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Properties of poly(lactic-*co*-glycolic acid) nanospheres containing protease inhibitors: Camostat mesilate and nafamostat mesilate

Jiahui Yin [a], Yasuhiro Noda [a], Toshihisa Yotsuyanagi [b,*]

[a] *Department of Pharmaceutics, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya 467-8603, Japan*
[b] *Laboratory of Pharmaceutics, School of Pharmacy, Aichigakuin University, Nagoya 464-8650, Japan*

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Abstract

Poly(lactic-*co*-glycolic acid) (PLGA) nanospheres containing protease inhibitors, camostat mesilate (CM) and nafamostat mesilate (NM), were prepared by the emulsion solvent diffusion methods in water or in oil, and the w/o/w emulsion solvent evaporation method. The average diameter of PLGA nanospheres prepared in the water system were about 150–300 nm, whereas those prepared in the oil system were 500–600 nm. Among the three methods, these drugs were the most efficiently encapsulated up to 60–70% in PLGA nanospheres in the oil system. Other factors that may influence drug encapsulation efficiency and in vitro release such as drug load, molecular weight of polymer were also investigated. Both the CM- and NM-loaded nanospheres prepared in the water system immediately released about 85% of the drug upon dispersed in the release medium while the drug initial burst of nanospheres prepared by the emulsion solvent diffusion in oil method reduced to 30% and 60% for CM and NM, respectively. Poly(aspartic acid) (PAA), a complexing agent for cationic water soluble drugs, showed little effect on the encapsulation efficiency and release behavior for CM and NM. The DSC study and AFM pictures of nanospheres demonstrated that temperature-dependent drug release behavior was ascribable to the glass transition temperature of the polymer, which also affected the morphology of nanospheres upon dispersed in the release medium and influenced the drug release consequently.

Keywords: Camostat mesilate; Nafamostat mesilate; Poly(lactic-*co*-glycolic acid); Nanospheres; Emulsion solvent diffusion in oil method; In vitro release

1. Introduction

There has been considerable interest in developing biodegradable nanospheres as effective drug delivery systems (Jain, 2000; Soppimath et al., 2001). The controlled release of pharmacologically active agents to the specific site of action at the therapeutically optimal rate and dose regimen has been a major goal in designing such devices. Various polymers have been explored as sustained-release and protective carriers of drugs to a target site and thus increase the therapeutic benefit, while minimizing side effects. Among these polymers, the thermoplastic aliphatic poly(esters) like poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and especially poly(lactic-*co*-glycolic acid) (PLGA) have generated tremendous interest due to their excellent biocompatibility and biodegradability. Several of formulations using these polymers have received world wide marketing approval (Ogawa et al., 1989; Okada, 1997).

Gabexate mesilate (GM), camostat mesilate (CM) and nafamostat mesilate (NM) are protease inhibitors currently available to inhibit the biological activities of plasma kallikrein, thrombin, plasmin and trypsin. They have been used for the treatment of pancreatitis up to now (Muramatsu and Fuji, 1972; Nakahara, 1983; Takaku and Yazaki, 2005). Also, these protease inhibitors are thought to inhibit cell-surface enzymatic activity of cancer cells and their anticancer effect has been explored (Ohkoshi, 1995; Hiwasa et al., 1998). However, due to their highly basic nature of the guanidine group or amidine group, these protease inhibitors are given as cationic forms and water-soluble, and therefore suffer from low membrane permeability, high protein binding in blood and rapid elimination from plasma due to the hydrolysis property of ester group (Hiraku et al., 1982; Nishijima et al., 1983; Shibuya et al., 1984; Akizawa et al., 1989). More suitable formulations of these protease inhibitors

* Corresponding author.
E-mail address: yotsuyng@dpc.aichi-gakuin.ac.jp (T. Yotsuyanagi).

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are needed for which PLGA nanospheres encapsulation was explored. The encapsulation is envisaged as one of the ways to protect the drugs from protein binding and possible hydrolysis (against phenolic ester) before they arrive at the target site. Furthermore, the prolonged-release of drugs from PLGA nanospheres could reduce the repeated injection times or dosage amount over a long therapeutic period.

It has been reported that the spontaneous emulsification solvent diffusion method in water was applied to prepare PLGA nanoparticles encapsulating either water-soluble or insoluble drugs (Niwa et al., 1993, 1994). In this method, the poured droplets of acetone-chlorinated hydrocarbons with PLGA and the drug were finely emulsified spontaneously into nanometer-sized spheres in the dispersing aqueous system due to the rapid diffusion of acetone from the organic to the aqueous phase. Also, the w/o/w emulsion solvent evaporation method has been used to prepare water-soluble drug-loaded nanoparticles (Gaspar et al., 1998; Avgoustakis et al., 2002).

We firstly prepared protease inhibitor-containing nanospheres by these two methods. In addition, to improve the encapsulation efficiency of these drugs, an emulsion solvent diffusion in oil method was applied to prepare nanospheres in an oil-dispersing medium (Kawashima et al., 1998, 2000). Because GM was very susceptible to hydrolysis (Nishijima et al., 1983; Ohta et al., 1986), it was omitted in this study. We also investigated the effects of molecular weight of polymer, the fed amount of the drugs and the additive (poly(aspartic acid) (PAA) as a complexing agent) on the encapsulation efficiency and in vitro release profile of the drugs. The thermal behavior of the drug-loaded nanospheres was also examined.

2. Materials and methods

2.1. Materials

Poly(lactic-*co*-glycolic acid) (PLGA) with average molecular weight of 20,000, whose copolymer ratio of D,L-lactide to glycolide was 75:25 (referred as PLGA20k(75:25) hereinafter), was obtained from Wako Pure Chemical Industries Ltd. (Osaka). PLGA (85:15) with average molecular weight of 50,000–75,000 (referred as PLGA50k(85:15) hereinafter) and PLGA (50:50) with average molecular weight of 50,000–75,000 (referred as PLGA50k(50:50) hereinafter) were obtained from Aldrich Chemical Co. (USA). Poly(vinyl alcohol) (PVA, M_w: 13,000–23,000) with 87–89% hydrolyzed and poly(aspartic acid) (PAA, M_w: 15,000–50,000) were also obtained from Aldrich Chemical Co. (USA). Camostat mesilate (CM, *N,N*-dimethylcarbamoylmethyl-*p*-(*p*-guanidinobenzoloxy) phenylacetate methanesufonate) was extracted from a commercially available product (camostat mesilate 100 mg/tablet, Ono Pharmaceutical Corp., Osaka, Japan). Nafamostat mesilate (NM, 6-amidino-2-naphthyl *p*-guanidinobenzoate dimethane-sulfonate) was supplied by Toronto Research Chemicals Inc. (Canada) (see chemical structures in Fig. 1). Caprylate and caprate triglyceride (Triester F-810) and hexaglycerin condensed ricinoleate (Hexaglyn PR-15) were obtained from Nikko Chemicals Co. (Tokyo). Sorbitan monooleate (Span 80) was supplied by Tokyo Kasei Industry Co. (Tokyo). All other chemicals and solvents were of reagent grade.

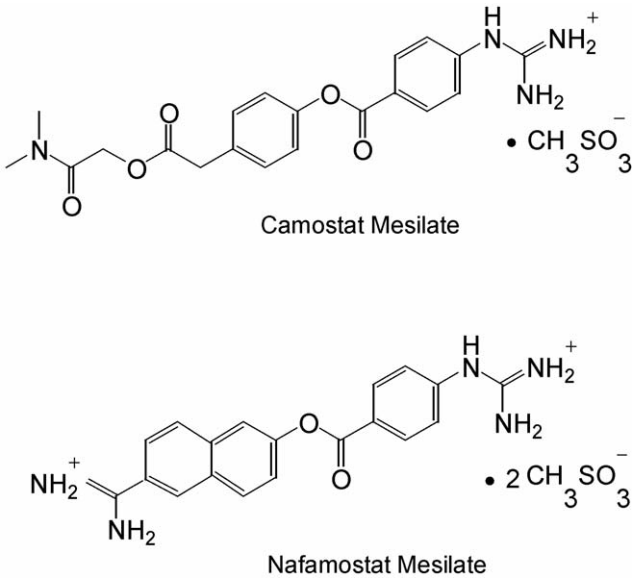

Fig. 1. Chemical structures of camostat mesilate and nafamostat mesilate.

2.2. Methods

2.2.1. Preparation of drug-loaded nanospheres

2.2.1.1. Emulsion solvent diffusion in water method. PLGA (60 mg) and 1.5 mg of CM or NM were dissolved in the mixed organic solvent of acetone (7.5 ml), dichloromethane (250 μl) and 0.22 μm filtrated water (750 μl). Water was added to dissolve CM or NM in this organic phase. The resultant organic solution was poured into 25 ml of aqueous PVA solution (2.0%, w/v) with magnetic stirring at room temperature. In the formulation with the addition of PAA, 75 μl of PAA aqueous solution was added in the organic phase and the charge ratio of PAA to CM was 1.2:1. The emulsified system was stirred under reduced pressure for 3–4 h until the evaporation of the organic phase was complete. Then the entire dispersion system was centrifuged (100,000 × *g* for 50 min, XL-90, Beckman, USA). The sediment was dispersed in distilled water and centrifuged twice under the same running condition. The redispersed suspension was dried using a freeze dryer (EYELA FD-5, Rika Mechanicals, Tokyo) overnight.

2.2.1.2. W/o/w Emulsion solvent evaporation method. An aqueous solution of CM (250 μl, 6 mg/ml) was emulsified by probe sonication at 20 W for 1 min in 2 ml dichloromethane, in which PLGA (60 mg) had been dissolved. This w/o emulsion was transferred to 20 ml aqueous solution of PVA (2.0%, w/v) and the mixture was sonicated at 20 W for 2 min (Ultrasonic disruptor UD-201, Tomii Fine Mechanicals, Tokyo). The w/o/w emulsion was gently stirred at room temperature under reduced pressure to evaporate the organic solvent. The following centrifugation, redispersing and freeze-drying procedures were the same as above-mentioned.

2.2.1.3. Emulsion solvent diffusion in oil method. CM (2.5–10 mg) or NM (2.5 mg) and PLGA (100 mg) were dissolved in 5 ml of a mixture of acetone and methanol (4:1, v/v) with the addition of 100 mg Span 80. In the formulation with the addition of PAA, 40 μl of its aqueous solution was added in the organic phase and the charge ratio of PAA to drug was 1.2:1. The resultant polymer-drug solutions were emulsified in a mixture of 60 ml of Triester F-810 (containing 2% Hexaglyn PR-15) and 40 ml of hexane under stirring at 400 rpm (Tornado, SM-102, Osaka). After the system was agitated for 3 h under reduced pressure at 30 °C, the entire suspension was added to 20 ml hexane and centrifuged (40,000 × *g* for 10 min, Himac CR20G, Hitachi, Tokyo). The sediment was dispersed with hexane and centrifuged under the same running condition. Additional dispersing in PVA solution and distilled water and centrifugation were carried out sequentially. The sediment after the last centrifugation was redispersed with filtered distilled water and then freeze-dried overnight.

2.2.2. Physicochemical properties of drug-loaded nanospheres

The average particle size of nanospheres dispersed in the aqueous medium was measured by dynamic laser scattering (DLS-7000, Otsuka Electronic, Osaka).

The morphological examination of nanospheres was performed using an atomic force microscopy (AFM) (Nanoscope III, Digital Instruments Co., USA) and scanning electron microscopy (SEM) (S-4300, Hitachi, Osaka). Samples for AFM and SEM were mounted on mica and a small metal cylinder, respectively. After drying, the sample was transferred to the sample holder for observation. For SEM, the sample was coated with platinum and palladium at 15 mA for 2 min using an ion sputter (E102, Hitachi, Osaka) before the transfer.

Differential scanning calorimetry (DSC) of CM, polymer and drug-loaded nanospheres was performed using DSC-60 (Shimadzu, Kyoto) in order to characterize their physical state after encapsulation. About 5 mg of a sample was weighed, crimped into an aluminum pan and analyzed at a scanning rate of 5 °C/min with temperature raise from −15 °C (initial holding time 2 min). The glass transition temperature (T_g) was obtained by extrapolating the linear portion of the thermograms above and below the glass transition point and determining the crossing point.

2.2.3. Drug content in nanospheres and encapsulation efficiency

The recovery of PLGA nanospheres was defined as the weight ratio of freeze-dried nanospheres to the initial loadings of PLGA and drug. The weighed dry CM or NM-loaded nanospheres were dissolved in acetonitrile, and then distilled water was added to precipitate the polymer. The drug solution filtrated through a membrane filter (0.22 μm) was properly diluted and analyzed by high performance liquid chromatography (Column, Inertsil ODS-80A, GL Sciences Inc.; UV detector, SPD-6A, detected at 265 nm for CM and 254 nm for NM respectively, Shimadzu; mobile phase, methanol/sodium 1-heptanesulfonate/sodium lauryl sulfate/acetic acid (200:100:50:1, v/v/v/v); flow rate, 0.4 ml/min; injection volume, 20 μl). Ethyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate were used as internal standard substances for CM and NM respectively. The drug encapsulation efficiency and content in the nanospheres are represented by Eqs. (1) and (2), respectively

$$\text{drug encapsulation efficiency} = \frac{\text{amount of drug in nanospheres}}{\text{amount of drug fed to the system}} \times 100\% \quad (1)$$

$$\text{drug content} = \frac{\text{amount of drug in nanospheres}}{\text{amount of nanospheres recovered}} \times 100\% \quad (2)$$

2.2.4. In vitro drug release study

The CM or NM-loaded nanospheres of 20 mg were dispersed in 20 ml of 0.05 M phosphate buffer (pH 7.4) in a sealed jacketed beaker at 37 °C. The suspension was stirred continuously at a constant rate. Aliquots of 200 μl of suspension withdrawn at various times were ultracentrifuged (15,000 × *g* for 15 min), and the drug content in the sediment was assayed, from which the amount of drug released was calculated. This is because there is possible degradation of the drugs after released in the medium.

3. Results and discussion

3.1. Morphology and size of drug-loaded nanospheres

The average diameter of the drug-loaded nanospheres prepared by the emulsion solvent diffusion in water method fell in the range of 200–300 nm by DLS (Table 1), irrespective of the

Table 1
Nanosphere recovery, drug content and drug encapsulation efficiency of nanospheres prepared by the emulsion solvent diffusion in water method

Drug	Polymer	Mean diameter ± S.D. (nm)	Nanosphere recovery (%)	Drug content (%)	Drug encapsulation efficiency (%)
CM	PLGA50k(85:15)	260 ± 42	94.1	0.04	1.5
	PLGA50k(50:50)	274 ± 45	93.9	0.03	1.3
	PLGA20k(75:25)	283 ± 49	85.1	0.5	17.7
	PLGA20k(75:25) with PAA[a]	252 ± 37	81.5	0.5	17.6
NM	PLGA50k(50:50)	296 ± 56	90.9	0.02	0.8
	PLGA20k(75:25)	228 ± 46	84.8	0.3	9.0

Nanospheres were prepared with PLGA (60 mg) and drug (1.5 mg).
[a] Charge ratio of PAA to CM is 1.2:1.

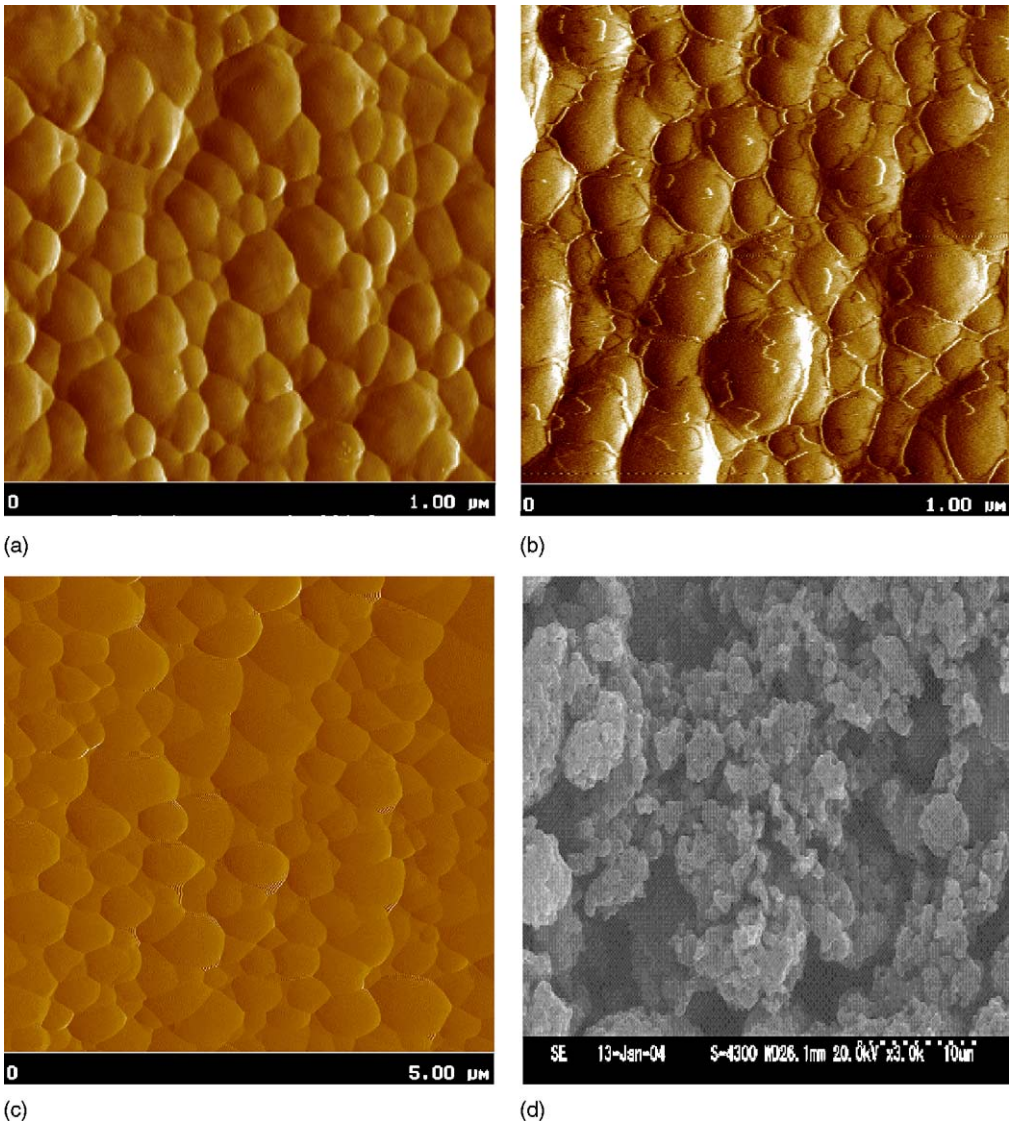

Fig. 2. Morphology of camostat mesilate-loaded PLGA nanospheres prepared by the emulsion solvent diffusion methods. (a) AFM, PLGA20k(75:25); (b) AFM, PLGA50k(50:50), prepared by the emulsion solvent diffusion in water method; (c) AFM PLGA20k(75:25); (d) SEM with PLGA50k(50:50), prepared by the emulsion solvent diffusion in oil method. The scale bar of AFM was shown as a white line at the bottom, which was exactly the length of the picture. The length bar of SEM photo was shown as dotted line.

molecular weight of the polymer. The size agreed with those observed by AFM in Fig. 2a for PLGA20k(75:25) and Fig. 2b for PLGA50k(50:50). The surface of the nanospheres prepared with high molecular weight polymer PLGA50k(50:50) appeared to be rugged while those with low molecular weight polymer PLGA20k(75:25) appeared to be smooth. Similar phenomena were observed for microspheres prepared by using high and low molecular weight PLGA (Graves et al., 2004). On their suggestion, a smooth surface would be expected because less viscous solution of the low molecular weight polymer could result in smooth surface of emulsion droplets during the encapsulation of drugs and the evaporation of the organic solvent. Nanospheres prepared by the w/o/w emulsion solvent evaporation method with PLGA20k(75:25) had an average diameter of 156 nm (Table 2), which could be ascribed to the production of fine oil droplets containing PLGA when w/o/w emulsions was prepared by probe sonication.

On the other hand, the drug-loaded nanospheres prepared by the emulsion solvent diffusion in oil method with low molecular weight polymer PLGA20k(75:25) exhibited larger average diameters between 500–600 nm by DLS (Table 3). Fig. 2c is the AFM picture of these nanospheres, verifying the result of DLS examination and showing the nanospheres have a same smooth surface as those prepared by the solvent diffusion in

Table 2
Nanosphere recovery, drug content and drug encapsulation efficiency of nanospheres prepared by the w/o/w emulsion solvent evaporation method

Drug	CM
Polymer	PLGA20k(75:25)
Mean diameter ± S.D. (nm)	156 ± 28
Nanosphere recovery (%)	86.0
Drug content (%)	0.7
Drug encapsulation efficiency (%)	23.0

Table 3
Nanosphere recovery, drug content and drug encapsulation efficiency of nanospheres prepared by the emulsion solvent diffusion in oil method

Drug	Formulation	Mean diameter ± S.D. (nm)	Nanosphere recovery (%)	Drug content (%)	Drug encapsulation efficiency (%)
CM	A: 2.5 mg[a]	592 ± 117	75.9	2.3	71.4
	B: 5 mg[a]	537 ± 110	71.2	4.6	69.9
	C: 10 mg[a]	557 ± 97	72.6	6.2	51.5
	D: 2.5 mg[a] with PAA[b]	491 ± 78	74.6	2.1	64.2
	E: 2.5 mg, polymer blend[c]	588 ± 85	72.7	2.3	70.7
NM	A: 2.5 mg[a]	549 ± 108	76.9	1.9	60.0
	D: 2.5 mg[a] with PAA[b]	478 ± 69	73.0	1.9	56.8

[a] Amount of drug in polymer (PLGA20k(75:25)) 100 mg.
[b] Charge ratio of PAA to drug is 1.2:1.
[c] Blend of PLGA20k(75:25) and PLGA50k(50:50) in ratio of 6:4.

water method. Blending of the high molecular weight polymer PLGA50k(50:50) with the low molecular weight polymer PLGA20k(75:25) at the ratio of 4:6 or addition of PAA had no effect on the size of the nanospheres. However, the nanospheres prepared with high molecular weight polymer PLGA50k(50:50) alone was found to aggregate, as shown by a SEM picture (Fig. 2d). The average diameter was 2–3 μm by DLS. The emulsion droplets in the oil system coalesced into large particles during agitation. The slower diffusion rate of the organic solvent into the dispersing oil medium than that in water caused a delay in the hardening of the emulsion droplets by precipitation of the polymer. Proper surfactant (dispersing agent) was recommended to prevent the coalescence of the emulsion droplets (Kawashima et al., 1998). However, the addition of Span 80 in the polymer solution and Hexaglyn PR-15 in the oil phase could not prevent the aggregation of the high molecular weight polymer. These aggregated nanospheres were not investigated further.

3.2. Recoveries of nanospheres and encapsulation efficiency of drugs

Table 1 shows the recoveries of nanospheres and encapsulation efficiency of drugs when prepared by the emulsion solvent diffusion in water method. No difference was found between the two different high molecular weight polymers, PLGA50k(85:15) and PLGA50k(50:50), which have the same molecular weight range but differ in the ratio of D,L-lactide to glycotide. The recovery of nanospheres prepared with low molecular weight polymer PLGA20k(75:25) was slightly lower than that with high molecular weight polymer PLGA50k(50:50), but the drug encapsulation efficiency was more than 10 times higher. The drug encapsulation efficiency in nanospheres containing NM was almost half of those containing CM. The electrostatic interaction between the carboxylic group in the polymer molecules and the positively charged group(s) of the drugs at the surface is likely to contribute in part to the encapsulation efficiency. There are two positively charged groups in the NM molecular structure, so the encapsulation efficiency of NM was almost half lower than that of CM This may be also the reason why low molecular weight polymer PLGA20k(75:25) could encapsulate more drugs due to the higher concentrations of carboxylic anion.

The drug encapsulation efficiency was less than 20%, which is an ordinary phenomenon observed for PLGA nanospheres or microspheres encapsulating water-soluble drugs by this method (Jalil and Nixon, 1990; Niwa et al., 1993; Kawashima et al., 1998). This is most probably due to the rapid diffusion of the drugs into the water phase when their solutions containing PLGA were poured into the aqueous medium.

It was reported that the drug encapsulation efficiency was enhanced by the addition of PAA as a complexing agent for water-soluble drugs (Govender et al., 2000). Also, PAA was widely applied in the research on polymeric micelles formed by block copolymers as novel core-shell typed particulates for drug delivery (Yokoyama et al., 1990; Nishiyama et al., 2001). Since the protease inhibitors used are positively charged, we also investigated the effect of PAA on the encapsulation efficiency of CM, but no effect was exhibited.

The w/o/w emulsion solvent evaporation method has also been used to prepare water-soluble drug-loaded nanospheres and it is supposed that the oil phase surrounding the internal water phase would prevent the leakage of drug. However, in this experiment, the drug encapsulation efficiency was 23% for CM-loaded nanospheres prepared with PLGA20k(75:25), indicating that no significant improvement was observed by using this method (Table 2), which could be ascribed to the same reason as above-mentioned.

In order to obtain nanospheres with higher drug encapsulation efficiency, a non-aqueous system may be a promising alternative. We therefore focused on examining the drug encapsulation efficiency of nanospheres prepared by the emulsion solvent diffusion in oil method. Significant improvement on the drug encapsulation has been reported because the diffusion of drugs into the dispersing medium was suppressed in the oil system (Kawashima et al., 2000; Horisawa et al., 2002). The experiment result is shown in Table 3. In the preparation of nanospheres containing CM, we also investigated the effect of fed amount of drug (formulation A–C with CM amount 2.5, 5 and 10 mg, respectively in PLGA20k(75:25) 100 mg), the additive–PAA (formulation D, PLGA20k(75:25)) and blend of polymer (formulation E, PLGA20k(75:25) and PLGA50k(50:50) were blended in a

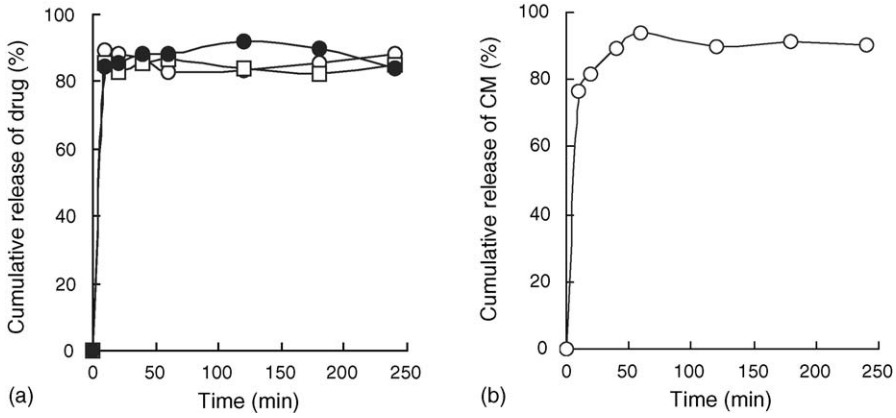

Fig. 3. In vitro release profile of camostat mesilate and nafamostat mesilate from PLGA20k(75:25) nanospheres in pH 7.4 phosphate buffer at 37 °C. (a) (○) CM, (●) CM, addition of poly(aspartic acid), (□) NM, prepared by the emulsion solvent diffusion in water method; (b) (○) CM, prepared by the w/o/w emulsion solvent evaporation method. All the plots were the average values of duplicate to triplicate runs.

ratio of 6:4). For all the formulations, similar nanosphere recovery was obtained, which was around 70–75%, lower than those prepared by the solvent diffusion in water method. This indicates that the precipitation of PLGA was more efficiently completed in the aqueous phase compared with that in the oil system. Except for formulation C, the drug encapsulation efficiency reached about 70% for CM and 60% for NM. Apparently, the drugs were efficiently entrapped in the lipophilic polymer by this method, although they are hydrophilic drugs. For formulation C with CM amount 10 mg, it decreased to 50%, indicating a saturation tendency in encapsulation efficiency. Neither the addition of PAA nor the blending of high molecular weight polymer PLGA50k(50:50) showed any effect on the CM or NM encapsulation efficiency.

3.3. In vitro drug release of nanospheres

Both the CM- and NM-loaded nanospheres prepared by the emulsion solvent diffusion in water method immediately released about 85% of the drug upon dispersion (Fig. 3a). The addition of PAA showed no effect on the CM release properties. CM-loaded nanospheres prepared by the w/o/w emulsion solvent evaporation method showed the same profile (Fig. 3b). This rapid initial release may be attributed to the large fraction of drug that was adsorbed or exposed on the surface of the nanospheres.

The comparison of in vitro release of CM and NM from nanospheres prepared by the emulsion solvent diffusion in oil method with PLGA20k(75:25) is shown in Fig. 4. Since CM may be hydrolyzed to some extent even in the nanospheres that were swollen by the release medium, the release was observed up to about 20 h (half life in the release medium: about 40 h at pH 7.4 and 37 °C). NM is much stable and the release experiment continued for about 100 h. The drug release exhibited a burst during the initial stage, i.e., 30% and 60% for CM and NM in 30 min, respectively, which was also thought to be the fraction of drug adsorbed or exposed on the surface of the nanospheres. NM has another positively charged amidine group beside the guanidine group. It may be assumed that more NM molecules stay on the surface of nanospheres in the dry state. Upon dispersed in release medium, the more hydrophilic property of NM than CM was thought to be responsible for its faster initial burst. After 1 day, 20% of CM and less than 10% of NM remained, respectively, in the nanospheres. There was no difference in encapsulation efficiency and release profile of CM with the addition of PAA. NM has a greater charge density than CM due to the amidine group,

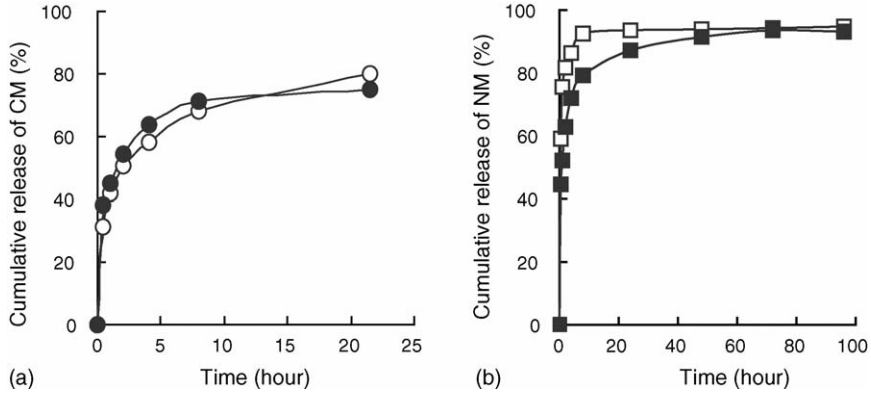

Fig. 4. Effect of poly(aspartic acid) on the in vitro release profile of camostat mesilate and nafamostat mesilate from PLGA20k(75:25) nanospheres prepared by the emulsion solvent diffusion in oil method. (a) (○) CM, (●) CM with addition of poly(aspartic acid); (b) (□) NM, (■) NM with addition of PAA. Release was performed in pH 7.4 phosphate buffer at 37 °C. All the plots were the average values of duplicate to triplicate runs.

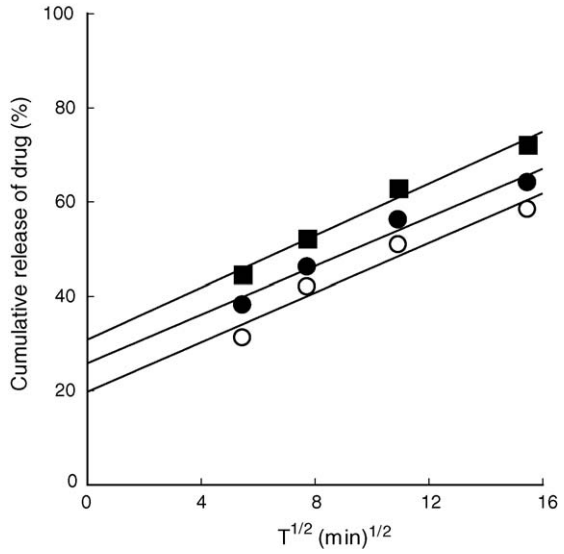

Fig. 5. Higuchi plot of release profile until 4 h of camostat mesilate and nafamostat mesilate from PLGA20k(75:25) nanospheres prepared by the emulsion solvent diffusion in oil method. (○) CM ($R^2 = 0.947$), (●) CM with addition of poly(aspartic acid) ($R^2 = 0.973$), (■) NM with addition of poly(aspartic acid) ($R^2 = 0.983$). Release was performed in pH 7.4 phosphate buffer at 37 °C.

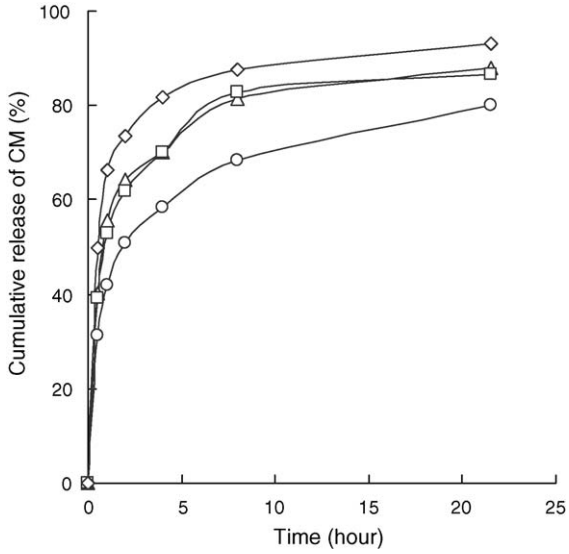

Fig. 6. In vitro release profile of camostat mesilate from different PLGA20k(75:25) nanospheres formulations in pH 7.4 phosphate buffer at 37 °C. (○) 2.5 mg drug-loaded (formulation A), (△) 5.0 mg drug-loaded (formulation B), (□) 10 mg drug-loaded (formulation C), (◊) blending of 40% of PLGA50k(50:50) (formulation E). All the plots were the average values of duplicate to triplicate runs.

which may be able to enhance interaction with PAA. Although the initial burst of NM decreased from 60% to 40% after the addition of PAA, the encapsulation efficiency and release profile of NM were not improved apparently, indicating that PAA is not a potential complexing agent for CM or NM. It was reported that PAA was used to improve the encapsulation efficiency of procaine hydrochloride and diminazene aceturate (Govender et al., 2000). The diminazene aceturate with two amidine moieties has a greater charge density than the former, and therefore the encapsulation efficiency was significantly improved by PAA. However, greater charge density may not always be major factor that can bring about complexation.

In Fig. 5, we plotted the cumulative release of drug (%) against square root of time according to the Higuchi plotting (Higuchi, 1962). Usually, the Higuchi plotting applies to the situation when the amount of drug release does not exceed 60% of total amount of drug in the matrix. Thus, the plot was performed until 4 h. The plot for NM without PAA addition was omitted since the initial burst reached about 60%. The drug release profile proved a linear relationship indicating a diffusion controlled drug release mechanism from the polymer matrix after the initial burst. We also investigated the morphology of CM-loaded nanospheres after 20 h of release test, as shown in Fig. 7a. The smooth surface before the test became to be very rugged and it was difficult to find complete round particles. It indicates that those drugs entrapped near the surface of nanospheres dissolve out continuously when the surface of the nanospheres begins to erode, although no significant degradation of polymer matrix was reported at this period (Horisawa et al., 2002). Twenty percent of CM remained after one day and 10% NM with addition of PAA remained after 4 days in the nanospheres (Fig. 4) and it is considered that those drugs loaded near the core of the nanospheres continued to dissolve out slowly with the degradation of the polymer matrix.

The release profile of 5 and 10 mg CM fed in PLGA20k (75:25) nanospheres were similar in percent but that of 2.5 mg CM fed in PLGA20k(75:25) showed slower release. The results suggest that the incorporated drug was not always uniformly distributed in the nanospheres (Fig. 6, Table 3). Blending of high molecular weight polymer in the low molecular weight polymer did not affect the drug encapsulation efficiency but increased slightly the initial burst, although it has been reported that blending high molecular weight polymer decreased the initial burst and the diffusion (Graves et al., 2004). This may be ascribed to the more compact internal structure containing the PGA-rich cluster in the nanospheres with the high molecular weight polymer, that makes drug loading relatively difficult.

For CM-loaded nanospheres prepared by the emulsion solvent diffusion in oil method, we show their AFM pictures after release one day at 37 °C in Fig. 7a and b. Compared to those prepared with low molecular weight polymer PLGA20k(75:25) (Fig. 7a), the nanospheres prepared with blend polymers remained round and the surface was less rough (Fig. 7b), indicating little surface erosion after one day release experiment. For the nanospheres prepared with low molecular weight polymer PLGA20k(75:25) and allowed to be dispersed in water at 25 °C (Fig. 7c), the particles remained their intact morphology. The in vitro release of these nanospheres at 25 °C was shown in Fig. 8. The drug was released very slowly after the initial burst and about 75% still remained in the nanospheres after 4 days.

3.4. Thermal behavior of nanospheres

To elucidate the large temperature-dependent drug release behavior at 37 and 25 °C, we measured the glass transition temperature (T_g) of polymers and nanospheres prepared with these polymers. As shown in Fig. 9, the thermal profiles showed

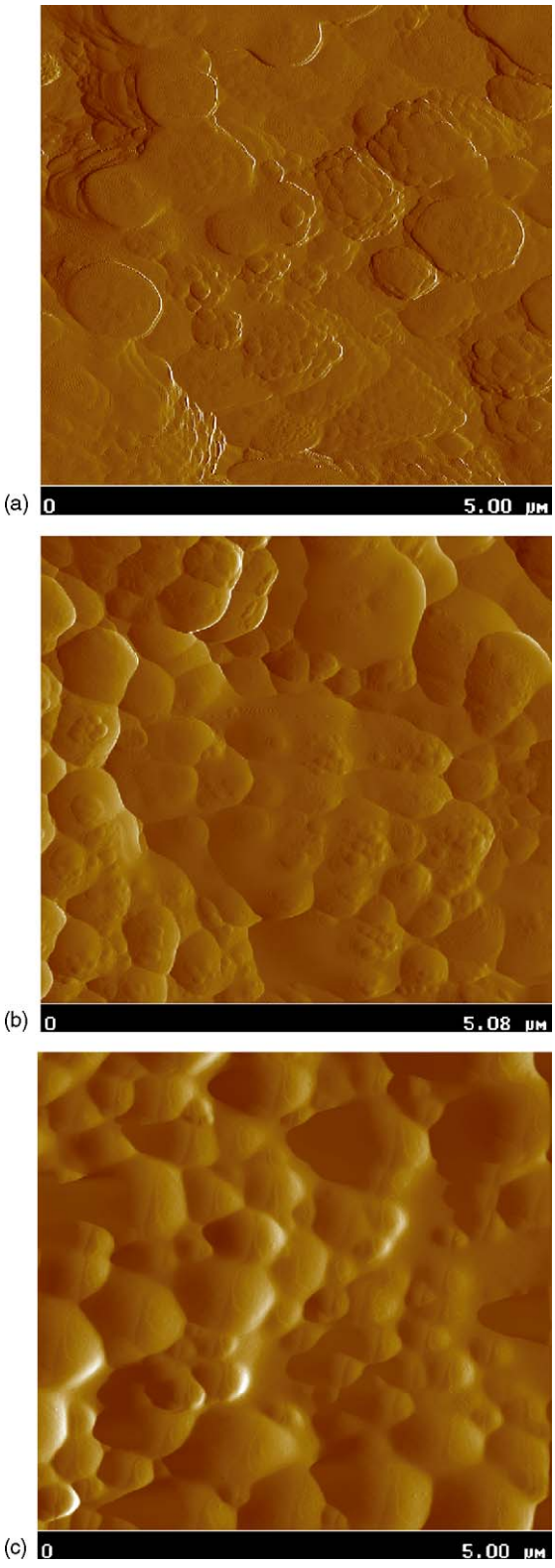

Fig. 7. The AFM of camostat mesilate-loaded nanoshperes prepared by the emulsion solvent diffusion in oil method after release dissolution test for 21 h. (a) PLGA20k(75:25) at 37 °C; (b) blend of PLGA20k(75:25) with PLGA50k(50:50) in 6:4 at 37 °C; (c) PLGA20k(75:25) at 25 °C. The scale bar of AFM was shown as a white line at the bottom, which was exactly the length of the picture.

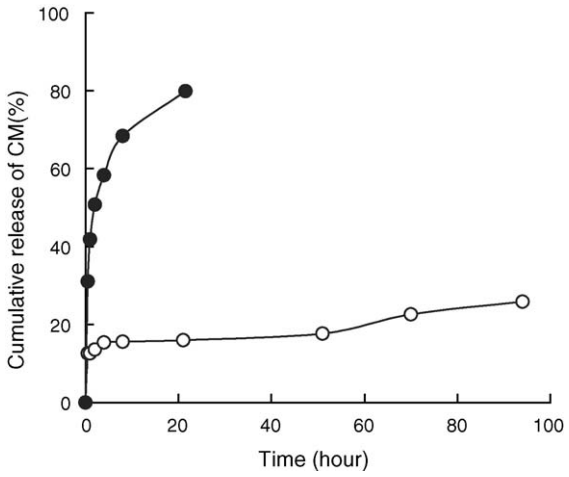

Fig. 8. Effect of experiment temperature on the in vitro release profile of camostat mesilate from PLGA20k(75:25) nanospheres prepared by the emulsion solvent diffusion in oil method in pH 7.4 phosphate buffer. (○) at 25 °C, (●) at 37 °C. All the plots were the average values of duplicate to triplicate runs.

two T_g (28.8–29.7 and 47.1 °C) for CM- and NM-loaded PLGA20k(75:25) nanospheres prepared in the oil system and little difference was found between CM and NM. This result indicates that loaded drugs did not affect the thermal behavior of the nanospheres. However, PLGA20k(75:25) and PLGA50k(50:50) raw polymers showed one T_g at 29.3 and 38.4 °C, respectively. There was no change of T_g of ca. 29 °C between CM-loaded PLGA20k(75:25) nanospheres and its raw polymer. Prepared in the water system, the T_g of PLGA20k(75:25) and PLGA50k(50:50) nanospheres decreased to 24.2 and 34.7 °C, respectively, which may be explained by the plasticization effect of water on the polymer (Park, 1994). It is noted here that the DSC profile of PLGA50k(50:50) nanospheres showed only

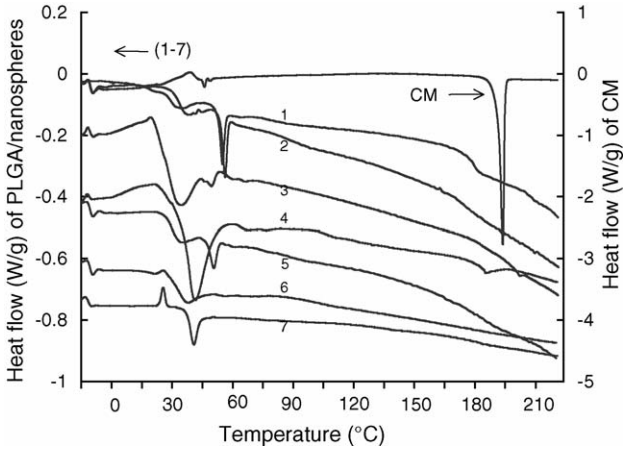

Fig. 9. DSC profiles of camostat mesilate, PLGA polymer and camostat mesilate-, nafamostat mesilate-loaded PLGA nanospheres. (1) CM-loaded PLGA20k(75:25) nanospheres prepared in the oil system; (2) NM-loaded PLGA20k(75:25) nanospheres prepared in the oil system; (3) CM-loaded PLGA20k(75:25) nanospheres prepared in the water system; (4) CM-loaded PLGA50k(50:50) nanospheres prepared in the water system; (5) CM-loaded nanospheres prepared in the oil system with blend of PLGA20k(75:25) and PLGA50k(50:50) in a ratio of 6:4; (6) PLGA20k(75:25) alone; (7) PLGA50k(50:50) alone.

one peak while that of PLGA20k(75:25) nanospheres showed two peaks. Two peaks were also found for the drug-loaded nanospheres prepared with blend of polymers. Accordingly, it was thought that the two peaks arose from PLGA20k(75:25) of the blend polymer. The two glass transition temperatures may be due to the orientation of PLA-rich and PGA-rich domains within the nanospheres occurred during the preparation process or drug–polymer interaction. The characteristic peak for the melting point of CM at 196 °C was absent in the CM-loaded nanospheres, indicating that the drug remains in a dissolved state in the polymer.

The glass transition temperature of the polymer during drug release is a very important feature when elucidating the release mechanism from controlled delivery system (Fan and Singh, 1989; Jain, 2000). If the polymer is in the glassy state, the mobility of the polymer molecules is very low. The free volume available for diffusion decreases, thereby decreasing the diffusivity. In contrast, if the polymer is in the rubbery state, the polymer molecules are much more mobile and the resulting drug diffusivity is orders of magnitude higher than that in the glassy state. In this study, for the nanospheres prepared with the same polymer PLGA20k(75:25), the temperature-dependent drug release behavior verified the effect of T_g on drug release. The release of CM from the nanospheres was retarded at 25 °C as the polymer was in glassy state. After 1 day's dispersion in release medium, these nanospheres remained their intact morphology (Fig. 7c). On the other hand, the polymer was in rubbery state at 37 °C and the release of CM from the nanospheres was much more rapid than that at 25 °C. After 1 day's dispersion in release medium, it was difficult to find complete round particles by AFM observation (Fig. 7a). At rubbery state, the surface of nanospheres was much easier to erode. The release of drug near the surface was accelerated once the surface becomes to erode. So the effect of T_g on the morphology of nanospheres in the release medium may be another factor influencing the release of CM from PLGA nanospheres. This output suggests that the application of high molecular weight polymer may restrain drug release to some extent, especially help to decrease the release amount at the initial period since the surface erosion of these nanopsheres may be retarded due to the polymer's higher glass transition temperature.

4. Conclusions

Water-soluble protease inhibitors CM and NM were efficiently encapsulated up to 60–70% in PLGA nanospheres by the emulsion solvent diffusion in oil method. The nanospheres prepared in the oil system was about 500–600 nm in size, about twice of the nanospheres prepared in the water system. PLGA20k(75:25) was better polymer material compared with PLGA50k(50:50). The high molecular weight polymer resulted in aggregated form in the preparation process. PAA, a complexing agent incorporated for cationic water-soluble drugs, did not work for CM and NM in terms of encapsulation efficiency and release behavior. The glass transition temperature of the polymers is a very important feature for the drug release from controlled delivery system. Selecting PLGA polymers with an appropriate molecular weight, proper PLA and PGA ratio and glass transition temperature may be useful to control the surface erosion of the nanospheres and drug release rate.

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