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Preparation of polylactide-co-glycolide and chitosan hybrid microcapsules of amifostine using coaxial ultrasonic atomizer with solvent evaporation

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Abstract

The objective of this study was to evaluate the effect of various processing and formulation factors on the characteristics of amifostine hybrid microcapsules. Amifostine-loaded hybrid microcapsules were prepared using PLGA and chitosan. In short, amifostine powder was dissolved in de-aerated water with or without chitosan. The amifostine solution was later emulsified into PLGA solution in dichloromethane containing phosphatidylcholine. The resultant emulsion was fed through the inner capillary of a coaxial ultrasonic atomizer. The liquid fed through the coaxial outer capillary was either water or chitosan solution. The atomized droplets were collected into PVA solution and the droplets formed microcapsules immediately. The hybrid microcapsules prepared with chitosan solution only as an outer layer liquid showed the maximum efficiency of encapsulation (30%). The median sizes of all three formulations were 33–44 μm. These formulations with chitosan showed positive zeta-potential and sustained drug release with 13–45% amifostine released in 24 h. When chitosan was incorporated into inner as well as outer liquid layers, the drug release increased significantly, 45% (compared with other formulations) released in 24 h and almost 100% released in 11 days. Hybrid microcapsules of amifostine showed moderately high efficiency of encapsulation. The cationic charge (due to the presence of chitosan) of these particles is expected to favour oral absorption and thus overall bioavailability of orally administered amifostine.

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Introduction

Amifostine, also known in the literature as ethiofos, WR-2721, and the trade name Ethyol, is an organic thiophosphate prodrug that serves as a cytoprotective agent useful in cancer chemotherapy and radiotherapy (Ali & Al Moundhri 2006; Bensadoun et al 2006; de Castro & Federico 2006). It has been studied extensively as a protector of normal tissue against the damaging effects of ionizing radiation and chemotherapy and was approved by the US Food and Drug Administration in 1997 as a parenteral formulation. Amifostine is rapidly cleared from the body and has a short distribution half-life of 0.9 min when administered as a bolus dose or as a 15-min intravenous infusion (Bonner & Shaw 2002; Cassatt et al 2002; Schuchter et al 2002). In an attempt to search for an alternative route of administration, several investigators have found that when compared with intravenous administration, subcutaneous administration provided a more effective dosing regimen, both in terms of a reasonable AUC and decreased toxicity (Godette 2001; Anne & Curran 2002; Bonner & Shaw 2002; Koukourakis et al 2002). Attempts have also been made to develop various formulations of amifostine such as transdermal patches (Lamperti et al 1990), subcutaneous implants (Srinivasan et al 2002), pulmonary inhalers and oral sustained-release microspheres (Fatome et al 1987). Despite these efforts, a non-injectable formulation of amifostine for clinical use is still not available.

In our attempts to develop an oral formulation of amifostine, we recognized the fact that encapsulation of therapeutically active compounds in envelopes of biodegradable polymers for controlled drug delivery has become a well-established technology. During the past

three decades, controlled-release microcapsules of numerous therapeutic agents have been developed and received worldwide marketing approval (Lewis 1990). Using either solvent evaporation (Pamujula et al 2004a) or spray drying (Pamujula et al 2004b) technique, we previously reported on the preparation of and characterization of loaded poly (lactide-co-glycolide) (PLGA) particles. PLGA is a biodegradable and biocompatible polymer and has been extensively used in encapsulation of a wide variety of drugs (Mandal 1998; Rajeev 2000; Homayoun et al 2003; Graves et al 2004; Pamujula et al 2004b). However, since these earlier amifostine/PLGA preparations required optimization, this report describes the results of our attempts at such optimization using modified coaxial atomization and chitosan. Following is a brief rationale for each of these two new approaches.

Compared with solvent evaporation and traditional spray drying (the two techniques used earlier by us), coaxial atomization is a relatively newer technique with certain advantages. Recently, it has been used to produce mono dispersed micro- and nanocapsules of several drugs (Loscertales et al 2002; Yeo et al 2003; Park & Yeo 2004; Yeo & Park 2004a; Yeo et al 2004). For example, using this technique, Yeo & Park (2004b) successfully encapsulated lysozyme into PLGA without any loss of lysozyme functional integrity. In a typical coaxial atomization procedure, two liquid phases are injected simultaneously at appropriate flow rates through two concentrically located tubes. Upon reaching the highly narrowed common tip of the two tubes (Figure 1), the concentric layered liquid phases are atomized and form fine droplets. These droplets are sprayed into a proper third liquid phase where the reservoir-type particles containing the drug are precipitated. The particles are formed by mid-air collision of the droplets from the two liquid phases followed by solvent exchange between the inner liquid containing the drug and the solvent in the outer liquid containing the polymer (Graves et al. In press).

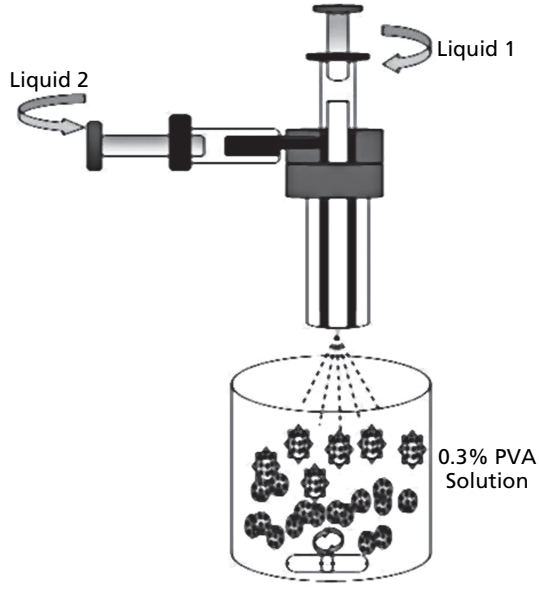

Figure 1 Schematic presentation of coaxial ultrasonic atomizer set-up.

Finally, chitosan, which is derived by deacetylation of chitin, a cellulose-like biopolymer from exoskeleton of crustaceans, has been used frequently in the formulation of pharmaceuticals because of its biocompatibility and biodegradability (Chopra et al 2006; George & Abraham 2006). Furthermore, chitosan is positively charged and hence binds readily to negatively charged surfaces, such as mucosal membranes, thereby acting as a bioadhesive. The same property also leads to the enhancement of the transport of polar drugs across epithelial surfaces.

Materials and Methods

Materials

The copolymer poly(DL-lactic/glycolic acid) (PLGA; 50:50; RG 502; inherent viscosity 0.2 dL g^{-1}, MW 14 000) was obtained from Boehringer Ingelheim (Ingelheim, Germany). Amifostine, low-molecular-weight chitosan (20–200 cP, 1% in 1% acetic acid), L-α phosphatidylcholine, polyvinyl alcohol (PVA, MW 30 000–70 000, 98–99% hydrolysed), dichloromethane and chloroform were obtained from Sigma Aldrich (St Louis, MO, USA).

Coaxial atomization

A schematic diagram of the instrument set-up is presented in Figure 1. The ultrasonic atomizer was equipped with a spray drier nozzle, 60 KHz frequency; dual micobore liquid feed (orifice diameter 0.020–0.040 inch); and a broad band ultrasonic generator (Sono-Tek, Milton, NY, USA). Nozzle power level was set at 4.8 W. Both liquid phases, the outer and the inner, were fed simultaneously through a syringe pump at a flow rate of 3 mL min^{-1}.

Using the instrument set-up described, amifostine/PLGA particles were prepared by feeding amifostine and PLGA as an emulsion through the inner capillary with or without chitosan. The outer phase was either simply water or an aqueous solution of chitosan. This set-up resulted in a 2×2 factorial design giving four separate formulations, which differed from each other in regard to whether the chitosan was present or absent in each of the inner and outer tubes (Table 1).

In short, 20 mg amifostine powder was dissolved in 0.1 mL de-aerated water with or without 0.25% chitosan. The amifostine solution was later emulsified into 9.9 mL PLGA solution (3%) in dichloromethane containing 0.05 mL phosphatidylcholine (8 mg mL^{-1}). Phosphatidylcholine is an emulsifier very commonly used in the preparation of PLGA microcapsules (Mandal et al 2002). The resultant emulsion was fed through the inner capillary tube of a coaxial ultrasonic atomizer while 10 mL of water or 0.25% chitosan was fed through the outer capillary. The liquids were sprayed into a 250-mL beaker containing 100 mL of 0.3% PVA solution where they immediately coalesced into microcapsules. The distance between the atomizer tip and the PVA solution was maintained at approximately 3 cm. The mixture was stirred

Table 1 Description of batch formula of the amifostine hybrid microcapsules

Formulation	Liquid 1 (10 mL) PLGA (300 mg) in DCM	Amifostine (20 mg) dissolved in	Liquid 2 (10 mL)
A	9.9 mL	0.1 mL Water	Water
B	9.9 mL	0.1 mL Water	0.25% Chitosan
C	9.9 mL	0.1 mL 0.25% Chitosan	Water
D	9.9 mL	0.1 mL 0.25% Chitosan	0.25% Chitosan

DCM, dichloromethane.

magnetically at 500 rev min^{-1} for 3 h at room temperature to allow complete evaporation of the solvent. Particles were finally collected by centrifugation at 13 000 rev min^{-1} and washed four times (50 mL each) with de-ionized water to remove any residual PVA on the surface of the particles. These particles were freeze-dried ($-20°C$; 6×10^{-4} mbar) (Labconco, Kansas City, KS, USA) to obtain a free-flowing powder. Each formulation was prepared in triplicate.

Particle size and morphology

Particle size and distribution was determined by a Mastersizer 2000 laser scattering device (Malvern Instruments Ltd, Malvern, UK). This technique measures the size of particles dispersed in a medium by the scattering pattern of a laser light shown through the medium. The size calculations assume the presence of spherical particles, which was confirmed via scanning electron microscopy (SEM), described later. The samples were analysed in a water medium and the Frauenhofer method was utilized to calculate the size distributions. For each sample, a background run of de-ionized water was performed. A sample of particles (5 mg) was added to the de-ionized water in a small volume sample dispersion unit. After subtraction of the background, the particle size distribution calculation was performed. Each measurement was performed in triplicate.

The surface and internal morphology of the particles were examined by a Hitachi 3000N variable pressure SEM (Hitachi, Gaithersburg, MD, USA) following mounting of samples on metal stubs. The analytical parameters included an accelerating voltage of 10 KV, a working distance of 13.5 mm and a vacuum of 40 Pa in variable pressure mode. Since the samples were analysed in variable pressure mode, the backscatter detector BSE2 was used. The internal morphology was evaluated following freeze-fracture of dried particles. The dried samples were rapidly frozen at $-196°C$ using liquid nitrogen and sectioned immediately using a surgical blade.

Zeta-potential

The zeta-potential of particles was measured using a Malvern Zetasizer 2000 (Malvern Instruments, Malvern, UK). The experiments were performed in de-ionized and de-aerated water, and all measurements were performed in triplicate.

Glass transition temperature (Tg)

To characterize their physical state after encapsulation, differential scanning calorimetry (DSC) of amifostine, PLGA, chitosan and amifostine/PLGA particles was performed using a DSC 2920 (TA Instruments, New Castle, DE). About 5 mg of a sample was weighed, crimped into an aluminium pan and analysed using a single heating scan from $-50°C$ to $250°C$ at a scanning rate of $3°C\ min^{-1}$. The glass transition temperature (Tg) was calculated using TA universal analysis software by extrapolating the linear portion of the thermograms above and below the glass transition point and determining the midpoint.

Amifostine content of particles

For each formulation, a 5-mg sample was dissolved in 200 μL of dichloromethane. Ten millilitres of 0.15% Tween 80 was added to the solution and the resultant precipitate was removed by ultracentrifugation (35 000 rev min^{-1} at 15°C) and the amifostine concentration was determined by HPLC. This method resulted in 99% recovery of amifostine.

HPLC analysis of amifostine

The analysis of amifostine was performed using a rapid and sensitive HPLC method (Pamujula et al 2004b). The chromatographic system consisted of a Waters Model 600 programmable solvent delivery module, Waters Model 717plus auto sampler (Waters, Milford, MA, USA) and a BAS LC-44 Model MF-9000 electrochemical detector (Bioanalytical Systems, West Lafayette, IN, USA). The chromatography was performed under the following specific conditions: column, μ bondpack C-18 (Waters 10 μm, 3.9×300 mm); mobile phase, 96 mM monochloroacetic acid at pH 2.8; 3mM hexane sulfonic acid, 3.5% acetonitrile; and 1 μM 2-mercaptoethylamine. The mobile phase was vigorously purged with helium gas for 15 min before use; the flow rate was 1 mL min^{-1}. The detector (Hg/Au electrode) oxidation potential was set at $+0.2$V; the injection volume was 20 μL. Standard calibration curves ($r^2 > 0.99$) for amifostine in the range 0.125–8.000 μg mL^{-1} concentrations were prepared. The concentration of amifostine in each sample was determined by intrapolating the peak height to the amifostine standard curve and each sample was analysed in triplicate.

In-vitro dissolution studies

Dissolution studies of microcapsules were performed by measuring the percentage of amifostine remaining within the microcapsules at a predetermined sampling time. For each formulation, 30 samples (5 mg each) were placed in 1.5-mL tubes and incubated in 1 mL of phosphate buffer (pH 7.4; 0.1 M) with constant shaking (20 rev min^{-1}) at 4°C. The studies were conducted at this temperature, rather than the physiologic temperature (37°C), because amifostine is not stable over 24 h at the physiologic temperature. The sink conditions were maintained throughout the dissolution study because amifostine is freely soluble in phosphate buffer. The total amount of amifostine remaining in microcapsules was determined at ten different time periods ranging from 1 h to 11 days. At each specified sampling time, three samples were filtered through a 0.2 μm Millipore filter paper, freeze dried, and extracted for amifostine using the method described earlier. The amount of amifostine in each sample was determined by HPLC.

Statistical analysis

Statistical analysis was performed using the SigmaStat, version 2.0 software package (SPSS Inc., Chicago, IL). The efficiency of encapsulation, glass transition temperature (Tg), particle size and zeta-potential data from four formulations (A–D) were compared separately using one-way analysis of variance. Cochran's test was used to determine the homogeneity of variance of the data. $P < 0.05$ was considered as evidence of a significant difference. In the event of significant difference, the mean values were further compared using Student–Newman–Keuls multiple range test (SNK) to determine which formulation was significantly different to the others.

Results and Discussion

SEM pictures of the particles from various formulations are shown in Figure 2. While particles from all four formulations

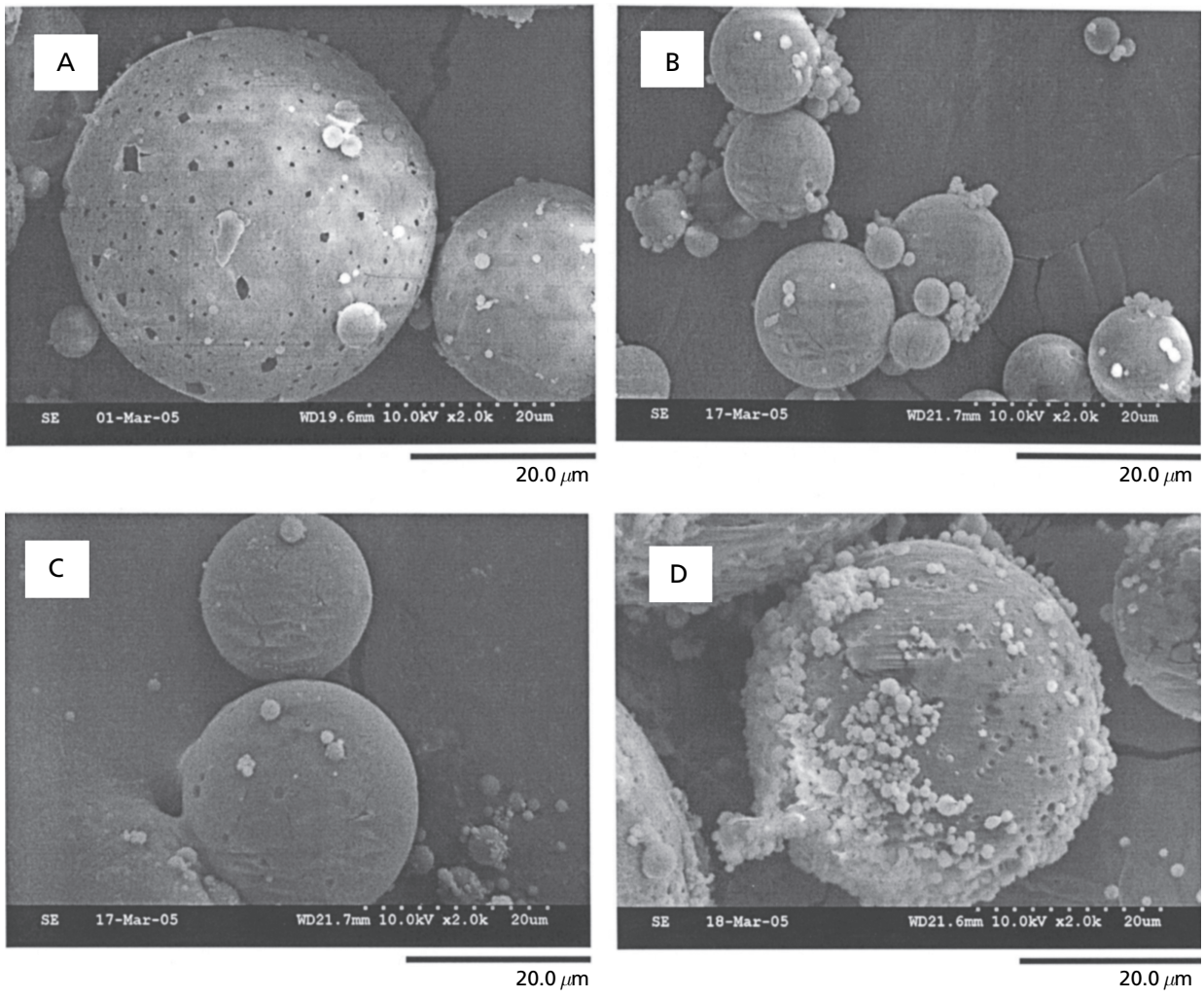

Figure 2 Typical SEM photographs of formulations A, B, C and D (A, B, C and D, respectively).

were spherical, particles from formulation D showed accumulation of smaller microcapsules on the surface of the larger particles. In addition, particles from formulations A and D also showed greater pitting on the surface than the other two formulations. The presence of this pitting on the surface may be due to the evaporation of the trapped organic solvent during the in-water drying process. The SEM pictures shown in Figure 2 for formulations A and D are relatively larger in size than the other two batches. As a result, these two microcapsules had larger amount of dichloromethane trapped within the matrix, which crossed the surface membrane during the in-water drying and left the pitted morphology. A comparison of the internal morphology of the formulations showed no apparent differences. All four formulations consisted of two different internal morphologies: particles with a honeycomb structure (Figure 3A), and those with a honeycomb structure with a large cavity at the centre (Figure 3B). The honeycomb structures are known to form due to the formation of w/o emulsion (aqueous amifostine/dichloromethane PLGA) in the inner liquid phase followed by evaporation of dichloromethane during the solvent evaporation. The presence of a large cavity at the centre of some of the microcapsules is also likely due to the known cavitation effect of ultrasound (Yang et al 2007).

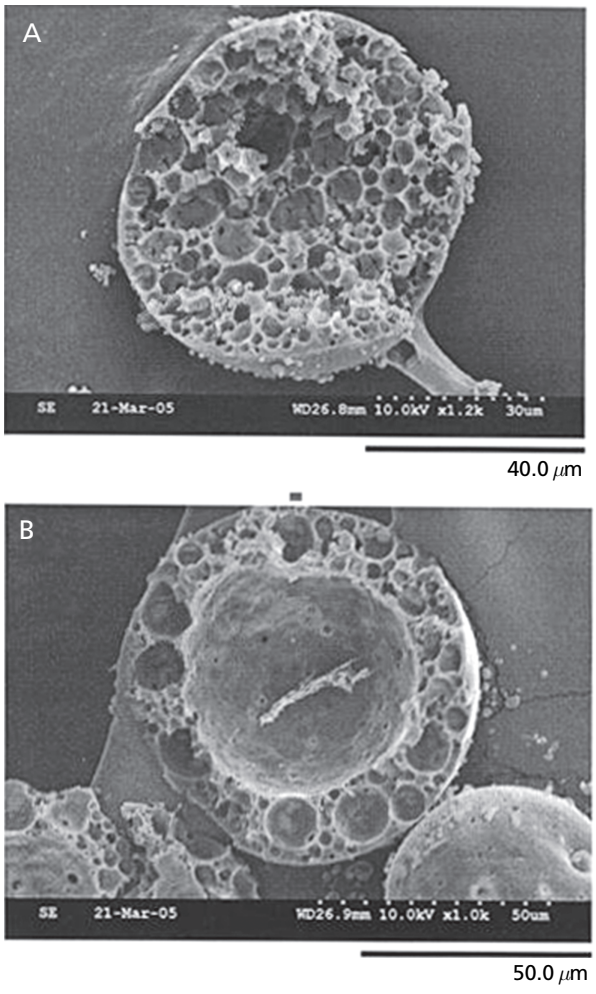

Figure 3 Typical cross sectional view (SEM photographs) of the formulations. Two types of internal structures were observed: I, honeycomb structure and II, honeycomb structure with a large cavity at the centre. Only one photograph of each type is included.

In regard to particle size, formulations B and D prepared with 0.25% chitosan in the outer liquid phase and formulation A with no chitosan showed unimodal particle size distribution (Table 2). However, formulation C, which contained 0.25% chitosan as the internal layer, showed a bimodal pattern (figure not included). Further analysis of the distribution pattern of this batch also revealed that the bimodal distribution was due to the presence of a higher percentage of smaller particles. While the median particle size of formulation D was highest (44.2 μm), it was formulation B that contained the greatest volume comprising both smallest and the largest particles.

Particle charge plays an important role in determining the absorption characteristics of particles following oral administration. Typically, positively charged particles are absorbed better than the neutral or negatively charged particles (El-Shabouri 2002). As expected, all formulations containing chitosan showed (Table 2) positive zeta-potential ($+6.5$ mV, $+4.8$ mV and $+3.6$ mV respectively for formulations B, C and D) while formulation A with no chitosan showed a high negative zeta-potential (-28.6 mV). Given these results, we will continue to use chitosan in all future optimization efforts.

A comparative analysis of Tg of the four formulations, PLGA, chitosan and amifostine showed (Table 2) that the Tg of formulations B and D were 7–8°C lower than that of formulations A and C, and 3°C lower than that of PLGA (RG 502, Tg: 35.5°C). This decrease in Tg may be due to the presence of an excess amount of chitosan on the particle surface in formulations B and D, causing the entrapment of residual solvent in the core of the particle. This residual solvent causes a plasticizing effect on the PLGA, resulting in a significant decrease ($P < 0.05$) in the Tg. On the contrary, while formulation C also contained chitosan, because the chitosan was in the inner liquid phase, it did not result in solvent entrapment. The 2–3°C increase in Tg was the result of the anti-plasticizing effect of the drug on PLGA. None of the formulations showed any characteristic peak for phosphatidylcholine because the amount of this emulsifier in the matrix is insignificant. None of the formulations showed any peak corresponding to the melting endotherm of amifostine (141°C). When a drug is mixed with a polymer during the preparation of microcapsules, there are generally three probabilities (Mandal et al 2002): firstly, the drug and polymer form a solid solution, in which case there is a shift in the position of the Tg of the polymer due to the plasticizing/anti-plasticizing effect of the drug, and the endotherm for the melting of the drug is absent in the microcapsules; secondly, the drug is dispersed in the polymer in an amorphous form, in which case the Tg of the polymer is not affected and the melting endotherm for the drug is absent in the microcapsules; and thirdly, the drug is dispersed in the polymer in a crystalline form; there is no change in the position of the Tg of the polymer or the melting endotherm of the drug. In the case of the amifostine-loaded hybrid microcapsules prepared

Table 2 Physicochemical properties of the amifostine hybrid microcapsules

Formulation	Efficiency of encapsulation (%) (s.e.m.)	Tg (°C) (s.e.m.)	Size (volume average) (μm) (80% confidence)	Zeta-potential (mV) (s.e.m.)
A	1.0 (0.1)	37.2 (2.4)	37.9 (13.0–79.0)	−28.6 (1.6)
B	30.0 (3.7)	30.9 (1.4)	33.2 (6.1–73.7)	+6.5 (1.5)
C	24.0 (2.1)	38.6 (1.0)	35.4 (4.3–170.3)	+4.8 (1.4)
D	9.3 (0.5)	30.8 (1.4)	44.2 (11.3–86.9)	+3.6 (1.6)
Results of SNK test	B > C > D > A	C = A > B = D	D > A > C = B	B > C = D > A

s.e.m., standard error of mean, n = 6. SNK, Student–Newman–Keul's multiple range test.

in this study, it appears that the drug and polymer formed a solid solution, with the drug having an anti-plasticizing effect. This was observed in formulations A and C. In formulations B and D, this anti-plasticizing effect could not be observed because the plasticizing effect of residual solvent countered it, resulting in an overall decrease in Tg.

A comparison of the efficiency of encapsulation of four formulations indicated a significance difference ($P < 0.05$) among them (Table 2). Formulation B showed maximum efficiency followed by formulations C, D and A, respectively. It is clear that the presence of chitosan has a significant, but variable, positive effect on encapsulation of amifostine. However, because of lower encapsulation efficiency when chitosan was either absent or present in both the inner and the outer liquid phases, this approach will not be utilized in future optimization. It seems that the presence of chitosan in the outer liquid phase may prolong the particle hardening time resulting in greater loss of amifostine into the external PVA solution.

These results clearly demonstrate that while the presence of chitosan in one of the two liquid phases is beneficial, its presence in both phases is not. For example, the presence of chitosan in both phases reduced encapsulation efficiency, increased the median particle size, caused greater particle aggregation and yielded the smallest positive surface charge. Future attempts at optimization of amifostine PLGA particles will therefore focus on selective use of chitosan.

The dissolution profiles of various formulations are shown in Figure 4, except for A; because of only 1% encapsulation efficiency and poor amifostine loading, there was no justification for studying the dissolution profile for this formulation. While formulations B and C showed no significant initial burst, formulation D showed almost 20% drug release in the first hour. Except for this initial release in formulation D, all three formulations with chitosan did demonstrate sustained release of amifostine over the study period of 11 days.

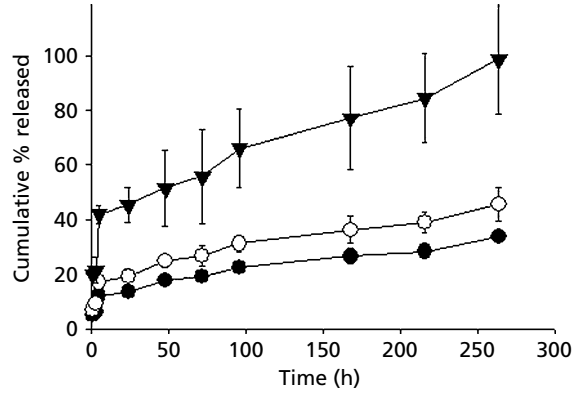

Figure 4 Dissolution profiles of formulation B (●), C (○) and D (▼). Bars represent the means ± s.e.m., n = 3.

Conclusion

In our continued efforts to develop an oral formulation of amifostine, the results of this and the previous three reports (Pamujula et al 2004a, b, 2005) have demonstrated the proof of principle that the development of an oral, slow-release biocompatible formulation is indeed quite feasible. This latest report further demonstrates that the judicious use of chitosan is likely to yield amifostine-loaded particles with desirable properties, such as high loading, positive surface charge and sustained release without a large initial burst. These properties, along with our data on bioavailability of earlier amifostine-loaded PLGA formulations, provide reasonable assurance that, upon oral administration, most amifostine will be absorbed as encapsulated particles through the gastrointestinal tract.

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