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Multiple emulsion technology for the design of microspheres containing peptides and oligopeptides

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Abstract

This paper reviews the preparation and characterization of small poly(lactic-co-glycolic acid) microspheres (mean size lower than 10 μm) containing small peptides and prepared by a water-in-oil-in-water emulsion solvent evaporation technique. These microspheres were shown to encapsulate very efficiently a 33 amino acid peptide (V3 BRU) and in vitro release kinetics studies showed that such microspheres could be employed for both oral vaccination and controlled release. The encapsulation of a seven amino acid peptide (pBC 264) led on the contrary to a very low encapsulation efficiency. In order to increase the encapsulation of pBC 264, two strategies were adopted: (i) taking into account the solubility of pBC 264 at different pH, the inner aqueous phase was maintained at a basic pH where the peptide was soluble, while the external aqueous phase was acidic; (ii) ovalbumin was added to stabilize the inner emulsion. These two strategies allowed us to increase significantly the encapsulation rate of pBC 264. Nevertheless, the in vitro release kinetics of the peptide were strongly influenced by the presence of ovalbumin which seems to form pores in the microsphere structure. By contrast, when ovalbumin was replaced by Pluronic® F68, microspheres did not have pores, thus the release profile and the extent of the burst were much smaller. When microspheres were stereotactically implanted in the rat brain, in vivo release profiles were in good agreement with the release observed in vitro. In conclusion, these microspheres are well suited for the slow delivery of neuropeptides in the brain, a feature expected to facilitate the study of long term effects of these compounds.

Keywords: Microspheres; Multiple emulsion solvent evaporation technique; Poly(lactic-co-glycolic acid); Peptides V3 BRU; Cholecystokinin; Brain delivery; Release kinetic

Contents

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1. Introduction

Peptides and protein drugs are becoming a very important class of therapeutic agents, as a result of our gaining more understanding of their role in physiology and pathology as well as the rapid advances in the field of biotechnology/genetic engineering [1]. The development of DNA-recombination techniques has made these compounds available on a larger scale than in the past. In addition, efforts of synthetic chemists have in many cases given rise to analogues of endogenous compounds with greater potency than the parent compound. However, available peptides and proteins are generally characterized by a short biological half-life. They are easily degraded by enzymes and most peptides poorly pass through biological barriers due to their poor diffusivity and low partition coefficient, which is unfavourable to diffusion in lipid membranes [2]. For these reasons, the entrapment of such drugs within microparticulate drug delivery systems, using various kinds of biodegradable polymers, has been studied extensively during the past two decades [3–7]. Microspheres are able to protect these molecules against degradation, to control their release from the site of administration and in some particular cases, to improve their passage through biological barriers. One of the most common methods of preparing microspheres is the oil-in-water (O/W) solvent evaporation method [8–12]. An O/W emulsion system involves the dispersion of an organic solution of polymer and drug into a continuous aqueous phase. Lipophilic drugs were successfully retained within microspheres prepared with this method, whereas poor encapsulation efficiency was observed with hydrophilic drugs [13,14], which partitioned out from the organic dispersed phase into the aqueous continuous phase [15]. This is the reason why a procedure based on a multiple emulsion water-in-oil-in-water (W/O/W) solvent evaporation method has been developed first by the industrial group of Takeda Chemical Industries [16–18] and then by others [19,20]. This innovative methodology allowed the preparation of multiphase microspheres generally ranging from 10 to 100 μm. We have adapted this procedure to prepare smaller microspheres with a diameter of around 5 μm [21]. This paper describes the conditions in which these microspheres were obtained, characterized and loaded with peptides of different length.

2. General considerations on peptide encapsulation in microspheres of poly(lactic-co-glycolic acid) (PLGA)

Encapsulation of peptides into polymeric material requires that (i) microspheres can be prepared from a biodegradable polymer, (ii) the procedure does not lead to peptide degradation and (iii) the encapsulation efficiency is high enough.

Most of the recent studies related to peptide and protein microencapsulation by the W/O/W solvent evaporation technique were carried out with poly(lactic acid) (PLA) or PLGA copolymers. PLA and PLGA are indeed the most investigated and advanced polymers with regard to available toxicological and clinical data. PLA and poly(glycolic acid) are non-toxic, biocompatible and biodegradable polymers [22] approved by the Food and Drug Administration for human use [23,24]. Low molecular weight polymers ($< 20\,000$) are produced by the direct condensation of lactic and/or glycolic acids in the presence or absence of catalysts. High molecular weight products are formed by the ring opening method using catalysts such as dialkyl zinc, trialkyl aluminium, and tetraalkyl tin in which the lactide and/or glycolide rings form a cyclic dimer [25]. Polymers with a particular molecular weight can be manufactured by the choice of adequate polymerization conditions, (e.g. amount of catalyst, time of reaction etc) [26]. The physical characteristics of PLA/PLGA include strength, hydrophobicity, and pliability. The degradation products consist in glycolic and lactic acid that are eliminated from the body [27,28]. Because the rate of hydrolysis of the polymer chain is dependent only on significant changes in temperature or pH or on the presence of catalyst, very little difference is observed in the rate of degradation at different body sites. This is obviously an advantage in regard to drug delivery formulations [28]. The degradation rate is controlled by factors including polymer molecular weight, polymer crystallinity and, for PLGA, the lactide/glycolide ratio [29]. Lactic acid contains an asymmetric carbon atom and has two optical isomers. Therefore, its polymers consist of L-, D-, and D,L-lactic acid in which the L-, or D-polymers have a crystalline form, and the D,L- polymers are amorphous and more rapidly degradable [30]. In addition, because of the methyl group, the lactic acid polymer is more hydrophobic than the glycolic one [31].

Stability issues are also critical when dealing with peptides, although degradation of small peptides is rather a rare event. In the process of preparing microspheres, peptides may, however, be exposed to extreme stresses. Manufacturing steps should not include excessive exposure to heat, shear forces, pH extremes, organic solvents, freezing and drying. During storage, the incorporated peptide may become hydrated and in this type of environment it may become more susceptible to denaturation and aggregation [32]. Also when a polymer begins to degrade following administration, a highly concentrated microenvironment is created in and around the microspheres due to polymer breakdown byproducts (acidic monomers). Peptides may be susceptible to aggregation, hydrolytic degradation and/or chemical modification in such an environment. Finally, they may undergo reversible or irreversible adsorption to the polymers which can affect drug delivery rate and ultimately can lead to the denaturation, aggregation and/or inactivation.

Peptides are highly heterogenous in size in their non-aggregated state. In addition, unless their N and C termini are blocked through cyclisation, amide formation or esterification, they are highly hydrophilic. The partition coefficients of several peptides and proteins are listed in Table 1. Due to their hydrophilicity, peptides and proteins are thus poorly encapsulated in matrix polymeric systems which are more often composed of hydrophobic polymers (e.g. microspheres of PLGA) [21].

Table 1
Partition coefficient of several therapeutic peptides and proteins: from [2]

Peptides/proteins	Partition coefficient (log K_p)
GRP	− 1.6
Insulin	− 1.66
α-MSH	− 1.3
LHRH	− 1.35
TRH	− 1.42
AVP	− 2.15
Glucagon	− 1.2
Substance P	− 0.56
N-Tyr-CRF	− 0.61
N-Tyr-β endorphin	− 1.86
N-Tyr-somatostatin	− 1.09
N-Tyr-DSIP	− 1.3
N-Tyr-GRF	− 1.7
Met-enkephalin	− 1.51
Leu-enkephalin	0.05
N-Tyr-FMRF	0.39
N-Tyr-MIF-1	0.06
Cyclosporin	3

All these properties have given rise to a new process of preparation of microspheres, that is the so-called W/O/W solvent evaporation method.

3. Development of a preparation process for the encapsulation of peptides

The multiple emulsion solvent evaporation method, —also called the in-water drying method—consists in dissolving the drug in distilled water or in a buffer solution [e.g. phosphate-buffered saline, Tris...] (inner water phase) and the polymer in a volatile organic solvent that is not miscible to water (organic phase) (Fig. 1). The inner water phase (containing or not a surfactant) is then poured into the organic phase. This mixture is generally emulsified forming the first inner emulsion or the primary emulsion (W/O) (Fig. 1). This W/O emulsion is then poured under vigorous mixing using mechanical stirring [21], into an aqueous phase (outer water phase) that contains an emulsifier forming the double W/O/W emulsion (Fig. 1). The resulting multiple emulsion is continuously stirred and the solvent is allowed to evaporate inducing polymer precipitation and thereby, the formation of solid drug-loaded microspheres.

The presence of an emulsifying agent in the outer water phase is critical for the successful formation of individual spherical microspheres [9]. The role of the emulsifier is to prevent the coagulation of microspheres during solvent removal. The stabilizer added to the outer water phase is most often poly(vinylalcohol) (PVA) but typical suspension agents including polyvinylpirrolidone, alginates, methylcellulose and gelatin might also be used [33].

Solvent evaporation can take place at atmospheric or reduced pressure, under various temperatures. The complete evaporation of the solvent can be obtained in three different ways:

1. An interrupted process: the evaporation, performed at room temperature, is stopped when the solvent is not completely eliminated, and the partially solid microspheres are transferred into a low concentrated emulsifier solution or an emul-

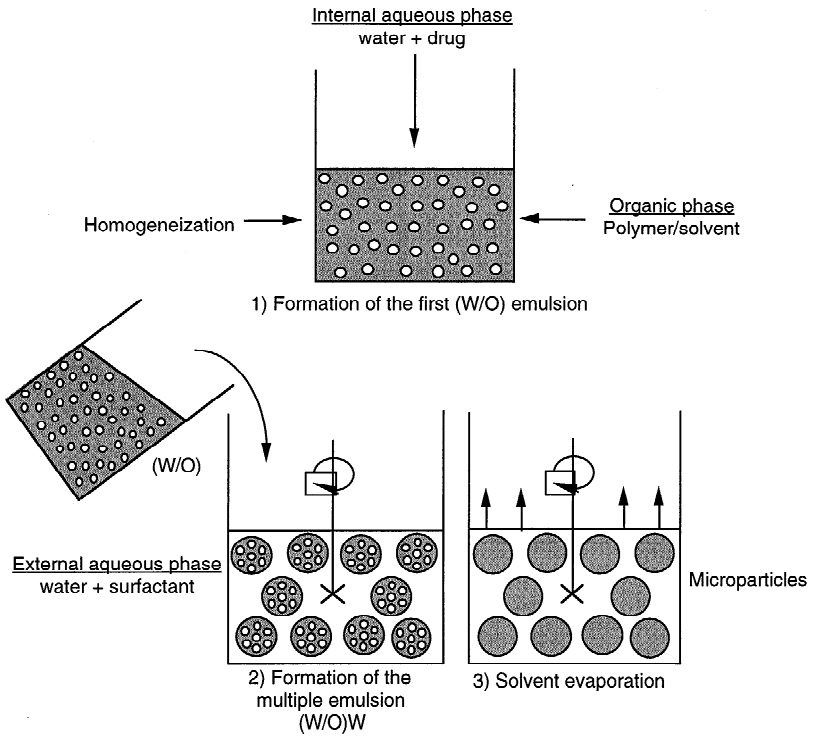

Fig. 1. Preparation of microsphere-containing peptides by the multiple emulsion solvent evaporation technique.

sifier-free medium where the evaporation is pursued [19,34].

2. Continuous process: the preparation is continuously stirred at room temperature until solvent evaporation is completed [21].
3. The multiple emulsion is placed into a rotatory evaporator and warmed to approximately 30°C [35].

The solid microspheres are isolated by filtration or centrifugation, washed several times in order to eliminate the emulsifier agent and dried under vacuum or freeze-dried.

The volatile organic solvent used in the preparation of the microspheres by the multiple emulsion solvent evaporation technique needs to possess a low boiling point to overcome the problems associated with residual solvent. Many solvents can be used for the preparation of microspheres: acetonitrile, ethyl acetate, chloroform, benzene, methylene chloride (MC). This latest solvent is considered as the most suitable for the preparation of microspheres by a W/O/W solvent evaporation method [36]. MC has indeed a high volatility that facilitates easy removal by evaporation, and also shows a good solubility towards a range of encapsulating polymers. The limit proposed by the U.S. Pharmacopeia is 500 ppm. Preparing such microspheres, Yamamoto et al. [37] succeeded in reducing the MC residue to below 100 ppm by a well programmed heating during the lyophilization process. It is particularly noteworthy that chlorinated solvents are generally undesirable for manufacturing processes, and they may be banned in the future for environmental and health reasons. However, recent studies have shown that there is no link between exposure to MC and cancer in humans [38]. In addition, MC does not deplete the stratospheric ozone layer and has proven low potential to create low-level ozone [38]. Finally, there are a number of non-chlorinated solvents potentially suitable (e.g. ethyl acetate or acetonitrile), and solvent selection has received some attention in recent years [36,39].

Some modified procedures of this methodology were also proposed. One of these methods is the solvent extraction technique which is very similar to

the solvent evaporation process except that the W/O/W emulsion is added to a third solution (e.g. isopropanol), which is a non-solvent of the polymer, but miscible with both water and the organic solvent [20,40]. Under these conditions, the solvent contained in the polymer droplets is extracted into the aqueous medium, the aim being to accelerate solvent removal [20,40].

Chemical and mechanical parameters strongly influence the diameter of the obtained microspheres. The most commonly used technique for assessing the size distribution of microspheres appears to be the Coulter counter® method, although light or electron microscopy were also shown to be interesting. The microsphere size tends to decrease with increasing mixing speed to form the double emulsion [41]. Ogawa et al. [35] have studied the effect of the homogenizer stirring speed on the final size of the microspheres. As the agitation rate increases, the size distribution clearly shifts to smaller diameters. In the same study, these authors have shown that an increase in the volume of the external aqueous phase resulted in a decrease of particle size [35]. On the contrary, Jeffery et al. [42] observed that particle size increased when increasing the volume of the outer aqueous phase. This effect was attributed to a reduction in the agitation that occurred because of the decrease in mixing efficiency associated with larger volumes. A reduction in the mixing efficiency probably produced an increase in the size of the emulsion droplets which subsequently resulted in the formation of larger microspheres.

Also, the particles size increases when the polymer concentration in the organic phase increases [31]. Increasing the concentration of the dissolved polymer increased the viscosity of the organic phase [21], which resulted in turn in a reduction of the stirring efficiency. The higher the viscosity of polymer solution, the more difficult was the formation of small emulsion droplets.

Particle mean diameter also depends upon the mixing methods used to prepare the first inner W/O emulsion and the second W/O/W emulsion [43]. When both are prepared by vortex-mixing, the obtained microspheres are large. However, when the inner W/O emulsion is prepared by sonication and the second W/O/W emulsion by vortex-mixing, a microfine inner emulsion is formed. The resulting microspheres become smaller and very homogenous. Finally, when the inner emulsion is formed with a homogenizer and the second emulsion with a vortex mixer, the particle diameter is intermediate between the sizes of the microspheres obtained by both methods.

Many studies [21,42] have shown that when the concentration of the surfactant in the outer water phase increases, a significant reduction in particle size is achieved. At high surfactant concentration, the rate at which the emulsion stabilizer molecules diffuse at the emulsion droplets–aqueous phase interface may increase, resulting in a greater presence of stabilizer at the surface of the emulsion droplets. This would provide an improvement in the protection of droplets against coalescence, resulting in the formation of smaller emulsion droplets for higher surfactant concentrations. As the solvent evaporates from the system, these droplets harden to form the microspheres. Therefore, the size of the finally obtained microspheres is dependent upon the size and stability of the emulsion droplets formed during the agitation process. At low surfactant concentration, small emulsion droplets are not stable, and the resulting microspheres are larger in size than those prepared with higher surfactant concentrations. By contrast, when adding a hydrosoluble stabilizing agent (gelatin, ovalbumin) to the inner water phase, particle size increases due to the rise in the diameter of the internal globules.

Other characterization studies have dealt with morphology, drug loading and release from microspheres. We will discuss these parameters in regard to the data obtained for the encapsulation of small peptides and oligopeptides.

4. Encapsulation of peptides (case study, V3 BRU)

We have applied the process of preparation described above (PLGA microspheres with a lactide:glycolide ration was of 75:25 and a molecular weight of 128 000) to the encapsulation of the V3 BRU [21] which is a 33 amino acid synthetic peptide of the gp120 of human immunodeficiency virus (HIV) in PLGA microspheres [44]. The partition coefficient of this molecule in the MC–water system gave a value of log K_p of -1. It has been found that this highly water soluble V3 BRU peptide was

completely entrapped within microspheres (displaying a diameter ranging between 1 and 10 μm) obtained by the W/O/W solvent evaporation method. Indeed loading efficiency was of 46 μg/100 mg of polymer. Since the peptide had a very low solubility in MC, its diffusion across this phase to reach the outer aqueous phase was reduced. In addition, V3 BRU was kept intact during the whole process of preparation [21]. On the contrary, only a small amount of the peptide was encapsulated when using the single solvent evaporation method (loading efficiency of 125 μg/100 mg of polymer). In addition, by increasing the initial amount of peptide a loading efficiency of 920 μg/100 mg of polymer could be obtained [21]. In this case, the peptide is supposed to leak out from the organic phase (where it was dispersed) to the aqueous phase where regarding the partition coefficient, it was much more soluble.

Electron microscopy observations after freeze fracture have shown that when microspheres were prepared in the presence of the peptide, most of the cavities (internal globules) exhibited a granular aspect which was supposed to correspond to the agglomeration of peptide molecules [21] (Fig. 2). Within each microsphere, the cavities are separated one from the other by the polymer core exhibiting characteristic oriented striations due to the plastic deformation occurring in the direction of the fracture [21] (Fig. 2).

Release profiles of V3 BRU from PLGA microspheres were investigated in phosphate buffer and in the presence of artificial gastrointestinal secretions. In phosphate buffer, microspheres exhibited a biphasic release with a faster release phase, which was followed by a slower one [21]. The faster release was probably due to a burst effect, resulting from the release of the small fraction of the peptide adsorbed onto the surface of the microspheres. The peptide fraction that was entrapped within the internal globules was then released very slowly [21]. The release of V3 BRU peptide from microspheres and its degradation in gastric and intestinal medium was also carried out. In gastric medium, the peptide was not released at all from microspheres, whereas about

Fig. 2. Freeze fracture electron microscopy of one microsphere. Many cavities, a few of which are smooth but most of which exhibit particles, are separated by the polymer core (arrow). Bar = 2 μm. From [21].

half of the entrapped V3 BRU was released after 4 h in the intestinal medium. V3 BRU was totally protected against degradation in simulated gastric medium and partially in the intestinal medium. These results show that the V3 BRU loaded PLGA microspheres system may be considered as a potential candidate for oral vaccination against HIV.

5. Encapsulation of oligopeptides (case study, a derivative of cholecystokinin) (CCK)

Peptide CCK which is widely distributed in the central nervous system and in the gastrointestinal tract plays a critical role as neuromodulator in the central nervous system and seem to control emotional and adaptation processes through specific binding sites located in various brain regions. CCK octapeptide (CCK-8), the predominant form in the brain fulfils the criteria of a neurotransmitter or a neuromodulator [45]. Numerous studies suggest that CCK is involved in satiety [46], motivated behavior [47], memory [48,49], nociception [50,51] and anxiety [52,53]. Moreover, in humans, intravenous injection of CCK-4, the C-terminal fragment of CCK-8, was shown to induce panic attacks which were suppressed by selective CCK-B antagonists [54]. However, the site and the mechanism of action of CCK-4 remain unclear. To study the long term effect of CCK agonist, a CCK-8 analog, pBC 264 (Boc-Tyr(SO_3H)-gNle-mGly-Trp-Nle-Asp-Phe-NH_2), which is a very potent and selective agonist for CCK-B receptors was developed [55]. This compound has been shown to give responses at low doses in behavioral and electrophysiological tests following local administration [56–58]. However, chronic local stimulation of CCK-B receptors is hindered by the rapid elimination of pBC 264 from the brain after central administration [59]. In order to study the long term effects resulting from local stimulation of CCK-B receptors, the possibility of slowly delivering pBC 264 into a particular brain structure for a long period of time has been considered using its encapsulation into PLGA microspheres.

pBC 264 was encapsulated into microspheres prepared by the multiple emulsion technology. Microspheres prepared using different PLGA copolymers showed a similar size with unimodal distribution and diameters ranging between 1 and 10 μm. In contrast to V3 BRU, the encapsulation efficiency of pBC 264 in microspheres was very low. However, when introducing in the inner aqueous phase a stabilizing agent such as ovalbumin to a concentration of 2%, the encapsulation efficiency was significantly improved [60]. An additional and dramatic improvement of the encapsulation rate was also observed when the pH of the internal aqueous phase was basic (pH 8 corresponding to the optimal solubility of pBC 264) and when the pH of the external aqueous phase was acid (pH 2.5 corresponding to a lower solubility of pBC 264) [60]. In this case, the internal aqueous phase was playing the role of a real ''trap'' for the peptide. Therefore, the simultaneous stabilizing effect of the ovalbumin and the adjustment of the pH allowed us to obtain an optimal entrapment efficiency (Fig. 3). Actually, albumin was shown to form an interfacial film between water and MC that may be considered as responsible for the stabilization of the inner emulsion [61]. The development of such an albumin interfacial film was shown to be of utmost importance for the efficient entrapment of other active compounds [61]. During the process of preparation, ovalbumin was believed to prevent the destruction of the internal globules, therefore avoiding the leakage of the peptide in the outer aqueous phase. The inner emulsion should also be stabilized due to the rigid structure formation (hydrophobic barrier) surrounding the drug cores following interaction between

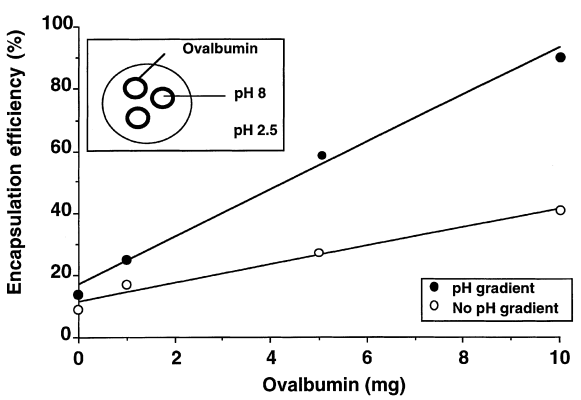

Fig. 3. Encapsulation efficiency of 1 mg of pBC 264 in 800 mg of PLGA microspheres as a function of albumin and pH gradient (adjustement of the internal aqueous phase to the pH of higher solubility of pBC 264 and the external aqueous to the pH of lower solubility of pBC 264). Adapted from [60].

basic compounds and dissociated polymers as has been shown with leuprorelin, thyrotropin-releasing hormone (TRH) and water-soluble acidic drugs combined with basic amino acids [62]. This improved stability resulted in a better entrapment of pBC 264.

Release kinetic experiments were performed in phosphate buffer solution (pH 7.4). pBC 264 was quickly released from microspheres exhibiting a dramatic burst effect (Fig. 4) [60]. The rate of peptide release was independent of the ratio of lactide/glycolide (75:25 or 50:50) and slightly attenuated when microspheres were prepared with high molecular weight copolymers (molecular weights were 19 700, 64 000 and 128 000) probably because of the rigidity of the matrix structure that reduced the overall porosity of the system [60]. Thus, the obtained microspheres did not exhibit a typical controlled release profile. The presence of ovalbumin as a stabilizer was shown to be responsible for the observed burst effect. Electron microscopy observations clearly showed that ovalbumin induced the formation of pores at the surface of the microspheres [60]. Actually, as ovalbumin is totally insoluble in MC, it did not interact with the polymer as suggested by differential scanning calorimetry (DSC) measurements [60]. The protein was assumed to be located at the interface between the first and second emulsion. Therefore, ovalbumin might have formed aggregates on the surface of the microspheres. The elution of the ovalbumin aggregates from the surface during the microspheres washing process is probably responsible for the formation of pores. These pores could constitute a pathway for the observed fast release of pBC 264.

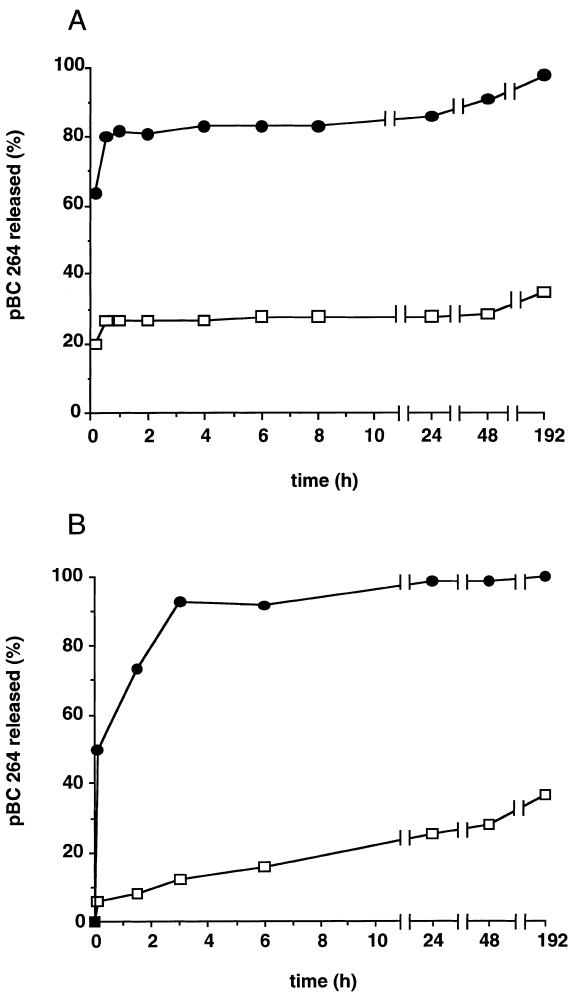

Fig. 4. Release kinetic of pBC 264 in phosphate-buffered saline pH 7.4 (A) and in vivo in brain tissue (B) from PLGA microspheres prepared with either ovalbumin (●) or Pluronic® F68 (□). Adapted from [62,63].

To reduce the porosity of the microspheres, other surfactants were used as stabilizers of the internal aqueous phase (PVA or Pluronic® F68) [63]. The influence of the surfactant on the stability of the first emulsion as well as the encapsulation efficiency and the release of pBC 264 were examined. The encapsulation efficiency was very low for the formulation containing PVA (Table 2). Pluronic® F68-containing microspheres displayed a lower encapsulation efficiency as compared to ovalbumin-stabilized microspheres (Table 2) [63]. The most important factor for the successful encapsulation of hydrophilic compounds in microspheres prepared by the W/O/W solvent evaporation method is the stability of the first inner emulsion [61]. From the stability studies (Table 2), it was shown that, when PVA was used as a stabilizer, the internal emulsion was unstable and the droplets coalesced. Thus, it was assumed that in these conditions, the peptide leaked out to the external aqueous phase. A similar phenomenon occurred with Pluronic® F68 but to a lesser extent since the stability of the emulsion was improved. Pluronic® F68 and PVA are hydrophilic surfactants that are not suitable for stabilizing W/O emulsions. On the contrary, proteins have a tendency to localize at the interfaces, increasing the stability of emul-

Table 2
Effect of different stabilizing agents, added to the inner water phase, on the encapsulation efficiency, on the mean diameter of PLGA microspheres and on the stability of the inner emulsion

Stabilizing agent (%)	Encapsulation efficiency (%)	Loading efficiency (μg/100 mg of polymer)	Mean diameter (μm)[a]	Phase separation time (min)[b]
PVA, 2	9.6	12	5.8	15
Pluronic® F68, 3	36	45	6.0	45
Ovalbumin, 2	95	119	7.2	>300

Loaded amount of pBC 264 was 1 mg from [62].
[a] Measured using a Coulter counter.
[b] The time required for initial macroscopic phase separation of the primary emulsion, measured at room temperature. Perylen (10 μg/ml) was dissolved in the organic phase to facilitate the macroscopic examination of the phase separation.

sions, therefore reducing the leakage of the peptide to the external aqueous phase.

Release of pBC 264 from microspheres prepared with Pluronic® F68 is shown on Fig. 4 and compared to the release obtained from ovalbumin-stabilized microspheres. The burst observed was much smaller when the microspheres were prepared in the presence of Pluronic® F68 (Fig. 4). The release profile and the extent of initial peptide burst was significantly influenced by the structure of microspheres. As mentioned above, the microspheres prepared with ovalbumin had a porous structure and, thus, provide a fast release profile with a large peptide burst. In contrast, microspheres prepared in the presence of Pluronic® F68, did not have pores and were characterized by a smooth surface. With Pluronic® F68 it may be hypothesized that the inner drug cores were more finely dispersed throughout the polymer matrix without any aqueous channels. Therefore, the differences in the initial burst from the tested formulations seemed to result from morphological differences.

The effect of the polymer concentration on the in vitro release of the peptide was also studied with the microspheres prepared in the presence of Pluronic® F68. The increase in PLGA concentration gave a highly viscous polymer solution. The higher the viscosity of the polymer solution, the more difficult was the formation of small emulsion droplets, resulting in PLGA microspheres with larger size. The initial burst release of pBC 264 decreased as the concentration of PLGA in the organic phase was increased [63]. This may be explained by the fact that an increase in the amount of polymer could produce microspheres with a more compact polymer phase and a dense core, thus decreasing the initial peptide burst.

For in vivo studies, microspheres were implanted into the rat brain in the anterior part of Nucleus Accumbens (N. Acc.). After the administration of fluorescently labeled microspheres, observations of histological coronal sections confirmed that the microspheres were effectively localized in the anterior part of N. Acc., and 1 h after implantation the microspheres remained located around the injection site [64]. The behavioral testing was carried out by placing rats in the open-field [64]. The behavioral effect was quantified by recording for 6 min: (a) the latency (or time) (s) to move out from the corner where the animal was placed and to cross two squares; (b) locomotion scores of the total number of squares crossed; (c) total number of rears; (d) number of defecation boli left on the field; and (e) number of grooming bouts. No behavioral change was observed 24 h or 8 days after administration of unloaded microspheres as compared to the control group, whatever the parameters measured in the open-field test [64]. Finally, implanted microspheres were well tolerated and minimally reactive, since only a localized inflammatory reaction was observed. This reaction was similar to the one induced by the administration of carboxymethylcellulose [64]. Two different kinds of microspheres differing only by the stabilizing agent added to the inner aqueous phase (ovalbumin or Pluronic® F68) were implanted into the rat brain. The in vivo studies showed that pBC 264 was almost completely released from the microspheres prepared with ovalbumin within the first

hour whereas only 35% of peptide was released after 8 days when microspheres prepared with Pluronic® F68 as the stabilizing agent were administered [63] (Fig. 4). This was in agreement with the in vitro results [62,63]. Intact pBC 264 was found to be released from PLGA microspheres containing Pluronic® F68 both in vitro and in vivo [62,63]. In addition, the brain diffusion of pBC 264 was very limited and its presence in the blood could not be detected. The peptide was essentially found concentrated near the site of administration in the N. Acc. showing that chronic behavioral studies using pBC 264 microspheres were made possible using the right formulation [64].

6. Conclusion

This paper reviews the different aspects of the encapsulation of peptides in PLGA microspheres using the multiple emulsion solvent evaporation method. This methodology was found efficient for the encapsulation in small PLGA microspheres (1–10 μm) of the V3 BRU peptide and an oligopeptide derived from CCK (pBC 264). The encapsulation of V3 BRU led to the conclusion that the system was totally adequate for this peptide in term of encapsulation and release. On the contrary, the encapsulation of pBC 264 was only made possible when a stabilizing agent was introduced in the inner aqueous phase and a pH gradient between the internal and external aqueous phase was created. Release kinetics of pBC 264 both in vitro and in vivo after brain administration were totally correlated. The choice of Pluronic® F68 as stabilizing agent allowed us to obtain a controlled delivery of pBC 264.

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