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Long-term Release of Clodronate from Biodegradable Microspheres

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ABSTRACT This paper describes the formulation of a biodegradable microparticulate drug delivery system containing clodronate, a bisphosphonate intended for the treatment of bone diseases. Microspheres were prepared with several poly(D,L-lactide-co-glycolide) (PLGA) copolymers of various molecular weights and molar compositions and 1 poly(D,L-lactide) (PDLLA) homopolymer by a water-in-oil-in-water (w/o/w) double emulsion solvent evaporation procedure. Critical process parameters and formulation variables (ie, addition of stabilizing agents) were evaluated for their effect on drug encapsulation efficiency and clodronate release rate from microparticles. Well-formed clodronate-loaded microspheres were obtained for all polymers by selecting suitable process parameters (inner water/oil volume ratio 1:16, temperature-raising rate in the solvent evaporation step 1°C/min, 2% wt/vol NaCl in the external aqueous phase). Good yields were obtained in all batches of clodronate microspheres (above 60%); drug encapsulation efficiencies ranged between 49% and 75% depending on the polymer used. Clodronate release from all copolymer microspheres was completed in about 48 hours, while those from PDLLA microspheres required about 20 days. The change of microsphere composition by adding a surfactant such as Span 20 or a viscosing agent such as carboxymethylcellulose extended the long-term release up to 3 months. Clodronate was successfully entrapped in PLGA and PDLLA microspheres, and drug release could be modulated from 48 hours up to 3 months by suitable selection of polymer, composition, additives, and manufacturing conditions.

KEYWORDS: PLGA microspheres, solvent evaporation method, long-term release, Clodronate

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INTRODUCTION

Bisphosphonates (BPs) are a class of drugs characterized by a P-C-P bond. Consequently, they are analogues of pyrophosphates that are more resistant to chemical and enzymatic hydrolysis. A number of bisphosphonates have been approved for clinical use in Paget's disease, hypercalcemia of malignancy, and osteoporosis [1-3]; these conditions require continued BP administration. The BP action mechanism is not completely understood, but the most likely hypothesis is that the drug is incorporated in the bone matrix and absorbed in osteoclasts, blocking the resorption process [4].

After oral administration in chronic drug therapy, poor absorption (1% of the oral dose) and adverse gastrointestinal effects in humans and high intra- and intersubject variability of absorption in both animal and human studies have been observed [5]. Parenteral administration of BPs has several limitations: Intravenous administration must be performed by slow infusion of the drug, which is diluted in high amounts of solvent (200-500 mL) to avoid kidney failure, and intramuscular and subcutaneous administration, which lead to rapid and good BP absorption, can cause local tissue damage and irritation at the site of injection [6,7]. For these reasons, BPs are good candidates for study, with the goals of improving their bioavailability and decreasing their side effects.

Attempts have been made to use absorption enhancers and prodrugs, mainly for oral administration, but without any real improvement in drug absorption or lessening of side effects [8]. Local implantation of a BP-loaded biodegradable microparticulate delivery system appears to warrant an assessment of the treatment of localized bone disease (ie, hypercalcemia in tumors). Chitosan microspheres loaded with BPs have had good results after local injection in the tibialis muscle, as described in the literature [9]. The

main advantage of these formulations is that the drug targets a specific site; moreover, the slow release of drug from microparticles would prevent local irritation and tissue necrosis associated with an intramuscular BP injection.

Our study evaluates the formulation of a polymeric microparticle delivery system intended for parenteral administration of clodronate to treat bone diseases. Among biodegradable polymers, poly(D,L-lactide) (PDLLA) and poly(D,L-lactide-co-glycolide) (PLGA) were selected to formulate microparticulate drug delivery systems containing BPs. The properties of polylactide homopolymer and its polylactide-co-glycolide copolymer, in terms of biodegradability and biocompatibility, are well documented because they have been commercially used for more than 2 decades as constituents of bioresorbable surgical sutures. They have been extensively studied for drug delivery, and their use in this area has been approved by the US Food and Drug Administration [10]. Recently these polymers have been used as constituents of biodegradable implant material in dentistry and orthopedics [11]. PDLLA and PLGA are thermoplastic aliphatic poly(esters) whose properties vary depending on polymer molecular weight and composition. In this study, several PLGA copolymers with various molecular weights and molar compositions, ranging between 50:50 and 75:25 molar ratio, and 1 PDLLA homopolymer were selected. The PDLLA and the copolymers used in this work are characterized by the capital letter H in their identification code and carry predominantly free carboxylic acid groups on 1 end of the chain. Therefore, they are more hydrophilic than are the corresponding standard PDLLA and PLGA esterified by a C_2H_5 group on the carboxyl moiety. This characteristic is important when the polymer is involved in the microencapsulation process of a hydrophilic compound.

The bisphosphonate evaluated in this study is clodronate. Its high hydrophilicity makes it difficult to microencapsulate it in a hydrophobic polymer, such as PLGA or PDLLA. Moreover, the drug's low molecular weight and its hydrophilicity make it difficult to obtain sustained release from the microparticulate system.

Water-in-oil-in-water (w/o/w) double emulsion solvent evaporation has been used to prepare microspheres. This is the technique traditionally used to prepare microspheres loaded with hydrophilic compounds (eg, peptides and proteins) [12]. Several formulation variables were modified and evaluated for their effect on encapsulation efficiency and clodronate release rate from microparticles.

MATERIALS AND METHODS

Materials

Clodronate (dichloromethylene) bisphosphonic acid monosodium salt was a gift of Chiesi Farmaceutici SpA (Parma, Italy). The polymers employed were purchased from Boehringer Ingelheim (Ingelheim, Germany); their characteristics are reported below.

- PLGA copolymers 50:50 lactide/glycolide molar ratio: RG 502 MW = 12 000 Da; RG 503 MW = 34 000 Da; RG 503H MW = 34 000 Da; RG 504 MW = 48 000 Da.
- PLGA copolymers 75:25 lactide/glycolide molar ratio: RG 752 MW = 22 000 Da; RG 755 MW = 68 000 Da.
- PDLLA homopolymer: R 202 H MW = 16 000 Da.

Poly(vinyl alcohol) (PVA; 87%-89% hydrolyzed, MW = 85 000-146 000) and Span 20 were purchased from Aldrich Chemical Company Inc (Milwaukee, WI). Carboxymethylcellulose (CMC) (viscosity of 1% aqueous solution 30-70 cP) was purchased from BDH (Milano, Italy).

All reagents were analytical grade.

Clodronate microsphere preparation

A w/o/w emulsion/solvent evaporation method was used to produce clodronate-loaded microspheres, which were then prepared as follows. The drug was dissolved in water; this solution was then added to 5 g of methylene chloride containing 500 mg of polymer while being stirred at 9500 rpm using an IKA Ultraturrax T25 equipped with a S25N dispersing tool (IKA Laboratory Technology, Staufen, Germany) at 10°C. This water/oil emulsion was poured into 100 mL of 1% (wt/vol) PVA solution and gently stirred using a Vibromixer E1 (Chemap AG, Volketswil, Switzerland) at 60 vibrations/sec to obtain double emulsion; the emulsion was then brought to 40°C and stirred for 1 hour to allow solvent evaporation. Microspheres were collected by centrifugation at 4000

rpm for 20 minutes, washed twice with water, collected on a millipore 0.8-µm membrane, and dried under vacuum.

In a preliminary step, several process parameters were evaluated to optimize microsphere morphology and drug loading:

- Volume ratios between the phases of the inner water/oil emulsion (1:5 or 1:16). Organic phase volumes were kept constant, modifying the volume of aqueous internal phase (1 mL or 300 µL).
- Temperature-raising rates in the evaporation solvent process (1°C/min; 10°C/min).
- Electrolyte (NaCl 2% wt/vol) in the outer aqueous phase.

Process parameter evaluation was performed on PLGA RG 503 as microsphere-forming polymer and drug/polymer theoretical weight ratio of 1:10 water/water.

- Several parameters in phase composition were further evaluated to investigate drug content and drug release behavior:
- Molecular weight and molar composition of the polymer used.
- Presence of a viscosity increasing agent (CMC) in clodronate solution or of an emulsifier agent (Span 20) in the polymeric solution.
- Two theoretical drug contents (9% or 18%).

Table 1 shows the batches of clodronate-loaded microspheres prepared maintaining these conditions at a constant 1:16 inner water/oil emulsion phase volume ratio, 1°C/min temperature-raising rate, and NaCl 2% wt/vol in the outer aqueous phase.

Table 1. Batches of Clodronate-Loaded Microspheres

Batch Number	Polymeric Composition	CMC (1% wt/vol)	Span 20 (1% wt/vol)	Theoretical Drug Content (%)
502/9	RG 502	-	-	9
503/9	RG 503	-	-	9
503H/9	RG 503H	-	-	9
504/9	RG 504	-	-	9
752/9	RG 752	-	-	9
755/9	RG 755	-	-	9
202H/9	R 202H	-	-	9
502/CMC/9	RG 502	+	-	9
502/SPAN/9	RG 502	-	+	9
504/CMC/9	RG 504	+	-	9
504/CMC/18	RG 504	+	-	18
504/SPAN/9	RG 504	-	+	9
504/SPAN/18	RG 504	-	+	18

CMC indicates carboxymethylcellulose.

Microsphere characterization

Surface morphology

Microsphere shape and appearance were evaluated by scanning electron microscopy (SEM) (Scanning Electron Microscope Jeol JX 840-A JEOL Ltd, Tokyo, Japan). Samples for SEM analysis were prepared by gold-sputtering the microspheres in an argon atmosphere.

Size distribution

Particle size analysis was performed by the light diffraction method with a Coulter apparatus, model LS230 (Coulter Corp, Hialeah, FL); this instrument works on laser diffraction optics and on another system based on polarized light of 3 wavelengths termed PIDS. The size range of the LS230 version is from 0.04 µm to 2000 µm. The samples of microspheres were suspended in filtered water, sonicated for 30 seconds, and subsequently analyzed. Three analyses were performed for each sample of microspheres.

Drug content

The amount of clodronate loaded into microspheres was determined by ultraviolet spectrophotometry (UV Spectrophotometer DU-7 500 Beckman, Fullerton, CA) [13]. Twenty milligrams of microspheres were dissolved in 5 mL of CH_2Cl_2, and the water-soluble drug (clodronate) was extracted with 5 mL of 1.5×10^{-3}M HNO_3. After solvent evaporation by a rotavapor (IKA Laboratory Technology, Staufen, Germany), the aqueous medium was recovered and complexed with a $CuSO_4$ solution. The clodronate-Cu^{2+} complex was analyzed at a 238-nm wavelength, and the concentration was determined using a calibration curve between 10 and 50 µg/mL of clodronate in Cu^{2+} (2.5×10^{-3}M) in 1.5×10^{-3}M HNO_3. Drug content was assayed in triplicate.

In vitro drug release

In vitro release tests were carried out by a dialysis method: 30 mg of microspheres were placed into dialysis tubs (cutoff 12 000-14 000 D). They were then were poured in 5 mL of water in bottles closed by screw stoppers at 37°C and shaken twice a day. At fixed time intervals, 1 mL of release medium was removed and replaced with 1 mL of fresh water to

maintain sink conditions. The same method was used for dissolution tests on clodronate-free drug as a control. Each sample withdrawn was added to a $CuSO_4$ solution in nitric acid, and the clodronate concentration was monitored spectrophotometrically during the formation of the clodronate-Cu^{2+} complex using the same calibration curve employed in the drug content analysis.

RESULTS AND DISCUSSION

W/o/w double-emulsion process is the method traditionally used to encapsulate water-soluble macromolecules as peptides and proteins into PLGA polymers; when the drug is a hydrophilic compound of low molecular weight-such as clodronate-this technique has to be modified to obtain well-formed microspheres with satisfactory drug loading. As a result, because of the high diffusion rate of the drug to the external aqueous phase in microsphere forming process, which could lead to microparticles with very low payloads. Results of SEM analyses suggest a relationship between microsphere morphology and the microsphere manufacturing process (Figure 1).

The first interesting parameter influencing microsphere morphology was the volume ratio between the aqueous and organic phases of the inner

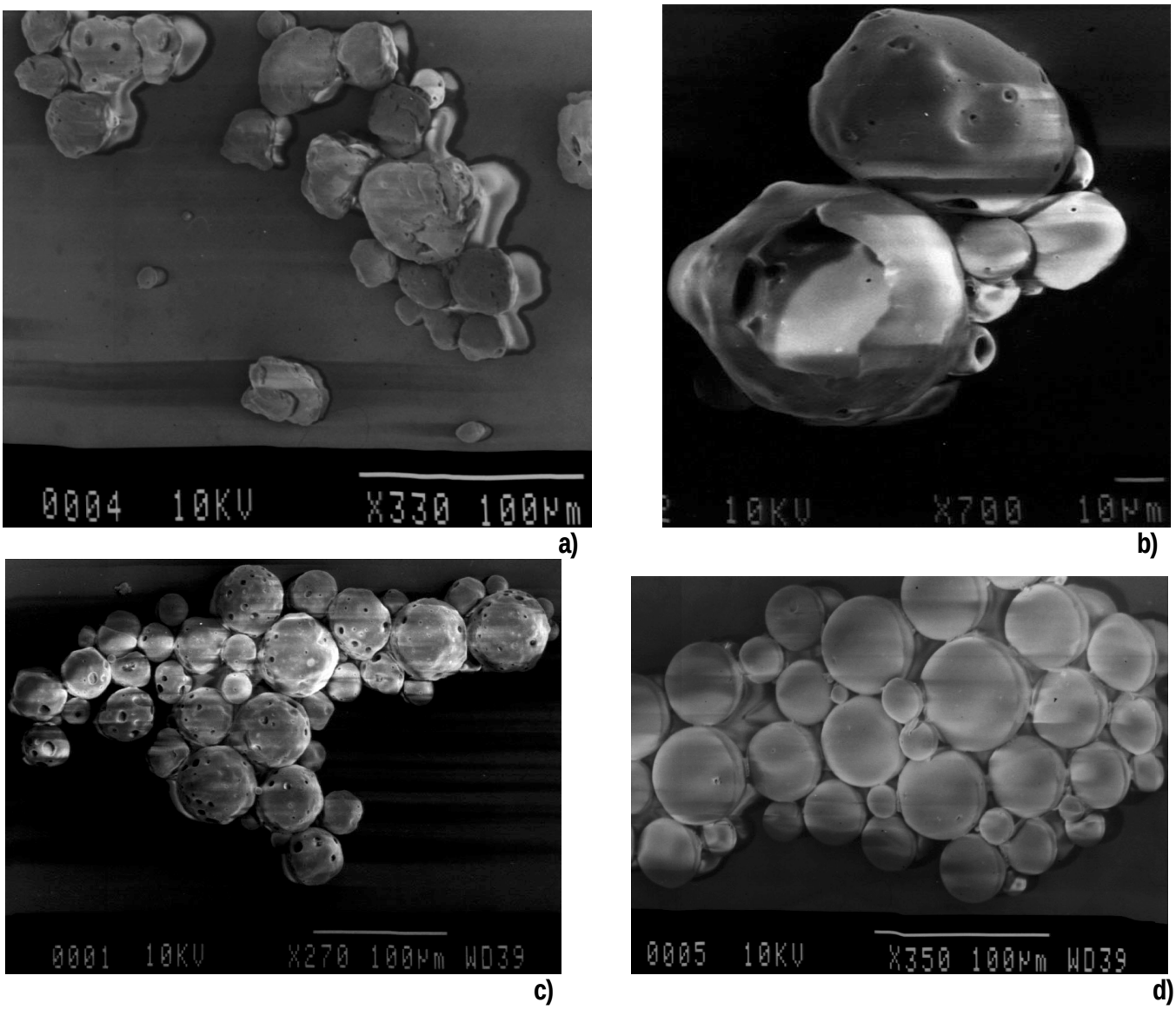


Figure 1. Photomicrographs of RG 503 microparticles. Obtained with: A, 1:5 inner water/oil emulsion volume ratio; B, temperature-raising rate 10°C/min; C, 1:16 inner water/oil emulsion volume ratio and temperature-raising rate 1°C/min; and D, 1:16 inner water/oil emulsion volume ratio, temperature-raising rate 1°C/min and NaCl 2% wt/vol in the external aqueous phase.

water/oil emulsion used in their preparation; the higher this ratio, the more difficult it was to obtain well-formed microspheres. Figure 1A shows microspheres made using 1:5 inner water/oil emulsion volume ratio. The sample revealed that microspheres were aggregated and had an irregular shape.

Low temperature-raising rates in the solvent evaporation step were also important as a means of avoiding a local explosion inside the droplets, which would lead to a disruption of the microsphere structure, as shown in Figure 1B (temperature-raising rate 10°C/min).

When the inner water/oil emulsion volume ratio is lowered to 1:16 and the temperature-raising rate is maintained at 1°C/min, microspheres appear spherical but have a porous surface structure (Figure 1C). The high microsphere surface porosity was possibly caused by a fast transfer of the inner aqueous phase containing clodronate, which occurred as soon as the second emulsification step took place; unfortunately, as a result of the high clodronate water solubility, these 3 batches of microspheres resulted in very low loadings (drug content less than 1%). The presence of additives, such as electrolytes, in the external aqueous phase was advantageous in first obtaining a stable w/o/w emulsion and then obtaining homogeneous microparticles with high drug loading [14]. Therefore, NaCl was selected as a suitable additive that might act as physical barrier due to its high osmotic pressure. Another possibility is that the NaCl competed with the surfactant for water molecules at the water/oil interface, generating a rigid interfacial layer that could be a more effective mechanical barrier to drug transfer. SEM of batch 503/9 showed that microspheres prepared with a 1:16 inner water/oil emulsion volume ratio and NaCl in the external aqueous phase, and subjected to a slow evaporation process of the organic solvent, appear spherical with a smooth surface (Figure 1D). Moreover, the presence of electrolytes led to microspheres with good drug loading (Table 2; batch 503/9, drug content 5.73%). This preliminary investigation confirmed that the presence of NaCl was necessary to obtain microspheres with good morphology and a high drug content [14].

The process parameters were then selected. Inner water/oil volume ratio 1:16, temperature-raising rate in the solvent evaporation step 1°C/min, and 2% wt/vol NaCl in the external aqueous phase were employed in the preparation of microspheres with all polymers assessed in the study. Table 1 shows the batches of microspheres and the drug loading.

Well-formed microspheres were obtained with all polymers used (data not reported). Granulometric analyses performed on these batches suggested that microsphere size increased with increasing molecular weight of the polymer. Figure 2 shows, as an example, size distribution of batches 502/9 and 504/9. Microspheres prepared with stabilizing agents showed the same behavior.

Smaller microparticle size was revealed in the 2 batches with the highest theoretical drug content (batches 504/CMC/18 and 504/SPAN/18, both having $d_{50\%} < 6$ µm, $d_{90\%} < 15$ µm); these results were also confirmed by SEM photomicrograph, shown as an example in Figure 3.

Table 2 shows the yields of production, drug contents, and encapsulation efficiencies of the batches of clodronate-loaded microspheres. Good yields (not less than 60%) were obtained. Drug encapsulation efficiencies ranged between 49% and 75% depending on the polymer employed (Table 2); it is interesting to note that the amount of active agent encapsulated did not seem to depend on polymer molecular weight. However, when copolymers with the same 50:50 molar composition and same MW are compared, the more hydrophilic polymer entrapped a higher amount of drug (batch 503H/9 compared to batch 503/9).

Table 2. Parameters Related to Efficiency of Microsphere Manufacturing Process

Batch Number	Yield of Production (%)	Actual Drug Content (%)	Encapsulation Efficiency (%)
502/9	76.36	5.22	58.03
503/9	76.52	5.73	63.70
503H/9	76.15	6.75	74.98
504/9	78.18	5.27	58.61
752/9	74.54	4.59	51.01
755/9	67.27	6.35	70.60
202H/9	78.18	5.39	59.94
502/CMC/9	77.67	4.70	52.22
502/SPAN/9	63.52	4.46	49.55
504/CMC/9	68.64	5.25	58.33
504/CMC/18	78.03	8.92	49.55
504/SPAN/9	60.00	5.66	62.89
504/SPAN/18	90.70	9.69	53.83

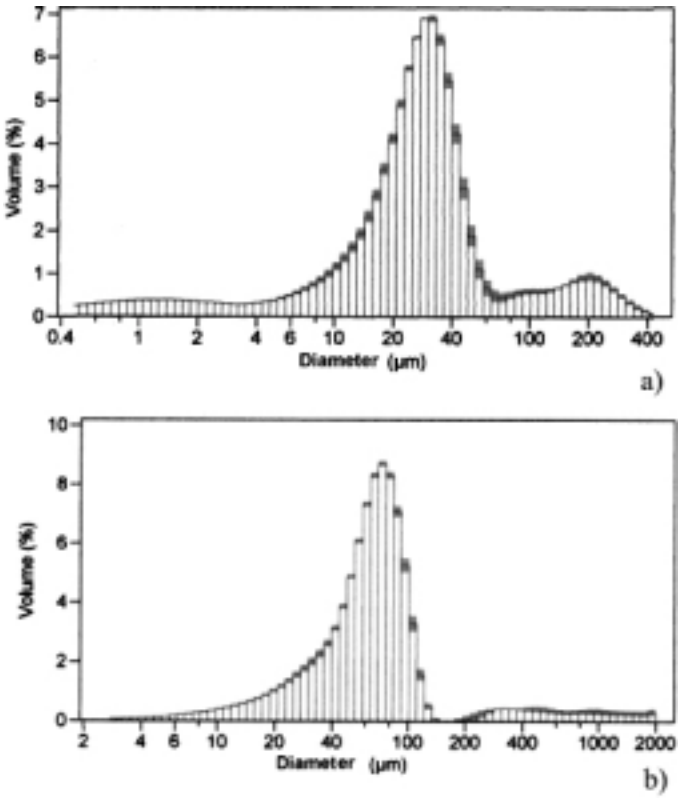


Figure 2. Particle-size distribution of batches. A, 502/9 ($d_{50\%}$ 26.85 μm, $d_{90\%}$ 93.58 μm). B, 504/9 ($d_{50\%}$ 64.42 μm, $d_{90\%}$ 113.1 μm).

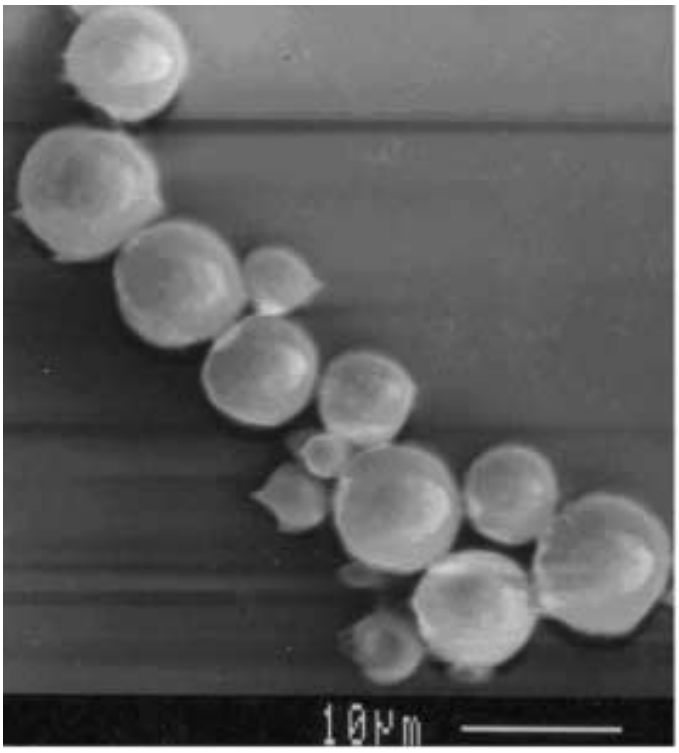


Figure 3. Scanning electron microscope photomicrograph of batch 504/SPAN/18.

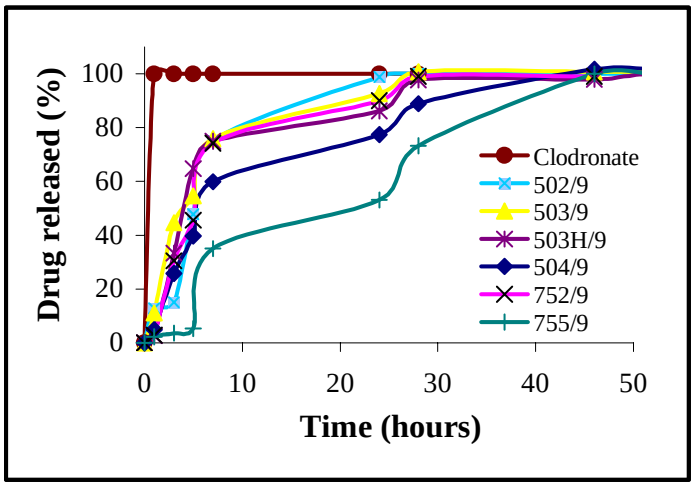
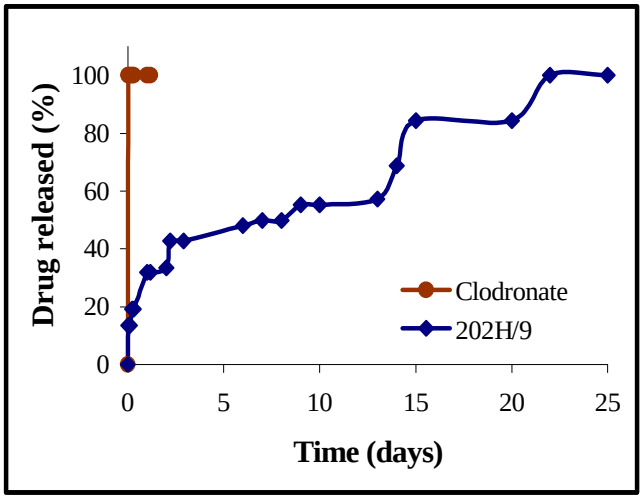


Figure 4. In vitro release profile of clodronate from microspheres produced with poly(D,L-lactide-co-glycolide) copolymers (n = 3, SD < 5%).

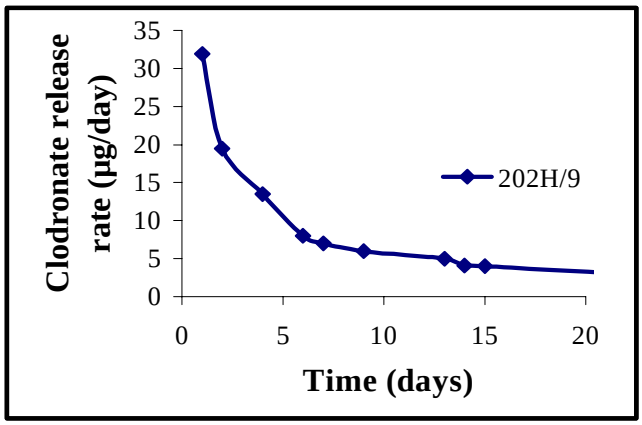
The increased viscosity of the inner aqueous solution obtained with the CMC addition is reported by some authors as method to raising drug loading when microspheres are prepared with a w/o/w multiple emulsion method [15]; on the contrary, the results reported in Table 2 show that CMC did not affect microsphere drug content (batch 502/CMC/9 compared to batch 502/9 and batch 504/CMC/9 compared to batch 504/9). Neither encapsulation efficiency was affected by increasing the theoretical drug loading (batches 504/CMC/18 and 504/SPAN/18 compared to batches 504/CMC/9 and 504/SPAN/9).

Results from in vitro release tests show that clodronate release from all copolymer microspheres was complete in about 48 hours, as shown in Figure 4. The drug release rate from microspheres seems to be affected mainly by copolymer MW. A significantly slower drug release rate is shown from batch 504/9 and batch 755/9, compared to batches 502/9 and 752/9, respectively.

Clodronate release from batch 202H/9 has a different behavior because of polymer characteristics (Figure 5A). In fact, R202H is a PDLLA homopolymer with higher hydrophobicity than copolymers. The drug release is the result of a mixed process of polymer diffusion and erosion according to the Higuchi equation modified for biodegradable polymers with a homogeneous mechanism (Figure 5B) [16]. Clodronate release was completed in about 20 days (Figure 5A).



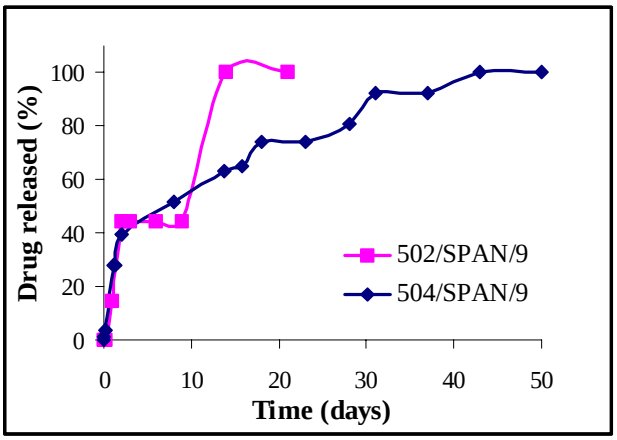
a)



b)

Figure 5. A, In vitro release profile of clodronate from poly(D,L-lactide) microspheres (batch 202H/9) (n = 3, SD < 5%). B, Fitting of clodronate release rate profile from batch 202H/9 (r = 0.994).

The change of microsphere composition by adding a surfactant such as Span 20, or of a viscosing agent such as CMC, substantially modified the drug release profile. The addition of stabilizing agent Span 20 prolonged clodronate release for up to 20 days using the PLGA copolymer RG 502 (batch 502/SPAN/9) and for beyond 45 days using RG 504 (batch 504/SPAN/9), as shown in Figure 6A. This behavior is probably the result of a better drug dispersion in the polymeric matrix with respect to batches prepared without emulsifier and to a more rigid barrier against water penetration and drug diffusion. Drug release from batch 502/SPAN/9 was faster than from batch 504/SPAN/9 because RG 502, being a low molecular-weight copolymer, erodes more quickly than RG 504



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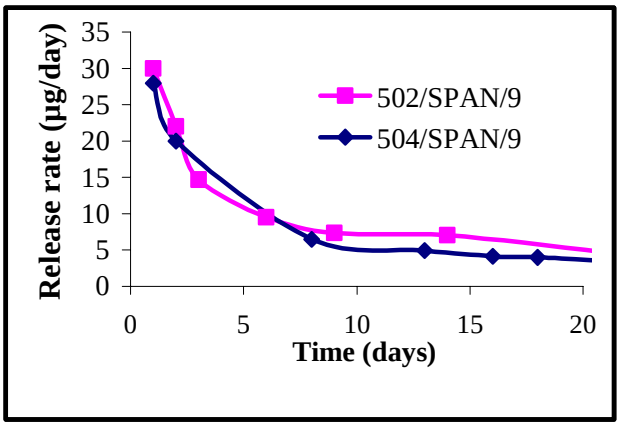
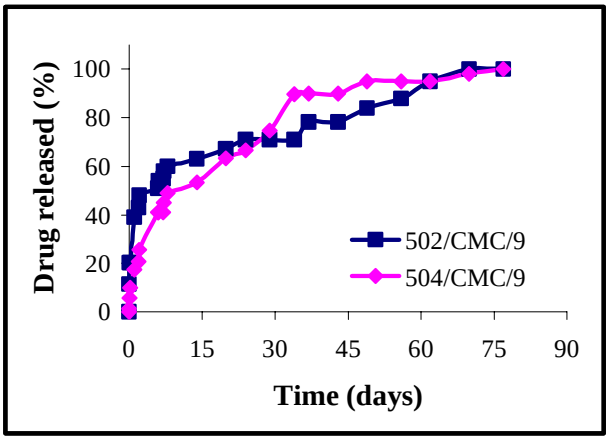


b)

Figure 6. A, In vitro release profile of clodronate from microsphere batches 502/SPAN/9 and 504/SPAN/9 (n = 3, SD < 5%). B, Fitting of clodronate release rate profiles from batches 502/SPAN/9 (Higuchi fitting, r = 0.996) and 504/SPAN/9 (Weibull fitting, r = 0.999).

copolymer. For this reason, drug release from RG 502 depended both on diffusion through the polymer and on erosion of the polymer; this hypothesis was confirmed by fitting analyses of release rate data according to the modified Higuchi equation used above (R = 0.996) (Figure 6B). The drug release rate from RG 504 copolymer cannot be fitted to the Higuchi equation, probably because of the higher molecular weight of the copolymer RG 504, which led to a longer erosion time. In fact, the release rate profile can be fitted to the Weibull model (R = 0.999) (Figure 6B). The presence of CMC in microspheres (batches 502/CMC/9 and 504/CMC/9) prolonged release for almost 3 months, both for RG 502 and RG 504 copolymers (Figure 7A); the release rate profile can be fitted to the Weibull model, reported in the Figure 7B.



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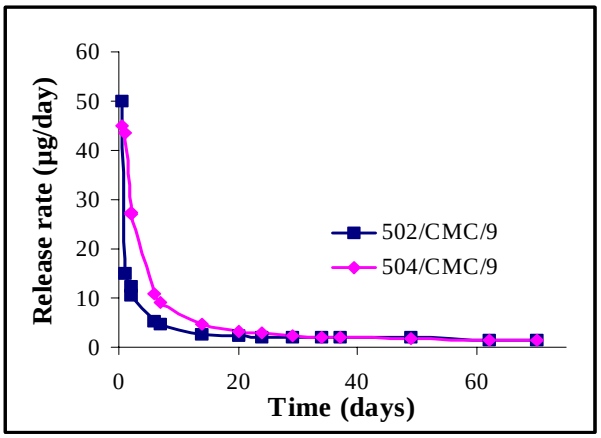

b)

Figure 7. A, In vitro release profile of clodronate from microsphere batches 502/CMC/9 and 504/CMC/9 (n = 3, SD < 5%). B, In vitro clodronate release rate profiles from batches 502/CMC/9 and 504/CMC/9 versus time (Weibull fitting, r = 0.999).

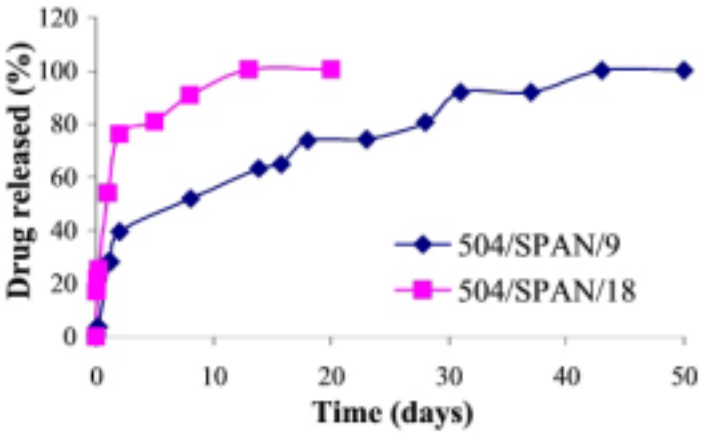

Figure 8. In vitro release profile of clodronate from microsphere batches 504/SPAN/9 and 504/SPAN/18 (n = 3, SD < 5%).

These results suggest that clodronate was released mainly by diffusion from the PLGA/CMC microspheres; hence, drug release behavior was governed mainly by the presence of CMC. Figure 7A shows that the release profiles of batches 502/CMC/9 and 504/CMC/9 were almost identical.

The batches produced with higher theoretical drug content (batches 504/CMC/18 and 504/SPAN/18) showed significantly faster release in comparison to batches 504/CMC/9 and 504/SPAN/9; in fact, the drug release was completed in 1 week for batch 504/CMC/18 and in 2 weeks for batch 504/SPAN/18. This result could be related to an increased number of channels formed by diffusion of the hydrophilic drug. Figure 8 shows, for example, the drug release profiles of batches 504/SPAN/9 and 504/SPAN/18.

CONCLUSIONS

The results obtained in this study show that clodronate can be successfully entrapped in PLGA and PDLLA microspheres using the w/o/w emulsion solvent evaporation method by adding NaCl to the external aqueous phase. CMC and Span 20 can be usefully employed to modify clodronate release from microspheres. Drug release from microparticulate systems can be modulated from 48 hours up to 3 months by selection of polymer molar composition, its molecular weight, and a suitable additive.

These "in vitro" results correlate to an "in vivo" administration of these drug delivery systems. Moreover, they can overcome the limitations of parenteral and oral administration of clodronate in treating bone diseases.

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