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Research paper

Preparation and in vitro characterization of amifostine biodegradable microcapsules

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Abstract

The purpose of this project was to develop sustained release microcapsules of amifostine. The microcapsules were prepared using solvent evaporation technique. The effect of several formulation variables on the characteristics of the microcapsules was studied. The formulation variables studied were drug loading, polymer (polylactide-co-glycolide) (PLGA) concentration, and the amount of gelatin in the initial aqueous phase. The drug loading was studied at three different levels (5, 10, and 25 mg); the PLGA concentration was studied at two levels (500 and 1000 mg); and the amount of gelatin used ranged from 2 to 14 mg. In general, the microcapsules were less than 155 μm in diameter with median size between 50 and 80 μm. While the use of higher amounts of PLGA significantly increased the median size of the microcapsules, using higher amounts of amifostine had no significant effect, irrespective of the amount of PLGA. The use of gelatin, within the range 2–14 mg, did not show any significant effect on the particle size distribution. Scanning electron microscopy (SEM) of the microcapsules revealed that all nine formulations yielded spherical particles. The use of 500 mg PLGA with 10 or 25 mg amifostine yielded microcapsules with porous surfaces. The surface pores, however, were not present in microcapsules prepared using 1000 mg PLGA. The efficiency of encapsulation decreased significantly from 63 to 24% when the amount of amifostine increased from 5 to 25 mg in the formulations using 500 mg PLGA. Similarly, the efficiency of encapsulation decreased from 87 to 23% when the amount of PLGA was doubled to 1000 mg. An increase in the amount of amifostine in the formulation using 500 mg PLGA also resulted in a significant increase in initial drug release (from 20 to 62%) within the first hour. These results were consistent with the porous morphology of these microcapsules. In general, all batches of microcapsules showed 24–96 h sustained drug release.

Keywords: Amifostine; Ethyol; Biodegradable; Microcapsule; Radioprotection

1. Introduction

Amifostine, also known in the literature as ethiofos, ethyol (MedImmune, Gaithersburg, MD, USA), or WR-2721, is an organic thiophosphate, which has been extensively studied as a cytoprotective agent [1–3]. It has been studied as a protector of normal tissue against the damaging effects of ionizing radiation and/or chemotherapy and was approved by the United States Food and Drug Administration in 1997. Amifostine's current indicated use in clinical practice is limited to that of reduction in the cumulative renal toxicity associated with repeated administration of cisplatin in patients with advanced ovarian or non small cell lung cancer [4,5]. Amifostine, however, continues to be evaluated as a cytoprotector in a variety of other clinical settings involving radiotherapy and/or chemotherapy [6,7]. Even if amifostine were shown to be successful in these trials, its clinical use is likely to remain severely limited due to the following facts: (1) amifostine in its presently available formulation must be administered intravenously in order to be effective [8]; (2) serious adverse effects of amifostine such as hypotension, nausea, and vomiting are significantly augmented upon intravenous route of administration [9,10]; (3) amifostine does not cross the blood–brain-barrier even when administered systemically; and (4) amifostine in its present formulation is not effective when administered orally [11,12]. Most, if not all, of the above-mentioned barriers to amifostine use can, however, be overcome by utilizing alternative formulation strategies, which are based upon modern techniques in drug

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delivery including microencapsulation using biodegradable polymers [12]. The most widely investigated biodegradable polymers are aliphatic polyesters based on lactic acid and glycolic acid. The copolymers of these two acids have attracted much attention because the biodegradation rate of the copolymer is easily controlled by altering their ratios [13–15]. These polymers have been used with numerous drugs and have been shown to be biocompatible [16].

The long-term objective of this project is to develop orally active amifostine microcapsules. In our previous report [17], we demonstrated that amifostine microcapsules prepared by the solvent evaporation technique using high molecular weight PLGA (RG506) released the drug in 3 days. However, these microcapsules had very low efficiency of encapsulation. Now we report on increasing the efficiency of encapsulation of amifostine by modifying the method of preparation.

2. Materials and methods

2.1. Materials

The copolymer poly(DL-lactic/glycolic acid), PLGA 50:50 (RG 502; inherent viscosity 0.2 dl/g) was obtained from Boehringer Ingelheim (Germany). The surfactant L-α phosphatidylcholine was obtained from Avanti Polar-lipids Inc. (Birmingham, AL, USA). Amifostine, polyvinyl alcohol (PVA), gelatin (Type A), chloroform, and dichloromethane were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Experimental design

Three samples were prepared where the PLGA amount was fixed at 500 mg while the amount of amifostine used varied at 5, 10, or 25 mg. An additional three samples prepared had the same variation in the amifostine amount but the amount of PLGA used was fixed at 1000 mg. Lastly, three additional samples were prepared to study the effect of gelatin incorporation. These samples were prepared using a fixed amount of both the PLGA (500 mg) and the amifostine (25 g) while the amount of gelatin used varied at 2, 7, or 14 mg.

2.3. Preparation of biodegradable microcapsules

Controlled release biodegradable microcapsules of amifostine were prepared using PLGA and the solvent evaporation technique [18]. Six preparations, labeled A–F, were prepared by dissolving a specific amount of amifostine powder in 200 μl of deaerated water followed by emulsification in 1 ml of dichloromethane containing a specific amount of PLGA (Table 1). The polymer solution was previously mixed with 20 μl of lipophilic surfactant L-α-phosphatidylcholine in chloroform (16 mg/ml).

Table 1
Description of batch formula and efficiency of encapsulation

Formulation	Amount of			Efficiency of encapsulation (%) (± SD)
	PLGA (mg)	Gelatin (mg)	Amifostine (mg)	
A	500	–	5	63.49 (5.56)
B	500	–	10	51.21 (2.07)
C	500	–	25	24.07 (3.60)
D	1000	–	5	86.47 (6.30)
E	1000	–	10	65.55 (5.71)
F	1000	–	25	23.45 (7.49)
G	500	2	25	28.94 (4.74)
H	500	7	25	29.24 (2.42)
I	500	14	25	37.39 (4.75)

The emulsification was carried out by sonication at output 4 (50 W) for 30 s (ultrasonic probe, Sonic and Materials Inc., Danbury, CT, USA). The resulting emulsion was further emulsified in 0.5 ml of an aqueous solution of polyvinyl alcohol (PVA; 1%) by vortexing for 15 s and then diluted in 100 ml PVA aqueous solution (0.3%). The mixture was stirred magnetically, under nitrogen, (at 500 rpm) for 2 h to allow complete evaporation of the solvent. Amifostine microcapsules were finally collected by centrifugation at 3000 rpm and washed four times with deaerated water to remove any residual PVA on the surface of the microcapsules. The microcapsules were later freeze-dried (−70 °C; 6×10^{-4} mbar) (Labconco, Kansas City, KS, USA) to obtain a free-flowing powder. Each formulation was prepared in triplicate. Three additional samples, labeled G–I, were prepared following the same procedure except the initial aqueous phase. In these samples, the aqueous phase also contained gelatin along with amifostine (Table 1).

2.4. In vitro characterization of microcapsules

2.4.1. Determination of amifostine content

For each formulation, a 10 mg sample was dissolved in 1 ml of dichloromethane. This was followed by the addition of 10 ml of 0.15% aqueous solution of Tween-80 and ultracentrifugation (35 000 rpm at 15 °C) to completely separate the precipitated copolymer. The efficiency of extraction and recovery of amifostine was measured independently in triplicate and was found to be at least 98%.

2.4.2. Analysis

The analysis of amifostine was performed using a rapid and sensitive high-performance liquid chromatography (HPLC) method [15]. The chromatography was performed under the following conditions. Column: μBondpack C-18 (Waters; 10 μm, 3.9 × 300 mm); mobile phase: 5 mM heptanesulfonic acid and 15% acetonitrile in 0.1 M chloroacetic acid at pH 3.0; flow rate: 1.0 ml/min; injection

volume: 20 μl; detector: electrochemical (Glassy carbon electrode; oxidation potential set at +1.4 V). Under these conditions, the retention time of amifostine was 5.2 min. The concentration of amifostine in each sample was determined by intrapolating the peak height to the amifostine standard curve. Each experiment was performed in triplicate.

2.4.3. Particle size and morphology

Particle size distribution was determined by a Coulter LS130 analyzer (Beckman Coulter Inc., Fullerton, CA, USA). This technique measures the size of particles dispersed in a medium by the scattering pattern of a traversing laser light. The samples were analyzed in a water medium and the Frauenhofer method was utilized to calculate the size distributions. The particle size calculations assume the presence of spherical particles and are based on calculated volumes of spheres. For each sample, a background run of deionized water was performed. A sample of microcapsules (2 mg) was added to the deionized water in a micro sample cell and counting was performed for 120 s. After subtraction of the background, the particle size distribution calculation was performed. Each experiment was performed in triplicate. Morphology of the microcapsules was examined by scanning electron microscopy (SEM) (Amray AMR 1000A, Bedford, MA, USA). Samples for SEM were mounted on metal stubs and coated with gold to a thickness of 200–500 Å.

2.4.4. 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, 24 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 rpm) 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 total amount of amifostine remaining in microcapsules was determined at 0, 1, 8, 10, 24, 48, 72, and 96 h. At each of the specified sampling times, three samples were filtered through 0.2 μm Millipore filter paper, freeze dried, and extracted for amifostine. The amount of amifostine in each sample was determined by HPLC.

2.5. Statistical analysis

The efficiency of encapsulation of amifostine and the amount of drug released from various formulations during the in vitro study were compared using SAS software package. A P value of less than 0.05 was considered as evidence of a significant difference.

3. Results and discussion

Six different formulations (A–F) were prepared, following a partial factorial design, to study the combined effects of the amount of PLGA and amifostine on the microcapsules characteristics. In an effort to improve the efficiency of encapsulation of amifostine, three additional formulations (G–I) were also prepared by incorporating 2, 7, or 14 mg gelatin within the amifostine aqueous phase. All microcapsules were evaluated for total drug content, i.e. efficiency of encapsulation, particle size and morphology, and in vitro drug release, i.e. dissolution characteristics.

3.1. Efficiency of encapsulation

Efficiency of encapsulation of amifostine was determined by measuring the total amount of amifostine actually present in each 10 mg sample, i.e. core loading experimental, and comparing this value with the total amount of amifostine expected in each of the samples, i.e. core loading theoretical. An increase in the PLGA amount from 500 to 1000 mg showed a significant increase in the efficiency of encapsulation, when the formulations contained either 5 mg (formulations A vs. D) or 10 mg (formulations B vs. E) amifostine. Irrespective of the amount of PLGA, efficiency of encapsulation was inversely related to the amount of amifostine. For example, the efficiency of encapsulation decreased from 63 to 24% when the amount of amifostine increased from 5 to 25 mg in the formulations using 500 mg PLGA (A vs. C). Similarly, when the formulations were prepared with 1000 mg PLGA, the efficiency of encapsulations decreased from 87 to 23% (D vs. F). It is known that the efficiency of encapsulation in formulations, such as the ones reported here, depends on the rate and extend of diffusion of the drug into the external aqueous phase during the in-water solvent evaporation following microencapsulation. The diffusion of the drug, in turn, is dependent on the rate of precipitation of the polymer, which depends on the polymer/solvent interaction and the aqueous solubility of the organic phase. In an effort to reduce the rate and extent of drug diffusion, the viscosity of amifostine phase was increased by adding gelatin (2, 7, or 14 mg). All of these formulations (G–I) were chosen to use 500 mg PLGA and 25 mg amifostine because it is at this combination that the efficiency of encapsulation was minimum (batch C). As the data show, the presence of gelatin at all three levels significantly increased ($P < 0.05$) the efficiency of encapsulation from 24% (C) to 29% (G and H) and 37% (I). These results support the hypothesis that an increase in the viscosity of amifostine aqueous phase also increased the efficiency of encapsulation by reducing drug diffusion during the in-water solvent evaporation.

3.2. Particle size and morphology

Particle size distribution data for various formulations are listed in Table 2. In general, the microcapsules were less

Table 2
Particle size distribution of amifostine microcapsules

Formulation	Median size (μm)	80% Confidence (μm)
A	50.54	19.19–89.81
B	55.95	20.80–109.10
C	51.91	24.56–90.60
D	79.29	27.81–140.70
E	80.10	36.79–154.70
F	79.87	30.01–150.50
G	64.28	28.68–119.10
H	62.97	26.08–88.53
I	62.41	26.40–93.48

than 155 μm in diameter with median size of 50 μm (formulation A) and 80 μm (formulation E). Formulations prepared with 1000 mg PLGA (D–F) were relatively larger in size compared with the formulations prepared with 500 mg PLGA (A–C). Thus, the use of higher amount of PLGA not only significantly ($P < 0.05$) increased the median size of the microcapsules, but also increased the range of 80% confidence. The maximum size of the microcapsules from formulations A–C were between 90 and 109 μm, whereas the maximum size of the microcapsules from formulations D–F were between 141 and 155 μm. The use of higher amount of PLGA increased the viscosity of the polymer solutions, which presumably also increased the initial size of the emulsion droplets resulting in higher particle size. A change in the amount of amifostine from 5 to 25 mg did not show any significant effect ($P > 0.05$) on the median size of the microcapsules, irrespective of the amount of PLGA. Regarding the formulations containing gelatin (G–I), although they showed slightly higher median particle size (63 μm) the ranges of 80% confidence were similar to the formulations prepared with no gelatin. These observations suggest that the use of gelatin, in the range of 2–14 mg, did not have any significant effect on the particle size distribution.

Results of the scanning electron microscopy for all nine formulations (A–I) are shown in Fig. 1. All formulations produced particles that were spherical in shape. The microcapsules prepared with 500 mg PLGA and different amounts of amifostine (Fig. 1A–C) showed slightly different surface morphology based on the amount of amifostine. An increase in the amount of amifostine from 5 to 25 mg resulted in porous surface (Fig. 1C). The appearance of these pores may be due to the presence of a large amount of highly water-soluble amifostine on the surface of the microcapsules during polymer precipitation, followed by diffusion into the aqueous phase during solvent evaporation. In contrast, the surface of the microcapsules,

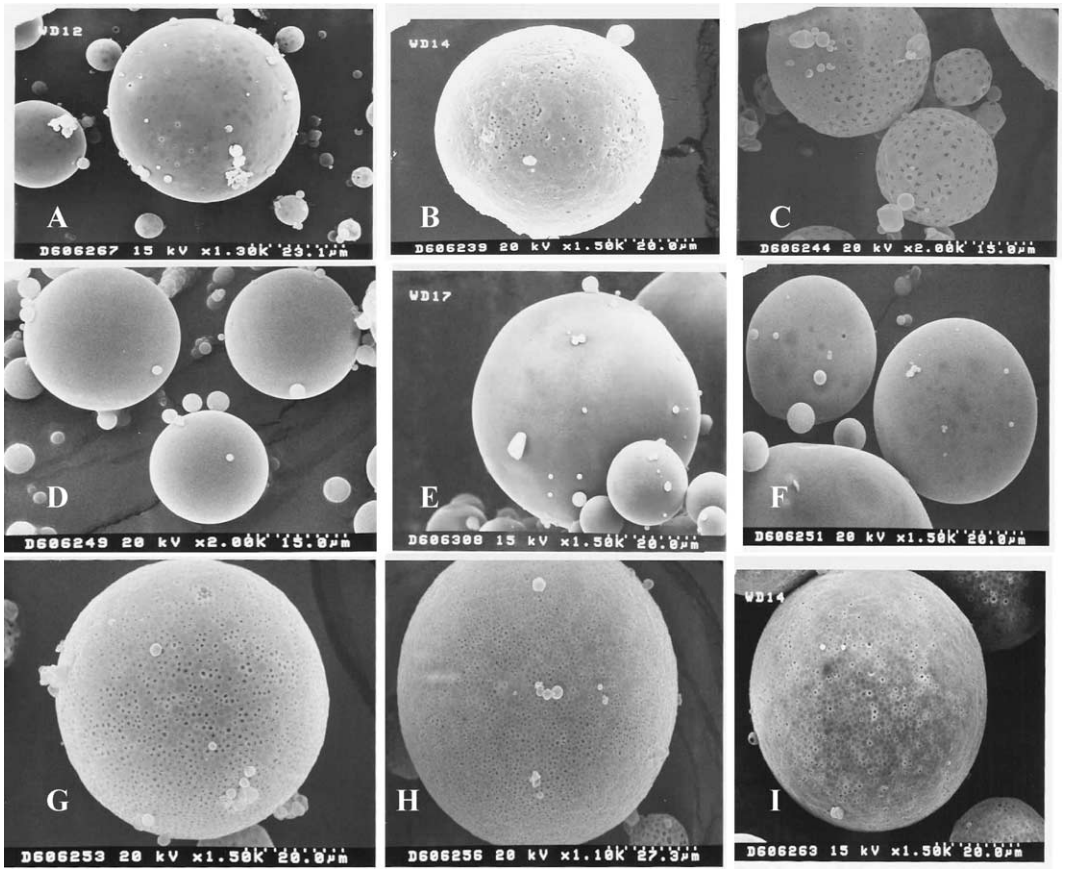

Fig. 1. Typical SEM photographs of the microcapsules. Batch A (A); batch B (B); batch C (C); batch D (D); batch E (E); batch F (F); batch G (G); batch H (H); and batch I (I).

prepared with 1000 mg PLGA, was smooth, irrespective of the amount of amifostine (Fig. 1D–F). All three formulations with the gelatin (Fig. 1G–I) also showed surface pores, similar to the formulation C. The amount of amifostine in these formulations (Fig. 1G–I) was same (25 mg) as in formulation C. This observation shows that a combination of 500 mg PLGA and 25 mg amifostine resulted in porous microcapsules irrespective of the presence of gelatin. Therefore, the presence of gelatin, from 2 to 14 mg, did not contribute to any significant change in the surface morphology of microcapsules.

3.3. In vitro dissolution

Figs. 2–4 show the dissolution profiles of nine formulations (A–I). An increase in the amount of amifostine, in the formulations using 500 mg PLGA (A–C), significantly increased ($P < 0.05$) the initial drug release (Fig. 2). The amount of amifostine released within the first one hour ranged between 20 ando 62%. These results are consistent with the morphology of the microcapsules. For example, batch C showed significant number of pores, which resulted in higher initial drug release. The later two batches (B and C) also showed more than 90% drug release within 24 h. However, batch A showed only 55% drug release during the same period followed by a second burst effect at the end of 48 h with drug release continuing up to 96 h. An increase in the amount of amifostine, in the formulation using 1000 mg PLGA (D vs. E and F) also increased ($P < 0.05$) the initial drug release (Fig. 3) but without following any particular order. Although batch C was prepared with the highest amount of amifostine (25 mg), the final microcapsules showed only 23% efficiency of encapsulation. This significantly high drug loss may be due to amifostine diffusion during the in-water solvent evaporation, resulting in the presence of less amount of drug near the surface of the microcapsules. The drug particles present at or near the surface are normally released during the initial

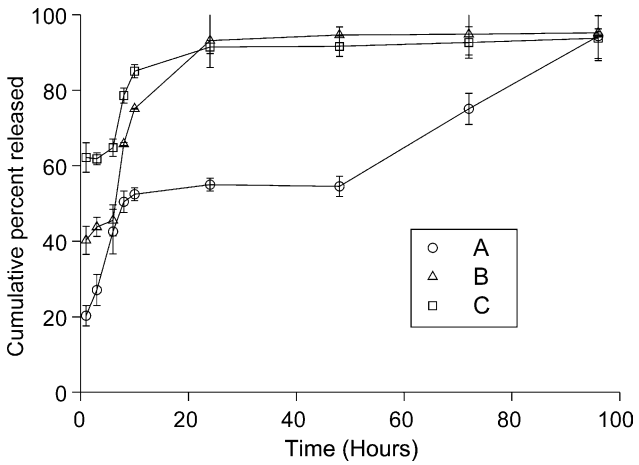

Fig. 2. Dissolution profiles of the microcapsules prepared with 500 mg PLGA.

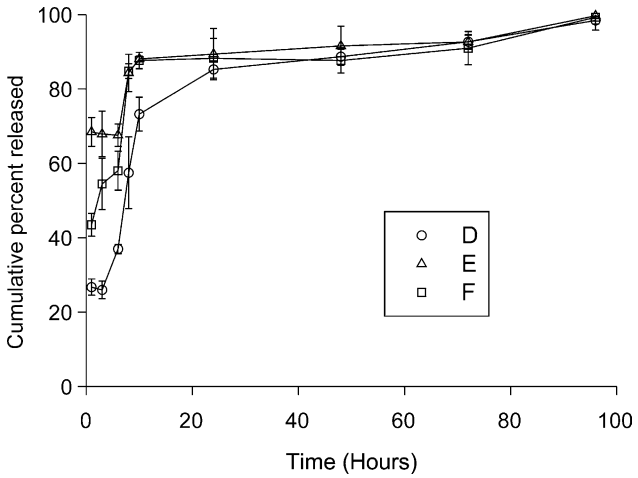

Fig. 3. Dissolution profiles of the microcapsules prepared with 1000 mg PLGA.

burst effect, so in spite of the higher amount of drug (25 mg loading), batch F showed significantly low initial drug release ($P < 0.05$) compared with batch E. All three batches (D–F) showed approximately 90% drug release within 48 h and the release continued up to 96 h. All three batches of microcapsules containing gelatin (G–I) showed significantly high initial drug release (69–76%; $P > 0.05$), irrespective of the amount of gelatin. These batches also showed over 80% release within the first 24 h, indicating that the incorporation of gelatin has a marked effect on the initial release of amifostine. Although the reasons for such facilitations are not clear at present, it is clear that the incorporation of gelatin in a PLGA-based preparation of amifostine is not conducive to slow release.

In conclusion, the efficiency of encapsulation decreased with an increase in amifostine loading. However, it increased significantly with an increase in the amount of PLGA used and in the presence of gelatin. Regarding particle size, the batches prepared with higher amount of

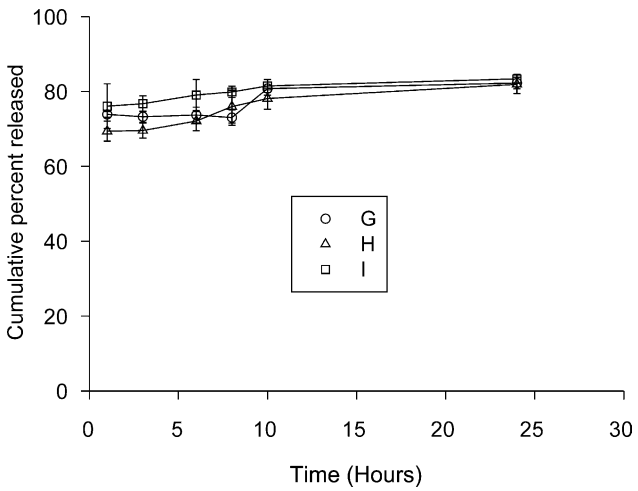

Fig. 4. Dissolution profiles of the microcapsules containing gelatin.

PLGA yielded higher particle size. A change in the amount of amifostine (5 to 25 mg) or gelatin (2 to 14 mg) did not change the median particle size. Regarding the nature of surface, the microcapsules containing lower amount of PLGA and higher amount of amifostine showed porous surface. These surface pores were not present when the microcapsules were prepared using a higher amount of PLGA. Hence, both the amount of amifostine and the PLGA significantly affect the surface morphology. Regarding amifostine release, microcapsules in general showed 24–96 h sustained drug release. These microcapsules are designed for oral delivery followed by absorption through Peyer's patches, Thus our goal is to deliver the microcapsules intact through this route into the body and to release the drug over a period of time. Slow release will provide long-term amifostine concentration to the specific organs. These microcapsules are currently under investigation for radioprotection and tissue distribution characteristics.

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