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RESEARCH PAPER

Preparation and Characterization of Heparin-Loaded Polymeric Microparticles

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ABSTRACT

Microparticles containing heparin were prepared by a water-in-oil-in-water emulsification and evaporation process with pure or blends of biodegradable (poly-ε-caprolactone and poly(D,L-lactic-co-glycolic acid)) and of positively-charged non-biodegradable (Eudragit® RS and RL) polymers. The influence of polymers and some excipients (gelatin A and B, NaCl) on the particle size, the morphology, the heparin encapsulation rate as well as the in vitro drug release was investigated. The diameter of the microparticles prepared with the various polymers ranged from 80 to 130 μm and was found to increase significantly with the addition of gelatin A into the internal aqueous phase. Microparticles prepared with Eudragit RS and RL exhibited higher drug entrapment efficiency (49 and 80% respectively) but lower drug release within 24 h (17 and 3.5% respectively) than those prepared with PCL and PLAGA. The use of blends of two polymers in the organic phase was found to modify the drug entrapment as well as the heparin release kinetics compared with microparticles prepared with a single polymer. In addition, microparticles prepared with gelatin A showed higher entrapment

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efficiency, but a significant initial burst effect was observed during the heparin release. The in vitro biological activity of heparin released from the formulations affording a suitable drug release has been tested by measuring the anti-Xa activity by a colorimetric assay with a chromogenic substrate. The results confirmed that heparin remained unaltered after the entrapment process.

Key Words: *Eudragit; Heparin; Microparticles; PCL; PLAGA; w/o/w emulsion*

INTRODUCTION

Microencapsulation has received considerable interest in recent years because it offers the capacity of controlling the pattern of drug release and pharmacokinetics, thereby optimizing drug therapeutic response and decreasing their deleterious side effects.[1–4] Moreover, another prime advantage concerns the preservation of drugs that are rapidly destroyed in the body, particularly biologically sensitive macromolecules. A number of microencapsulation techniques have been developed. Among these, the water-in-oil-in-water (w/o/w) emulsification and evaporation method is well suitable for the encapsulation of water-soluble drugs. A variety of drug classes such as vaccines, polypeptides, proteins, and low molecular drugs have been encapsulated previously in micro- and nanoparticles by this technique.[5–7]

Heparin is the anticoagulant of choice widely used for the treatment and the prevention of deep vein thrombosis and pulmonary embolism.[8] However, owing to its short physiological half-life and no bioavailability when administered orally, it has to be administered by the parenteral route. The major disadvantages of parenteral administration of heparin are bleeding complications, cutaneous necrosis, and the requirement of careful patients monitoring.[9] Therefore, administration of heparin by the oral route would be highly desirable for patients. Several attempts to develop oral formulations of heparin have been investigated. Heparin complexes with spermine and lysine salts,[10] hydrophobic organic bases,[11,12] and EDTA[13] or oil–water emulsions[14] have been developed. The experimental results in animals have shown an increase in the absorption of heparin after their oral administration. However, the mucosal damage of the gastrointestinal tract induced by the toxicity of adjuvants such as EDTA and the lack of stability of the oil–water emulsions hampered their practical use. Other dosage forms such as liposomes[15] and proteinoid microspheres[16–18] have also been investigated, and showed a slight increase in the absorption of heparin. However, the anticoagulant activity of heparin lasted only for 1.5–2 hr, and was much shorter than that of a similar dose administered subcutaneously. Recently, a novel absorption enhancer, sodium *N*-[8-(2-hydroxybenzoyl)amino]caprylate (SNAC)[19–21] coadministered orally with heparin has been shown to improve the heparin absorption across the small intestine and the colon. Nevertheless, for all oral heparin formulations previously reported, high doses of heparin (up to 150,000 IU) were used.

Owing to the high solubility of heparin in water, the double emulsion and evaporation method has been chosen as one of the most appropriate technique for the preparation of microparticles. Two different biodegradable polyesters, poly-ε-caprolactone (PCL) and poly(D,L-lactic-*co*-glycolic acid) 50/50 (PLAGA) as well as two positively charged non-biodegradable copolyesters of acrylic and methacrylic acid (Eudragit® RS and RL) were used alone or blended for the microparticles preparation. First, the encapsulation efficiency and the in vitro heparin release were optimized by varying the type of polymer(s) into the organic phase as well as by using various excipients in the internal aqueous phase. Microparticles, thus prepared were then compared in terms of size, morphology, encapsulation efficiency, and drug release. Second, the biological activity of heparin released after 24 hr from the formulations affording a suitable drug release was studied by measuring the anti-Xa activity by a colorimetric assay.

MATERIALS AND METHODS

Materials

Poly(D,L-lactic-*co*-glycolic acid) 50/50 (PLAGA, MW 40,000 Da) and poly-ε-caprolactone (PCL, MW

42,000 Da) were respectively purchased from Medisorb Technologies International (Welmington, DE, USA) and Aldrich Chemicals Company (Steinheim, Germany). Eudragit® RS PO and RL PO (MW 150,000 Da) were kindly supplied by Röhm GmbH (Darmstadt, Germany). Standard heparin (injectable solution, 25,000 IU/5 mL) was supplied by Leo (Saint Quentin-en-Yvelines, France). Polyvinylalcohol (PVA, MW 30,000, 88% hydrolyzed), gelatin A (from porcine skin, 175 bloom), gelatin B (from bovine skin, 75 bloom) were provided by Sigma chemical Co. (St. Louis, MO, USA). The reagents used for the measurement of the anti-Xa activity (Stachrom® Heparin) were purchased from Diagnostica Stago (Asnières-sur-Seine, France). All other chemical reagents were of analytical grade and used as supplied.

Preparation of Microparticles

Heparin-loaded microparticles were prepared by the w/o/w emulsion and evaporation method. Briefly, 1 mL of aqueous heparin solution (5000 IU) was first emulsified in methylene chloride (10 mL), containing the polymer(s) (0.25 g) by vigorous magnetic stirring at 1500 rpm for 3 min. The resulting water-in-oil (w/o) emulsion was then poured into 1500 mL of a PVA aqueous solution (0.1%). A w/o/w emulsion was formed by extensive stirring with a three-bladed propeller for 2 hr at room temperature (except 3 hr for PLAGA microparticles), until the organic solvent was totally removed. Upon solvent evaporation, the polymer precipitates and the microparticle core solidifies. Microparticles were then collected by filtration, washed extensively with deionized water and dried at room temperature. Blank microparticles as well as microparticles prepared with gelatin (5%), NaCl (2%) or blends of polymers (ratio 1/1) were prepared in the same conditions.

Determination of Drug Entrapment Efficiency

The amount of heparin entrapped within polymeric microparticles was determined with a modified Azure II colorimetric method similar to that described by Lam et al.[22] by measuring the amount of non-entrapped drug in the external aqueous solution recovered after filtration and washing of the microparticles. Typically, aliquots (500 μL) of each aqueous sample were reacted with 4.5 mL of the Azure II solution (0.01 mg/mL) at room temperature and assayed in triplicate at 530 nm by UV spectroscopy. The drug entrapment efficiency was expressed as the percentage of heparin entrapped with respect to the theoretical value, while the drug loading was presented as the amount of heparin entrapped per gram of polymer.

Particle Size and Morphology Analysis

The particle size distribution was analyzed with a Malvern Mastersizer (Mastersizer S, Malvern Instruments, France) using laser diffraction.

The external and internal morphology of microparticles was analyzed by scanning electronic microscopy (SEM). Microparticles were fixed on supports with carbon-glue and coated with gold–palladium under an argon atmosphere using a gold sputter module in a high-vacuum evaporator. The samples were observed with a Cambridge model S240 electron microscope (Leica Cambridge Ltd., Cambridge, UK) at 20 KV.

Drug Adsorption Onto the Microparticle Surfaces

Ten milligrams of blank microparticles were suspended in 10 mL of PVA aqueous solution (0.1%) containing 3.3 IU/mL of heparin under gentle magnetic stirring for 3 hr. The amount of adsorbed heparin corresponded to the difference between the initial heparin concentration and the amount of free heparin in the supernatant recovered after filtration of the microparticles. The experiment was performed in triplicate.

In Vitro Drug Release

One hundred milligrams of unloaded or heparin-loaded microparticles were suspended in a 100 mL saline phosphate buffer (PBS, 0.011 M, NaCl 0.15 M, pH 7.4) flask containing tween® 80 (0.1%). The microparticle suspension was gently stirred (150 rpm) at 37°C in a water bath. One milliliter of suspension was withdrawn at appropriate intervals and filtered with a 0.22 μm Millipore® filter. The filtrate was assayed for drug release and replaced by 1 mL of fresh buffer. The amount of heparin in the release medium was determined by the colorimetric method described previously. Each microparticle batch was analyzed in triplicate.

Bioactivity Assay

Heparin-loaded microparticles (100 mg) were incubated in a 100 mL PBS flask at 37°C in a water bath under magnetic stirring at 150 rpm. After 24 hr, 1 mL of the suspension was removed and filtered through a 0.22 μm Millipore® filter. The biological activity of heparin recovered in the filtrate was checked by the measurement of the anti-Xa activity by a colorimetric method with a chromogenic substrate according to the procedure described by the supplier of the kit.

RESULTS AND DISCUSSION

Size and Morphology of Microparticles

As presented in Table 1, the size of heparin-loaded microparticles prepared with each polymer or blends of polymers ranged from 96 to 129 μm, with a relatively monodispersed distribution. In addition, heparin-loaded microparticles were larger than unloaded microparticles, except those prepared with PLAGA whose size remained unchanged. Moreover, the smallest microparticles were obtained when they were prepared with Eudragit®. Since the particle size is related to a great extent to the stability of the first emulsion, this could be explained by the tensioactive properties of the Eudragit® confered by their quaternary ammonium groups, which stabilized the first emulsion and hampered the fast coalescence of the droplets. This fact was confirmed by the obtention of colloidal dispersions rather than microparticles when Eudragit RL, alone or in combination, was used for the drug-free formulations preparation. This could be due to the higher ammonium groups content (1/20) in Eudragit RL than in Eudragit RS (1/40). On the contrary, it was possible to prepare microparticles with Eudragit RL, alone or in combination, as soon as heparin was added in the internal aqueous phase: the size ranged from 79 to 128 μm. This fact could be explained by the neutralization of quaternary ammonium groups induced by the ionic interactions between the positively charged ammonium groups and heparin, a macromolecular polyanion, involving a loss of the tensioactive characteristics of the polymer. The influence of some excipients within the internal aqueous phase has been evaluated for heparin-loaded microparticles prepared with Eudragit RS, PCL, and PLAGA (Table 2). No significant influence was observed on the particle size with NaCl and gelatin B, compared with unloaded microparticles. On the contrary, microparticles prepared with gelatin A were larger. Since its isoelectric point value is 8.6, gelatin A was in cationic form in the internal aqueous heparin solution at pH 6.5 and interacted with the drug. As a matter of fact, the increase of the microparticle size resulted from the interactions of gelatin A with heparin as well as from the swelling of microparticles confered by gelatin. A similar mechanism was

Table 1

Microencapsulation Efficiency, Drug Loading and Mean Diameter of Heparin-Loaded (a) and Drug-Free (b) Microparticles Prepared by the Double Emulsion Method with a Single Polymer (250 mg) or Blends of Polymers (125/125 mg)

Polymer	Drug Loading* (IU/g polymer)	Entrapment Efficiency* (%)	Mean Diameter (a) (μm)	Mean Diameter (b) (μm)
RS	9952 ± 798	49 ± 4	96	71
RL	15960 ± 688	80 ± 3	80	–
PCL	4566 ± 700	24 ± 4	128	119
PLAGA	5360 ± 749	27 ± 4	125	125
RS/PCL	7277 ± 722	36 ± 4	129	94
RL/PCL	9032 ± 466	45 ± 2	103	–
RS/PLAGA	10520 ± 908	52 ± 4	87	86
RL/PLAGA	12750 ± 785	64 ± 4	128	–
RS/RL	13310 ± 303	67 ± 2	88	–
PCL/PLAGA	3506 ± 929	17 ± 5	82	100

*Data expressed as mean ± SD ($n \geq 3$); –: Not determined.

Table 2

Microencapsulation Efficiency, Drug Loading and Mean Diameter of Heparin-Loaded Microparticles Prepared with Eudragit RS, PLAGA, and PCL by Adding Excipients (gelatin A, gelatin B, and NaCl) within the Internal Aqueous Phase

Polymer	Excipient	Mean Size (μm)	Drug Loading (IU/g polymer)	Entrapment Efficiency (%)
RS	None	96	9952 ± 798	49 ± 4
	Gelatin A (5%)	124	13320 ± 811	67 ± 4
	Gelatin B (5%)	80	9260 ± 300	46 ± 2
	NaCl (2%)	85	3186 ± 395	16 ± 2
PLAGA	None	125	5360 ± 749	27 ± 4
	Gelatin A (5%)	282	11740 ± 787	59 ± 4
	Gelatin B (5%)	130	7505 ± 492	37 ± 2
	NaCl (2%)	108	4517 ± 666	23 ± 3
PCL	None	128	4566 ± 700	24 ± 4
	Gelatin A (5%)	201	11580 ± 928	58 ± 5
	Gelatin B (5%)	125	4826 ± 537	24 ± 3
	NaCl (2%)	91	3416 ± 378	17 ± 2

Data shown as mean ± SD ($n \geq 3$).

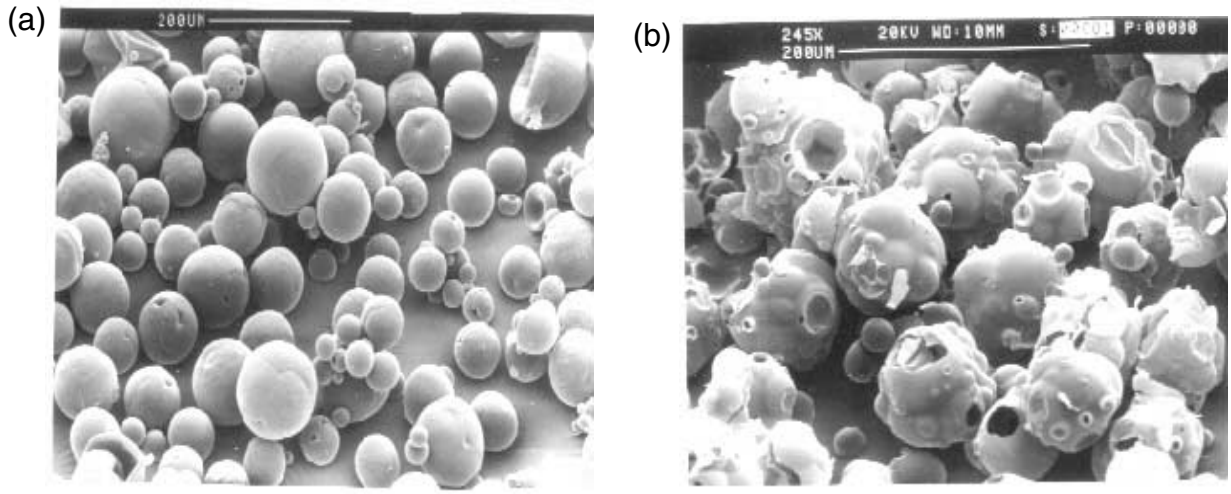

Figure 1. SEM photographs of heparin-loaded microparticles prepared with (a) PCL and (b) Eudragit RS.

not supposed to occur with gelatin B whose isoelectric point value is 4.7. In the internal aqueous heparin solution at pH 6.5, it was in anionic form and, therefore, cannot form a complex with the polyanionic drug.

Figure 1 displays the morphology and shape of microparticles prepared with the biodegradable and non-biodegradable polymers. Eudragit® microparticles appeared porous, with an irregular shape and seemed brittle, while those prepared with both PCL and PLAGA were smooth and spherical. Two possible hypothesis could explain the irregular morphology of Eudragit microparticles. Firstly, when heparin reacted with the quaternary ammonium groups of Eudragit RL or RS, the latter were not able to act as surfactant any longer, thus involving the coalescence of the emulsion droplets. Secondly, since the drug-free Eudragit RS microparticles were smaller than heparin-loaded microparticles, and had smoother and pore-free surfaces

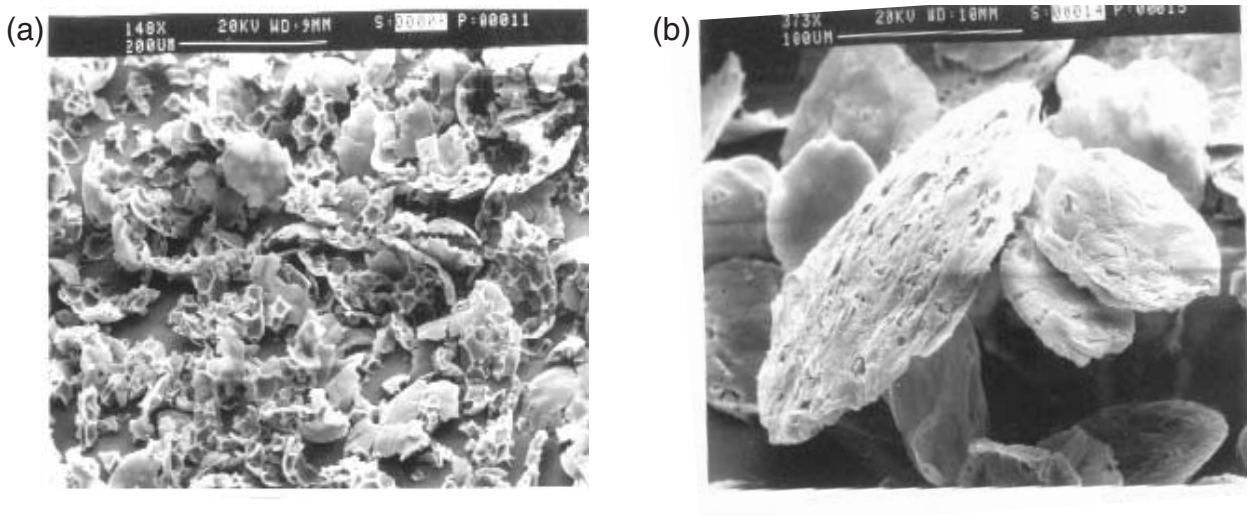

Figure 2. SEM photographs of heparin-loaded microparticles prepared with (a) blends of Eudragit RS and PLAGA and (b) PCL after heparin release for 24 hr.

(pictures not shown), the change in both size and morphology resulted from the presence of heparin. Indeed, sulfate groups of heparin may create an ionic network with quaternary ammonium groups of Eudragit within which air may have been incorporated in the primary emulsion. This could lead to the formation of large pores during solvent evaporation and easily breaking particles. Moreover, this statement was corroborated by the fact that Eudragit microparticles loaded with heparin were completely broken up after the dissolution tests (Fig. 2b). Also, this hypothesis was correlated with that of Witschi and Doelker.[23] Indeed, these authors stated that with increasing tetracosactide concentration within microparticles prepared with PLA and PLAGA by the w/o/w method, the particles lost their spherical shape, due to very large pores owing to the incorporation of air in the primary emulsion and appeared aggregated.

Encapsulation Efficiency

As reported in Tables 1 and 2, entrapment efficiency within polymeric microparticles was significantly affected by the nature of the polymer and the use of excipients into the internal aqueous phase. The encapsulation efficiency ranged from 16 to 80%, and afforded the highest rate when Eudragit RL and RS were used for the microparticles preparation. Owing to the polyanionic nature of heparin, electrostatic bindings between the drug and the quaternary ammonium groups of the two polymers led to an increase of heparin immobilization compared with PCL and PLAGA polymers. This hypothesis is furthermore strengthened by the fact that a higher drug loading was observed with Eudragit RL, which carries more quaternary ammonium groups than Eudragit RS. In addition, when Eudragit RS or RL was mixed (50/50) with PCL or PLAGA, heparin entrapment was higher than when PCL or PLAGA was used alone. Moreover, adsorption experiments performed with blank Eudragit, PCL, and PLAGA microparticles reacting with an aqueous heparin solution, showed negligible drug adsorption with PCL and PLAGA ($\leq$5%), while more significant adsorptions were observed with Eudragit RL and RS ($\approx$20%). These results demonstrated that heparin was mainly encapsulated within PCL, and PLAGA microparticles, whereas it could be both encapsulated and adsorbed by electrostatic bindings with Eudragit. The differences of entrapment efficiency observed with each polymer can also result from the difference in their molecular weight: the higher the molecular weight, the more viscous the polymeric solution and consequently the first emulsion. Since an increase of the viscosity of the first emulsion is related to a reduction in the partitioning of the drug into the external aqueous phase,[24] it was therefore not surprising that higher

drug-loading was obtained with both type of Eudragit that presented the highest molecular weight (150,000 Da). In addition, this hypothesis is corroborated with the results observed with microparticles prepared with gelatin A since the increase in the viscosity of the internal aqueous phase led to a higher heparin-loading within microparticles.

On the other hand, the lower entrapment efficiency obtained with PCL and PLAGA can also be explained by the high solubility of heparin in water. It was known that diffusion and drug loss across the droplet interface occurs mainly during the first minutes of emulsification, since the polymer precipitates rapidly hindering any further drug leakage.[25] Organic solvents with high water solubility result in rapid polymer precipitation and hardening of polymer solution. However, the limited solubility of methylene chloride in water can be expected to prolong the solvent-swollen conditions, involving a slower precipitation of the polymer and consequently an increase of the drug leakage into the external aqueous phase.[26] The addition of NaCl within the internal aqueous phase decreased the drug encapsulation rate, especially for Eudragit RS microparticles, probably owing to the counter-ion effect of NaCl on heparin.[27] Indeed, the authors stated that the efficient charges of heparin were decreased by adding NaCl into an aqueous heparin solution. Consequently, the electrostatic interactions between heparin and Eudragit RS decreased and the drug entrapment efficiency was reduced.

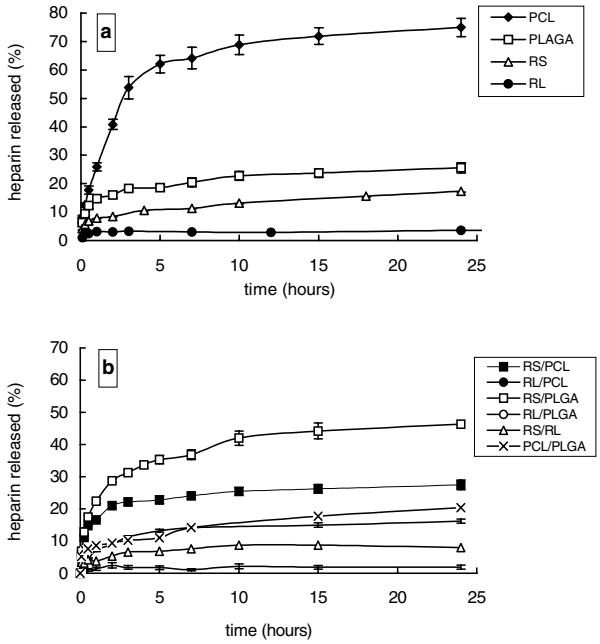

Figure 3. Release profiles of heparin from microparticles prepared either (a) with a single biodegradable or non-biodegradable polymer, or (b) with blends of two polymers. Experiments were performed in phosphate buffer at 37°C and pH 7.4. Data are shown as mean ± SE ($n = 3$).

Heparin Release

Figures 3 and 4 illustrate the in vitro release profiles obtained for each formulation prepared with one or two polymers, and with excipients respectively, by presenting the percentage of heparin release with respect to the amount of encapsulated heparin. As reported on Fig. 3a, low heparin releases were observed with microparticles prepared with Eudragit RS and RL. This result was not surprising, taking into account the strong ionic interactions between the polymers and heparin; such strong interactions could not be disrupted at the pH experiment. Furthermore, this hypothesis is corroborated by the fact that the release of heparin was lower from microparticles prepared with Eudragit RL compared to Eudragit RS. Indeed, due to higher quaternary ammonium groups content in Eudragit RL than in Eudragit RS a higher electrostatic binding was expected between Eudragit RL and heparin. The difference in drug release observed with PCL and PLAGA microparticles was curiously unexpected. Indeed, owing to the higher hydrophobicity of PCL, a lower heparin-release would have been expected from PCL microparticles. Consequently, since heparin-loaded microparticles prepared with PCL and PLAGA afforded both similar size and encapsulation efficiency, the higher drug release obtained with PCL microparticles could be due to a higher diffusion coefficient of heparin in PCL as opposed to PLAGA. Figure 3b displays the heparin release from microparticles prepared with blends of polymers. As expected, microparticles prepared with Eudragit RL in combination with the three other polymers afforded the lowest heparin release compared with Eudragit RS. However, as opposed to Fig. 3a, a higher heparin-release occurred when PLAGA was added to Eudragit RS when compared with PCL. Since microparticles prepared with Eudragit RS in combination with PLAGA

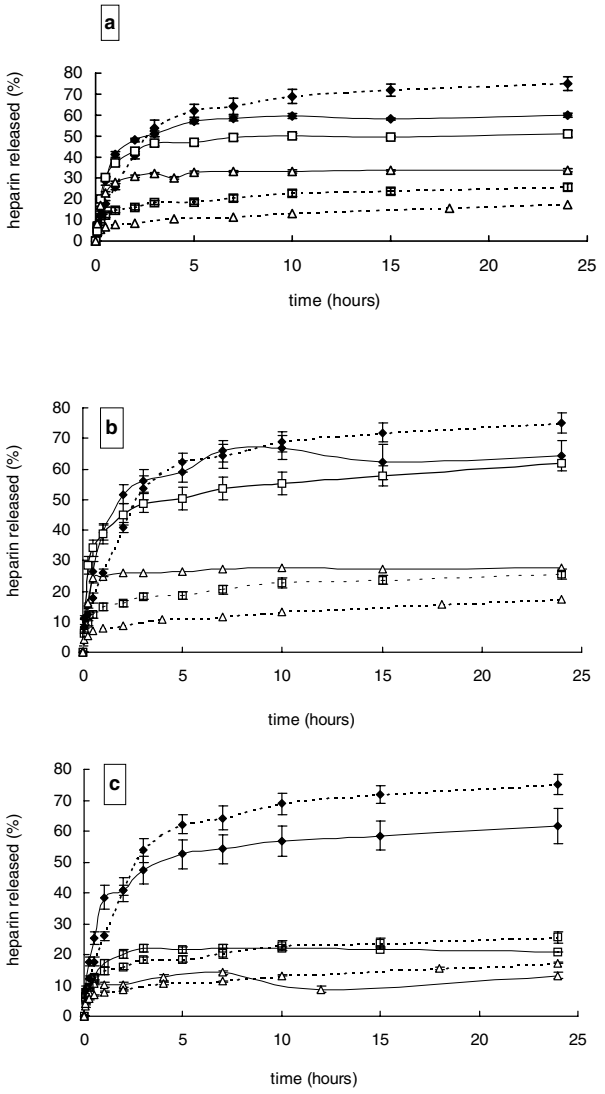

Figure 4. Release profiles of heparin from microparticles prepared by adding gelatin A (a), gelatin B (b), and NaCl (c) within the internal aqueous phase. Experiments were performed in phosphate buffer at 37°C and pH 7.4. Microparticles prepared with and without excipients were respectively presented in solid and broken lines. PCL (◆), PLAGA (□), Eudragit RS (△).Data are shown as mean ± SE ($n = 3$).

were completely broken up after the dissolution test (Fig. 2b), the contact surface was dramatically increased and the heparin release was consequently easier and more important (by a two-fold factor at 24 hr). On the contrary, microparticles prepared with PCL preserved a compact structure and appeared as flat discs (Fig. 2a), involving a lower

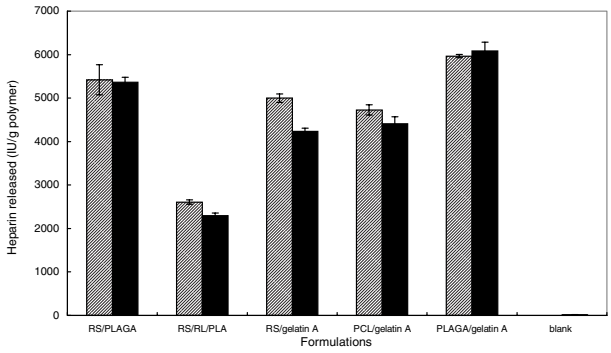

Figure 5. Comparison of the amount of heparin released after 24 hr from polymeric microparticles determined by the colorimetric method with azure II (black) and by the anti-Xa activity with a chromogenic substrate (hatched). Blank corresponded to unloaded microparticles. Data are shown as mean ± SE ($n = 3$).

drug-release. The influence of some excipients on the drug release has been evaluated for heparin-loaded microparticles prepared with Eudragit RS, PCL, and PLAGA (Fig. 4). A significant burst release occurred when gelatin A and B were added in the internal aqueous phase, as compared with microparticles prepared without excipient (Figs. 4a and 4b). As gelatin is highly soluble in water at 37°C, it attracts water, then increasing probably the solubility of the drug, whose diffusion may become easier. Moreover, gelatin induces the swelling of the microparticles, thus improving the diffusion of the drug as well. On the contrary, the heparin release was lower and slower when NaCl was added within the internal aqueous phase, in comparison with formulations prepared without excipient (Fig. 4c).

It was important to verify whether or not heparin was still keeping its biological properties due to the nature of the encapsulation process, which involves both strong steps of shear and evaporation, as well as an interface contact with the organic solvent. The amount of heparin released from heparin-loaded microparticles affording both suitable encapsulation efficiency and drug release has been determined by a biological method based on the measurement of the anti-Xa activity. As reported in Fig. 5, a good correlation was obtained for the amount of heparin released after 24 hr and determined by the colorimetric method with Azure II and the biological one as well. These results were consequently related to the preservation

of the anticoagulant activity of heparin after the encapsulation process.

CONCLUSION

Polymeric particles can be expected to be used as potential carriers of heparin prepared by the emulsification and evaporation process. Furthermore, since heparin has been found to be poorly absorbed when orally administered, due to its short half-life, polymeric microparticles are expected to protect heparin from digestion in the gastrointestinal tract and consequently enhance its peroral bioavailability. Our results establish consequently the feasibility of new oral dosage form for the delivery of heparin and may have broader implications in the absorption of other macromolecules.

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