Why Does PEG 400 Co-Encapsulation Improve NGF Stability and Release from PLGA Biodegradable Microspheres?

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Purpose. The aim of this work was to understand the mechanism by which co-encapsulated PEG 400 improved the stability of NGF and allowed a continuous release from PLGA 37.5/25 microspheres.

Methods. Microparticles were prepared according to the double emulsion method. PEG 400 was added with NGF in the internal aqueous phase (PEG/PLGA ratio 1/1 and 1.8/1). Its effect was investigated through interfacial tension studies. Protein stability was assessed by ELISA.

Results. A novel application of PEG in protein stabilization during encapsulation was evidenced by adsorption kinetics studies. PEG 400 limited the penetration of NGF in the interfacial film of the primary emulsion. Consequently, it stabilized the NGF by reducing the contact with the organic phase. In addition, it avoided the NGF release profile to level off by limiting the irreversible NGF anchorage in the polymer layers. On the other hand, the amount of active NGF released in the early stages was increased. During microparticle preparation, NaCl could be added in the external aqueous phase to modify the structure of microparticles. This allowed to reduce the initial release rate without affecting the protein stability always encountered in the absence of PEG.

Conclusions. PEG 400 appeared of major interest to achieve a continuous delivery of NGF over seven weeks from biodegradable microparticles prepared by the double emulsion technique.

KEY WORDS: NGF; PEG; PLGA; microencapsulation; protein stability; interfacial tension.

INTRODUCTION

Sustained delivery of neurotrophic factors from biodegradable microparticles has received much attention as a potential treatment for neurodegenerative diseases (1–4). We have recently reported, using a W/O/W emulsion solvent extraction-evaporation method, the preparation of nerve growth factor (NGF)-loaded poly(d,l-lactide-co-glycolide) (PLGA) biodegradable microparticles characterized by an encapsulation yield of 100% (5). Unfortunately, the *in vitro* release profile was characterized by a marked burst effect followed by a plateau, as often observed in other studies dealing with protein release from PLGA microparticles (6). To decrease the initial release rate, the microparticle structure can be modified by the addition of a salt like sodium chloride (NaCl) in the dispersing aqueous phase. Then the microparticles become denser and the diffusion of the drug outside the polymer matrix is slower (7,8). However, we had recently demonstrated the addition of NaCl led to a marked denaturation of the NGF released in the early stage (6). The presence of NaCl in the dispersing phase was believed to modify the osmotic equilibrium of the double emulsion and to create a water out-flow which is detrimental to the protein stability.

Basically, several steps appeared critical for protein stability during microparticle preparation. This situation has limited the expansion of these drug delivery systems for clinical use. For example, in the double emulsion process, the contact of protein with the coating polymer-containing organic phase is a major cause of denaturation. Several excipients have been proposed to stabilize proteins during microparticle preparation (9,10,11). The present study focused on PEG 400. This compound was added in the internal aqueous phase of the W/O/W double emulsion. Its effect on protein stability and release was thoroughly investigated by carrying out experiments based on the pendant drop methodology. Its ability to protect NGF against denaturation when salts were added in the external aqueous phase was also studied. The final aim of this work was to prepare PLGA biodegradable microspheres which would provide a continuous release of active NGF.

MATERIALS AND METHODS

Materials

Uncapped PLGA were obtained from Phusis (PLGA 37.5/25, Saint-Ismier, France). The mean molecular weight (Mw) was 17,500 (I = 1.6). Nerve growth factor (2.5S murine, 28 kDa) was supplied by Promega (Charbonnières, France), human serum albumin (HSA, fraction V, 69 kDa), mannitol and sucrose by Sigma-Aldrich (St Quentin, France), and ^{125}I-NGF (Receptor grade 2.5S NGF murine) by NEN (Les Ulis, France). Citric acid, acetone, methylene chloride, polyvinyl alcohol (PVA) (Rhodoviol® 4/125, 88% hydrolyzed) were purchased from Prolabo (Paris, France), and polyethylene glycol (PEG 400) from Cooper (Melun, France).

Microsphere Preparation

NGF-loaded microspheres were produced according to a W/O/W emulsion solvent extraction-evaporation method. A 0.15 ml internal aqueous phase (16 mM citrate buffer, 0.1% w/v HSA, pH 6.0) containing 2.5 mg of additional HSA (5% with respect to the amount of PLGA), 10 μg of NGF (0.02%) including ^{125}I-labelled NGF was emulsified in an organic solution (1.5 ml of methylene chloride and 0.5 ml of acetone) containing PLGA (50 mg). The emulsion was set up in a polytetrafluoroethylene (PTFE) tube using sonication (sonicator Microson XL2007, 100W, 23 kHz, with a 3.2 mm microprobe, Misonix, Servilab, Le Mans, France) for 15 seconds. Then, the primary emulsion was poured into a 5% (w/v) external aqueous solution of PVA (30 ml) under mechanical stirring at 500 rpm for 1 minute. The resulting emulsion was poured into deionized water (400 ml) and stirred with a magnetic stirrer for 25 minutes. Finally, the microparticles were filtered, washed five times with

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100 ml of deionized water, freeze-dried and stored at +4°C. When PEG was introduced in the microencapsulation process (0.05 ml and 0.09 ml or PEG/PLGA ratio of 1/1 and 1.8/1), it was mixed with the internal aqueous phase maintaining a total volume of 0.15 ml. In some cases, NaCl was added to the dispersing phase of the double emulsion and to the extracting phase at a 10% concentration (w/v).

All the experimental details concerning the determination of the NGF encapsulation yield are described elsewhere (5).

Size Determination and Scanning Electron Microscopy (SEM) Analysis

The size determination was carried out using a Coulter® Multisizer (Coultronics, Margency, France).

Freeze-dried microspheres were mounted onto metal stubs using double-sided adhesive tape, vacuum-coated with a carbon film (MED 020, Bal-Tec, Balzers, Lichtenstein) and directly analyzed under SEM (JSM 6301F, JEOL, Paris, France). To characterize the internal morphology, the adhesive tape with particles stuck to it was folded on itself and then roughly unfolded to fracture the microparticles.

In Vitro Release Studies

10 mg of microparticles were placed in a tube with 4 ml of PBS buffer pH 7.4. PVA (0.005% w/v) was added in the release medium to improve microparticle dispersion. The tubes were closed and the medium was subjected to gentle magnetic agitation using an immersible stirrer (Variomag HP15 equipped with telemodul 20P, Labortechnik GmbH, Servilab, Le Mans, France) in a thermostated bath (37°C). At selected time points, the tubes were centrifuged (7,500 rpm–20°C). Between 1 and 3 ml of release medium were removed for the measurement of the released NGF fraction by radioactivity counting and replaced with fresh buffer. Each experiment was done in triplicate.

Stability Studies

After 2 hours of microparticle incubation, the fraction of NGF released in a denatured form was determined by comparing the amount of released NGF as measured by radioactivity counting and by immuno-enzymatic assay (ELISA). The two-site ELISA was performed using a mouse monoclonal anti-mouse NGF antibody 27/21 (Boehringer Mannheim Biochemica, Meylan, France), as previously described (6). As the epitope recognized by the monoclonal antibody overlapped the TrK receptor site responsible for the NGF activity, the ELISA-recognized NGF was considered fully bioactive (12).

To study the protective effect of various additives on protein stability, NGF aqueous solutions (10 μg in 0.15 ml of citrate buffer, pH6, HSA 2.5 mg) were emulsified in 1 ml of organic phase without PLGA. The organic phase corresponded either to methylene chloride or to a methylene chloride/acetone mixture (3/1). PEG 400 (50 μl), a mannitol (4.5 mg)/sucrose (4.5 mg) mixture or additional HSA (7.5 mg) were added to the aqueous phase (0.15 ml total volume). The emulsions were carried out in glass tubes using a Vortex® for 1 minute, then the organic phase was evaporated at 37°C under gentle agitation for a 4-hour period while keeping the aqueous phase volume constant. The NGF concentration was assessed by ELISA and compared to that of an NGF aqueous phase kept at 37°C for 4 hours. It was previously checked that PEG alone did not interfere in the ELISA response and that both a mixture of PEG/NGF and NGF alone gave the same response.

Determination of Glass Transition Temperatures

Thermal analyses were conducted using a differential scanning calorimeter (Mettler TC11, Mettler-Toledo, Viroflay, France). 2–3 mg samples were sealed into aluminum pans. The glass transition temperature (midpoint) was measured from the second heating run performed at 10°C/min between −10°C and +100°C.

Interfacial Tension Measurements

To obtain information on the organization of the interfacial film formed during primary emulsification, a first set of experiments was conducted at the methylene chloride/water interface, using the dynamic pendant drop method (13,14). A drop of methylene chloride was formed in an aqueous solution containing PEG 400 (4 g/l) and/or HSA (2 g/l). In a second set of experiments, PLGA was dissolved in the organic phase (5g/l). The apparatus used (DT 1000, IT Concept, Longessaigne, France) allows the determination in real time (8 measurements per second) of both the interfacial tension, the drop surface area and the drop volume, at the same time. The evolution of the interfacial tension with time allowed the determination of the adsorption kinetics of PEG, albumin, and PLGA. This was performed keeping the drop area constant during the analysis of the adsorption process. This avoided drop detachment due to decreasing interfacial tension.

RESULTS

Preformulation studies showed PEG 400 was a good candidate to protect NGF against denaturation by contact with an organic phase during emulsification (Table I). This was not the case for both albumin at high concentration and sugars. In addition, the methylene chloride/acetone mixture seemed less detrimental to NGF stability than methylene chloride alone. Moreover, PEG 400 did not affect microparticle structure, microparticles prepared without NaCl had the same internal structure irrespective of the presence of PEG in the internal

Table I. NGF Stability in Aqueous Solution after Emulsification in Various Conditions

NGF aqueous solution emulsified in	NGF recovery in ELISA-recognized form (%)
Methylene chloride/acetone	13
Methylene chloride	6
Methylene chloride/acetone + mannitol (3% w/v) and sucrose (3% w/v) in the aqueous phase	18
Methylene chloride/acetone + additional albumin (5% w/v) in the aqueous phase	9
Methylene chloride/acetone + PEG 400 (33% v/v) in the aqueous phase	105

aqueous phase (Figs. 1A, 1B). On the other hand, the microparticles prepared with NaCl in the dispersing phase (Fig. 1C), as expected, exhibited a denser structure with smaller internal cavities. The surface of microparticles prepared with PEG and NaCl was smooth and no pores were detected (Fig. 1D). The PLGA glass transition temperatures (Tg) of microparticles prepared without or with PEG were 39.9°C and 39.6°C, respectively, as compared to 38.8°C for the starting PLGA.

In all the cases, the NGF encapsulation yields were in the range of 95–100%. In contrast, the introduction of NaCl and/or PEG in the microencapsulation process modified significantly the protein release profile. The addition of PEG (50 μl) to the internal aqueous phase increased strongly both the burst effect and the amount of released NGF over a 7 day period. In addition, the release did not level off (Fig. 2). The concomitant addition of NaCl in the dispersing phase allowed us to obtain a more progressive NGF release profile: 11% of the protein were released in the first 24 hours and 59% after 4 weeks. The increase of PEG volume (90 μl) in the aqueous phase during microparticle preparation increased the initial release (Fig. 2). The use of NaCl in the absence of PEG in the microencapsulation process induced a pronounced NGF denaturation since only 28% of the released NGF in the first 2 hours were recognized by ELISA (Table II). However, an effective NGF protection was

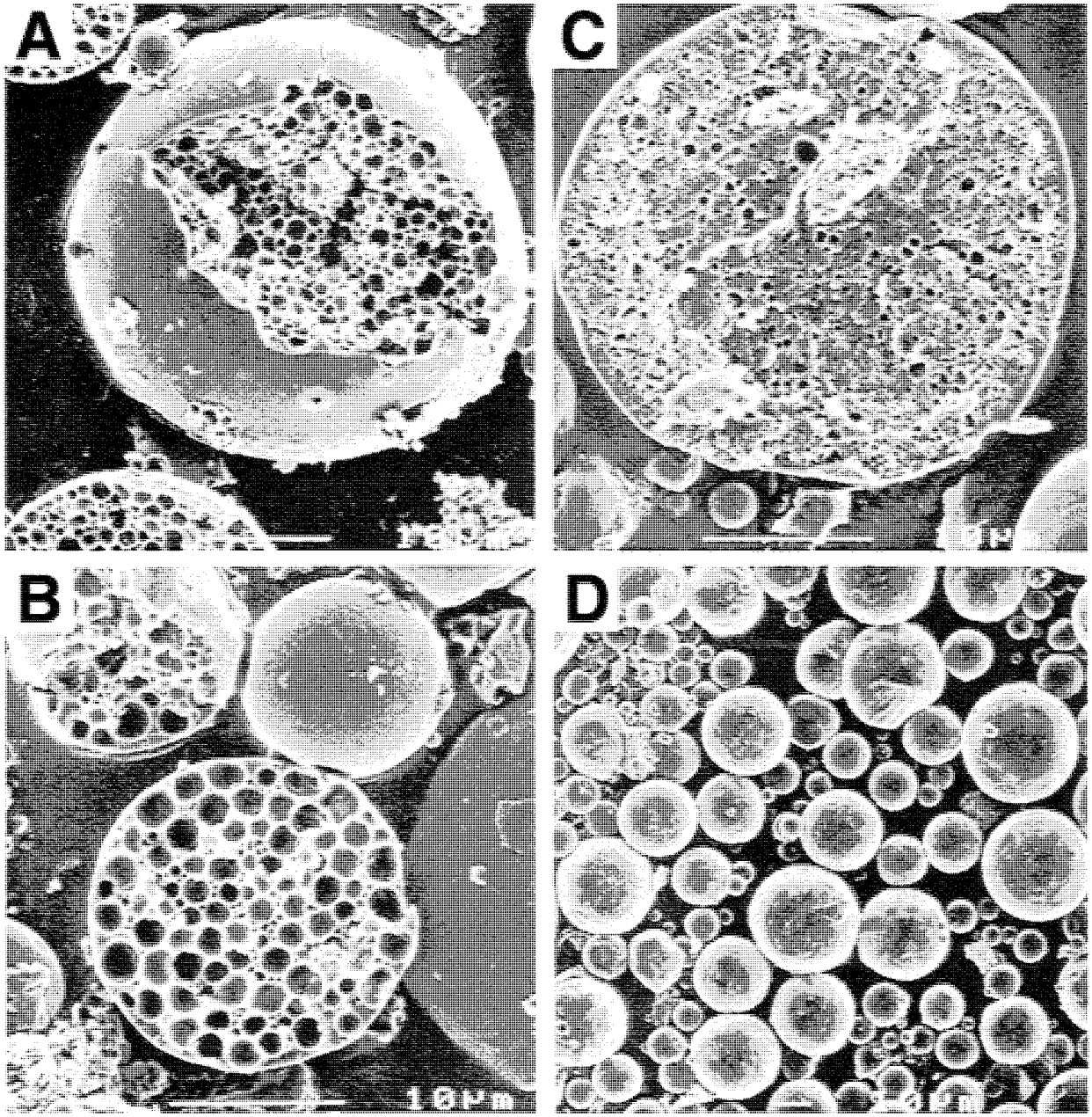

Fig. 1. Scanning electron microscopic analysis of fractured NGF-loaded microparticles (A,B,C) or non fractured NGF-loaded microparticles (D) prepared according to different conditions: (A) without additives; (B) with PEG (50 μl) in the internal aqueous phase of the W/O/W double emulsion; (C,D) with PEG (50 μl) in the internal aqueous phase and NaCl in the dispersing aqueous phase.

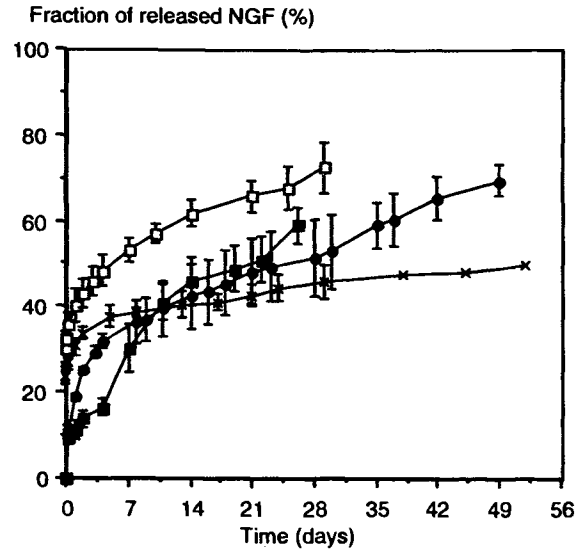

Fig. 2. NGF *In vitro* release from microparticles prepared according to different conditions: (X) without additives; (□) with PEG (50μl) in the internal aqueous phase of the W/O/W double emulsion; (■) with PEG (50 μl) in the internal aqueous phase and NaCl (10% w/v) in the dispersing aqueous phase; (●) with PEG (90 μl) in the internal aqueous phase and NaCl (10% w/v) in the dispersing aqueous phase.

provided by using a high quantity of PEG (90 μl) in the aqueous phase (150 μl). Higher amounts of PEG induced protein desolvation. This did not alter its stability but the resulting release profile was not modified with respect to 90 μl of PEG (results not shown). The size distribution of the microparticles prepared with PEG (90 μl) and NaCl was 27 ± 11 μm (expressed in volume).

To elucidate whether the effect of PEG 400 on NGF stability and NGF release profile could be attributed to interfacial phenomena, adsorption kinetics studies were performed. Figure 3A shows the adsorption kinetics of HSA and PEG at the methylene chloride/water interface. Without any surfactant agent, the interfacial tension value of methylene chloride/water interface was 28.4 mN/m. The HSA adsorption kinetics showed a lag time (200s) followed by a rapid decrease of the interfacial tension. An equilibrium value (8 mN/m) was reached after 2000s. When PEG was added to pure water, the interfacial tension value dropped to 20 mN/m at the beginning. The equilibrium was rapidly reached (<600s) with a low decrease of the interfacial tension (<3 mN/m). When albumin and PEG were

Table II. Effect of the Addition of NaCl and PEG 400 on NGF Stability During Microparticle Preparation

Concentration of NaCl in the dispersing phase (% w/v)	Volume of PEG in the internal aqueous phase (150 μl) (μl)	Fraction of NGF released over a 2h period in a ELISA-recognized form (%)
0	0	94 ± 3
10	0	28 ± 8
	50	40 ± 3
	90	88 ± 8

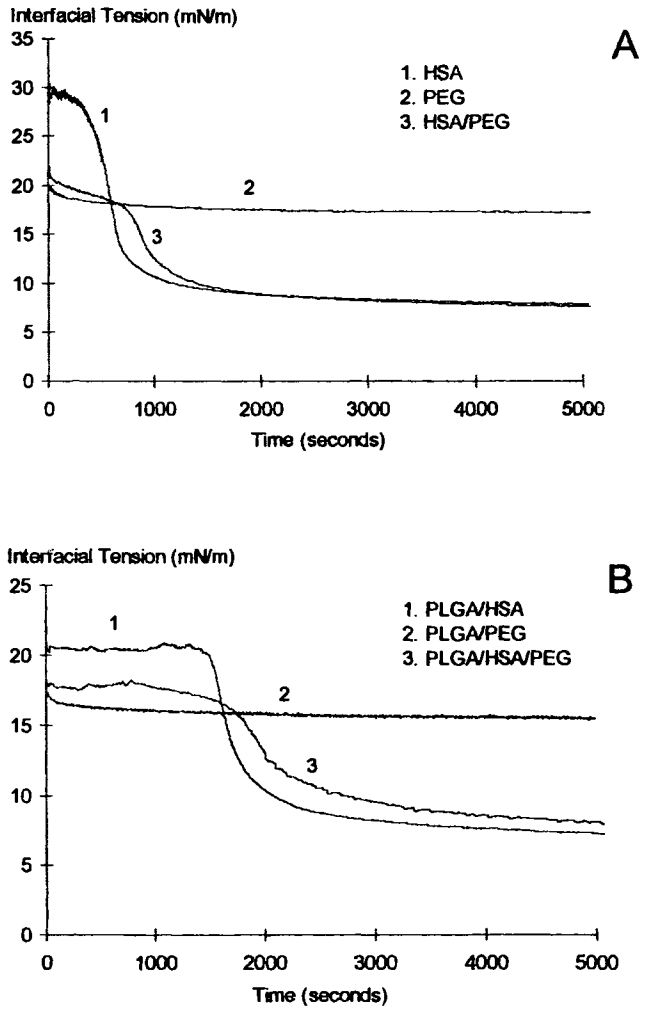

Fig. 3. Adsorption kinetics of HSA and PEG dissolved in the aqueous phase at the O/W interface: (A) without PLGA in the organic phase; (B) with PLGA in the organic phase.

added simultaneously in the aqueous phase, the initial interfacial value was 21 mN/m and slowly decreased during the first 600s. Then, an abrupt fall was observed leading to an equilibrium value close to the one measured for an aqueous phase containing only albumin. When the organic phase was supplied with PLGA, several modifications of the charts were noticed (Fig. 3B). The albumin adsorption kinetics were characterized by an initial value of 20 mN/m and remained unchanged over a 1500s period. Then, a rapid decrease of the interfacial tension was observed before equilibrium. The profile of the PEG adsorption kinetics was unchanged with the presence of PLGA in the organic phase but the initial interfacial tension was 17 mN/m instead of 20 mN/m in the absence of PLGA. When HSA and PEG were added together in the aqueous phase, a lag time was noticed at a tension of 17 mN/m. Then, the interfacial tension decreased more slowly than in the case of an albumin-containing aqueous phase. Finally, equilibrium was reached at a value slightly over 8 mN/m. It could also be mentioned the interfacial tension of the methylene chloride/pure water interface was 22 mN/m when PLGA was added in the organic phase and remained unchanged over time.

DISCUSSION

The objective of the current study was to design microspheres displaying a continuous release of active NGF over time. The presence of a plateau in the release profile could be related to the wall structure of the internal cavities of the microparticles. The wall structure itself depended on the interfacial film structure of the primary emulsion. We had previously demonstrated, using rheological measurements, that bovine serum albumin (BSA) and PLA50, previously dissolved in water and methylene chloride, respectively, formed a homogeneous interfacial film at the organic phase/water interface (13). The protein penetrated irreversibly the interfacial layer and interacted with the PLA50 chain (15).

An additional fraction of the encapsulated protein was believed to adsorb on the mixed layer according to a multilayer model, as described by Verrecchia *et al.* (16). The resulting structure constituted the internal surface of the matrix cavities and suggested desorption kinetics played a key role on protein release kinetics, including the burst effect.

The results of stability studies of NGF in solution showed that contact with an organic phase led to pronounced protein denaturation. Thus, during microparticle preparation, the protein strongly engaged in the interfacial film of the primary emulsion must be mainly denatured due to the detrimental effect of the organic medium. To avoid such protein denaturation, the use of PEG 400 appeared to offer an interesting alternative. To evidence the role of PEG, adsorption kinetics studies were performed using the pendant drop method in a simplified situation, the organic phase consisting of pure methylene chloride. The aim was to obtain information concerning the influence of PEG on protein adsorption at an O/W interface. When PLGA and PEG were added to the organic phase and the aqueous phase, respectively, they adsorbed at the interface as soon as the droplet was formed. The HSA adsorbed more slowly at the interface in the presence of PLGA and/or PEG than in their absence. When both PLGA and PEG were added, the decrease of the interfacial tension due to the adsorption of HSA, after more than 20 minutes, was less marked than in the other cases. The final equilibrium reached was slightly over the equilibrium reached in the case of the PLGA/HSA system. Thus, during microparticle preparation, it appeared both PLGA and PEG were adsorbed at the interface of the primary emulsion as soon as it formed. As the microparticle solidification required less than 10 minutes, proteins did not penetrate this layer as readily as in the absence of PEG. PEG reduced the protein anchorage in the polymer layer that prevented the release profile to level off even at the late stages of incubation. In parallel, it increased indirectly the fraction of protein weakly adsorbed on the polymer surface of the inner reservoirs during microparticle preparation. Consequently, the protein fraction available to be released at the early stage from microparticles prepared with PEG was greater than in the case of microparticles prepared without PEG. As it was checked that PEG did not induce any polymer plasticization or microparticle morphology modification, the important fraction of protein available explained clearly the marked burst effect and the release profile obtained in the case of microparticles prepared in the presence of PEG. Finally, the protein displacement from the interface explained the protective effect of PEG against NGF denaturation during stability studies. Thus, it could be speculated that the NGF released at the late stage of microparticle incubation was non-denatured while PEG 400 was co-encapsulated. HSA did not display such a protective effect against NGF denaturation since it formed a complex with NGF and was unable to successfully displace the NGF from the interface (17). In the prospected experimental conditions, sucrose and mannitol also appeared inefficient probably due to their lack of tensioactive properties.

To decrease the initial release rate, the microparticle structure had to be modified to reduce the diffusion of the drug through the polymer matrix. In the present study, this was achieved by the addition of NaCl in the microparticle preparation process. The addition of NaCl in the dispersing phase of the W/O/W emulsion induced a water out-flow from the aqueous dispersed phase to the aqueous dispersing phase, through the organic layer. Consequently, the microparticles obtained had a denser structure with smaller internal cavities than those prepared without NaCl. Unfortunately, the water out-flow induced a severe protein denaturation (6). Therefore, the use of NaCl to reduce the burst effect required the previous stabilization of NGF. In the present work, we had speculated this denaturation proceeded through protein dehydration followed by irreversible precipitation and aggregation. High concentrations of PEG in water, having a large exclusion volume, are known to concentrate proteins in solutions until their solubility is exceeded (18); then precipitation occurs without denaturation (19). This was consistent with the observed precipitation of NGF in the aqueous phase containing more than 90 µl of PEG. At 90µl, a reversible precipitation of non-denatured protein was believed to occur during the water out-flow. But it should be noted lower amounts of PEG did not fully protect the protein due to the partitioning of this compound between the organic and aqueous phases (20). Finally an additional protective effect due to a specific binding between polar but uncharged PEG and proteins, could not be discarded, as previously suspected in various protein/surfactant systems (21).

In optimal stability conditions (90 µl PEG/150 µl total aqueous phase), the initial release was not negligible but remained less marked than in the case of microparticles prepared without NaCl.

The quality of the protein fraction which is able to be released at the late stages of microparticle incubation could not be studied *in vitro* since protein can undergo physical and chemical changes during extended storage in the release medium (22). In the case of microparticles prepared by the double emulsion technique, the protein fraction released in the first 2 hours cannot be taken as being representative of the whole encapsulated protein. Therefore, an *in vivo* bioassay is now in progress by our group to check the stability and the activity of the NGF released several weeks after microparticle implantation in rat brain.

CONCLUSIONS

PEG 400 displaced protein from the W/O interface and protected NGF from denaturation by contact with the organic phase during the primary emulsification step. This compound counteracted the NGF denaturation process induced by the presence of NaCl in the external aqueous phase of the W/O/W double emulsion. Thus, using the double emulsion solvent extraction-evaporation technique, the co-encapsulation of PEG

400 allowed the preparation of biodegradable microspheres displaying a continuous release of NGF.

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