Preparations of biodegradable nanospheres of water-soluble and insoluble drugs with D,L-lactide/glycolide copolymer by a novel spontaneous emulsification solvent diffusion method, and the drug release behavior


Department of Pharmaceutical Engineering, Gifu Pharmaceutical University, Mitahora-Higashi, Gifu, Japan

(Received 27 May 1992; accepted in revised form 8 December 1992)

Nanospheres with D,L-lactide/glycolide copolymer (PLGA) were prepared as a biodegradable polymeric carrier for both water-soluble and insoluble drugs by a novel spontaneous emulsification solvent diffusion method. Indomethacin and 5-fluorouracil (5-FU) were employed as poorly water-soluble and water-soluble model drugs, respectively, to investigate the encapsulation efficiency. The drug and PLGA, dissolved in an acetone–dichloromethane (or acetone–chloroform) mixture, were poured into an aqueous solution of polyvinyl alcohol with stirring using a high-speed homogenizer when necessary. The dispersed droplets were finely emulsified into nanometer-sized spheres. The marked decrease of the interfacial tension between organic and aqueous phases and the spontaneous mixing caused by a rapid diffusion of acetone from the organic to aqueous phase resulted in the formation of submicron-sized PLGA spheres. The recovery of indomethacin entrapped in the nanospheres (mean diameter: 400–600 nm) increased to 75% at maximum. The rapid deposition of polymeric film on the droplet was required for improving the encapsulation of 5-FU to prevent leakage from the droplet. The mean diameter of nanospheres formulated with 5-FU were successfully decreased to 200–300 nm even without high-speed homogenizing. The drug release behavior from nanospheres suspended in buffered solution exhibited a biphasic pattern. The initial burst of release might be due to the rapid release of drugs deposited on the surface and in the water channels of nanospheres. At a later stage, the drug release rate was reduced. During the release test, PLGA was not degraded for 100 h irrespective of the molecular weight. The molecular weight of polymer was a main factor in controlling the drug release rate from the nanospheres.

Key words: Spontaneous emulsification; Solvent diffusion method; Nanosphere; D,L-Lactide/glycolide copolymer (PLGA); Biodegradation; Indomethacin; 5-Fluorouracil

Correspondence to: T. Niwa, Department of Pharmaceutical Engineering, Gifu Pharmaceutical University, 5-6-1 Mitahora-Higashi, Gifu 502, Japan.
Introduction

A wide variety of polymeric particulate carriers have been devised for protecting the active molecules against the host and for controlling drug release in body fluids, e.g., blood, lymph and digestive juice. Special attention has been paid to the biodegradability of the polymer to avoid chronic toxicity encountered in the administration of nonbiodegradable polymer, especially when parenterally administered. Microparticles made of biodegradable polymers, such as poly(lactide-co-glycolide) (PLGA) [1], poly(D,L-lactide) (PLA) [2] and poly(glycolide) (PGA) [3] have been devised as delivery systems for controlled-release vaccines, cytostatics and insulin. Prolonged-release injectable microcapsules with PLGA encapsulating leuprolide acetate, a highly potent analogue of luteinizing hormone-releasing hormone, were successfully prepared on the industrial scale [4]. However, their sizes were too large to direct the drug to target tissues via systemic circulation.

The colloidal drug carriers, such as liposome and polyalkyl-cyanoacrylate nanoparticles, facilitate transport of the potential drug from injection sites to the target tissues via the vascular system because of their submicron sizes. Jani et al. reported that nanoparticles with diameter smaller than 500 nm, following oral administration, could cross the M cells in Peyer's patch and the mesentery on the surface of gastrointestinal mucosa with retention of their intact drug-loaded vesicular structure, delivering the drug to the systemic circulation [5]. Moreover, Damge et al. demonstrated that polyalkylcyanoacrylate nanoparticles with insulin decreased the glycemia in fed diabetic rats after oral administration [6].

Although Jeffery et al. produced PLGA microcapsules with diameter smaller than 3 μm using an oil-in-water emulsion solvent evaporation system (drying-in-water system) with vigorous homogenization [7], the nanometer-sized drug carrier made of PLGA has not been prepared by this method.

The present authors intended to develop a novel method to prepare PLGA nanospheres loaded with water-soluble and insoluble drugs by modifying the emulsion-solvent diffusion method of preparing spherical polymeric microsponges or microballoons previously established by us [8,9]. Indomethacin and 5-fluorouracil (5-FU) were selected as poorly water-soluble and water-soluble model drugs, respectively. The mechanism of formation of nanospheres was clarified by characterizing the physicochemical properties of the nanospheres, such as particle diameter, surface topography and drug content. Furthermore, the drug release properties of nanospheres and the degradation behavior of PLGA in the nanospheres were investigated.

Materials and Methods

Materials

PLA with average molecular weight of 110,150 (abbreviated as PLA-110,150 hereafter), PLGA with average molecular weights of 12 279, 66 671 and 127 598, whose copolymer ratio of D,L-lactide to glycolide is 85:15 (abbreviate as PLGA(85·15)-12 279, PLGA(85·15)-66 671 and PLGA(85·15)-127 598, respectively) and PLGA(50·50)-65 475 were supplied by Du Pont Company (USA). Polydispersity values of all polymers ($M_w/M_n$) are 1.5–1.9, where $M_n$ and $M_w$ are the number- and weight-based mean molecular weight, respectively. The weight average molecular weight ($M_w$) was determined by gel permeation chromatography (GPC) by the supplier. PLGA(85·15)-66 671 was mainly used to establish the optimum conditions for preparation of nanospheres. Polyvinyl alcohol (PVA-217, Kuraray Company, Tokyo), indomethacin (Sumitomo Pharmaceuticals Company, Osaka) and 5-fluorouracil (Kyowa Hakko Kogyo Company, Tokyo) were used as supplied.
Preparation of nanospheres

The concept of the preparation method was based on the 'emulsion-solvent diffusion technique' [8,9] developed by the present authors. The system consisted of a good solvent phase for the drug and the polymer, and aqueous dispersion phase dissolved with dispersing agent. PLGA (120 mg, Medisorb, Du Pont Co.) and the drug (12 mg) were dissolved in the mixed organic solvent of acetone (0-25 ml) (miscible with water) and dichloromethane or chloroform (0.5, 15 ml) (immiscible with water). The resultant organic solution was emulsified into nanodroplets in 50 ml of aqueous PVA solution (2.0%, w/v) under stirring at 15 000 rpm using a homogenizer (Physcotron, Nichion Irriakikai, Japan) when necessary for a few minutes at room temperature. Then the emulsified system was stirred by a magnetic stirrer under reduced pressure. During evaporation of the water-immiscible organic solvent (dichloromethane or chloroform) from the droplets of mixed organic solution (for 3-4 h), the dispersed nanodroplets solidified in the aqueous solution. The whole dispersed system was filtered with a membrane filter (pore size: 1.0 μm, FR-100, Fuji Photo Film Co., Ltd., Japan) to separate microspheres. The nanospheres dispersed in the filtrate were sedimentated by ultracentrifugation (156 200 g × 1 h; CP-56G, Hitachi Koki Co., Tokyo) and recovered by removing the water. The schematic procedure for preparation is shown in Fig. 1.

Physicochemical properties of nanospheres

The shape and the surface topography of nanospheres dried under cooling at 5°C were observed by means of a scanning electron microscope (JSM-T330A, Nihon Denshi Co., Ltd., Tokyo, Japan). The particle diameters and their distributions of nanospheres dispersed in the system filtered by a 1.0 μm membrane filter were measured by means of a dynamic light scattering method (LPA-300, Otsuka Electronics Co., Ltd., Osaka, Japan). The recovery (%) of nanospheres was represented by a ratio of the weight of the resultant spheres passed through a filter (1.0 μm pore size) to the weights of polymer and drug loaded. The amount of microspheres of more than 1 μm in diameter was excluded from the recovery values. The measurement of the zeta potential of nanospheres was carried out by means of electrophoresis (Lazer Zee Meter Model 501, Pen Kern, USA) in distilled water and phosphoric buffered solution (0.1 M, pH 7.4; ionic strength = 0.679).

Drug content in nanospheres and encapsulation efficiency

The weighed nanospheres after drying under reduced pressure were dissolved in acetonitrile, to which methanol was added to preferentially precipitate the polymer. The drug in the filtrate passed through a membrane filter (pore diameter, 0.22 μm) was properly diluted and analysed spectrophotometrically at 232 and 265 nm for indomethacin and 5-FU (UV-160A, Shimadzu Co., Ltd., Japan), respectively. The drug recovery and content in the nanospheres are represented by equations (1) and (2), respectively.

\[
\text{Drug recovery} \% = \frac{\text{amount of drug in nanospheres}}{\text{amount of drug fed in the system}} \quad (1)
\]

\[
\text{Drug content} \% = \frac{\text{amount of drug in nanospheres}}{\text{amount of nanospheres recovered}} \quad (2)
\]

![Fig. 1. Schematic procedure for preparation of PLGA nanospheres.](image-url)
The theoretical drug content is 9.1%, as calculated from the amounts of drug and polymer loaded.

**Measurement of interfacial tension**

The interfacial tension between the dichloromethane or chloroform phase without dissolving PLGA and drug and 2% of aqueous PVA solution was measured by means of the drop-weight method [10] thermally controlled at 20°C. The data were evaluated from the average value of four measurements.

**Drug release property of nanospheres**

The drug release properties from nanospheres were investigated using a diffusion cell comprising separable donor and acceptor compartments, which were assembled into one unit with an inserted polycarbonate filter (pore diameter, 0.1 μm; diameter, 25 mm; Nuclepore membrane; Nuclepore Co., Ltd., USA) between them. Forty ml of phosphate-buffered solution (0.1 M, pH 7.4) were placed into each compartment as a dissolution medium. The nanospheres separated by ultracentrifugation were immediately dispersed throughout the dissolution medium in the donor compartment. The concentration of drug in the acceptor medium transferred through the membrane from the donor medium was periodically monitored spectrophotometrically at 232 nm using a spectrophotometer for indomethacin, and at 254 nm using a high-performance liquid chromatograph (pump, PU-980; detector, 875-UV; Japan Spectroscopic Co., Tokyo) for 5-FU. The whole system for the dissolution test was controlled thermally at 37°C. As a reference, the diffusion test of the free drug through the polycarbonate membrane was undertaken under the same conditions as employed for the nanospheres. It was confirmed that drug degradation did not occur during the test. After the drug release test of nanospheres, the drug concentration in a mixture of the same volume of donor and acceptor media was measured to determine the initial amount of drug loaded.

To investigate the degradation behavior of the PLGA chain in the nanospheres during the drug release test, a dissolution test of drug-free nanospheres dispersed in the donor medium was conducted. Aliquots of the donor medium were taken at suitable intervals to measure the molecular weight change of the PLGA consisting of nanospheres, using gel permeation chromatography (GPC). The measurements were performed with a high-performance liquid chromatograph system (Japan Spectroscopic Co., Ltd., Tokyo) at 35°C (oven, 860-CO) at a flow rate of 1.0 ml/min (pump, 880-PU) detected with a refractive index meter (detector, 830-R1). Two-hundred μl of the PLGA solution dissolved in THF was separated with three Shodex KF-805L columns connected in series (Showa Denko Co., Ltd., Japan). The weight-average molecular weight was calibrated with an integrator (Labchart 180, System Instruments Co., Ltd., Japan) by the use of standard polystyrene (Shodex Standard S series).

**Results and Discussion**

**Spontaneous emulsification solvent diffusion method for the formation of PLGA nanospheres**

It was not possible to produce nanospheres with diameter less than 1 μm using the widely used dichloromethane (or chloroform)-in-water emulsion solvent evaporation (drying-in-water) system as reported by Jeffery [7]. Although vigorous agitation of the system by a high-pressure homogenizer was useful to reduce the particle size [11], the minimum average size in the present work was 1.2–1.5 μm measured by the laser-based time of the transition analysis system (cis-1, Galai, Israel). When acetone was formulated into the organic phase of the drug and polymer, the mean diameter of resultant spheres decreased dramatically to the order of submicrons, compared to that of spheres prepared without using acetone as shown in Fig. 2, in which the amount
Fig. 2. Effects of addition of acetone in polymer solution on mean diameter of nanospheres with indomethacin (○, △) and interfacial tension (●, ▲) of organic solvent without dissolving PLGA and drug/2% of aqueous PVA solution. Key: (△,▲) CH₂Cl₂; (○,●) CHCl₃ system. Amount of CH₂Cl₂ (CHCl₃): 15 ml. Dispersed medium: 2.0% PVA solution.

of dichloromethane or chloroform in the organic solution was fixed to 15 ml. When the organic polymer solution comixed with more than 5 ml of acetone was homogenized in the aqueous medium mechanically, the mean diameter of PLGA nanospheres containing indomethacin decreased consistently to 400-500 nm. Moreover, 5-FU (a water-soluble model drug) was successfully encapsulated in the PLGA nanospheres without high-speed homogenizing, when the volume of dichloromethane was reduced to 0.5 ml in the mixture, as discussed later. Homogenization of the system without acetone produced only coarse spheres having the diameters of 2–3 μm [7].

It was found that the interfacial tension between the dichloromethane or chloroform phase and the aqueous medium decreased with increasing amount of acetone in the organic phase in the equilibrium state, as shown in Fig. 2. In the actual preparation process, the two-phase system (i.e., organic and aqueous media) was not in equilibrium. Therefore, the concentration gradient of acetone at the interface of the two phases existed even at lower concentrations of acetone in the organic phase. Such a concentration gradient could produce longitudinal variations of interfacial tension depending on the concentration of acetone, as shown in Fig. 2, at the interface. This variation induces the interfacial turbulence or spontaneous agitation of the interface between two unequilibrium liquid phases, which is governed by the so-called Marangoni effect [12]. It is further reported in the literature that solute (acetone in the present system) transferring out of the phase of higher viscosity (organic phase), steep concentration gradients (acetone) at the interface and interfacial tension variation sensitive to solute concentration are the important factors promoting the interfacial turbulence. Such complex interfacial hydrodynamic phenomena, i.e., the perturbation of the interface arising from the rapid diffusion of acetone across the interface between organic and aqueous phases spontaneously produced a much larger interface area resulting in much finer droplets. Therefore, this technique was named the “spontaneous emulsification solvent diffusion method”, developed by modifying our previous method [8,9]. The diffusion and evaporation of the organic solvents from the organic dispersed droplets and the counterdiffusion of water into the droplets reduced the solubility of PLGA and deposited it in the droplets, forming the nanospheres. When the nanoencapsulation was carried out using the aqueous dispersing phase containing acetone and the dispersed simple organic phase (without acetone), the diameter of nanospheres increased two-fold and the recovery drastically decreased to 1/40. This result agreed with the previous report [12], in which the interfacial turbulence was usually greater for solute transfer from organic to aqueous phase than for transfer in the opposite direction. It was clearly indicated that the addition of acetone to the organic phase was essential for effectively forming the nano-sized spheres.

The amount of acetone in the organic polymer solution required to maximize the recovery of nanospheres was different depending on the type of organic solvent, i.e., in dichloromethane or chloroform (Fig. 3). The difference might be interpreted as due to the solubility difference of solvent in water. The solubility of dichloromethane in water is higher than that of chloroform. Therefore, part of the dichloromethane in the mixed solution could transfer with acetone into
the outer aqueous phase, contributing to the higher recovery with less volume of acetone than in the chloroform system.

Figure 4 shows the effects of PVA concentration in the aqueous dispersing medium on the recovery and the particle size of PLGA nanospheres containing indomethacin. The volumes of chloroform and acetone in the organic phase were fixed at 15 and 12.5 ml, respectively. The recovery of nanospheres increased with increasing concentration of PVA dissolved in the aqueous medium, whereas the particle size of nanospheres was independent of the concentration of PVA. PVA was assumed to work as a protective colloid for the emulsion droplets during the preparation. The PVA molecules adsorbed on the surface of droplets prevented the coalescence of droplets. However, it was found that the suspension with the solidified nanospheres was physically stable in the absence of any surfactant, e.g., PVA or poloxamer. The excellent stability of dispersion was attributed to the electric repulsion force brought about by the high zeta potential of nanospheres (−49.6 and −10.6 mV for indomethacin, −37.6 and −10.1 mV for 5-FU in distilled water and phosphoric buffered solution, respectively), due to the carboxylic groups of the polymer and indomethacin.

Physicochemical identification of PLGA nanospheres and improving drug content in nanospheres

The scanning electron microphotographs of typical nanospheres with indomethacin (A) and 5-FU (B) prepared in the present study are shown in Fig. 5. Discrete spherical nanospheres having a diameter of less than 600-800 nm (A) and 200-300 nm (B) were produced. In the case of 5-FU, smaller nanospheres were produced without using a high-speed homogenizer owing to the complete self-emulsification into the aqueous phase when the organic phase was mixed. The surface of nanospheres appeared to be a rigid film structure. The disappearance of the melting point of the drug on the differential scanning calorimetric thermograms of the dried nanospheres indicated that the drug was dispersed uniformly at the molecular level (i.e., amorphous) in the PLGA polymer.

The mean diameters of nanospheres with indomethacin measured by means of a dynamic light scattering method were around 340-640 nm and the standard deviations were rather low (monodispersity), as shown in Table 1. These values agree with the observation by SEM (Fig. 5). The recovery and the drug content of nanospheres with indomethacin depended mainly on the copolymer ratio of polymer (Table 1). When D,L-PLGA (85·15) was formulated, the drug recovery and the drug content of nanospheres reached the maximum. The poor solubility of D,L-PLGA (50·50) in the organic phase employed...
resulted in the low recovery of nanospheres. Some drug might have leaked out during the preparation process due to the diffusion of acetone from the organic phase droplet in the aqueous dispersing medium. By controlling the pH of the aqueous medium to less than the $pK_a$ of indomethacin ($=4.5$), the leakage of drug was prevented in some degree, and the drug content in nanospheres increased to 7.0% (theoretical value $=9.1$%).

Water-soluble drug, e.g., 5-FU, was poorly encapsulated in the nanospheres when prepared with the same solvent system employed for indomethacin because considerable leakage of drug into the aqueous phase occurred. To improve the entrapment of 5-FU in the nanospheres, the solvent composition for the polymer was modified. When the volume of dichloromethane decreased to 0.5 ml and the volume of acetone increased, the entrapment ratio of 5-FU in the nanospheres was improved, as shown in Table 2. It was also required to admix methanol to dissolve 5-FU in the dispersed organic phase. The higher the acetone to dichloromethane ratio, the more rapidly acetone diffuses into the aqueous phase, causing rapid polymer deposition on the outer surface of droplets. Perfect spontaneous emulsification of the organic phase into the aqueous medium was
TABLE 2

Entrapment of 5-fluorouracil in various polymeric nanospheres prepared in dichloromethane-acetone-methanol system (polymer, 120 mg; drug, 12 mg)

<table>
<thead>
<tr>
<th>Solvent composition</th>
<th>Polymer (Mw)</th>
<th>Mean diameterb</th>
<th>Recovery of nanospheres (%)</th>
<th>Drug recovery in nanospheres (%)</th>
<th>Drug content in nanospheres (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH2Cl2/acetone/methanol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5/5.0/5.0</td>
<td>PLGA(85.15)</td>
<td>12 279</td>
<td>283 ± 37</td>
<td>70.3</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>66 671</td>
<td>213 ± 13</td>
<td>82.7</td>
<td>0.85</td>
</tr>
<tr>
<td>0.5/25.0/5.0</td>
<td>PLGA(85.15)</td>
<td>12 279</td>
<td>195 ± 34</td>
<td>96.6</td>
<td>1.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>66 671</td>
<td>207 ± 13</td>
<td>83.9</td>
<td>5.47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>127 598</td>
<td>199 ± 11</td>
<td>93.7</td>
<td>15.0</td>
</tr>
</tbody>
</table>

*aMw, weight average molecular weight measured by GPC.
bMeasured by photon correlation spectroscopy.

also attained by this modification of the solvent composition for the organic polymer solution, without mechanical homogenization. The film like wall of PLGA deposited at the interface between the droplet and the aqueous medium prevented the leakage of water-soluble drug, resulting in improved drug content in nanospheres. Dichloromethane was more suitable than chloroform for the rapid deposition of the polymer and for the improvement of the trapped percentage of water-soluble drug due to its higher water solubility than chloroform [13].

The drug recovery in nanospheres increased with increasing molecular weight of the polymer employed. This result was also explained by the increased deposition rate of polymer due to the decrease in solubility of polymer with increasing molecular weight.

**Drug release behavior of PLGA nanospheres**

The release behavior of indomethacin from PLGA nanospheres is illustrated in Fig. 6, which indicates the biphasic pattern. At the initial stage, the burst of drug release appeared with the nanospheres with lower-molecular-weight polymer. By increasing the molecular weight of the polymer to 127 598, this burst was significantly avoided. At a later stage, the drug was released more slowly, the rate of which might be controlled by the degradation speed of the polymer of nanospheres. Therefore, the degradation of the polymer was monitored by measuring the molecular weight of the polymer composed of nanospheres with gel permeation chromatography (Fig. 7). It was notable that the degradation of the polymer did not substantially occur, at least for the monitored period (100 h) in the present system. The initial burst might be due to the rapid release of drugs deposited on the surface and in the water channels in nanospheres because the molecular weight of the polymer did not decrease during this period. Therefore, when the rigid matrix structure was produced in the na-

![Fig. 6. Release profiles of indomethacin from PLGA nanospheres in phosphate buffer (0.1 M, pH 7.4). Key: (O) solution; nanospheres with (●) PLGA(85-15)-12 279; (△) PLGA(85-15)-66 671; (▲) PLGA(85-15)-127 598.](attachment:fig6.png)
nospheres of higher molecular weight, e.g., PLGA(85·15)-127 598, the burst of drug release was avoided. At a later stage, the drug was released more slowly, the rate of which was determined by the diffusion of the drug in the rigid matrix structure. It was reported that the release behavior of hexapeptide from PLA-4000 and PLA-6000 microspheres showed a lag time of 2–3 weeks, during which the drug was not released after the initial burst [14]. However, the drug was released continuously from the nanospheres because of their large specific surface area compared to microspheres.

For 5-FU, even if the molecular weight of PLGA increased to 127 598, the initial burst of drug release could not be avoided, as shown in Fig. 8. It was assumed that most of the 5-FU was also released before the degradation of PLGA nanospheres. The rather rapid release of 5-FU might be due to the higher solubility of 5-FU in water and the smaller diameter of nanospheres than those of indomethacin. The initial burst within a few hours should be avoided for the clinical application of nanospheres. However, in the present release test intact nanospheres with drug-dissolved water channels were employed, because the nanospheres were coalesced during drying. Further investigations to find a suitable drying method to avoid the coalescence of nanospheres are required in the future.

In conclusion, both poorly and well water-soluble drugs (indomethacin and 5-fluorouracil) were successfully encapsulated in PLGA nanospheres (diameter, 200-600 nm) by means of the novel spontaneous emulsification solvent diffusion method using an O/W emulsion system. By coadmixing acetone into the dispersed organic phase, the interfacial tension between the organic and aqueous phase was significantly reduced and perturbation at the interface occurred due to the diffusion of acetone through the organic phase to the aqueous medium. These actions may be the main factors for promoting spontaneous emulsification. The polymer and the drug were coprecipitated in the dispersed nanodroplets during the diffusion and the evaporation of organic solvent from the system, resulting in the PLGA nanospheres. The present method is of advantage to avoid heating and homogenization of the system, and is easy to scale up to the industrial level. The drug was released from the nanospheres in aqueous medium, following the biphasic kinetics of the initial burst and the prolonged release which depended on the molecular weight of the polymer employed.

The present nanospheres can be expected to be used as carriers of physiologically active agents, e.g., hormone, peptide and antigen, because of their biodegradability, and to be distributed into the systemic circulation after parenteral (intramuscular, subcutaneous, intravenous) administration. Furthermore, their discrete submicron-sized structure is attractive for application to oral
or transmucosal (ophthalmic, nasal and pulmonary) administration.

Acknowledgements

The authors wish to thank Du Pont Japan Ltd. for the supply of PLGA.

References