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Protein micro- and nano-capsules for biomedical applications†

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Micro- and nano-scale systems have emerged as important tools for developing clinically useful drug delivery systems. In this tutorial review, we discuss the exploitation of biomacromolecules for this purpose, focusing on proteins, polypeptides, nucleic acids and polysaccharides and mixtures thereof as potential building blocks for novel drug delivery systems. We focus on the mechanisms of formation of micro- and nano-scale protein-based capsules and shells, as well as on the functionalization of such structures for use in targeted delivery of bioactive materials. We summarise existing methods for protein-based capsule synthesis and functionalization and highlight future challenges and opportunities for delivery strategies based on biomacromolecules.

Key learning points

(1) Many therapeutically active compounds cannot be delivered using conventional strategies due to undesired pharmacological properties and/or interactions with cellular components during their delivery.

(2) To address this challenge, micro- and nano-scale systems have emerged as promising delivery systems enabling controlled transport and delivery, with protein-based systems being particularly attractive components in this context.

(3) The fabrication of protein or hybrid protein/polymer micro- and nano-capsules requires control of compartmentalisation on the micro and/or nano-scale. Several approaches are available to achieve this objective including emulsification and ultrasonic emulsification.

(4) A key challenge is to engineer the building blocks assembly pathways to encode the information required for the formation of capsules with desired properties. Chemical synthesis and functionalization approaches can play a key role in this area.

(5) While many challenges remain to fully realise the utility of biopolymer based delivery vehicles, their versatility, biocompatibility and low immunogenicity makes these materials a key addition to the palette of available delivery systems and open up new possibilities for biomedical applications. In addition, it is possible to obtain protein-based particles with a reduced size (<100 nm), as required for many applications, while keeping proteins fully functional and accessible as well as avert protein co-aggregation.

1. Introduction

To address the challenges associated with the controlled delivery of active components for biomedical applications, encapsulation methodologies with a high performance and tenability are required.[1] A powerful motivation for this line of research is given by the observation that many clinically relevant drugs cannot be administered using conventional release methods due to unfavourable solubility and toxicology characteristics and inappropriate interactions with other chemical species during delivery.[2–4] A class of materials that has emerged as being particularly promising in this context is that of biocompatible polymers, protein capsules and hybrid polymer–protein capsules. These structures consist of a polymeric shell with dimensions on the micro- or nanoscale encapsulating active species in their interior. Multiple synthetic pathways are available to generate these materials,[5] and a crucial challenge is the engineering of the precursor components and the assembly pathway to generate capsules with the desired properties.[6] For instance, chemical modification of precursor components can lead to several distinct capsule structures. Furthermore, significant structural complexity can be accessed by combining different materials in the core and shell parts of the capsules, for example in the form of hollow protein capsule

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shells,[7] oil-filled protein capsule shells,[8] water-filled protein capsule shells[9] and protein coated nucleic acid particles.[10] The chemical properties of the building blocks can be used to tune the material properties and structure of the final capsules.[11] Protein-based capsules can offer significant advantages over purely synthetic systems, and the use of human proteins as the precursor materials often results in delivery materials with minimal immunogenecity.[12] Several types of protein-based capsules, their derivatives, and other biocompatible protein complexes have gained prominence for the specific delivery of drugs to sites of disease while sparing healthy tissues. Protein modification and/or coupling with other functional molecules as well formulations of different types of proteins can be used and enhance the versatility of this class of materials. For instance, the engineering of functionalized protein capsules, with an external hydrophobic layer, can allow the incorporation of specific functionalities at the surface of the capsule to include specificity to a predetermined target, enhance transdermal transport properties, encode a release switch mechanism or even enhance the biological effect of the active species delivered through the capsules, for example by displaying antigens that will result in an increased immune response in the case of vaccines.[13] The present tutorial review focuses on the synthesis pathways available for the generation of protein capsules with controlled properties. We discuss the most prevalent approaches, their advantages and limitations and highlight the key challenges and future opportunities in this area.

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Dr Ulyana Shimanovich accomplished her PhD in Chemistry in 2012 at Bar-Ilan University, Israel, under the supervision of Prof. A. Gedanken. In October 2012 she moved, for postdoctoral research, to the group of Dr Tuomas Knowles, at the University of Cambridge, UK. From October 2013 until September 2014, Ulyana is Fellow of "Women in Science Fellowship (Bar-Ilan, Israel)". Her current research interests include synthesis and design of new biomolecules and micro/nano-scale diagnostic and therapeutic agents for applications in biomedicine and material science.

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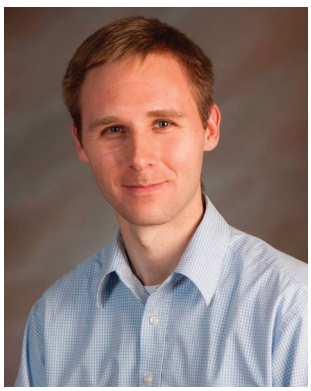

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There are a rich variety of methods described in the literature for the preparation of protein capsules and shells. One of the most widely adopted methods to obtain micro- to nano-scale capsules is based on micro-emulsion technology.[14] This approach builds on the use of two immiscible fluids, commonly an oil phase and an aqueous phase, the former typically carrying the active substance (a hydrophobic substance for encapsulation in the organic phase and a hydrophilic substance for accumulation in the protein shell) to be encapsulated and the latter one carrying the protein precursor molecules used to form the shell. A micro-emulsion formed from these two immiscible phases acts as a template for the synthesis of the capsules around the interface of the phases. This approach allows phase separation and a range of related physical or chemical phenomena to be exploited, including variations in surface tension, repulsive and attractive forces or specific chemical interactions.[15] The properties of the capsules are closely controlled by the sizes and characteristics of the template emulsions, and a wide range of approaches for the controlled generation of micro-emulsions have been exploited. These include bulk single emulsions,[16] bulk double emulsions,[17] polymerization techniques,[18] phase separation coacervation technique,[19] spray drying and spray congealing,[20] solvent extraction,[21] microfabrication, and homogenization and ultrasonic emulsification.[22] These methods result in protein capsules with fundamentally different properties, including size and stability. When devising new synthetic strategies or evaluating existing ones it is key to carefully evaluate the impact of core functionalization on the thermodynamic and kinetic stability of the resulting protein capsules. A significant influence on the stability of the final structures originates from the properties of the materials that are used as precursor components in the synthesis. For emulsification techniques that rely on cavitation, notably sonication-based approaches, sonochemical processes can contribute to the cross-linking of the capsule proteins without the need for specific chemical cross-linking agents. In the following section we discuss existing emulsification methods that are used for protein capsule formation.

2. Current methods for protein micro/nanocapsules production

In order to act effectively as delivery agents, protein micro- and nano-capsules should satisfy key criteria in respect of:

(a) Stability of the preparation after synthesis with a life compatible with clinical practice.

(b) Controllable particle size and monodispersity in biological media used for injection.

(c) Biocompatibility and controllable biodegradability as well as controllable release rate of the active reagent.

(d) Targeted delivery to specific sites of disease requires amenability to chemical surface functionalization.

A range of approaches have been developed to fulfil these criteria and are depicted in Fig. 1.

Single and double emulsion techniques[16]

To synthesise micro- or nano-containers from biopolymers by using single emulsions (Fig. 1a), the precursor molecules, commonly proteins, are dissolved or dispersed in the aqueous medium followed by emulsification in a non-aqueous medium such as oil. Next, to ensure structural integrity, chemical or physical cross-linking of the dispersed droplet is carried out (Fig. 1b). The cross linking can be achieved either intrinsically by means of heat, or by using the chemical cross-linkers, including glutaraldehyde, formaldehyde or diacid chloride *etc.* Intrinsic cross-linking[23] has the advantage of minimizing potential toxicity and undesired reactivity with other chemical components resulting from the presence of chemical cross linkers.

More complex structures can be obtained through the use of double emulsions (Fig. 1c).[17] Similarly to the case of single emulsions, the aqueous protein solution is dispersed in a lipophilic organic continuous phase. The primary single water-in-oil emulsion[24] obtained in this manner is then added to an aqueous solution of a second biopolymer that will then form the outer shell of the capsule. This process hence results in the formation of a double emulsion. The biggest advantage of the capsules created by the double emulsion method over capsules created *via* the single emulsion method is the possibility of controlling independently the inner and outer surface of the capsule. Capsules created *via* such a double emulsion method allow encapsulation of water-soluble species and are therefore well suited for the delivery of water-soluble drugs, peptides, proteins and design of vaccines.

Phase separation coacervation technique[19]

Creation of protein capsules of specific sizes can also be achieved by phase separation in solution rather than emulsification. Colloidal systems, including polymer solutions, have the propensity to undergo coacervation where a homogeneous solution phase separates into two liquid phases, one of which is essentially pure solvent, and the other contains the solute. In the context of the creation of protein-based capsules, this phenomenon is exploited based on the principle of decreasing the solubility of the biopolymer (protein) to induce the formation of a protein rich phase, called the coacervates (Fig. 1d). In this method, the drug particles to be delivered are initially dispersed in a solution of the protein and then an incompatible polymer is added to the system leading to the phase separation of the protein and subsequent accumulation of the drug into the protein rich phase. In order to enhance the stability of the capsules formed through coacervation, in some cases, for unstable proteins, a cross-linking agent is used prior to adding the incompatible polymer.

Spray drying and spray congealing[20,21]

These methods are based on microdroplet spray generation from a solution of the protein and the drug (Fig. 1e). The spray can then undergo solvent removal in spray drying or rapid cooling followed by freeze-drying in the case of spray congealing. One of

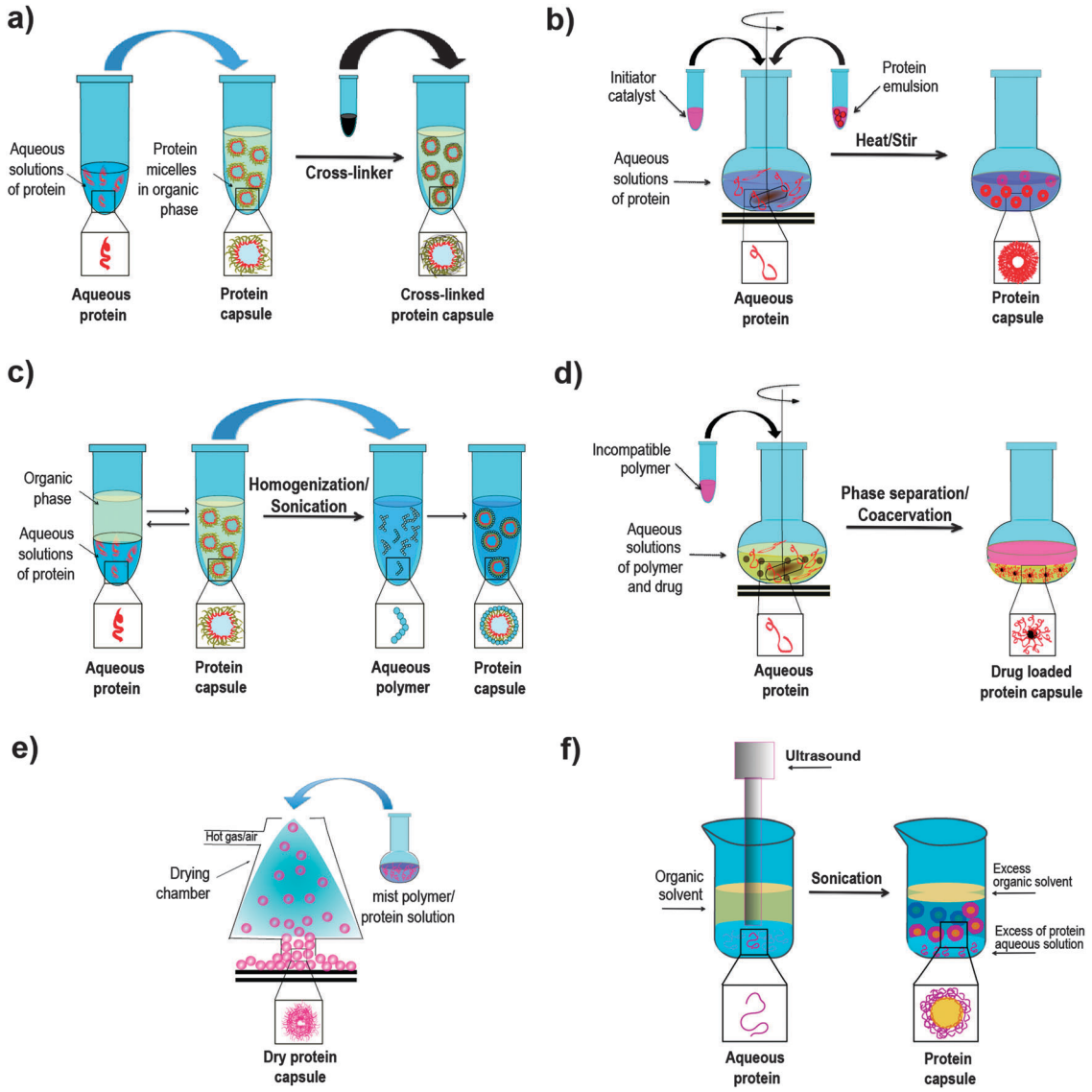

Fig. 1 Schematic representation of emulsification methods used for protein capsule preparation: (a) single emulsification method; (b) polymerization (cross-linking) method; (c) double emulsification method; (d) phase separation/coacervation method; (e) spray drying/spray congealing method; and (f) ultrasonic emulsification method.

the major advantages of the process is the feasibility of operating under aseptic conditions due to the absence of a liquid phase, which could mediate contamination. This process has been used for protein loaded lipid capsules.[25,26]

Ultrasonic emulsification technique[27]

The creation of protein capsules *via* the ultrasonic emulsification method involves mechanical mixing, by the action of high-intensity ultrasonic waves to a macroscopic oil/water interface. As a result, an oil-in-water emulsion is created in which the protein molecules, initially present in the aqueous phase, localise at the interface of the droplet. The preparation of capsules using ultrasound emulsification from aqueous protein solutions and immiscible hydrophobic solvents is a well-known technique and it has been the subject of recent reviews.[22,28,29] Those reviews provide comprehensive information about the mechanism of formation of protein capsules.[27]

The interfaces obtained through the emulsification methods described above can be used to direct polymerization of soluble precursor monomer molecules to form polymeric structures on the surface of the emulsion. This approach (Fig. 1b) is commonly applied to the generation of capsules from synthetic polymers, but also holds great potential in the area of protein-based capsules. Polymerization emulsification can be combined with the emulsification approaches described above, including, suspension, precipitation, emulsion and micellar polymerization processes. An alternative approach to stabilize protein-based capsules is to use them as templates to direct the polymerization of the synthetic polymers on their surface, leading to a coating that enhances the robustness of the capsules.

Microfabrication method[30]

Monodispersed emulsions can be formed using the microfabrication method, which enables precise control of the particle size at micro/nano scale dimensions. The technique enables manipulation of ultralow interfacial tensions at the surface of emulsion droplets that results in a highly monodisperse emulsion. One of the most attractive features of the microfabrication method is precise manipulation of emulsion composition.

3. Properties of protein capsules

3.1 Size

The size of the capsules is a key parameter that affects the ability of such particles to interface with biological systems and influences the rate at which species can be released from protein capsules. In methods involving emulsification, the properties of the oil–water interface, including surface tension and viscosity, can be used to tune the size of the droplets which are used to template the capsules.[31,32] In cases where the hydrophobic phase remains encapsulated in the final structure, its level of hydrophobicity can also influence the stability of the capsule. For instance it has been found that food oils yield more stable particles than dodecane, which is more hydrophobic (Fig. 2).[27,28] Furthermore, intrinsic differences between high viscosity food oils such as olive oil and low viscosity oils such as soya bean oil can be used to tune the size of the resulting capsules: capsules generated from a food oil with higher viscosity values leads to an increase in the size of the protein capsules, but commonly also reduces their stability and fraction of protein incorporated in such capsules.

A further parameter of interest is the ratio of hydrophobic solvent to water in the precursor mixture. Low fractions of the hydrophobic phase to the aqueous phase yield smaller capsules, which are less stable relative to bigger capsules formed with a higher organic solvent fraction (Fig. 2).[27]

Moreover, when the capsules are used to carry a drug like small molecule, the chemical and physical nature of the encapsulated drug can affect not only the capsules' sizes, but also the hydrophobicity of the internal part of the capsule. For example, the size dependence of BSA protein (Bovine Serum Albumin) capsules has been shown to be affected by the length of encapsulated RNA molecules.[31] These changes in size are shown in Fig. 3a. The results demonstrate that the size of the RNA loaded capsules increases with an increase of the number of nucleotides in the RNA chain, *i.e.* with an increase in the size of the encapsulated molecule. In parallel, the hydrophilicity of the inner part of the protein capsule increases upon encapsulation of a hydrophilic molecule such as RNA. For studies of changes in the environment within capsules, environmentally sensitive dyes such as Nile Red have proven to be very valuable due to their ability to change their fluorescence spectrum upon the change in solvent polarity. The red signal, indicating an hydrophilic environment, emits from the shell of unloaded capsules (Fig. 3b, top image). As soon as the protein spheres were loaded with a hydrophilic RNA molecule, the inner environment became more hydrophilic, and a red-shifted

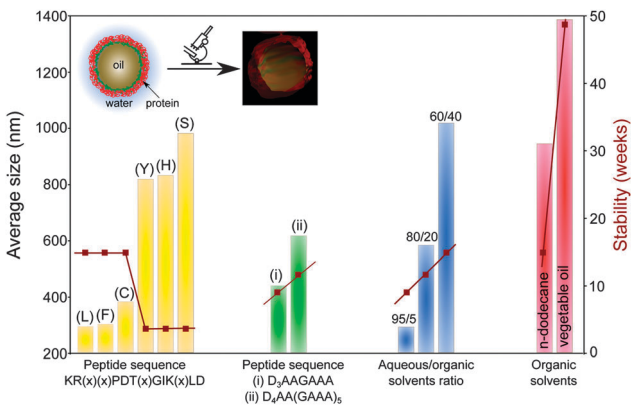

Fig. 2 Schematic representation of size and stability studies for protein aqueous microspheres as a function of peptide sequence, size of amphiphilic peptides, aqueous/organic solvent ratio and type of organic solvent. The scheme and microscopic image of a protein aqueous microsphere is shown in the left corner. Scheme: the protein is localised at the water/oil interface with hydrophobic residues (green) towards the oil phase and hydrophilic (red) towards the water. Microscopy image: Nile Red coloured protein microsphere filled with oil (green) in its inner part and protein (red) forming the shell of the sphere. Schematic representation based on data from reference.[27]

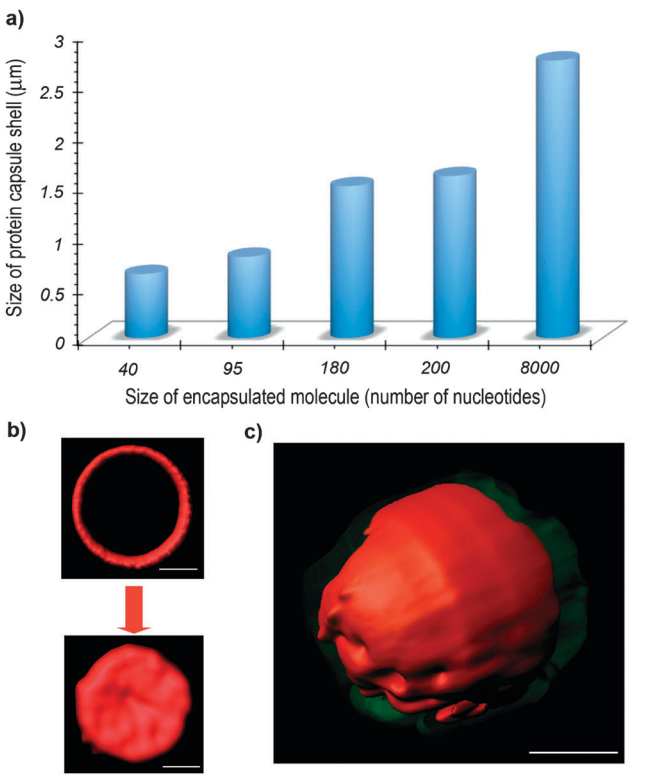

Fig. 3 Length of encapsulated RNA influences the size of protein capsules. (a) Graphic represents size dependence of the protein capsules as a function of the length of encapsulated RNA chain. (b) Fluorescent microscopy images of Nile Red colored BSA spheres. Unloaded spheres are presented on the top and RNA loaded on the bottom side.[31] (c) 3D confocal image of a fitc-BSA sphere loaded with Cy3RNA molecules. Scale bars = 1 micrometer.

emission signal of Nile Red dye, indicating a hydrophilic environment, was observed from the shell as well as from the inner part of the capsules (Fig. 3b, bottom image).

Furthermore, capsules can be formed from a mixture of two different proteins.[32] Unlike in the case of capsule formation from a mixture of nucleic acids and proteins, where RNA loaded BSA spheres were created, the mixture of two different proteins forms uniform protein capsules where two proteins co-localize in the shell of the capsule.

An additional approach that enables control of the size of protein-based particles has been recently reported.[33] Incorporation of PEGylated surfactants (polyethyleneglycol-based surfactants) into BSA based protein particles leads to decrease in particle size from 200 to 100 nm. Particle formation in the presence of PEGylated surfactants is physically restricted by the steric effect of the polymer (PEG) resulting in decrease in the average protein particle size.

3.2 Stability

In general, an emulsion of oil and water, once formed, can be unstable with respect to separation into two bulk phases, a reflection of the fact that oil and water notoriously do not mix. The stability of such emulsions is dependent on the protein sequence and size, and may be enhanced by the presence of surfactant molecules that are added to the precursor mixture. Proteins are amphoteric enough to localize at the oil–water interface and stabilize the emulsion, leading to an increase in the lifetime of uncross-linked oil-filled protein spheres and preventing the droplets from merging and reforming two bulk phases. Disulfide bonding has been thought to be the most prevalent contributor to protein capsule stability by formation of cross-linking between proteins. However, a recent report shows that the replacement of cysteine residues involved in disulfide bonding by hydrophobic residues can result in stable capsules (Fig. 2).[27] Thus, stabilization of protein capsules may also be achieved through a hydrogen network formed at hydrophobic residues instead and/or in addition to covalent cross-linking. In addition, the length of amphiphilic peptides determines the final size and stability of the resulting protein capsules. A longer amphiphilic peptide yields a larger and more stable protein aqueous sphere (Fig. 2).[27] Furthermore, it has been found that the inner hydrophobic parts when exposed to the oil interface may lead to a change of protein conformation, which results in enhanced capsule stability (Fig. 2).[27]

The stability of mixed protein capsules can also be enhanced through the addition of a biocompatible synthetic polymer to the mixture of proteins. For example, the silk fibroin capsules, prepared by the phase separation method, were shown to be stabilised by polyvinylalcohol polymer (PVA).[34] This stabilization can in part originate from the change in the secondary structure observed for silk fibroin following the addition of PVA. A further agent that has been shown to stabilize protein capsules is polyethylene glycol (PEG).[35] In addition to the enhanced stability, the interactions of the capsules with biological systems are modified by pegylation. In particular, such capsules are often less immunogenic, resulting in the blocking and delaying of the first step in the phagocytic process, oponisation. Thus, the pegylated capsule shells have an increased half-life in blood circulation; this effect has been observed to reach up to several orders of magnitude. A representative list of protein stabilizers is given in Table 1.

In view of their applications as delivery systems, an important parameter of interest of protein capsules is their ability to

Table 1 List of main protein stabilizers

Type of protein stabilizers	Protein stabilizers	Function	Protein storage
Cryoprotectants	DMSO Ethylene glycol Glycerol 2-Methyl-2,4-pentadiol (MPD) Propylene glycol Sucrose	Prevent formation of ice crystals	−20 °C
Protease inhibitors	PMSF (Phenylmethylsulfonyl fluoride): *inhibition of serine proteases* Benzamidine: *inhibition of serine proteases* Pepstatin A: *inhibition of acid proteases* Leupeptin: *inhibition of cysteine proteases* Aprotinin: *inhibition of serine proteases* Antipain: *inhibition of cysteine proteases* EDTA and EGTA: *inhibition of metalloproteases*	Inhibit protein activity and present proteolytic cleavage of proteins	−20 °C, 4 °C
Antimicrobial agent	Sodium azide Ethylenediaminetetraacetic acid (EDTA)	Inhibit microbial growth in protein solutions	4 °C
Metal chelators	Orthophenanthroline	Inhibit metal-induced oxidation of free thiols helping in maintaining the protein in a reduced state	4 °C
Reducing agents	Dithiothreitol (DTT) 2-Mercaptoethanol Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP)	Maintain the protein in a reduced state	−20 °C, 4 °C

interact with biological systems and in particular their biocompatibility. A key objective, therefore, is to develop chemistry, which is compatible both with the structural requirements as well as minimizing the toxicity of the capsules. The use of potentially toxic covalent cross-linkers, for example, will lead to capsule stabilization but, at the same time, can induce an increase in toxicity of the resulting capsule. In order to circumvent this limitation, for emulsification methods such as template based polymerization, suspension polymerization, *etc.*, that commonly require the use of toxic chemical cross-linkers, the toxicity can be alleviated by removal of the unreacted cross-linkers post synthesis, leading in many cases to a reduction in toxicity of the protein capsules. This additional step is not required for synthesis methods that do not involve chemical cross-linkers, including double emulsification, solvent extraction, and ultrasonic emulsification. In such cases, capsules can be stabilized by native cross-linking in the form of covalent bonds in inter-molecular disulfide bonds[36] between two cysteine residues in adjacent protein molecules or hydrophobic forces, or hydrogen bonding networks,[37] that are strong enough to hold entire 3D protein capsules intact. The methods that lead to creation of such a native supportive system for the protein capsules are therefore of great interest due to the inherent non-toxicity and biocompatibility. In order to favor the creation of such native cross-linking networks some form of activation is commonly required. In the case of ultrasonic emulsification, the activation originates from the presence of superoxide radicals formed upon the collapse of cavitation bubbles, favoring S–S bond creation; a further effect of sonication is enhanced mass-transfer from the flows generated by exposure to ultrasound. The activation as well as capsule formation occurs in a one-step sonochemical reaction.

A summary of the different emulsification methods that have been used for the synthesis of different types of protein capsules and their basic properties are presented in Table 2.

Many of the capsules synthesized by emulsification methods are found to be compatible with a role as delivery vehicles and in many cases are either less toxic or non-toxic. Remarkable differences from one emulsification method to another are found in the capsules' stability and average size (Table 2).

The use of ''free of cross-linkers'' methods (methods that do not involve the use of chemical cross-linkers) is relatively new in the field of interfaces. Thus, it is of great importance to unveil the mechanism of protein capsules formation that does not use cross-linkers, in particular for those capsules formed using sonochemical procedures. The detailed mechanism of formation of protein capsules is discussed below.

4. Mechanism and structural changes accompanying the formation of protein capsules

In order to optimize rationally the properties of protein capsules, the mechanism leading to their formation is required. A significant effort has recently been focused in particular on the formation of cross linker free capsules, especially in the case of capsules that were formed sonochemically.[22,27–29]

Insights into the structural changes in the protein following capsule formation have been gained by Fourier Transform Infra-Red spectroscopy (FTIR) and molecular dynamic simulations.[27]

These studies showed that protein molecules accumulate at the oil/water interface and undergo conformational changes in their secondary and tertiary structures. In particular, an increase of β-sheet content was found in silk fibroin microspheres while molecular simulation has shown a twist of globular 3D structure (see protein conformation images on the top of the chart – Fig. 4). The proteins are able to adjust their globular structures upon binding to the interface by orienting the hydrophobic residues towards the organic phase and the hydrophilic residues towards water; this transition results in a new energetically favorable 3D structure. The increase in β-sheet content of the protein due to the ultrasonic spheridization is reminiscent of amyloid-like behaviour, when protein molecules aggregate to form densely hydrogen-bonded fibrillar structures.[38,39]

The protein conformations observed within the β-sheet rich nanospheres might therefore have structural analogies with *bona fide* amyloid fibrils. The possibility to form protein capsules from proteins like silk fibroin and cysteine-free peptides indicates the presence of further driving forces in addition to covalent cross-linking, namely hydrophobic, electrostatic interactions and enhanced mass transport, induced by ultrasound in liquid media.

Interestingly, this approach is not restricted to proteins. Indeed, this strategy has been successfully used for the formation of nanospheres made of nucleic acids,[40–42] DNA and RNA, where the stabilization of the spheres was proposed to be due to hydrogen bonding, hydrophobic and electrostatic interactions stabilized by counter ions (commonly Ca^{2+}, Na^{+} and Mg^{2+}).

The role of radicals in covalent cross-linking during ultrasonic capsule formation was studied by preventing the creation of radicals in the reaction cell through performing the synthesis under an inert (Ar) atmosphere. In all three cases where these phenomena were probed for proteins as well as for nucleic acid capsules, it was observed that capsules were successfully formed even under an inert atmosphere and without the presence of disulfide residues.[40–42] These results demonstrated that capsule stabilization can occur even in the absence of covalent cross-linking. The formation of a hydrogen bonding network connecting proteins in the capsule can be promoted by organic solvents.[43] In this work, the formation of a core–shell structure made of Zein protein induced by the presence of citral (3,7-dimethyl-2,6-octadienal) in the precursor mixture was reported. The solvent ratio of binary solvents (ethanol 60–90% and citral) affects the formation of core–shell structures. It was shown that solvent polarity affected the ability of core material (zein protein) to form small diameter droplets inside the shell. The solvent with relatively high polarity (60% aqueous ethanol) increased interfacial tension, leading to solidification of entire zein spheres structure with no core. By contrast, low polarity of the solvent (90% aqueous ethanol) decreased interfacial tension, and allowed separation of the core material from

Table 2 Summary of existing emulsification methods for protein capsules synthesis and protein capsules' properties

Protein class	Protein type	Emulsification method	Stability	Capsules' size	Toxicity	Compatibility as a "drug carrier"
Globular	*Bovine Serum Albumin* (*BSA*)	Template based polymerization	High	200 nm–5 μm	Low	Compatible
		Coacervation	High	150–200 μm	Non-toxic	Compatible
		Single/Double emulsification	High	200 nm–1 μm	Non-toxic	Compatible
		Ultrasonic emulsification	High	100 nm–3 μm	Non-toxic	Compatible
Globular	*Casein*	Coacervation	High	150–200 μm	Non-toxic	Compatible
		Single emulsification	Average	2–50 μm	Non-toxic	Compatible
		Suspension polymerization	Low	200 nm–1 μm	Low	Compatible
		Ultrasonic emulsification	High	2–3 μm	Non-toxic	Compatible
Fibrous	*Collagen*	Phase separation	Average	1–2 mm	Average	Compatible
		Coacervation	High	100–200 μm	Non-toxic	Compatible
		Solvent extraction	Average	100 nm–1 μm	Non-toxic	Compatible
		Template based polymerization	High	100 nm–1 μm	Non-toxic	Unknown
Globular alpha-protein	*Hemoglobin* (*Hb*)	Template based polymerization	High	1–5 μm	Low	Compatible
		Ultrasonic emulsification	Average	1–2 μm	Low	Unknown
Globular plasma-protein	*Human Serum Albumin* (*HSA*)	Template based polymerization	High	200 nm–5 μm	Low	Compatible
		Single emulsification	Average	10–60 μm	Low	Compatible
		Coacervation	High	500 nm–20 μm	Low	Compatible
		Ultrasonic emulsification	High	1–3 μm	Low	Compatible
Fibrous	*Insulin* (*INS*)	Template based polymerization	High	200 nm–5 μm	Low	Compatible
		Coacervation	Low	600 nm–1 μm	Average	Unknown
		Suspension polymerization	High	20–100 μm	Low	Compatible
Fibrous	*Lysozyme* (*HEWL*)	Template based polymerization	High	200 nm–5 μm	Low	Compatible
Fibrous	*Nonfimbrial adhesion* (*NFA*)	Suspension polymerization	Unknown	50–250 nm	Unknown	Unknown
Fibrous	*Silk fibril* (*SF*)	Coacervation	Low	200 nm–1.5 μm	Low	Unknown
		Suspension polymerization	High	100 nm–5 μm	Non-toxic	Compatible
		Phase separation	High	500 nm–1.5 μm	Unknown	Unknown
		Ultrasonic emulsification	High	500 nm–1.5 μm	Low	Compatible
Globular	*Soy protein isolate* (*SPI*)	Coacervation	High	1–60 mm	Non-toxic	Compatible
		Phase separation	High	10–30 μm	Non-toxic	Compatible
		Template based polymerization	High	150 nm–2 μm	Non-toxic	Compatible
		Single emulsification	Average	10–60 μm	Non-toxic	Compatible
		Ultrasonic emulsification	High	1–2 μm	Non-toxic	Compatible
Globular	*Whey protein* (*WP*)	Electrospraying	High	100 nm–1 μm	Low	Compatible
Globular	*Zein protein* (*ZP*)	Phase separation	High	10–30 μm	Non-toxic	Compatible
		Single emulsification	Average	10–60 μm	Non-toxic	Compatible
		Template based polymerization	High	150 nm–2 μm	Non-toxic	Compatible

shell material, thus leading to zein core–shell structure formation.

The phenomenon of formation of hydrogen bonding networks makes the incorporation of two different proteins in a single capsule shell structure possible. The evidence for the presence of a dense network of hydrogen bonds was reported for the BSA-silk fibroin capsules.[44] The protein capsule shell made of a mixture of BSA and silk fibroin proteins showed the characteristic FTIR absorption bands of the two proteins with spectra of varying intensities due to the composition and interactions of different groups of proteins, confirming the blending of the two proteins.

Another evidence of hydrophobic interactions in protein capsule shell structure was reported by Subirade and co-workers.[45] The whey protein granular capsule shells were stabilized by adding calcium (Ca) alginate to the precursor protein solution. The strong hydrophobic interactions created between hydrophobic side-chains of whey protein buried in the core of the whey sphere and hydrophilic side chains facing alginate resulted in enhanced stabilization of protein capsule structure.

5. Incorporation of functionalities at the surface of the protein capsules

Tailoring of the surface chemistry of protein capsules allows for control over the pathway and targeting of the protein capsules inside the living systems as well as modification of the immunogenicity of the particles.[46] However, incorporating specific surface modifications into the protein emulsions is challenging

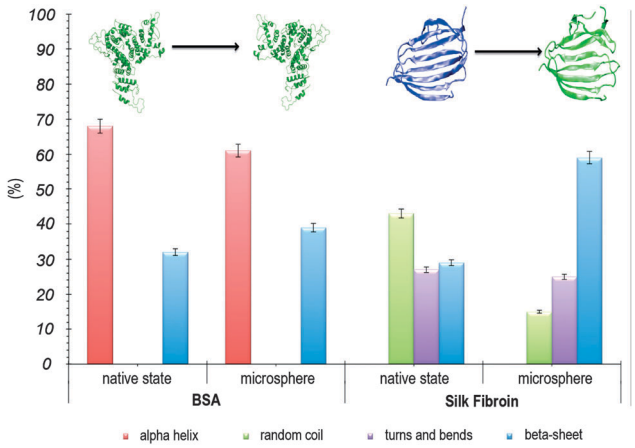

Fig. 4 Chart summarizing BSA and silk fibroin secondary structure changes due to protein shell formation based on FTIR measurements. The secondary structure before (left hand side) and after (right hand side) conversion of protein into protein capsule shells is presented at the top of the chart, respectively to BSA and SF proteins. Schematic representation based on data from reference.[27]

and can compromise the structural characteristics of the capsule and the process itself can require chemical conditions that might lead to loss of biocompatibility and gain of toxicity of the protein capsules for cells and organisms.

A number of methods have been described for the incorporation of functionalities on the surface of protein emulsions: (1) construction of the protein capsules from a mixture of two proteins that have two different functionalities;[32] (2) coating of protein capsules with biocompatible polymers;[47] (3) chemical conjugation of polysaccharides to proteins,[48] and (4) conjugation of target ligands, including folate, to the surface of protein spheres.[49] The list of bioactive ligands that are attractive candidates for functionalization strategies has been reviewed elsewhere.[50] Below we review some representative examples of functionalization of protein capsules.

A convenient approach for introducing new functionality into a capsule is through the use of an additional protein with the required properties. This can either be achieved through co-assembly of distinct proteins from solution, or capsule formation from a tandem construct of fused proteins. In this manner, the same capsule shell can have a number of functionalities. A specific example of this type of surface functionalization by conjugation of a second protein to the main protein capsule shell was described by Rahimipour and co-workers.[51] In this case the protein BSA was chosen as the main structural protein for capsule shell construction, whereas the KLVFF peptide was used for capsule's surface functionalization. The KLVFF peptide is a known inhibitor of the amylogenic Aβ-40 protein aggregation phenomenon that leads to Alzheimer's disease. The BSA protein-based capsule activated with the KLVFF peptide induced the formation of amorphous Aβ-40 aggregates with no fibrillar structure upon the incubation with Aβ on their surface.[51]

Protein capsules suitable for targeted delivery may also be synthesized *via* alteration of surface charge of the protein capsule by coating it with uncharged (*e.g.*, PVA-polyvinylalcohol) or positively charged (*e.g.*, PEI-polyethileneimine) biocompatible synthetic polymers. Moreover, coating of protein spheres with magnetic (Fe_2O_3) nanoparticles enables specific delivery when applying an external magnetic field. Both of these types of surface coatings have been used to functionalize the surface of RNA loaded proteinaceous BSA microspheres.[47] When coated with PVA or PEI, the native negative charge of the BSA is buried by the polymer layer, eliminating the adhesion of uncoated capsules to the cell membrane and allowing efficient delivery of RNA into human osteosarcoma U2OS cancer cells and Trypanosoma parasites.[47] In this context, the metal coating (Fe_2O_3) could be used to enhance contrast in electron microscopy and allowed the spatial localization of the capsules to be determined inside the parasites and mammalian cancer cells.

An additional way of incorporating functionality into the protein shells is the biochemical conjugation of a polysaccharide and a protein prior to capsule formation through emulsification. An example of this strategy was recently provided by Wong and co-workers[48] who demonstrated the incorporation of a fluorescently labelled saccharide into the precursor protein followed by capsule formation of the construct.

The direct chemical conjugation of folic acid to the surface of the BSA and human serum albumin (HAS) protein nanocapsules has also been described.[49] In this case, the folic acid was pre-activated with a heterobifunctional cross-linker and then coupled with primary amines on HSA nanocapsules. Folic acid receptors are over-expressed in many diseases and these folate-coated HSA nanocapsules have great potential to be used for drug delivery in a number of disease settings.

6. Future directions and concluding remarks

The field of protein capsules has seen remarkable development in the last decade. Indeed, it is now possible to assemble protein capsules of defined sizes and with pre-defined functionalities. Such methods have enabled the design and assembly of protein capsules for tissue specific drug-delivery. We anticipate that these methods, together with recent advances in methods for the site-specific modification of proteins,[46] will expand significantly the use of functionalised protein micro- and nano-capsules for targeted drug-delivery, imaging or vaccination. In the latter case, the requirement of presenting the low immunogenic oligosaccharide in a protein carrier for an optimal immune response[52] makes oligosaccharide modified immunogenic protein nanospheres particularly attractive vaccine candidates. The availability of a protein carrier for surface modifications, the enhanced permeation and retention effect in combination with active targeting by the protein capsule shell carriers would enhance the therapeutic effect of delivered agents without increase in their toxicity. Thus, protein capsules have the potential to become one of the safest and most effective therapeutic vectors.

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