drug delivery using tyrosinase from *Agaricus bisporus*. *RSC Adv.* 3, 1460–1467
43. Schneider, K.P. *et al.* (2011) Bioresponsive systems based on polygalacturonate containing hydrogels. *Enzyme Microb. Technol.* 48, 312–318
44. Rabanel, J.M. *et al.* (2012) Drug-loaded nanocarriers: passive targeting and crossing of biological barriers. *Curr. Med. Chem.* 19, 3070–3102
45. Desai, N. (2012) Challenges in development of nanoparticle-based therapeutics. *AAPS J.* 14, 282–295
46. Alexis, F. *et al.* (2008) Factors affecting the clearance and biodistribution of polymeric nanoparticles. *Mol. Pharm.* 5, 505–515
47. Jain, R.K. and Stylianopoulos, T. (2010) Delivering nanomedicine to solid tumors. *Nat. Rev. Clin. Oncol.* 7, 653–664
48. Jahanshahi, M. and Babaei, Z. (2008) Protein nanoparticle: a unique system as drug delivery vehicles. *Afr. J. Biotechnol.* 7, 4926–4934
49. Shen, Z. *et al.* (2011) Improved drug targeting of cancer cells by utilizing actively targetable folic acid-conjugated albumin nanospheres. *Pharmacol. Res.* 63, 51–58
50. Ulbrich, K. *et al.* (2011) Interaction of folate-conjugated human serum albumin (HSA) nanoparticles with tumour cells. *Int. J. Pharm.* 406, 128–134
51. Loureiro, A. *et al.* (2015) Functionalized protein nanoemulsions by incorporation of chemically modified BSA. *RSC Adv.* 5, 4976–4983
52. Loureiro, A. *et al.* (2015) Folic acid-tagged protein nanoemulsions loaded with CORM-2 enhance the survival of mice bearing subcutaneous A20 lymphoma tumors. *Nanomedicine* 11, 1077–1083
53. Abe, K. *et al.* (1997) Surfactant–chymotrypsin complex as a novel biocatalyst in organic media. *J. Ferment. Bioeng.* 83, 555–560
54. Moser, K. *et al.* (2001) Passive skin penetration enhancement and its quantification in vitro. *Eur. J. Pharm. Biopharm.* 52, 103–112
55. Tahara, Y. *et al.* (2008) A solid-in-oil nanodispersion for transcutaneous protein delivery. *J. Control. Release* 131, 14–18
56. Sato, K. *et al.* (1988) Effect and mode of action of aliphatic esters on the in vitro skin permeation of nicorandil. *Int. J. Pharm.* 43, 31–40
57. Arellano, A. *et al.* (1999) Influence of propylene glycol and isopropyl myristate on the in vitro percutaneous penetration of diclofenac sodium from carbopol gels. *Eur. J. Pharm. Sci.* 7, 129–135
58. Leopold, C.S. and Lippold, B.C. (1995) An attempt to clarify the mechanism of the penetration enhancing effects of lipophilic vehicles with differential scanning calorimetry (DSC). *J. Pharm. Pharmacol.* 47, 276–281
59. Hess, B. *et al.* (2008) GROMACS 4: algorithms for highly efficient, load-balanced, and scalable molecular simulation. *J. Chem. Theory Comput.* 4, 435–447
60. Marrink, S.J. *et al.* (2007) The MARTINI force field: coarse grained model for biomolecular simulations. *J. Phys. Chem. B* 111, 7812–7824
61. Ita, K.B. (2014) Transdermal drug delivery: progress and challenges. *J. Drug Deliv. Sci. Technol.* 24, 245–250
62. Rudzinski, W.E. and Aminabhavi, T.M. (2010) Chitosan as a carrier for targeted delivery of small interfering RNA. *Int. J. Pharm.* 399, 1–11
63. Jain, A. *et al.* (2013) Peptide and protein delivery using new drug delivery systems. *Crit. Rev. Ther. Drug Carrier Syst.* 30, 293–329
64. Mukherjee, S. *et al.* (2009) Solid lipid nanoparticles: a modern formulation approach in drug delivery system. *Indian J. Pharm. Sci.* 71, 349–358
65. Fan, T. *et al.* (2014) Design and evaluation of solid lipid nanoparticles modified with peptide ligand for oral delivery of protein drugs. *Eur. J. Pharm. Biopharm.* 88, 518–528
66. Yagi, N. *et al.* (2007) Synthesis and evaluation of a novel lipid–peptide conjugate for functionalized liposome. *Bioorg. Med. Chem. Lett.* 17, 2590–2593
67. Ma, G. (2014) Microencapsulation of protein drugs for drug delivery: strategy, preparation, and applications. *J. Control. Release* 193, 324–340
68. Jana, S. *et al.* (2014) Carbopol gel containing chitosan–egg albumin nanoparticles for transdermal aceclofenac delivery. *Colloids Surf. B Biointerfaces* 114, 36–44
69. Herwadkar, A. and Banga, A.K. (2012) Peptide and protein transdermal drug delivery. *Drug Discov. Today Technol.* 9, e147–e154
70. Bos, J.D. and Meinardi, M.M.H.M. (2000) The 500 Dalton rule for the skin penetration of chemical compounds and drugs. *Exp. Dermatol.* 9, 165–169
71. Kalluri, H. and Banga, A. (2011) Transdermal delivery of proteins. *AAPS PharmSciTech* 12, 431–441
72. Tahara, Y. *et al.* (2010) Transcutaneous immunization by a solid-in-oil nanodispersion. *Chem. Commun.* 46, 9200–9202
73. Yoshiura, H. *et al.* (2007) Factors affecting protein release behavior from surfactant–protein complexes under physiological conditions. *Int. J. Pharm.* 338, 174–179
74. Pissuwan, D. *et al.* (2011) A solid-in-oil dispersion of gold nanorods can enhance transdermal protein delivery and skin vaccination. *Small* 7, 215–220
75. Martins, M. *et al.* (2014) Design of novel BSA/hyaluronic acid nanodispersions for transdermal pharma purposes. *Mol. Pharm.* 11, 1479–1488
76. Hirakawa, Y. *et al.* (2015) Transcutaneous immunization against cancer using solid-in-oil nanodispersions. *MedChemComm* 6, 1387–1392
77. Kitaoka, M. *et al.* (2013) Needle-free immunization using a solid-in-oil nanodispersion enhanced by a skin-permeable oligoarginine peptide. *Int. J. Pharm.* 458, 334–339
78. Kitaoka, M. *et al.* (2015) Transcutaneous peptide immunotherapy of Japanese cedar pollinosis using solid-in-oil nanodispersion technology. *AAPS PharmSciTech* 16, 1418–1424
79. Humphrey, W. *et al.* (1996) VMD: visual molecular dynamics. *J. Mol. Graphics* 14, 33–38