Microspheres as delivery systems for the controlled release of peptides and proteins

REVIEW

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ABSTRACT

Microencapsulation of peptides and proteins has recently become a relevant alternative to develop novel drug-delivery systems. Among techniques designed to microencapsulate substances of different nature, the interfacial polymerization, spray-drying and solvent evaporation techniques are those most widely studied for encapsulation of biomolecules. The solvent double emulsion-evaporation procedure is most commonly applied for this kind of product that is available in the market. On the other hand, this method of protein encapsulation is a rather complex process, involving several factors that determine the properties of the final product and the stability of the encapsulated molecule. In this paper, we offer an updated overview on the use of microspheres as systems for the controlled release of proteins and peptides, the main techniques used for microencapsulation of such biomolecules, and the parameters considered for characterizing the microspheres and the encapsulated protein. Experimental conditions influencing their stability during the solvent double emulsion/evaporation microencapsulation procedures, and strategies used to preserve and stabilize the physicochemical and biological properties of the encapsulated proteins are also discussed.

Keywords: microspheres, PLGA, controlled release, protein delivery

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RESUMEN

Las microesferas como sistemas de liberación controlada de péptidos y proteínas. Recientemente, la microencapsulación se ha convertido en una alternativa importante para desarrollar sistemas novedosos de liberación de péptidos y proteínas. Entre las numerosas técnicas diseñadas para microencapsular sustancias de diversa naturaleza, la polimerización interfacial, el secado por atomización y la evaporación de solvente, han sido las más estudiadas para encapsular biomoléculas. La de doble emulsión-evaporación de solvente es la más utilizada con fines investigativos y para la obtención de los productos que se encuentran en el mercado. La encapsulación de proteínas por este método es un procedimiento complejo, en el cual intervienen numerosos factores que definen las características del producto final y la estabilidad de la molécula encapsulada. Este artículo ofrece información actualizada acerca de las microesferas como sistemas de liberación controlada de péptidos y proteínas; las principales técnicas para microencapsular estos fármacos; los parámetros para caracterizar las microesferas y la proteína encapsulada; así como un análisis de las condiciones experimentales que más inciden en la estabilidad de estas biomoléculas, frente al proceso de microencapsulación por la técnica de doble emulsión-evaporación de solvente; y por último, las estrategias de estabilización que se han desarrollado para conservar las propiedades fisicoquímicas y biológicas de las proteínas encapsuladas.

Palabras clave: microesferas, PLGA, liberación controlada, liberación de proteínas

*I*ntroduction

Due to their relevant function in the body, proteins have been studied for several decades as potential therapeutic agents. Nevertheless, until the biotechnological revolution it was impossible to effectively address their application, based on improved production processes to provide sufficient amounts of protein to develop pharmaceutical products.

Today there are several proteins, used as active pharmaceutical ingredients in different pharmaceutical presentations, available in the market (Table 1). However, proteins bear certain properties as therapeutic agents, such as physicochemical instability in some body fluids (*e.g.*, saliva and gastric secretions), which limit some administration routes (e.g. the oral route). Additionally, their huge size as biomolecules hampers the use of a transdermal administration. All these reasons have established the parenteral route as that of choice for proteins. Besides the overall limitations inherent to this route, there are also others arising from the behavior of proteins as the active ingredient (a short half-life in the body requiring repeated administrations, adverse events determined by their mechanism of action, or undesired side-effects, and even immunogenicity in certain cases [1-4]).

Due to these limitations, the search continues for new and appropriate protein administration systems that take advantage of their therapeutic potential with minor inconveniences. Among the strategies explored, methods comprising chemical modification of biomolecules with polyethylene-glycol (PEGylation),

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Table 1. Some therapeutic proteins available in the international market as pharmaceutical presentations

Protein	Product	Company	Indication
Erythropoietin	Epogen	Amgen	Anemia associated to kidney failure
Interferon alpha-2b	Intron A Heberon alfa R	Schering-Plough HeberBiotec	Hepatitis C Hepatitis B and some types of cancer
Interferon alpha-2b	Roferon A	Roche	Lung cancer, hepatitis B, Kaposi's sarcoma, hairy cell leukemia
Interferon beta	Avonex	Biogen	Multiple sclerosis
Insulin	Humulin	Eli Lilly	Diabetes mellitus
Streptokinase	Estreptasa Heberkinasa	Aventis HerberBiotec	Acute myocardial infarction Acute myocardial infarction
Granulocyte Colony-stimulating Factor	Neupogen	Roche/Amgen	Neutropenia caused by cytostatic treatment
Growth hormone	Genotropin Nutropin	Pfizer Genetech	Growth hormone deficiency in children Growth hormone deficiency in children
Interleukin-2	Proleukin	Chiron/Roche	Kidney cancer
Platelet-derived Growth Factor	Regranex	Johnson & Johnson	Neuropathic ulcers in lower limbs of diabetic patients

and protein encapsulation into lipid- or polymer-based systems (liposomes and microspheres, respectively) are among the most successful applications.

PEGylation involves conjugating polyethylene-glycol (PEG) to proteins, a strategy that started in the late 1960's from the work of Davis and coworkers [5]. In the beginning, PEGylation was intended to diminish the immune response against proteins, further demonstrated as increasing *in vivo* resistance to proteases, thermal stability and half-life in the organism, the latter being the most currently exploited advantage.

Liposomes are colloidal vehicles composed of a lipid bilayer containing hydrophilic and hydrophobic substances, with unique structures that provide attractive properties. They alter the biodistribution of encapsulated drugs, augmenting their efficacy and decreasing their toxicity. Liposomes can also be used to passively target pharmaceuticals to injured tissues. In diseases of increased capillary permeability (cancer, infection, inflammation), liposomes concentrate more at affected zones than on healthy tissues, due to the inability of liposomes to permeate healthy capillaries [6].

In the last 20 years, biodegradable microspheres composed of biocompatible polymers have been studied as systems for the controlled release of proteins and peptides. Generally, the drug is distributed through the polymeric matrix and released by two main mechanisms: diffusion through the matrix, and polymer degradation which erodes the particles. Natural and synthetic polymers have been used, with lactide-glycolide co-polymers as the most relevant materials.

The advantages and limitations of these three systems are summarized in table 2 [4], with PEGylation as the most industrially advanced system with several products in the market. However, several research groups are approaching the microencapsulation of proteins and peptides for their controlled release, instead of the immediate release formulations available, to surpass the decrease in the biological activity of the protein and the heterogeneous product obtained with PEGylation.

Microencapsulation: concepts, aplications and main techniques to generate microparticles

Microencapsulation comprises the coating of solid, liquid or gaseous materials with a film of polymer or fats to generate free-flowing micrometric particles. The product obtained by this process is called "microparticle", "microcapsule" or "microsphere", which differentiate in morphology and internal structure. When these particles are below 1 µm in size, they are known as "nanoparticle", "nanocapsule" or "nanosphere", respectively [7].

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7. Remuñán C, Alonso MJ. Microencapsulación de medicamentos. En: Vilá-Jato JL. Tecnología Farmacéutica. Aspectos fundamentales de los sistemas farmacéuticos y operaciones básicas. Madrid: Ed. Síntesis, SA; 1997:577-609.

Table 2. Advantages and limitations of some of the systems for drug release mostly applied for peptides and proteins

System	Advantages	Limitations
PEGylation	Improved pharmacokynetic properties of the product Low fluctuation of the active ingredient's concentration Lower drug toxicity and immunogenicity Improved patient's quality of life Increased physicochemical stability of the drug	Diminished biological activity Loss of significant amount of proteins during processing Generally yielding an heterogeneous product (positional isomers)
Liposomes	Improved pharmacokynetic properties of the product Lower drug toxicity Passive targeting of the drug	Captured by the reticuloendothelial system Vascular weakening A hard-to-obtain long-lasting physicochemical stability
Microspheres	Steady serum levels of the drug for long periods of time Low fluctuation of the drug concentration Improved patient's quality of life	Incomplete release profile Protein instability when microencapsulated

When a solid material is encapsulated or coated, irregular microparticles are generated, resembling the original shape of the solid encapsulated material since the coating material is deposited. When microcapsules are composed of a gaseous core coated by a film of polymers or lipids. If the substance encapsulated is a liquid, solution, emulsion or dispersion, then, microcapsules or matrix (microspheres) particles are obtained (Figure 1). The resulting structure depends on the encapsulation procedure employed and the properties of their components.

Microencapsulation was formerly described in 1931, with gelatin microcapsules being generated by a procedure called "coacervation" [7]. This technique was varied during the 1940's, with ink encapsulation for carbon paper manufacturing as its most important application.

During the following years, microencapsulation was expanded to other fields of application, including:

- Agriculture, for microencapsulated fertilizers and pesticides
- Naval constructions, to coat screws and rivets and protect them from corrosion
- Cosmetics, for making products like deodorants, shampoos, sprays, to improve their stability or bioavailability
- Medicine and the pharmaceutical industry, taking advantage of microencapsulation to mask undesired organoleptic properties of certain substances (odor, flavor or color), protecting a component sensitive to environmental conditions, or protecting the environment and the user from a toxic component. Also, to cover particles that are irregular in shape and difficult to compress, to turn active ingredients from liquids into solids making them more easier-to-handle, and mainly to design formulations for the controlled-release of drugs for different uses.

Microencapsulation was originally applied to encapsulate drugs and control their release, by a company in the late 1950's [7]. Aspirin was among the first microencapsulated drugs, to avoid gastric irritation. In spite of the delayed application of this method, it was readily and widely introduced in the field of pharmaceuticals.

Coating material

The variety of microencapsulation materials was gradually expanded with the development of biomaterials, and new applications of this technique.

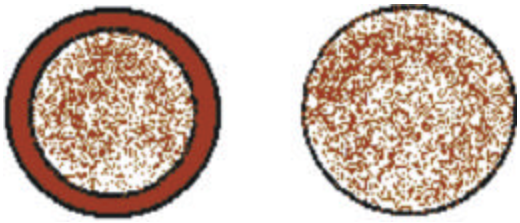

Figure 1. Types of structures of the particle resulting from the microencapsulation process of a liquid material.

In general, these materials fall into three main categories: lipids, proteins and polymers [7].

Fats

Carnauba wax, stearyl alcohol and stearic acid are fats with a characteristic melting temperature and are degraded by lipases in the stomach.

Proteins

Gelatin was the first material used for the purpose of microencapsulation, with current potential applications. Albumin and collagen have also been applied.

Polymers

This family of compounds is the most widely used for microencapsulation, with three major subsets: natural, semisynthetic and synthetic. Natural polymers are composed of animal or vegetal polysaccharides, like alginate, dextran, gum arabic and chitosan. Semisynthetic polymers comprise cellulose derivatives, with a variety of compounds with distinct solubility properties available in the market. For example, ethylcellulose and cellulose acetobutyrate are insoluble polymers, while cellulose acetophthalate shows pH-dependent solubility. Acrylic derivatives and polyesters are among the most widely used synthetic polymers. Acrylic derivatives include insoluble polymers with varying degrees of permeability, and pH-dependent solubility variants. On the other hand, polyesthers are biodegradable polymers that may be administered by the parenteral route. Among them, poly-ε-caprolactone, polylactic acid and the copolymers of lactic acid and glycolic acid (PLGA) have been preferentially used.

It is essential for the coating material to be soluble in the solvent of choice and appropriate for the application of the microencapsulated compound. It should produce homogeneous matrixes or membranes with good porosity, be stable when stored under environmental conditions, and inert to the other compounds they get into contact with. It must also lack toxicity and pharmacological activity if it is to be used for drug design. Lactic acid homopolymers and PLGA are the most widely employed materials for specific pharmaceutical purposes and for preparing injectable microspheres, due to their biocompatibility and capacity to achieve differential drug release profiles.

PLGA as polymeric matrix to microencapsulate pharmaceuticals

PLGA are polyesters obtained by linear polycondensation of hydroxyacids, or by ring-opening polymerization of their respective lactones [8] (Figure 2).

They are hydrophobic polymers soluble in organic solvents like dichloromethane, chloroform, ethylacetate, acetone and tetrahydrofurane. They could be crystalline or amorphous, depending on the lactic acid-glycolic acid ratio, with formulations for a controlled release mostly composed of amorphous polymers. These polymers differ in their monomer ratio, molecular weight (between 5 and 100 kDa, approximately) and the terminal group (-COOH or -COOR), with these three parameters determining their hydrophobicity, degradation kinetics, and ulti-

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mately the efficiency of encapsulation and release rate of the encapsulated material [9].

The term "biodegradable" for these polymers refers to the hydrolysis of their ester bonds by contact with artificial or biological fluids. This reaction generates lactic acid and glycolic acid that are completely metabolized in Krebs'cycle yielding water and CO_2 [9].

When used as matrixes to obtain microspheres, these polymers are degraded in two sequential steps. First, the ester bonds are excised by hydrolysis in a reaction named degradation, generating oligomers and monomers, decreasing the molecular weight of the original polymer. In the second step named erosion, the matrix mass is reduced, the excision speed is speeded up by the autocatalytic action of the resulting acidic degradation products [10].

Precisely, they are used for developing drug controlled-release systems because they are insoluble but degradable in water.

Additionally, PLGA are biocompatible due to their lack of toxicity when introduced into the body by several routes [11, 12]. In fact, these polymers have been traditionally used to manufacture biodegradable surgical threads, and orthopedic fixation devices like discs, pins and screws [13].

Microencapsulation techniques

Several methods have been developed to microencapsulate compounds of different nature, mostly divided into three main groups: physicochemical, mechanical and chemical [7].

1. Physicochemical methods: extensively studied at the laboratory-scale.

a) Simple coacervation. Based on inducing the partial desolvation of the coating material and its deposition on the surface of particles or droplets subjected to coating. Phase separation can be induced in several ways (by adding a non-solvent, by shifting pH or temperature, pH, or adding a salt or an incompatible polymer).

b) Complex coacervation. Phase separation occurs by the spontaneous electrostatic attraction between two or more polymers of opposite charges (polyanion and polycation) after mixing them in an aqueous medium.

2. Mechanical methods. The most industrially applied methods, based on their feasibility.

a) Extraction/solvent evaporation. By starting with an oil-in-water (o/w) or oil-in-oil (o/o) emulsion and incorporating a tensoactive agent into its outer phase. The dissolved coating material and the substance to be coated are in the inner phase, the substance is dispersed if it is hydrophilic (as in proteins) with the resulting double emulsions w/o/w or solid-in-oil-in-water (s/o/w), respectively. The substance is dissolved if it is hydrophobic. The polymeric material is precipitated by the extraction/evaporation of the solvent based on its partition coefficient in the emulsion phases. A w/o emulsion is employed when the coating material is soluble in water.

b) Spray drying. The active ingredient is dissolved in a solution containing the coating material. This mixture is sprayed into a chamber under a hot air flow. Through this process, the solvent evaporates and microspheres are obtained.

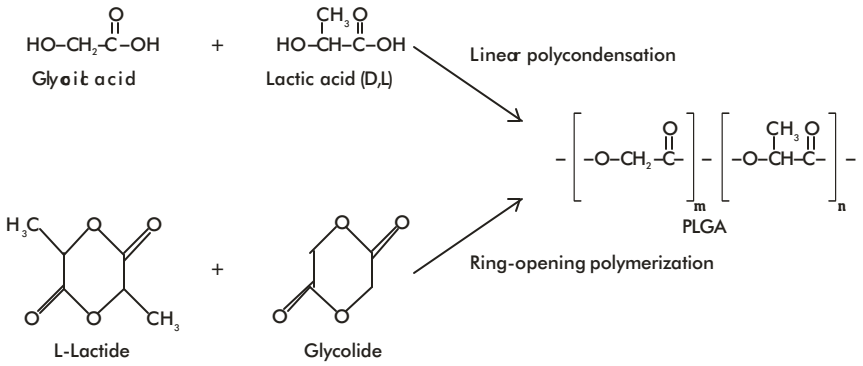

Figure 2. Ways to obtain poly (lactic-co-glycolic acid) copolymers.

c) Fluid bed coating: the particles of the substance to be coated are suspended in a fluid bed and a solution of the coating material is sprayed on the particles. Therefore, the coating material deposits on the particles and then solidifies by the action of air on the bed.

3. Chemical methods. The most recently developed methods, mostly applied at laboratory scale.

a) Interfacial polymerization: This process occurs at the interface of an emulsion, with the production of microcapsules at the interface. In the starting emulsion the monomer is dissolved in one phase and the initiator in the other, or in a system using two monomers, each one dissolved in one phase. In the latter case, an interfacial polycondensation takes place.

b) Heterogeneous polymerization: The coating reaction occurs in a disperse system, resulting in microparticles of different sizes depending on the specific technique employed. Emulsion polymerization (< 5 μm); surfactant-free emulsion polymerization (0.5-5 μm); activated seed swelling emulsion polymerization (10-30 μm); seed polymerization (1-20 μm); suspension polymerization (> 10 μm); dispersion polymerization (0.1-10 μm); precipitated polymerization (0.1-100 μm).

Microencapsulation of peptides and proteins in PLGA-based polymeric systems

Potential advantages

Formulations of proteins microencapsulated in biodegradable microspheres can offer one or several advantages [3, 14, 15]:

1. Reduce the frequency of administration, with a better acceptance by the patient.

2. Increase therapeutic benefit, eliminating fluctuations in serum concentrations of the protein.

3. Potential decrease in the total dosage required for the treatment, due to a higher bioavailability of the dose administered.

4. Potential decrease of adverse events, decreasing the amount of protein delivered in the body at the moment of administration.

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These advantages have encouraged research in this field, resulting in new microencapsulation methods and applications for novel formulations. Nevertheless, very few products developed by this technique are available in the market (four microencapsulated peptides and a protein, see table 3) [16].

The most frequent techniques used for microencapsulation of peptides and proteins

Protein molecules are fragile under experimental conditions, accounting for the main limitations of their microencapsulation conditions. Therefore, among the previously described encapsulation techniques, only a few are most frequently used to obtain microspheres loaded with protein substances. One of them, the simple coacervation method with the addition of a non-solvent compound [17-21], increases the efficiency for encapsulation, but solvent residues in the final product and difficulties for scaling-up have limited its application to obtain biopharma-ceutical products [22]. Besides, micrometric particles are hard to ge obtained [23].

The spray drying method increases the encapsulation efficiency also; it is relatively easy to scale up and facilitates conducting the process under aseptic conditions [24]. Although several authors have addressed the properties of the particles obtained by this method, only scarce data have been published on the stability of the encapsulated protein. During the process, the active ingredient is exposed to harsh conditions (organic solvents and high temperatures) that could considerably damage the activity of the encapsulated proteins, requiring the verification of their biological activity [25]. Other limitations consist of the losses in the final product at the expansion chamber of the microencapsulation device, and difficulties to control particle size [26].

The interfacial polymerization method advantageously preserves all or most of the biological activity of the protein, due to its mild microencapsulation conditions. Nevertheless, trace monomers in the final product have limited its application [27-30].

The solvent evaporation method, starting from a simple emulsion, has been mainly applied to encapsulate certain peptides [31, 32]. It was favored by the introduction of a w/o/w emulsion for highly hydrophilic drugs (including proteins) [33-36], being the preferred method at the laboratory scale for research purposes [37-40], and also at the industrial scale to obtain the majority of the commercialized products. This could be derived from the method's simplicity and the equipment required; the main limitations arise from the process conditions [9, 41]. The exposure to organic solvents, besides the vigorous shaking used to generate emulsions, could reduce the biological activity of the drug [38, 42, 43]. However, several proteins have been successfully encapsulated with adequate preservation levels of their biological activity [34, 44].

To generate microspheres loaded with an active ingredient, a solution containing a polymer dissolved in an organic solvent is used to emulsify or disperse a dissolved or solid drug, respectively. A second aqueous phase is added to the previous emulsion to form a double emulsion, further stirred to extract/evaporate the solvent and facilitate the formation of microspheres that are collected, washed and dried.

A variant of the double emulsion/solvent evaporation method at the liquid nitrogen temperature enabled the encapsulation of the human growth hormone with an almost intact biological activity, also known as the cryogenic method [15, 45]. Because of the complex equipment required, this method has not yet been generally implemented. Therefore, the solvent evaporation method remains as the most widely studied and assayed method for proteins.

Characterization of proteins-loaded PLGA microspheres

Routinely, a group of parameters are determined to characterize a microsphere sample, including: morphology, particle size, encapsulation efficiency, loading, release profile, residual solvent, among others. However, protein-loaded microspheres additionally have to be exhaustively characterized attending to the properties of the encapsulated protein, due to its lability, thereby experimenting structural changes that affect its physicochemical and biological properties, and ultimately its function as a therapeutic agent.

PLGA microsphere characterization parameters

Although most of these parameters must be part of the quality control of microsphere-based pharmaceutical formulations at the end of their manufacturing processes, microspheres are an intermediate product in the formulation process, and they should be also controlled as such.

Morphology

Morphological studies of microparticles reveal their relevant properties, such as shape, surface regularity, membrane continuity (for microcapsules), pore size and the uniformity of their distribution, particle size homogeneity, defects and/or aggregation, and give data

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Table 3. Pharmaceutical formulations of microsphere-encapsulated peptides and proteins available in the market

Product (Date)*	Active ingredient	Company	Application	Frequency of administration
Lupron Depot (1989)	Leuprolide	ABBOTT	Palliative treatment of advanced prostatic cancer	Monthly or quarterly
Sandostatin LAR (1998)	Octreotide	Novartis	Acromegaly and endocrine gastroenteropancreatic tumors	Monthly
Nutropin Depot (1999)	Somatropin (hrGH)	Genentech	Growth disorders in pediatric patients	Monthly or bimonthly
Trelstar Depot (2000)	Triptorelin	Debio Rechereche Pharmaceutique SA	Palliative treatment of advanced prostatic cancer	Monthly
Plenaxis (2003)	Abarelix	Praecis Pharmaceuticals	Palliative treatment of advanced prostatic cancer	Monthly

*Date of approval by the Food and Drug Administration (FDA) of the United States of America.

on size, although it is not the most appropriate method to determine this parameter.

These properties affect microparticle applications. For example, aggregation is undesirable, affecting the homogeneity of the product and blocking the syringe needles when injecting the particles. At the same time, the amount and size of pores could influence the release of the encapsulated protein or even modify the release mechanism.

The morphology of particles is studied by microscopy techniques, commonly by transmission electron microscopy [25, 39, 46, 47], and also by atomic force microscopy to characterize the surface of nanospheres [48, 49]; confocal microscopy, to obtain evidence on the acidity of microsphere cores derived from PLGA degradation [50] or to study the distribution of the protein inside the particle [51]; and fluorescence microscopy is used, to unravel the inner structure of the particle [52].

The morphological properties of microparticles can be determined by the microencapsulation technique, and particularly, by experimental conditions; for example, the type of solvent and evaporation rate [53, 54]. By adding salts to one or both aqueous phases in a double emulsion, particles with differential inner structures can be obtained [55].

Particle size

Particle size is a relevant parameter for microspheres; it should not be longer than 180 µm when it is to be administered by the parenteral route [56, 57]. Besides, their size must be reproducible batch-to-batch, influencing the release profiles and encapsulation efficiency, among other properties. Several experimental conditions affect particle size, irrespective of the method used, such as polymer type and molecular weight [58, 59], polymer/drug ratio [59, 60], polymer concentration in the organic phase [61, 62], polyvinyl alcohol concentration in the outer aqueous phase [60, 61, 63] and stirring rate [64-66].

Therefore, microsphere size and its distribution must be carefully determined. There are several methods available, including: centrifugation, analytical ultracen-trifugation, sedimentation, electrical conductivity, optical and electron microscopy, light scattering and laser diffraction, among others [16]. However, differences in their respective measuring principles and their requirements to build models from the experimental data generate inconsistent results between methods for a given sample.

Only electron microscopy covers the whole range of sizes obtained. Burgess and coworkers offered an extensive analysis on this topic, recommending the appropriate selection of the method according to the production process, particle size required for clinical use and particle segregation during formulation manufacturing and storage [16].

Loading and encapsulation efficiency

The efficiency of encapsulation is the fraction, expressed in percentage, of the protein encapsulated in respect to the total amount of protein used in the process [62]. This is an essential parameter, indicating the quality of the process, which is better or more efficient when a larger fraction of the drug is encapsulated. In the same way, the load is the amount of protein encapsulated per microsphere mass, expressed in percentage [62]. This parameter comprises a wide range of values, according to the protein dose required for administration, and that needs to be precisely determined, because this defines the amount of microspheres to administer in a single formulation dose.

To determine these parameters, the protein must be extracted by several methods to an aqueous phase, for proper quantification. The aqueous two-phase extraction method, which uses two immiscible liquid phases, the organic solvent precipitation method followed by filtration, and the accelerated hydrolysis of the polymer by incubating microspheres in NaOH [67]. Of these three procedures, the latter is the most widely applied, because it generates aqueous phases that are neutralized and further analyzed by several total protein quantization procedures: absorbance at 280 nm or the specific wavelength of maximal absorbance for the protein under study, Lowry's and Bradford's methods and also the most usual micro-BCA method.

All these parameters are profoundly affected by the encapsulation method and the experimental conditions where the particles are generated, including the volume of the inner aqueous phase and the concentration of the protein on it, polymer type and concentration in the organic phase, emulsification time, and additives in the different emulsion phases, which are among the relevant factors [62, 68, 69].

Residual humidity

The presence of water inside the particles can cause undesirable events, such as changes in the polymeric matrix by the hydrolysis of ester bonds in the polymer, or changes in the protein favored by the damp media [41]. For this reason, it is important to determine residual humidity in microspheres. The method of choice is Karl-Fisher's method, commonly used to determine humidity in lyophilized products [45].

Residual humidity derives mainly from the method used to generate them and also the drying procedure. Lyophilization is the most common laboratory-scale procedure to dry protein-loaded microspheres, because of being amenable to preserve the structure and properties of the encapsulated protein. Additionally, the vacuum-drying process in specific devices has been used for the industrial production of microsphere batches. Microspheres with low humidity have been obtained by both procedures.

Residual solvent

Solvents remaining in the pharmaceutical products are defined as volatile, organic chemical products used or produced while manufacturing excipients and drugs. Due to their toxicity, these substances have to be avoided in any medicine, but unfortunately, most of the time they cannot be completely removed during the technological manufacturing processes. Therefore, they must be quantified as part of the quality control of formulations, their content having to be below the limit established by regulatory agencies [70].

Gas Chromatography is the most common technique used to determine these substances in microspheres

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[45, 53, 70], although alternative procedures are being introduced for such purposes [71].

The encapsulation method could notably influence the amount of solvent remaining in microspheres, and also the type of solvent [53]. Another important factor is the drying process, including the conditions where it is carried out.

Release profile

The release profile is a highly relevant parameter when designing microspheres for the therapeutic administration of proteins. It is studied *in vitro* and could be related or not to the *in vivo* properties of the protein release process. Nevertheless, the *in vitro* study offers an idea of the potential of the system, indicating the affordability for the controlled.

The proteins encapsulated in PLGA microspheres are commonly released following a pattern of three main steps. First, the burst release phase, usually occurring during the first day and mainly determined by the protein in the surface, channels and pores of the microspheres, which were filled by the incubation media for a few hours at the beginning of the trial. Secondly, the slow release phase, releasing few or no protein at all. The third and last phase comprises a faster release of protein due to the erosion of particles [58]. Occasionally, the release can occur in two steps and the profile shows an asymptotic pattern [67] (Figure 3).

Several processes contribute to the release of the encapsulated proteins, such as the diffusion through pores and channels, and exposure of protein molecules to the incubation media, due to the superficial erosion of particles, also derived from the degradation of the polymeric matrix. The channels and pores are formed during the assembly of the particles or result from polymeric degradation [24].

Therefore, factors influencing the release profile include the properties of the polymeric matrix and the protein used, the structure of the microparticle, the encapsulation technique and the experimental conditions, as well as the co-encapsulation of additives for several purposes [72].

Factors other than those of microparticle properties that determine the release profile are related to the assay conditions, like composition and volume of the incubation media, temperature, the profiling device, the procedure and stirring rate, and the method used to change the incubation media (partially or completely)[46, 73, 74].

When conducting release profile studies in protein-loaded microspheres, highly variable results are usually obtained due to biomolecule degradation by the acidic media, which arises from polymeric matrix degradation products and their exposure to the aqueous media. This inconvenience can be solved by frequently changing the incubation media or measuring the amount of proteins retained by microspheres, instead of measuring the concentration of proteins in the incubation solution [16].

Sterility

The formulations prepared with these systems cannot be sterilized by steam or irradiated, because the properties of the polymeric matrix and the encapsulated molecule can be undesirably modified. Besides, the size of the particles hampers the use of sterilizing filtration for the final product. These limitations make it necessary to manufacture these pharmaceutical products under aseptic conditions, also requiring internal and external sterility verifications [16, 75].

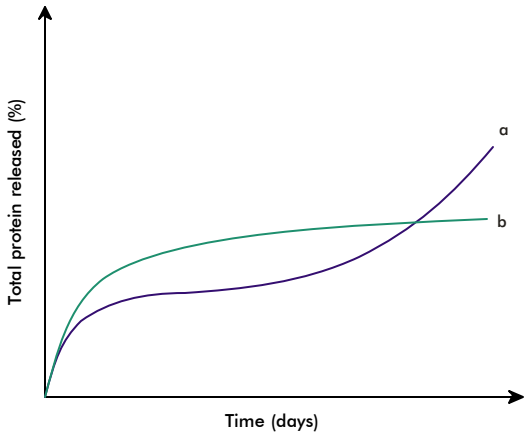

Figure 3. Release profile of encapsulated protein: a) in three steps, b) asymptotic.

Characterization of the encapsulated protein

The encapsulated protein should be characterized according to the nature of each particular molecule, and should reflect its functioning properties. Usually, chromatography, polyacrylamide gel electrophoresis and immunoenzymatic and biological activity assays are carried out for these purposes.

PLGA insolubility in water demands the design of a procedure to extract the encapsulated protein and to obtain aqueous samples that are appropriate to run the previously mentioned techniques, also preserving protein properties. It has been previously mentioned that there are several methods based on extracting the protein by using systems composed by two immiscible liquid phases [76], extraction by precipitation with organic solvents where the polymer is soluble [67, 77] and electrophoretic extraction [47, 78, 79]. Specifically, the extraction in the two-phase systems has been used to evaluate the properties of encapsulated peptides, with good results [40, 80, 81], while other authors have found that protein recovery can be negatively affected because the protein tends to be distributed between the interphase and the aqueous phase [67, 82]. This can also cause changes in the protein extracted and alter the results. Nevertheless, this extraction variant could be used, while demonstrating its applicability, to the system under study. A similar case comprises the precipitation methods, where proteins can expe-riment interactions with polymeric materials under the extraction conditions, leading to non-quantitative yields. In general, all these methods have specific advantages and disadvantages, and are selected according to the protein of interest.

Another group of techniques, potentially useful to study the properties of the encapsulated protein without extracting it is available; however, only the

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Fourier transform infrared spectroscopy method has yielded successful results [51, 83-85].

Effects of experimental conditions on the properties of microspheres obtained by the solvent double emulsion/solvent evaporation method

Protein microencapsulation is a complex process, the properties of the final product notably depending on a number of factors which are associated to the microencapsulation technique used.

The experimental parameters affecting the properties of protein-loaded microspheres generated by the double emulsion/solvent evaporation technique are shown in table 4 [68, 86].

The magnitude of the effect of each parameter listed depends at the same time on the nature of the encapsulated molecule. For this reason, it is essential to determine the effect of all or at least some of the factors characterizing the particles, in order to define the properties of a given sample. Experimental fractional or complete factorial designs are usually applied for this purpose, using a minimal number of experiments to characterize the main experimental variables providing microsphere properties and their respective magnitude [62, 87, 88].

Table 4. Main experimental parameters influencing the properties of protein-loaded microspheres, manufactured by the double emulsion/solvent evaporation technique*

Steps of the process	Parameter
First emulsion	Concentration of the polymer solution
	Composition and molecular weight of the polymer
	Organic solvent
	Volume of the organic phase
	Protein concentration in the aqueous inner phase
	Volume of the aqueous inner phase
	Emulsion equipment (e.g. for homogenization or sonication)
	Stirring rate
	Rate at which the aqueous phase is added to the organic phase
	Mixing time
	Temperature and pressure
Second emulsion	Volume of the outer aqueous phase
	Nature and concentration of the emulsifying agent
	Emulsion equipment
	Rate at which the first emulsion is added to the aqueous phase
	Stirring rate
	Mixing time
	Temperature and pressure
Solvent extraction	Volume of the extraction phase
	Presence of additives or stabilizers
	Stirring rate
	Extraction time
	Temperature and pressure
Collecting and washing	Collection system (e.g. filtration or centrifugation)
	Volume and composition of the washing solution
	Temperature and pressure
Drying	Method employed (e.g. lyophilization, fluid bed)
	Time
	Residual humidity
	Addition of excipients

*Adapted from reference 68.

Protein stability during the double emulsion/solvent evaporation microencapsulation process. Strategies for stabilization

As previously mentioned, the physicochemical properties of proteins can be modified during the microencapsulation process. The most frequent modifications comprise denaturing, covalent and non-covalent aggregation deamidation, oxidation and the incorrect assembly of disulphide bonds [24, 89-91].

Most of the time, these difficulties have been solved by designing a protein stabilization strategy against the aggressive conditions of the microencapsulation process, also determining their impact on the release profile [24, 41].

Some of the solutions used by several groups to avoid the loss of activity of microencapsulated biomolecules are listed in table 5.

Conclusions

Although several studies related to the microencapsulation of peptides and proteins are available, very few have led to currently marketed products. In order to increase the number of biopharmaceutical products based on microspheres, it is essential to achieve satisfactory results in several aspects, for example, the development of scalable microencapsulation methods, enabling the microencapsulation of these sensitive active pharmaceutical ingredients without affecting their physicochemical and biological properties; the design of simple and appropriate procedures to characterize the encapsulated biomolecules, etc. Although considerable research is needed to make easy-to-manufacture microsphere-based pharmaceutical products available, which comply with quality control requirements, they are very attractive release systems, considering their advantages leading to a rapid development in this field.

Table 5. Strategies and mechanisms to stabilize proteins against conditions of the microencapsulation double emulsion/solvent evaporation technique

Stress factors	Stabilization strategy	Stabilization mechanism
Water/organic solvent interphase	Addition of sugars, polyols, PEG	Isolates the protein from the interphase
	Increased protein concentration	Decreased interphase/protein ratio
	Addition of other proteins	Competition for the interphase
	Pre-encapsulate the protein into an hydrophilic core	Isolates the protein from the interphase
PLGA-protein contact	Addition of other proteins	Competition for the PLGA
	Pre-encapsulate the protein into an hydrophilic core	Shielding against PLGA
Shear	Addition of surfactants	Competition for the interphase
	Reducing the agitation time	Minimized time of exposure to shear
	Avoid ultrasound while generating the first emulsion	Decreased exposure to shear
Drying	Adding lyophilization protecting agents	Increased Gibb's free energy for protein incorrect folding
	Replacing lyophilization by another drying method	Lack of the freezing step

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