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Review article

Strategic approaches for overcoming peptide and protein instability within biodegradable nano- and microparticles

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Abstract

This paper reviews the major factors that are closely involved in peptide and protein degradation during the preparation of biodegradable nano- and microparticles. The various means usually employed for overcoming these obstacles are described, in order to bring to the fore the strategies for protein stabilization. Both processing and formulation parameters can be modified and are distinctly considered from a strategic point of view. We describe how partial or full protein stability retention within the carriers and during drug release might be achieved by individual or combined optimized strategies. Additionally, problems commonly encountered during protein quantification, stability determination and release are briefly reviewed. Artefacts that might occur during sampling and analytical procedures and which might hinder critical interpretation of results are discussed.

Keywords: Biodegradable polymers; Nanoparticles; Microparticles; Peptides; PLGA; PLA; Proteins; Stabilization

1. Introduction

The advent of recombinant DNA technology has made possible the commercial production of proteins for pharmaceutical applications from the early 1980s. The development of pharmaceuticals directly from this ever emerging technology has enabled to provide the market with products of exceptional high purity. In order to maintain such high standards of quality, manufacturers have to make sure that the protein drugs are fully active upon administration and that they contain no toxic by-products. Formulations must be able to release an intact active moiety and actually, the ability to demonstrate protein stability inside pharmaceuticals and during delivery becomes a requisite for reaching the product approval stage [1,2]. Nevertheless, protein stability certainly still remains one of the most important hurdles for their successful incorporation in biodegradable systems, such as nano- or microparticulate carriers. Indeed, in the field of protein nano- and microencapsulation, ensuring product consistency is a rather tricky matter, insofar as each component of such a formulation has not only to be recognized as safe, but conjugated complexes between drugs and polymeric macromolecules might also be considered as new chemical entities by health authorities [3]. With respect to the safety of the polymers used for encapsulation, it should be underlined that those derived from D,L lactic and glycolic acids (like poly(D,L-lactic-*co*-glycolic acid), PLGA) have gained some popularity, mainly because of their noteworthy properties in terms of tissue compatibility and biodegradability [4].

According to the FDA, a pharmaceutical product is considered as stable as long as it deteriorates by no more than 10% in 2 years. But as far as proteins are concerned, the term *stability* needs to be defined with

Abbreviations: BSA, bovine serum albumin; CD, cyclodextrin; DNA, deoxyribonucleic acid; EPO, erythropoietin; GH, growth hormone; GOD, glucose oxidase; HBcAg, hepatitis B core antigen; IGF-I, insulin-like growth factor-I; INF, interferon; NGF, nerve growth factor; PEG, poly(ethylene glycol); PEO, poly(ethylene oxide); PLA, poly(D,L-lactic acid); PLGA, poly(D,L-lactic-*co*-glycolic acid); PVAL, poly(vinyl alcohol); SDS, sodium dodecyl sulfate; TT, tetanus toxoid.

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more accuracy. *Chemical stability* involves typically the integrity of the amino acid sequence (primary structure) and the reactivity of the side chains. Most of the time, the activity of peptides depends on the primary and possibly secondary structures, whereas proteins possess an additional tertiary and sometimes even a quaternary structure, that allows the protein chains to fold and adopt a three-dimensional conformation [5]. The chemical stability of a pharmaceutical protein can be impaired during for instance proteolysis. Such a degradation pathway leads to two or more products of smaller molecular weight, which should be characterized. It is indeed not only necessary to recover high amounts of therapeutically active protein from the pharmaceutical, but even low amounts of degraded protein must also be safe, since degradation products might be therapeutically inactive, or cause unpredictable side effects, such as toxicity or antigenicity. *Physical stability* is generally defined as the ability of a protein to retain at least its tertiary structure, that is crucial for the biological activity too. Common chemical and physical degradation pathways have been reviewed and discussed, for instance, by Witschi [6] within the framework of the encapsulation of a peptide drug into microparticles. Briefly, chemical degradation includes deamidation, isomerization, hydrolysis, racemization, oxidation, disulfide formation and β-elimination. Physical degradation involves reversible or irreversible denaturation through a loss of tertiary structure and unfolding with further reactions like chemical degradation, aggregation and precipitation. Most of the time, the *pharmaceutical properties* of therapeutic proteins closely depends on the retention of their *biological activity*. Consequently, the pharmaceutical efficacy (and hence the three-dimensional integrity) should be assessed by monitoring the *biological activity* at the end of the formulation process. In the same way, the control of *antigenicity* is needed to guarantee the efficacy of protein therapeutics like vaccines or to exclude any protein degradation (e.g. aggregation often causes increased antigenicity) [7]. The antigenicity of a protein is dependent on the intactness of its antigenic determinants (well defined sub-units of the molecular structure). It must be emphasized that a protein might undergo a loss of enzymatic activity but preserve its antigenicity, because the active site region responsible for the expression of activity is often different from the epitope that reacts with a polyclonal antibody [8–10]. Additionally, results obtained from in vitro enzymatic activity measurements and antibody assays should be compared to those obtained during in vivo assessments, since in vitro–in vivo correlation is not always possible [11–16]. Ideally, these experiments should also be completed by structural analysis in order to make sure that protein stability is totally retained [1].

Nowadays, the assessment of protein stability during manufacture, storage or release is increasingly being integrated into research programs and in the past few years, the number of publications relating to encapsulated protein stability has considerably grown. Additionally, some excellent reviews have addressed many aspects concerning protein stability, such as the factors responsible for instability and the stabilization techniques [17–22]. For protein degradation to be at least reduced and preferentially avoided, an accurate stabilization rationale is required. However, it is most of the time not possible to adopt a general strategy for protein stabilization. For instance, an additive that efficiently stabilizes a protein might damage another one [23]. Thus, proteins are traditionally formulated by an empirical trial and error approach, each protein being different one from the other. Generally, the need to keep the protein stable within the formulation has right of way over any release considerations. But obviously, the ideal goal would be to achieve satisfactory protein stabilization along with an appropriate drug release by adopting the same strategic approach. Further additional tedious developments of the manufacturing process are thereby avoided [24,25].

The present article reviews the state of the art of manufacture techniques and discusses parameters that induce perturbations in entrapped proteins, as well as the strategies adopted for peptide or protein stabilization during the encapsulation and release process. The paper focuses on the studies performed with biodegradable nano- and microparticles. Since critical interpretation of results can be considered as being of major importance, sampling procedures and some analytical techniques for protein stability analysis are also discussed. Initial protein quality, properties and preformulation are addressed as they might drastically influence final protein stability in the carriers. Finally, the section concerning the use of additives presents typical examples of successful and failed stabilization trials of different commonly used model and therapeutic proteins. Protein stability issues during freeze-drying procedure and during storage are not addressed.

2. Sampling and analytical issues

The assessment of stability and the precise quantification of an encapsulated protein still remain difficult tasks and major obstacles are encountered during the sample preparation before analysis and during the analysis itself [5,15,16,26,27]. Protein integrity evaluation is indeed likely to be affected by artefacts during these operations [28], thereby preventing the scientist from critically ascribing detected protein denaturation to manufacturing conditions. In vitro, there is an utmost interest in measuring the extent of protein degradation within the carriers and during release. For such purposes, the protein needs to be removed from the polymeric matrix before analysis by using generally harsh conditions. Practically, protein is usually recovered either by an extraction-based method with help of potentially deleterious organic solvents or by hydrolysis of the carriers

with alkaline media. A comparison between these two methods generally reveals that extraction-based methods underestimate protein content values (mainly due to protein aggregation at interface) with respect to hydrolytic methods, which have a more pronounced adverse impact on protein stability. To date, new methods of protein recovery have been proposed, so as to avoid creation of interfaces or to perform both the recovery and the analysis in only one step. To overcome the problem of the presence of interfaces, the filtration method can be used as an alternative to the extraction method. After dissolution of the carriers in an organic solvent, the suspended insoluble protein is recovered by filtration [29], lowering also the risk of underestimation of drug content. In another example, microspheres were incorporated into a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE system) allowing electrophoretic extraction of the protein [13,15]. Direct dissolution of both the carrier and the protein drug in a single liquid phase is also possible and involves the use of aqueous mixtures containing water-miscible organic solvents such as acetonitrile or DMSO [30,31].

Additionally, the choice of an accurate analytical technique is of importance, since certain methods might not be able to discriminate between denatured and native protein. A simple spectrometric method for instance does not always allow discrimination between the monomeric protein form and its aggregates, whereas HPLC might separate these species and thus provides more accurate qualitative data [32]. However, HPLC cannot quantify exclusively the amount of active antigen, as is the case with ELISA techniques [33]. Nowadays, Fourier transform infrared (FTIR) spectroscopy has become a popular, non-invasive method, as it is able to characterize the secondary structure of entrapped proteins [31,34–38]. Only recently, the integrity of their primary structure was evaluated, thanks to a new MALDI-TOF mass spectrometry method [30,39]. The method was shown to require little sample material and only a simple dissolution of the carrier was needed prior to the analysis. This technique allowed elucidation of a new degradation pathway, i.e. peptide acylation within PLGA carriers resulting from a chemical interaction between peptide and degraded polymer [40]. Moreover, the technique was also useful for quantification and it should be underlined that no interference from PLGA was detected during the measurements. For all the reasons cited above, mass spectrometry should be considered as one of the most promising methods for protein analysis inside polymeric carriers. Finally, it is recommended to use several complementary analytical methods for the same protein formulation. Indeed, to perform only some types of assays that are insensitive to structural and conformational changes (e.g. antigen–antibody based tests (ELISA), enzymatic activity measurements, in vivo assays) might not be sufficient to conclude in the full retention of protein stability.

As discussed above, the sample preparation and analysis of the active form of a protein are often tedious, whereas simple total protein quantification is less problematic. For example, complete digestion of carriers followed by amino acid analysis was demonstrated to produce reliable results [41,42].

During in vitro release, the protein is subjected to potentially deleterious conditions, since the carriers are traditionally incubated in aqueous buffers at 37 °C for prolonged periods. Moreover, carriers made from PLGA generate acidic moieties over time, i.e. lactic and glycolic acids. Trapped inside the particulate carriers, these products are responsible for creating a low pH (as low as pH 3) microenvironment, that might affect the stability of the encapsulated protein [10,13,15,43]. Basic salts can be co-incorporated into the carriers, in order to counteract acid-induced protein degradation by buffering [43–45]. Additionally, reduced accumulation of acidic products might be achieved by the frequent replacement of the medium with fresh buffer or by using dialysis systems [13,15,26]. The composition of the release medium can also be a potential factor of instability. Lysozyme for instance was found to aggregate in phosphate buffer leading to incomplete release, whereas glycine buffer stabilized the protein [46]. Incomplete protein release is often ascribed to PLGA–protein contacts such as non-specific adsorption to the polymer or interactions of hydrophobic nature. Such phenomena lead generally to less active protein in the release medium than the total amount initially expected, mostly because aggregated or adsorbed protein is not released. In order to evaluate the respective contribution of non-covalent aggregation and of adsorption to incomplete release, some specific agents can be added to the release medium. Indeed, sodium dodecyl sulfate (SDS) can dissociate non-covalent aggregates and also displace protein molecules adsorbed on the polymer surface, whereas guanidine hydrochloride or urea can only dissociate non-covalent aggregates. The contribution of protein adsorption is thereby evaluated by deduction [16,31,47,48]. During in vivo assays, these problems are minimized, since the protein is directly assessed through its biological activity or immunogenicity.

3. Processing parameters affecting protein stability

Among all the possible techniques for protein encapsulation, the most widely used is certainly the water-in-oil-in-water ($w_1/o/w_2$) double emulsion method. This is a suitable method for hydrophilic drug incorporation, insofar as the drug is first dissolved in the inner aqueous phase (w_1) and then trapped within a polymer resulting in a matrix (sphere) or a reservoir (capsule) system. Briefly, an aqueous solution of the hydrophilic drug is emulsified into an organic solution of the polymer. Ethyl acetate and methylene chloride are usually selected as organic solvents. This primary w_1/o emulsion is poured into a second water phase (w_2)

containing a convenient stabilizer to form a $w_1/o/w_2$ double emulsion. The organic solvent is then removed by evaporation or extraction leaving hardened nano- or microparticles in an aqueous medium. The double emulsion method is appropriate for numerous proteins, although they might be subjected to several stress factors during the process. In certain cases, resulting carriers are able to entrap proteins that suffer neither from aggregation nor from molecular fragmentation [49] and to improve the activity retention of released protein [50].

The interface between water and a solvent is a well-known destabilizing factor [35,48]. Typically, proteins become especially prone to aggregation from the moment they migrate and adsorb at the interface. They undergo unfolding by presenting their hydrophobic area, usually buried in the molecular structure, to the organic solvent. The formation of the primary w/o emulsion, and to a lesser extent of the secondary w/o/w emulsion, is mainly responsible for protein denaturation [14]. It has been shown that glucose oxidase (GOD) loses 28% of its activity during the first step and 20% during the second emulsification step, whereas the cumulative activity loss after solidification, centrifugation and freeze-drying was less than 4%. The absence of an outer water phase in the w/o/o method, a variant of the w/o/w method, resulted in a reduced GOD leakage out of the core of the droplets, a higher entrapment efficiency in the resulting microspheres, but a reduced enzymatic activity compared to those obtained by the original method [24]. For the sake of comparison, microspheres generated by a w/o/o scheme were able to protect insulin, but only with an appropriate polymer [51]. Morlock et al. [52] showed that the first w/o emulsion step caused the formation of covalently bound aggregates of erythropoietin (EPO), whereas the subsequent steps of the process did not cause further degradation. The nature of the organic solvent has an impact on protein stability too. Ethyl acetate usually induces less denaturation than methylene chloride [53–55]. When these two solvents were used in a 1:1 ratio, both entrapment efficiency and activity retention of GOD were improved [24]. The protective effect of acetone, commonly employed for plasmatic fractionation, was successfully tested to enhance protein C and nerve growth factor (NGF) stability. It was demonstrated that the use of an acetone/methylene chloride blend was better than methylene chloride alone, probably owing to reduced surface tension between the organic and the water phases [8,56–58].

Protein degradation might also take place upon mechanical shearing and exposure to ultrasound, but it should be noted that proteins are generally more sensitive to interfaces than to sonication [10,14]. Shearing considerably augments the chances for dissolved proteins to adsorb onto air/water and water/organic solvent interfaces, thereby promoting hydrophobic interactions that might lead to further aggregation. The sonication of liquids produces acoustic cavitations, i.e. small bubbles of gas, which expand and collapse quickly with local rising of temperature and pressure, resulting in the so-called 'hot spots' and in the formation of free radicals [59–64]. The deleterious effect of shearing and sonication stress might be minimized by reducing the exposure time or the intensity of the stress, possibly in combination with others strategies such as the use of additives or appropriate organic solvent systems [8,56,57,65,66]. The choice of the apparatus used for the preparation of the w/o emulsion has an influence on the protein stability too. It was shown, for instance, that EPO aggregation was more favoured by sonication or vortexing than by homogenization, whereas vortexing affected less protein C activity than sonication [52,56].

In order to avoid the detrimental effect of the w/o interface of the first emulsion, the protein might be directly suspended in the organic phase, so as to form a s/o/w emulsion. In fact, protein powders can be suspended in various organic solvents without causing solvent-induced structural perturbations, provided they are suspended in an anhydrous state. In theory, a protein in pure organic solvent displays less conformational flexibility than in aqueous/organic mixtures leading to a more preserved secondary structure and impaired denaturation [67]. However, in order to obtain an anhydrous powder, the protein must be freeze-dried prior to encapsulation or spray-freeze dried if a reduced particle size is required. Lyoprotectants like mannitol or trehalose might reduce structural perturbation during this step [29,54,68–70]. The further dispersion of protein into the organic phase was not harmful for bovine serum albumin (BSA), with its secondary structure not significantly altered, whatever the speed rate of the homogenizer [71]. In contrast to the s/o/w method, the solid-in-oil-in-oil (s/o/o) methodology prevents protein leakage into the external water phase, improves the entrapment efficiency and totally avoids water/organic solvent interface [29]. The secondary structure of BSA within PLGA microspheres remained essentially unaffected provided that the protein was protected by a suitable additive [68]. Jiang and Schwendeman performed a comparative study that showed the superiority of the s/o/o method upon the w/o/w method for the preparation of formalinized-BSA loaded microspheres. They also demonstrated the relevance of combining this s/o/o method with other stabilization stategies, as, e.g. the co-encapsulation of additives [25,72]. The solid-in-oil (s/o) first step can also be combined with subsequent co-acervation by using silicon oil as co-acervating agent. However, this alternative to the non-aqueous s/o/o method did not efficiently protect the protein in the absence of additive [69].

Spray-drying is a valuable alternative method for protein-containing microparticle production. Nevertheless, during the process, the proteins might be exposed to organic solvents, organic/aqueous or air/liquid interfaces and intensive mechanical and thermal stress. Gander et al. have studied the effect of 10 different types of polymer solvents on BSA stability. It was claimed that BSA

undergoes neither cleavage nor aggregation whatever the solvent, whereas the in vitro protein antigenicity was reduced by 50% especially by water-miscible solvents. Interestingly, methylene chloride and ethyl acetate had no detrimental effect on BSA antigenicity [73]. The impact of the type of feeding liquid used for spraying was also investigated and it was shown that it did not denature insulin. Indeed, no differences were observed between a w/o emulsion, a s/o suspension and an acetic acid solution [74]. The w/o/w double emulsion and the spray-drying methods were compared for EPO encapsulation. It was found that spray-drying avoided EPO aggregation in contrast to the double emulsion [75]. However, when tetanus toxoid (TT) was spray-dried, no residual bioactivity of the protein entrapped in the microspheres was detected [44].

Techniques which are intended to circumvent problems usually encountered with the traditional methods have been proposed. Initially designed for lipophilic drug encapsulation, the emulsification solvent diffusion method was modified so as to enable hydrophilic drug entrapment. Basically, the protein and PLGA were dissolved in a blend of acetone (predominantly), methylene chloride and water. This unique phase was poured into a poly(vinyl alcohol) (PVAL) solution enabling acetone to rapidly diffuse into the aqueous phase and to produce nanospheres. The resulting nanospheres improved nafarelin stability during release in an acidic medium in comparison to a simple solution of the native peptide [76].

Cleland and co-workers have described a new cryogenic process, which is very similar to the spray-freeze drying, and which is derived from the spray-drying technique. It employs neither surfactants nor water, avoids solvent interfaces and elevated temperature. Briefly, the protein is suspended in a PLGA organic solution and then sprayed through a nozzle submitted to ultrasound into a vessel containing frozen ethanol overlaid with liquid nitrogen. When the temperature was set at about −70 °C, the ethanol melted and extracted the organic solvent from the droplets leading to hardened microspheres. The adopted strategy efficiently protected a chemically modified growth hormone (GH) during the encapsulation process and resulted in vivo in 20-fold longer GH serum levels relative to those observed with a protein solution injected subcutaneously [77]. It also provided some promising results in combination with well-chosen additives [78,79].

Another modified spray-drying technique involving two steps has been proposed for the production of BSA-loaded alginate microparticles. First, a water solution of sodium alginate and BSA was spray-dried so as to obtain 'temporary microparticles'. Then, a solution of calcium chloride and of chitosan was used to provoke the hardening of these protomicroparticles by an interfacial crosslinking reaction. Using this method, BSA was shown to be protected during the preparation and release processes [80]. BSA was also stabilized when this ionic gelation process was adapted to an emulsion-based method [81].

A novel method named gas antisolvent technique involves the atomization of a drug/polymer organic solution in a high pressure carbon dioxide (CO_2) environment. The organic solvent diffuses out of the droplets into the CO_2 phase causing protein-loaded microsphere formation by precipitation. This technique was performed in combination with appropriate polymer blends and successfully retained entrapped in vivo insulin activity [82].

Nanosphere manufacture by direct polymerization instead of using preformed polymers has been described for insulin loading. The nanospheres were made of a new temperature-sensitive acrylate-polyethylene glycol (PEG) copolymer and obtained by thermally initiated polymerization. Protein loading was subsequently accomplished through equilibrium partitioning and thanks to the swelling properties of the resulting nanospheres at low temperature. The stability of entrapped insulin against elevated temperatures was improved with respect to insulin in solution [83]. Another method involving a new system composed of polyethyleneimine and dextran sulfate was described for insulin-loaded particle manufacture. A simple crosslinking reaction initiated by zinc was responsible for nanoparticle formation. The resulting carriers enabled a prolonged hypoglycaemic effect in vivo [84].

Finally, another original but time-consuming and complex technique is based on the production of a heterogeneous polymeric composite constituted of protein-loaded agarose or starch-based hydrogel particles further dispersed in PLGA microspheres. These systems were shown to protect more efficiently insulin, BSA and horseradish peroxidase (HRP) from structural degradation and bioactivity loss during the manufacture process and release than conventional PLGA microspheres [85,86]. A slightly different concept was designed for stabilizing TT. The antigen was first inserted into a core made of gelatin and optionally poloxamer in order to provide a mild environment. Then, this core was covered by a PLGA coating, thanks to a oil-in-oil (o/o) procedure. TT was released in its full active form, but only when poloxamer was used in the formulation [87].

4. Formulation parameters affecting protein stability

This section includes the formulation factors that might affect protein stability, i.e. the protein properties, the polymer employed and the excipients added in the formula.

4.1. Protein properties/preformulation

The source and purity of protein can greatly affect protein stability. Indeed, the degree of protein denaturation is often dependent on the manufacturer and protein purification is certainly one of the most critical issues,

with the presence of contaminants being a major determining factor of protein instability. However, literature regarding this concern is rather scarce [16,17,55,80,88,89]. Moreover, since individual entrapped peptides and proteins differ in terms of physico-chemical properties and chemical/therapeutic function, each species is expected to demonstrate a different degree of sensitivity to stress [10,14,38,90] and react differently to the same stabilization strategy [91]. The proteins commonly encapsulated in polymeric carriers are listed in Table 1.

A very attractive goal in protein formulation is to allow the native molecule to be released from the dosage form, without any prior chemical modification. Nevertheless, the stability of native proteins is likely to be affected by the manufacturing processes, whereas chemical modification provides protein stabilization before encapsulation. Such an approach presents the advantage to alleviate the encountered constraints during the drug loading into the particulate carriers and to give more room for manoeuvre for subsequent procedure optimization (e.g. for release improvement). However, the modified protein is liable to be considered as a new chemical entity with modified physico-chemical properties and activity relative to the native protein. If the encapsulated protein is an enzyme, the chemical modification has to assure its specific activity retention. In this respect, it should be underlined that, to date, protein pegylation is the most promising protein modification chemistry. Indeed, such a chemical treatment has enabled to get relevant clinical results and to commercialize therapeutics against, e.g. hepatitis C and leukemia [92]. Chemical modification can be combined with entrapment too. Methoxypoly(ethylene glycol)-conjugated lysozyme, for instance, exhibited better stability than native lysozyme against manufacturing processes (e.g. exposure to organic solvents, homogenization) [93]. Carboxymethylated bovine serum albumin is devoid of free thiol groups, which is deeply involved in intermolecular covalent aggregation [94]. Pegylated interferon-α (INF-α) displayed better resistance against DCM/water interface-induced degradation and longer half-life than

Table 1
List of proteins entrapped in biodegradable carriers and subjected to investigation for stability retention by single or cumulated stabilization strategies

Protein family	Protein	Therapeutic class	References
Antigens	Diphteria toxoid	Vaccine	[14,33]
	E. Coli enterotoxin B subunit	Vaccine	[14]
	Hepatitis B core Ag	Vaccine	[12]
	Staphylococcal enterotoxin B-toxoid	Vaccine	[9]
	Tetanus toxoid	Vaccine	[14,29,33,44,55,72,87,112,118]
Enzymes	Asparaginase	Antitumoral agent	[5,110]
	Carbonic anhydrase	Model protein	[13,15]
	Chymotrypsin	Proteolytic enzyme	[115,119]
	Glucose oxidase	Model protein	[24]
	Heparinase	Model protein	[91]
	Horseradish peroxidase	Model protein	[50,86]
	Lysozyme	Mucolytic agent, antiviral agent Antiviral agent	[16,28,31,34,35,37,46,47,49,57,93,102]
	Protein C	Anticoagulant agent	[8,56]
	Superoxide dismutase	Model protein	[23]
	Trypsin	Proteolytic enzyme	[91]
	Urease	Model protein	[53]
Hormones	Calcitonine	Calcium regulator	[40,105,111]
	Growth hormone	Growth hormone	[14,36,54,77,104,121]
	Parathyroid hormone	Calcium regulator	[40]
	Insulin	Hypoglycaemic hormone	[30,43,51,74,82–85,96,116]
	Leuprolide	LHRH analogue	[40]
	Nafarelin	LHRH analogue	[76]
	Orntide	LHRH analogue	[109]
	Tetracosactide	Corticotrope hormone	[6]
Cytokines	Erythropoietin	Red cell proliferation and differentiation regulator	[52,75,106]
	Insulin-like growth factor-I	Osteogenesis promotor	[65]
	Interleukin	Cytokine, antitumoral agent	[10]
	Interferon	Cytokine, antitumoral agent	[54,95,117]
	Tumor necrosis factor	Antitumoral agent	[114]
	Vascular endothelial growth factor	Cytokine	[78]
Other proteins	Albumins	Model protein	[15,16,25,26,32,34,45,49,50,68,69,71,73,80,81,86,90,94]
	Nerve growth factor	Neurotrophic factor	[58,79,120]

native INF-α [95]. Encapsulated GH, previously complexed with zinc, showed an unaltered structure relative to the protein before encapsulation [36,77] and NGF was stabilized during encapsulation and release, thanks to the same approach [79]. Toxins are sometimes treated with formaldehyde to convert them into toxoids devoid of pathogenic effect but still having antigenic activity. However, this chemical treatment leads to another instability problem in this case, since these formalinized antigens might aggregate through a formaldehyde-mediated aggregation pathway [72].

Crystalline proteins are usually less prone to chemical degradation than the amorphous form and X-ray scattering can aid the investigation of crystal structure in PLGA particles [17,22,96]. Protein concentration is of importance too, since unmodified proteins exhibit a 'self-protecting' behaviour, especially when used at high concentrations in emulsion-based techniques of encapsulation. This interesting effect is probably due to the proportional distribution of the protein between the water phase and the water/organic interface, regardless of the initial amount of protein added in the water phase. Once a fraction of protein has adsorbed onto the interface, it aggregates and shields the non-adsorbed protein fraction that is thereby prevented from aggregation [13,44,54]. Increasing the concentration of lysozyme alone [23] or together with that of the polymer simultaneously [57] has been reported to augment biological activity.

In order to counteract protein degradation related to interfacial phenomena, some processing protocols avoid partial or total use of water. The protein is thus often suspended in organic solvents and protected through reduced conformational flexibility (see Section 3). An opposed approach is to solubilize directly the protein in organic solvents. This can be achieved by different means. Cleland and Jones assumed that native protein conformation could be maintained by precipitating the protein at its isoelectric point (pI). The molecule is then free of charge and can be readily solubilized in organic solvents [54]. Conversely, an alternative concept is based on the freeze-drying of the protein at a pH away from its pI value before formulating it. It was thought that this strategy could increase protein solubility and stability in various polar and water-miscible organic solvents like DMSO [97–101]. Using the dissolution approach, insulin was successfully formulated [82], whereas incomplete lysozyme release from microspheres was observed and ascribed to aggregation [47]. Protein solubility can also be increased via an ion-pairing mechanism. The protein is modified by adding an oppositely charged surfactant that binds to the protein, so as to obtain a neutral hydrophobic entity and to reduce thus direct contact between the protein and the organic solvent. Positively charged proteins and negatively charged surfactants should be employed, since cationic surfactants might have toxic side effects. This technique was shown to improve lysozyme conformational stability [102,103].

A new interesting concept is to encapsulate an aggregated protein in a reversibly dissociable form, in order to avoid the formation of irreversible aggregates during processing and to promote the sustained release of the native monomeric form. GH was successfully formulated, thanks to this approach [104].

Finally, it should be noted that a preformulation procedure consisting of using spray-freeze drying with a suitable additive was able to stabilize BSA before encapsulation (see Section 3) [69].

4.2. Effect of the polymer

The choice of the appropriate polymer for protein encapsulation often depends on the release properties that the pharmaceutical scientist wants to confer to the carrier. Usually, hydrophilic polymers facilitate water uptake and continuous drug release. It is possible to use blends of hydrophilic and hydrophobic polymers or amphiphilic copolymers. These two strategies might also be of interest for protein stabilization. Jiang and Schwendeman [25] used a blend of hydrophobic poly(lactic acid) (PLA) and hydrophilic PEG in order to avoid acid-induced BSA degradation caused by the hydrolysis products of PLGA during drug release. When the PEG content in the blend was less than 20%, BSA release was incomplete and insoluble non-covalent BSA aggregates were observed in the residual device. In contrast, when PEG content was between 20 and 30%, continuous release was improved and BSA remained structurally intact. These results were explained by the synergetic properties of both polymers. The slower degradation of the PLA (compared to PLGA) avoids too high a concentration of acidic species within the polymer matrix and the use of the hydrophilic PEG enables better water uptake, easier diffusion of the degradation products out of the polymer, thus maintaining a more neutral pH in the device during drug release. A PLA/PEG blend was also used for insulin encapsulation and the resulting microparticles were compared to those obtained with a PLGA/PEG blend. In vitro results showed reduced insulin degradation with the PLA/PEG blend [51]. In vivo tests showed that insulin retained more than 80% of the native hypoglycaemic activity [82]. This strategy might be combined with others approaches such as, e.g. the preparation through a o/o emulsion and the co-encapsulation of additives [25,72,82].

The copolymer poly-D,L-lactide-*co*-poly(ethylene glycol) (PLA-PEG) was tested for GOD entrapment. The highest protein activity was observed when the copolymer contained 10–30% PEG, which was a far better performance compared to that resulting from microspheres made from PLA or PLGA [24]. A diblock copolymer, monomethoxy-poly(ethylene oxide)-poly(lactic acid) (MPEO-PLA), was tested for protein C encapsulation by a w/o/w procedure. With optimized sonication times and an appropriate organic solvent, nearly all the protein released from the resulting

nanoparticles retained its activity [8]. However, the use of copolymers might have the opposite effect to that expected. PEG-PLA, for instance, did not succeed in avoiding calcitonin acylation during release [105]. Morlock et al. used an ABA triblock copolymer (consisting of PLGA as block A and poly(ethylene oxide) (PEO) as block B) in order to generate EPO-loaded microspheres by double emulsion that could exhibit continuous release profiles. This protein is very sensitive and can form high molecular weight aggregates, that are covalently bound and pharmacologically inactive. About 20% of the EPO was found as aggregates when using the ABA copolymer for the microspheres. This amount dropped to less than 5% with the addition of stabilizers. When only PLGA was used, the amount of aggregates was 5% [106] and addition of cyclic D,L-lactide dimers to PLGA did not improve EPO stability [75]. As a matter of fact, stability of proteins might be preserved in certain cases by using PLGA alone [50].

Interactions between entrapped proteins and PLGA are often responsible for protein aggregation and subsequently slower and incomplete release in vitro. These interactions are often hydrophobic in nature, but ionic interactions might also contribute to protein adsorption onto the polymer [50,107,108]. The presence of blocked (capped) or of free (uncapped) carboxyl end groups generally has an influence on protein release. Indeed, proteins formulated at a neutral pH (lower than their pI) are often positively charged and thus might interact with negatively charged PLGA containing free carboxyl end groups. This interaction depends on the content of free carboxyl end groups: the more numerous, the stronger the interaction [32,47,78,109]. L-asparaginase activity is influenced by such an interaction, since nanoparticles made of uncapped end groups were able not only to entrap a higher amount of protein but also to provide a longer continuous delivery of active enzyme over time compared to nanoparticles made of end-capped PLGA [110]. It should be mentioned that electrostatic interactions generally increase encapsulation efficiency [80] and that, for some proteins, non-specific adsorption onto the polymer surface might represent an useful delivery system instead of proper loading inside particle core [111]. It has been experienced with TT and promising in vivo results were obtained along with an appropriate polymer [112].

Polymer concentration might also influence protein stability as demonstrated in the case of lysozyme. When double emulsion was used, the retained biological activity of lysozyme increased from 59 to 83%, when PLGA concentration was increased from 4.5 to 37% in the organic phase. This improvement was ascribed to the probable higher rate of solidification of the microspheres resulting from the higher PLGA concentration [57].

Polymers other than the well-known systems described above have also been investigated. Polyanhydride microspheres were able to provide more active trypsin and heparinase after 12 h of incubation relative to the proteins simply dissolved in buffer under the same conditions [91]. The novel polymer sulfobutylated poly(vinyl alcohol)-*graft*-PLGA was used for TT with promising in vivo results [112]. Alginate carriers were shown to protect BSA [80,81] and poly(ϵ-caprolactone) has also been used for protein entrapment [113]. Simple or pegylated acrylate derived polymers were reported to be good protectors for TNF-α and insulin [83,114]. Agarose/starch-PLGA composite microspheres, as well as those made of the new polyethyleneimine–dextran sulfate system have also been successfully used as already disscussed in Section 2.

4.3. Effect of additives

The use of additives during the encapsulation process has been studied extensively and represents certainly the most widely employed strategy for stabilization of protein pharmaceuticals [19]. Typically, the appropriate additives for the protein under investigation are experimentally selected among various substances by screening. This tedious experimental screening is partly necessary due to the present inability to predict protein stability after addition of such excipients. The most tenable and generally well-accepted explanation of protein stabilization in aqueous solution by additives is the so-called preferential hydration of proteins. Briefly, a protein in an aqueous environment is in equilibrium between the native and the denatured states. Since the direct binding of a stabilizer to the protein molecule is not considered as thermodynamically favourable, stabilizers are excluded from the protein molecules. The protein is preferentially hydrated and folds back to its native state [17]. Stabilizers such as sugars, certain salts, amino acids, and polyols are excluded from protein molecules. Finally, other stabilization mechanisms might also be involved, due to the surface-active properties of some additives or electrostatic interactions.

4.3.1. Sugars

During a w/o/w procedure, sugars are often added to the inner aqueous phase. Trehalose was shown to partially improve the BSA secondary structure protection within PLGA microspheres and to facilitate BSA monomer release [34]. Trehalose and mannitol had a significant effect on the recovery of soluble non-aggregated INF-γ and GH after emulsification and ultrasonication [54], whereas no or very little protecting effect on insulin-like growth factor-I (IGF-I) against these stress factors was observed [65]. Trehalose also improved asparaginase biological activity [5]. No effect of trehalose, mannitol and sucrose was observed against o/w interface-induced degradation of lysozyme, whereas lactose and lactulose significantly improved its structural stability and activity, mostly if these additives were also added to the second aqueous phase [28,37]. Lysozyme and trypsin activity was not improved by addition of sucrose, which was unable to protect them from an emulsion-induced denaturation and from sonication [35,91]. Mannitol and sucrose dissolved together in the inner aqueous phase were

of slight effect on NGF activity [58] and neither mannitol nor lactose improved hepatitis B core antigen (HBcAg) immunogenicity during methylene chloride/water emulsification [12]. Surprisingly, sucrose and trehalose even decreased urease bioactivity, showing the opposite effect to that expected [53]. Co-encapsulation of maltose reduced α-chymotrypsin aggregation [115]. With respect to particles generated by spray-drying, trehalose was effective in retaining TT antigenicity [44] and in preventing BSA secondary structure degradation [69]. Trehalose protected efficiently NGF during the processing, but did not prevent its aggregation during in vitro release [79]. Cyclodextrins (CD) have also been used as stabilizers. These cyclic oligosaccharides exhibit a hydrophilic zone at the periphery of the molecular structure and a hydrophobic cavity that can chemically interact with proteins. Interaction of aromatic amino acids with the hydrophobic core of CD are likely responsible for the stabilization of the protein conformation against denaturation. However, their use for protein stabilization during encapsulation has not ever been crowned with success. When CD was co-entrapped in the internal aqueous phase, EPO covalent aggregate formation was significantly reduced during microparticle preparation by the double emulsion method [52] and lysozyme stability was improved [28]. However, CD showed no protecting effect on IGF-I [65] and HBcAg [12] and even promoted the loss of superoxide dismutase activity at high CD concentrations [23]. Cyclodextrins also showed low efficiency in retaining spray-dried TT antigenicity, probably due to antigenic epitopes being buried inside the molecular CD core [44]. Finally, carboxymethylcellulose (CMC) did not efficiently stabilize HBcAg and GH against methylene chloride-induced denaturation [12,54]. Based on the examples cited above, it can be stated that trehalose is statistically more effective than the other sugars, whereas mannitol is rather inefficient.

4.3.2. Surfactants

Surfactants have the ability to lower surface tension of protein solutions and prevent protein adsorption and/or aggregation at hydrophobic surfaces. Among them, non-ionic surfactants are generally preferred, insofar as ionic surfactants might bind to groups in proteins and cause denaturation. However, non-ionic surfactants such as polysorbate 20 or 80 were not good stabilizers for lysozyme and GH against the unfolding effect of the water/methylene chloride interface. It has been assumed that both the hydrophilic (PEG chains) and hydrophobic part (fatty acid chain) of the polysorbate molecules were preferentially partitioned in the methylene chloride phase, leading to low protection efficacy [35,54]. Conversely, when three non-ionic surfactants of different hydrophilic–lipophilic balances (HLB) were co-encapsulated with insulin by the w/o/w double emulsion method, only polysorbate 20 was able to improve insulin stability within particles and to limit formation of high molecular weight products during the sustained release period [116]. The gelling properties of the amphiphilic poloxamer 407 was successfully employed for urease encapsulation. The protein was likely protected during the microsphere preparation by a hydrated gelled structure due to the hydrophilic polyoxyethylene chains [53]. Poloxamer 188 was also successfully used when mixed with PLGA for prolonged release of active INF-α [117], but such a formulation had no effect on BSA secondary structure compared to PLGA alone [69]. Poloxamer 188 was not effective in preventing NGF aggregation during in vitro release from microspheres generated by spray-drying [79]. Interleukin-1α (IL-1α) was protected by phosphatidylcholine from damage during the double emulsion process, but underwent inactivation during microsphere incubation [10]. SDS significantly reduced insulin aggregation at the methylene chloride/water interface, whereas dodecyl maltoside did not, this surfactant being more efficient at air/water or solid/water interfaces [48]. It should be mentioned that surfactants are along with sugars proteins and polymers, the most used additives for protein encapsulation.

4.3.3. Proteins

Although the efficiency of proteinic additives for protein stabilization has been clearly demonstrated in several occasions even during encapsulation processes, their use in pharmaceuticals is at present not desirable from a strictly regulatory point of view. Additionally, such agents might contribute to complicate all subsequent protein characterization within the formulation. Among these additives, albumins and gelatins are those mainly used for protection purposes. The protective effect of albumins against protein unfolding and aggregation has been extensively documented and is likely due to their surface-active properties. Albumins (i.e. bovine, human or rat serum albumins) are thought to occupy the interfaces and shield the therapeutic proteins from contact with the solvents or hydrophobic surfaces. Significant protein protection was achieved when serum albumins were added to the inner aqueous phase during the primary emulsification step of multiple emulsion procedures. In this way, BSA (bovine serum albumin) was able to preserve more than 80% of IGF-I integrity upon w/o emulsification and ultrasonication [65]. Since BSA is known to aggregate itself at the interfaces, it improved lysozyme recovery [35] and caused a significant reduction of EPO covalent aggregate formation [52,106]. Albumins increased TT immunoreactivity from 8 to 82%, contrasting with ovalbumin (OVA) that was 2-fold less effective [14]. BSA improved the carbonic anhydrase release profile probably by reducing protein aggregation and thereby minimizing non-specific protein adsorption onto the polymer surface [13]. However, albumins were not always found to be good stabilizers. For example, albumins did not reduce loss of trypsin and NGF activity during the double emulsion process [58,91]. It was postulated that HSA (human serum albumin) formed a complex with NGF hampering the

displacement of NGF from the interface. When TT was encapsulated by spray-drying, BSA preserved up to 40% of TT antigenicity, whereas no activity was detected on formulations without additives. In addition, BSA was thought to scavenge protons during polymer degradation, avoiding any aggregation resulting from acidity [44]. The other proteinic additive used is gelatin, which is composed of a heterogeneous mixture of water soluble proteins. For microparticles generated by double emulsion, gelatin provided no apparent improvement of GOD activity when added to the internal aqueous phase [24], but it dramatically reduced HBcAg inactivation during exposure to methylene chloride. This protective effect was dependent on the gelatin molecular weight and concentration: the higher the molecular weight/concentration, the better the stabilization effect [12]. Gelatin also improved the antigenicity of TT during release [118], and succinylated gelatin significantly protected IGF-I against ultrasonication, preserving up to 90% of its integrity. When gelatin was associated with BSA, IGF-I integrity was fully preserved [65].

4.3.4. Polymers

PEGs, in addition to their role as chemical modifiers, are also known for their protective capacity during carrier manufacture. Indeed, PEG 400 dissolved in the inner aqueous phase provided a protective effect to NGF and asparaginase, as a result of protein displacement from the interface during the double emulsion process [5,58]. A mixture of PEG 400 and BSA in the inner aqueous phase fully preserved IGF-I integrity along with optimized sonication conditions, but significantly decreased the entrapment efficiency [65]. PEG reduced γ-chymotrypsin and HRP disturbance during lyophilization and emulsification. Parenthetically, PEG could be added either in the aqueous or the organic phase (with the polymer) [115,119]. PEG 2000 more effectively stabilized HBcAg against methylene chloride-induced degradation compared to PEG 6000 [12]. PEG was not able to prevent NGF aggregation during in vitro release from microspheres prepared by spray-drying [79]. Conversely, polyoxyethylene (POE) promoted rather than prevented EPO covalent aggregate formation during microparticle preparation [52]. The surface-active properties of partially hydrolyzed PVAL enabled lysozyme to be totally recovered with full activity retention [35]. The same polymer preserved more than 75% of TT immunoreactivity, whereas povidone performance was of 55% and only 8% was retained in the absence of stabilizer [14]. The following groups of additives are less important and less investigated than those already discussed.

4.3.5. Amino acids and alcohols

Certain amino acids might sometimes also act as stabilizers, but their use is rare. EPO aggregation was significantly reduced by L-arginine, the stabilizing effect being ascribed to possible ionic interactions with EPO [52]. In contrast, glycine and lysine did not provide any reduction of trypsin activity loss or HBcAg degradation [12,91]. When histidine was co-lyophilized with f-BSA, formaldehyde-mediated aggregation was strongly inhibited [72]. Glycerol increased the biological activity of asparaginase, when dissolved in the inner aqueous phase [5].

4.3.6. Salts

In order to minimize acid-induced degradation during protein release, co-encapsulation of alkaline salts might result in enhanced protein stability. These additives are either dissolved in aqueous solutions, or suspended in organic solvents. The addition of calcium bicarbonate improved the insulin in vitro release profile and significantly reduced covalent dimerization of unreleased insulin [43]. Other salts such as calcium carbonate, calcium orthophosphate, magnesium hydroxide or zinc carbonate have been investigated too [44,45]. Initially used to improve the release profile by modification of the structure of microparticles, sodium chloride-induced pronounced NGF denaturation, when present in the outer aqueous phase [120]. Complexation with zinc was able to stabilize spray-freeze dried BSA against aggregation, but did not reduce perturbations of the secondary structure [70]. Addition of zinc into microparticles enabled higher serum levels of GH after subcutaneous injection to be achieved [77,121].

5. Conclusion

The production of biodegradable nano- and microparticles containing a stable therapeutic peptide or protein still remains a major challenge, mostly in terms of technical obstacles. Beyond the traditional techniques usually used for stabilization of protein in solid state or in solution, some strategies are specific to protein encapsulation such as the development of new manufacturing methods or the choice of a suitable polymer. To enable protein stabilization, the optimization of each step of nano- and microparticle production is necessary. Nevertheless, a trial and error approach is needed to achieve such an optimisation, which is often tedious and time-consuming. In this respect, the use of specific additives is to date most of the time necessary to counter the denaturing factors. Often protein instability is closely related to the presence of water or interfaces during particle preparation and some new techniques such as oil/oil emulsion, certain spray-drying techniques or direct polymerisation procedures were developed to overcome these pitfalls. Ideally, proteins of therapeutic interest should be studied on a case-by-case basis, so as to bring to the fore processing steps and stress factors which damage them. In some cases, proteins can be chemically modified (e.g. by grafting PEG chains) so as to be less prone to denaturation during encapsulation. Interestingly, the use of a pegylated polymer or of a blend of PEG and polymer was also shown to resolve stability problems along with conferring

interesting release properties to the resulting carriers, avoiding further procedure adjustments. PEG is therefore a very interesting stabilising substance. Finally, it is necessary to make sure that the sample preparation before each protein stability analysis as well as the analysis itself is performed in conditions that do not damage the protein. For instance, protein extraction by polymer hydrolysis or using biphasic solvent systems should only be employed with care. Moreover, the use of analytical techniques such as FTIR or MALDI-TOF mass spectrometry certainly constitutes a step forward for protein analysis in more appropriate conditions.

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