International Journal of Pharmaceutics 370 (2009) 196–201

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Particle size control of poly(DL-lactide-co-glycolide) nanospheres for sterile applications

Yusuke Tsukada[a], Kaori Hara[a], Yohei Bando[a], C.C. Huang[b], Yasuo Kousaka[a], Yoshiaki Kawashima[c], Ryuichi Morishita[d], Hiroyuki Tsujimoto[a,*]

[a] Hosokawa Powder Technology Research Institute, 1-9 Shoudai, Tajika, Hirakata, Osaka 573-1132, Japan
[b] Hosokawa Micron Powder Systems, 10 Chatham Road, Summit, NJ 07901, USA
[c] Aichi Gakuin University, 1-100 Kusumoto, Chikusaku, Nagoya, Aichi 464-8650, Japan
[d] Division of Gene Therapy Science, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita 565-0871, Japan

ARTICLE INFO

Article history:
Received 5 August 2008
Received in revised form 22 October 2008
Accepted 24 November 2008
Available online 28 November 2008

Keywords:
PLGA nanospheres
ESD method
Drug delivery system
Pressure filtration
Sterilization
Particle size

ABSTRACT

Parameters affecting the particle sizes of poly(DL-lactide-co-glycolide) (PLGA) nanospheres produced by the Emulsion Solvent Diffusion (ESD) method were evaluated in this study, so that suitable PLGA nanospheres could be prepared to pass through a membrane filter with 0.2 μm pore size and used as a sterile product. Experimental results demonstrated that the particle sizes of PLGA nanospheres could be reduced by the following efforts.

(1) Increase stirring rate of poor solvent.
(2) Decrease feed rate of good solvent.
(3) Increase poor solvent ratio.
(4) Increase the temperature of poor solvent.
(5) Decrease polyvinyl alcohol concentration in poor solvent.
(6) Increase ethanol concentration in good solvent.
(7) Decrease PLGA concentration in good solvent.

After optimization, PLGA nanospheres with a mean particle size of 102–163 nm and the 100–98% of filtration fraction could be produced and passed the bacteria challenge tests. This study found PLGA nanospheres can be efficiently prepared as a sterile product.

1. Introduction

Nano-sized particles such as liposome (Ishida and Kiwada, 2004), polymer micelle (Kataoka et al., 2001), emulsion (Igarashi et al., 2001) and PLGA nanospheres (Kawashima et al., 1998; Murakami et al., 1999, 2000; Kawashima, 2006) have been widely used in drug delivery systems (DDS) to improve the drug absorption, reduce the side-effects by improving drug delivery efficiency to affected area or tumor tissue, and sustain pharmacological effects.

PLGA, a biodegradable and biocompatible material, has been clinically used not only as surgical suture thread since long but also as substrate material for sustained-release microcapsule products, e.g. Leuplin® (Takeda Pharmaceutical Company Ltd., Japan) (Toguchi et al., 1991; Okada et al., 1994; Kawamura, 2006).

Various methods have been proposed for making PLGA nanospheres (Fessi et al., 1989; Bodmeier and Cohen, 1990; Allémann et al., 1992). One of the promising techniques is the Emulsion Solvent Diffusion (ESD) method (Kawashima et al., 1998; Murakami et al., 1999, 2000; Kawashima, 2006) developed by Kawashima et al. With this method, nano-sized emulsion droplets are formed by self-emulsification generated at the time when water-miscible organic solvent with dissolved PLGA is added into water, which is a poor solvent for PLGA. Consequently, PLGA in the droplets precipitates as both solvents continue to counter diffuse; and, the drug encapsulated PLGA nanospheres can be easily prepared under moderate mechanical mixing with a stir at a speed below 1000 rpm if the organic solvent also contains drug. Therefore, the ESD method is more suitable for encapsulating polymer or heat-sensitive drugs like nucleic acid drug than other nano-precipitation methods, such as emulsification–diffusion method (Fessi et al., 1989) applying water-immiscible organic solvent and salting-out method (Allémann et al., 1992) using considerable amount of salt,

* Corresponding author at: Hosokawa Powder Technology Research Institute, 1-9 Shoudai, Tajika, Hirakata-shi, Osaka 573-1132, Japan. Tel.: +81 72 855 2231; fax: +81 72 855 2294.
E-mail address: hytsujimoto@hmc.hosokawa.com (H. Tsujimoto).

0378-5173/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2008.11.019

which require high-speed mechanical stirring for emulsification. In addition, the ESD method is much easier to scale up and can encapsulate low molecular weight drugs as well as peptides and nucleic acid drugs in the PLGA nanospheres as reported in the literatures (Tsujimoto et al., 2004, 2005; Yamamoto et al., 2004; Tsukada et al., 2006). We have succeeded in encapsulating not only low molecular weight drugs but also peptides and nucleic acid drugs in the PLGA nanospheres by the ESD method and reported their usefulness in the DDS applications (Tsujimoto et al., 2004, 2005; Yamamoto et al., 2004; Tsukada et al., 2006).

Sterilization procedures were investigated, since it is required to qualify PLGA nanospheres as a sterile pharmaceutical product for clinical trials. Heat sterilization is not suitable for PLGA, because of its low glass transition temperature (about 45 °C). Other unheated sterilization methods including electron irradiation and the pressure filtration by using a membrane filter with 0.2 μm pore size could be considered. However, both drugs and PLGA can decompose with the former method and it results in the change of product's properties, such as the release rate of a drug from PLGA capsule (Toguchi et al., 1991; Kawamura, 2006). Also, it is not easy to identify all components generated by the decomposition and evaluate their toxicity. Therefore, the latter method is better for sterilization of PLGA nanospheres.

This study initially evaluated the pressure filtration performance of PLGA nanospheres with a mean particle diameter of 200–300 nm prepared by the standard conditions of ESD method as stated in the previous reports (Tsujimoto et al., 2004, 2005; Yamamoto et al., 2004; Tsukada et al., 2006). However, only less than 10% of the nanospheres could pass through the membrane filter, which was not suitable for the practical use as a sterile product.

To establish the preparation conditions for making PLGA nanospheres proper for filter sterilization, this study evaluated the effects of following parameters on the particle sizes of PLGA nanospheres: (1) stirring rate of poor solvent, (2) feed rate of good solvent, (3) poor solvent ratio to the whole solvent, (4) temperature of poor solvent, (5) polyvinyl alcohol concentration in poor solvent, (6) ethanol concentration in good solvent, and (7) PLGA concentration in good solvent.

The filtration fraction of PLGA nanospheres prepared by the optimum operating conditions obtained in this study was determined, and its sterilization performance was validated by the bacteria challenge test. Furthermore, the primary particle size of PLGA nanospheres dispersed in water was also evaluated with transmission electron microscope (TEM).

2. Materials and methods

2.1. Materials

PLGA (wt. average molecular weight: 20,000, co-polymer ratio of DL-lactide to glycolide: 75/25, PLGA-7520, Wako Pure Chemical Industries Ltd., Japan) was used as the substrate of the nanospheres. Polyvinyl alcohol (PVA) (EG-05, Nippon Synthetic Chemical Industry Co., Ltd., Japan) was used as the dispersant for the production of PLGA nanospheres. High reagent grade acetone, acetonitrile, methanol and ethanol were used as good solvents for PLGA; and, Japanese Pharmacopoeia grade purified water was used as the poor solvent.

2.2. Preparation of PLGA nanospheres by ESD method

Fig. 1 showed the apparatus of a production system for preparing PLGA nanospheres and experimental conditions. The preparation procedures of nanospheres under basic condition are as follows.

80 mL of 1 wt% PVA aqueous solution (a poor solvent for PLGA) was put into a 500 mL cylindrical glass vessel, as shown in Fig. 1.

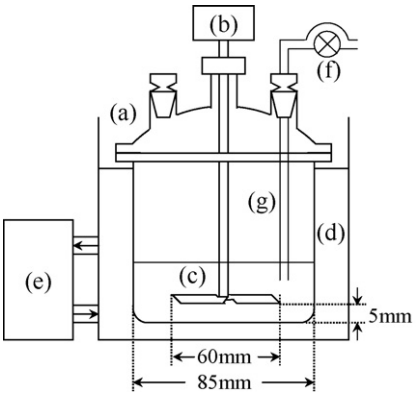

Fig. 1. Apparatus for preparing of PLGA nanospheres with ESD method: (a) separable cylindrical glass vessel (500 mL); (b) motor; (c) propeller agitator; (d) water bath; (e) thermo-regulator; (f) tubing pump; (g) feeding tube, and the experimental conditions. Experimental conditions (the basic condition is shown in parentheses): stirring rate, 10–1000 rpm (400); feed rate of good solvent into poor solvent, 2–360 mL/min (20); the ratio of poor solvent to whole, 40–73 vol% (43); temperature of poor solvent, 5–40 °C (40); PVA concentration in poor solvent, 1–10 wt% (1); ethanol concentration in good solvent, 0–38 vol% (33); PLGA concentration in good solvent, 6.7–133 mg/mL (33)

60 mL of 33 vol% ethanol/acetone solution containing 2 g of PLGA was dropped into the poor solvent stirred at 400 rpm and 40 °C at a rate of 20 mL/min. After the operation, a white suspension containing dispersed PLGA nanospheres were obtained as described in the literatures (Kawashima et al., 1998; Murakami et al., 1999, 2000; Kawashima, 2006). The organic solvents were then removed by vacuum evaporation at −60 kPa, 40 °C with a stirring rate of 200 rpm.

The operating parameters were varied as follows to evaluate their effects on the particle size of PLGA nanospheres.

(1) Stirring rate is from 10 to 1000 rpm,
(2) feed rate of good solvent is from 2 to 360 mL/min,
(3) poor solvent ratio to the whole solvent is from 40 to 73 vol% at 60 mL of good solvent,
(4) temperature of poor solvent is from 5 to 40 °C,
(5) PVA concentration in poor solvent is from 1 to 10 wt%,
(6) ethanol concentration in good solvent is from 0 to 38 vol% of ethanol/acetone at 60 mL of acetone, and
(7) PLGA concentration in good solvent is from 6.7 to 133 mg/mL.

2.3. Physical evaluations of PLGA nanospheres

Mass equivalent mean particle diameter of PLGA nanospheres was measured by the dynamic light scattering method using MICROTRAC UPA-150 from Nikkiso Co. Ltd., Japan. Information required for particle size measurements, such as relative density of PLGA nanospheres, refractive index of PLGA nanospheres, refractive index of solvent under the measuring conditions, and viscosity of nanospheres suspension, were obtained by using gas displacement method with Ultrapycnometer™1000 from Quantachrome Instruments USA, Beche line detection method with ECRIPSE80i™ from Nikon Japan, V block method with Refractometer PR-2 from Carl Zeiss Jena Germany, and cone plate type viscometer LVDV-II Pro CP from Brookfield Engineering Laboratory USA, respectively.

The PLGA nanospheres used for the measurements of the relative density and refractive index were prepared by the following procedure.

PLGA nanospheres suspension prepared as mentioned before was centrifuged at 48,000 × *g* and −20 °C for 20 min with CR-20G (Hitachi High-Technologies, Japan) to cause sedimentation of the nanospheres. After removing the supernatant, purified water was

added to mix with the sediment. The wet PLGA nanospheres were then freeze dried.

The viscosity of poor solvent was measured by the same viscometer as mentioned above.

The morphology of PLGA nanospheres to be used for pressure filtration evaluation was observed by TEM JEM-2010 from JEOL Japan. Uranium acetate was used as the staining agent in the TEM observation. Number equivalent mean particle diameter of the nanospheres was determined from a cumulative distribution curve consisting 300 circle equivalent diameters of particles obtained by TEM image analysis with Scion Image Beta 4.0.3, Scion Corp. USA. The particle diameter is also known as Heywood diameter and described as TEM observation diameter in this study.

The pressure filtration property of the nanospheres was evaluated by the following procedures. 50 mL of a diluted suspension containing 0.25 wt% PLGA nanospheres was put in a filtration equipment consisting a stainless-steel cylindrical barrel (42 mm ID by 160 mm long) with a 0.2 μm polyethersulfone membrane filter (Φ47 mm, Sartopore 2®, Sartorius Stedim Japan) at its bottom. The suspension was filtrated by pressurizing the internal of the equipment to 0.15 MPa with nitrogen gas. The filtration fraction of PLGA nanospheres was calculated by comparing PLGA concentrations in the suspension before and after the filtration operation. The PLGA concentration in the suspension was measured by the following procedure.

(1) Take the weight of a 30 mL glass bottle and then put 10 mL of the suspension in the bottle to measure the weight (W_{sus}) of the suspension.
(2) After freeze drying the suspension, 30 mL of acetone was added to dissolve the dry PLGA in the bottle. The solution was then filtered through a 0.2 μm PTFE membrane filter (ADVANTEC, Japan) and collected in another pre-weighted 30 mL glass bottle (W_{g1}). Furthermore, additional 10 mL of acetone was used to wash through the membrane filter and collected, so that the PLGA could be recovered as much as possible.
(3) After the collected solution being dried at 80 °C, the weight (W_{g2}) of the glass bottle was measured.
(4) The PLGA concentration in the suspension was calculated with the equation below.

$$\text{PLGA concentration (wt\%)} = \frac{(W_{g2} - W_{g1})}{W_{sus}} \times 100$$

The sterilization performance of the membrane filter under the filtration condition was evaluated by the bacteria challenge test based on FDA Aseptic Processing Guideline. Brevundimonas diminuta was used as a common biological indicator. Because PLGA nanospheres suspension had no antibacterial activities, the indicators were directly added during the tests. By closing the valve-inlet of the filtration equipment to stop the flow of the suspension, the contact time of the suspension with the membrane filter was adjusted to exact 300 min. The integrity of the membrane filter before and after the test was examined using the bubble point test (Sartocheck®, Sartorius Stedim Japan).

3. Results and discussion

3.1. Effects of operating parameters on the particle size of PLGA nanospheres

3.1.1. Relationship between the dispersion of emulsion droplets and the particle size of PLGA nanospheres

The dispersion of emulsion droplets appeared affecting the particle size of PLGA nanospheres. Fig. 2 showed the relationship between the stirring rate and mean particle diameter of

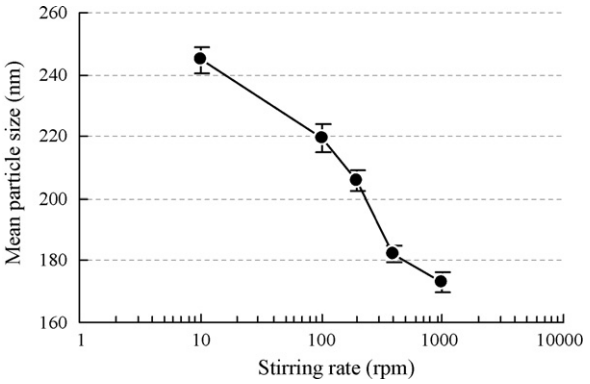

Fig. 2. Relationship between mean particle size of PLGA nanospheres and stirring rate. Data were shown as a mean ± S.D. (n = 3).

PLGA nanospheres. The mean particle diameter decreased with an increase in stirring rate. Because the emulsion droplets generated by ESD method were more likely to coalesce comparing to other droplets whose surfaces were stabilized by surfactants and counter diffusion of both solvents completed before the droplets sufficiently dispersed in the poor solvent, the increase in stirring rate could improve the droplet dispersion, prevent droplet coalescence and resulted in a smaller apparent particle size of PLGA nanospheres.

The dispersion condition of the droplets changed as well according to its concentration in the poor solvent. Therefore, we evaluated the effects of feed rate of good solvent and poor solvent ratio, which controlling the droplets concentration in poor solvent, on the particle size of nanospheres. Fig. 3 showed the changes of the particle size of nanospheres against feed rate; and, Fig. 4 showed that against poor solvent ratio. As seen in Fig. 3, the mean particle diameter showed a minimum at 20 mL/min of feed rate and a maximum at 2 mL/min. The mean particle diameter increased with feed rate quickly when over 20 mL/min due to high-droplet concentrations. However, at the feed rate of 2 mL/min, some amount of PLGA deposit was observed at the vent of feeding tube and inner surface of the experimental vessel after feeding the good solvent. This phenomenon did not happen when setting the vent above the level of PVA solution in the vessel. It was believed that the PLGA deposit was formed before good solvent completely left the vent of feeding tube at very low feed rate. As a result, the emulsion droplets tended to coalesce and formed large PLGA nanospheres.

Fig. 4 showed that particle diameter decreased as the ratio of poor solvent increased. It was because the increase in poor solvent ratio would reduce the droplet concentration in the poor solvent,

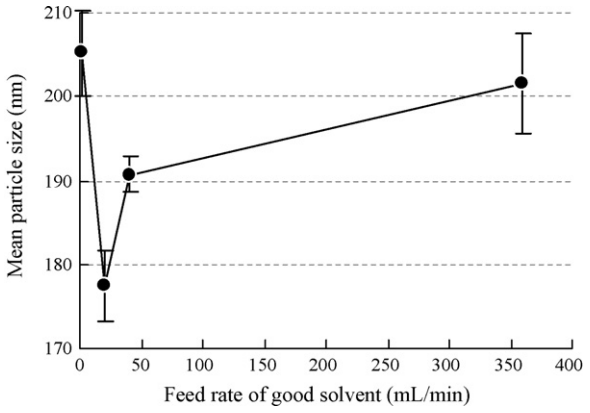

Fig. 3. Relationship between mean particle size of PLGA nanospheres and feed rate of good solvent. Data were shown as a mean ± S.D. (n = 3).

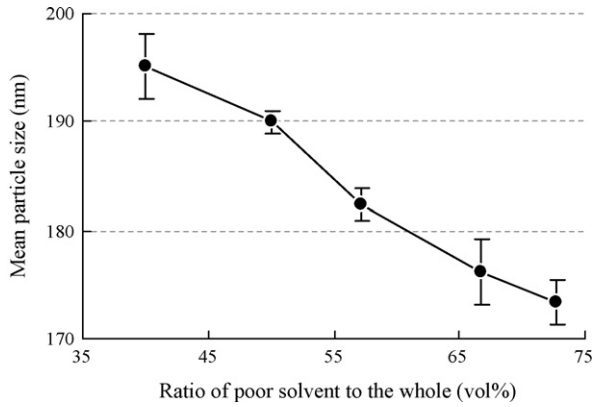

Fig. 4. Relationship between mean particle size of PLGA nanospheres and the ratio of poor solvent to whole. Data were shown as a mean ± S.D. (n = 3).

which prevented droplets from coalescence, and resulted in smaller sizes of PLGA nanospheres.

3.1.2. Relationship between the viscosity of poor solvent and the particle size of PLGA nanospheres

Not only the dispersion condition of the droplets but also the precipitation rate of PLGA can affect the coalescence of the emulsion droplets. The precipitation rate is determined by the counter diffusion rate of the solvents and the solubility of PLGA in good solvent. Consequently, we paid attention to the viscosity of poor solvent, which influenced the counter diffusion rate and could be controlled by the temperature of poor solvent or its PVA concentration.

Figs. 5 and 6 showed the effects of poor solvent temperature and PVA concentration in poor solvent on the mean particle diameter of PLGA nanospheres and the viscosity of poor solvent, respectively. Both the viscosity of poor solvent and the mean particle diameter of PLGA nanospheres decreased with an increase in poor solvent temperature and decrease in PVA concentration. The precipitation rate of PLGA in droplets increased because the decrease in the poor solvent viscosity facilitated the counter diffusion rate of solvents. And, it prevented emulsion droplets from coalescence and in turn resulted in smaller PLGA nanospheres. Using PVA as emulsifying agent with high-speed mechanical stirring as previously reported (Konan et al., 2002), an increase in PVA concentration decreases the particle size of PLGA nanospheres. However, with the ESD method, the particle size is easily influenced by the viscosity of the solvent or PLGA concentration rather than PVA concentration. As a matter of fact, sub-200 nm PLGA particle can be prepared without any PVA, although its re-dispersibilty after freeze-dried is slightly degraded.

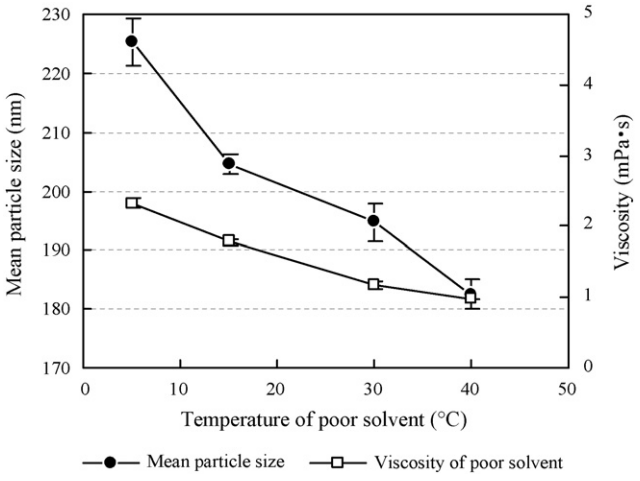

Fig. 5. The effect of poor solvent temperature on mean particle size of PLGA nanospheres and the viscosity of poor solvent. Data were shown as a mean ± S.D. (n = 3).

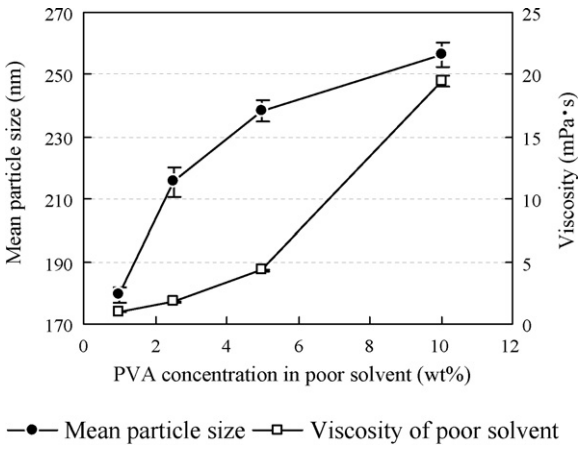

Fig. 6. The effect of PVA concentration in poor solvent on mean particle size of PLGA nanospheres and the viscosity of poor solvent. Data were shown as a mean ± S.D. (n = 3).

3.1.3. Relationship between the solubility of PLGA in good solvent and the particle size of nanospheres

Next, the influence of the solubility of PLGA in good solvent was evaluated as another parameter for controlling the precipitation rate of PLGA in emulsion droplets and the particle size of PLGA nanospheres.

Fig. 7 showed the change of particle size of PLGA nanospheres against ethanol concentration in the good solvent. The mean particle diameter of PLGA nanospheres decreased as ethanol concentration increases. This was because the increase in ethanol concentration would reduce the solubility of PLGA in the good solvent and improve the precipitation rate of PLGA in the droplets. It in turn prevented the droplets from coalescence and produced smaller PLGA nanospheres.

3.1.4. Relationship between PLGA concentration in good solvent, the particle size of nanospheres, and dispersion condition of PLGA nanospheres in water

The preceding paragraphs discussed the relationship between the operating parameters of ESD method and the particle size of generated PLGA nanospheres from the view point of the coa-

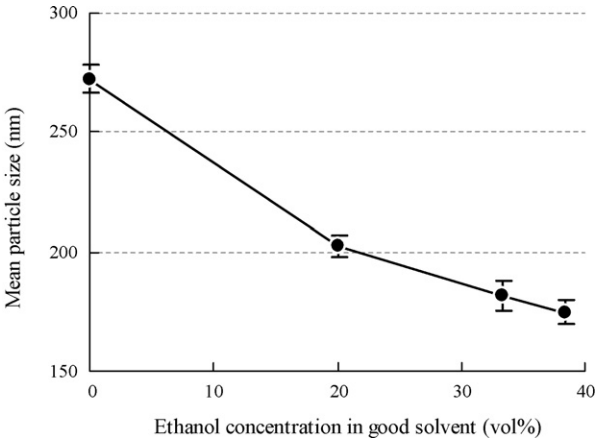

Fig. 7. Relationship between mean particle size of PLGA nanospheres and the ethanol concentration in good solvent. Data were shown as a mean ± S.D. (n = 3).

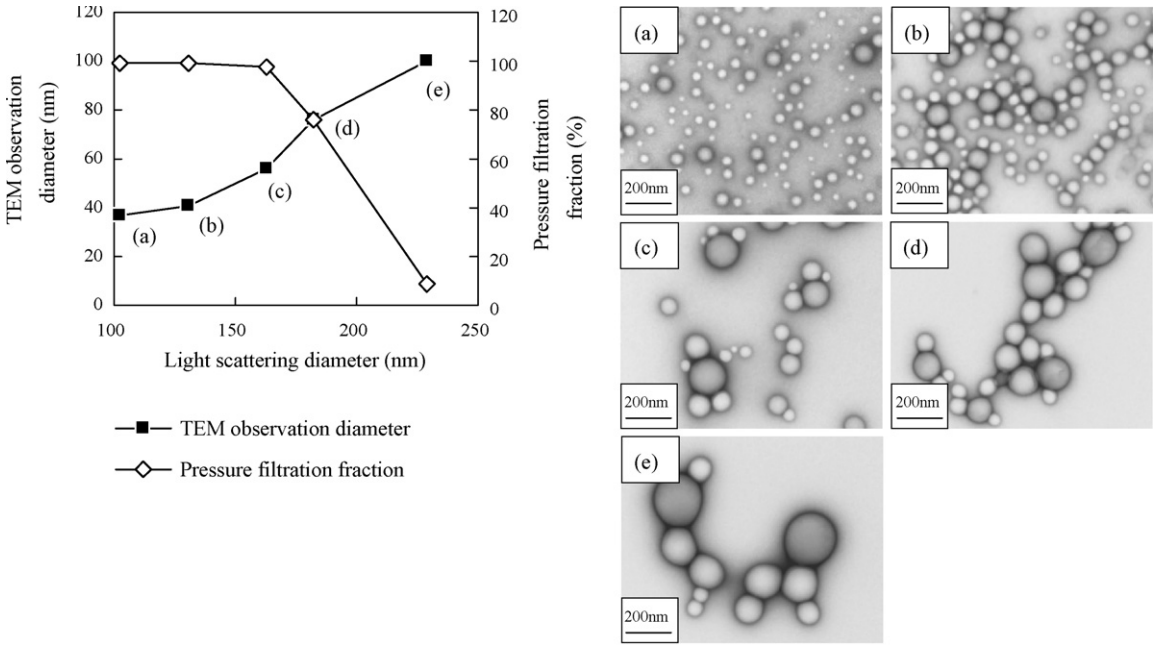

Fig. 8. TEM observation diameter and pressure filtration fraction as a function of light scattering diameter of PLGA nanospheres and their TEM images. Light scattering diameter was (a) 102 nm, (b) 131 nm, (c) 163 nm, (d) 182 nm, and (e) 229 nm respectively; PLGA concentration was (a) 6.7 mg/mL, (b) 17 mg/mL, (c) 33 mg/mL, (d) 67 mg/mL, and (e) 133 mg/mL, respectively under the optimum operating conditions (i.e. stirring rate: 1000 rpm, feed rate of good solvent: 20 mL/min, poor solvent ratio: 73 vol%, the temperature of poor solvent: 40 °C, PVA concentration in poor solvent: 1 wt%, and ethanol concentration in good solvent: 38 vol%).

lescence of emulsion droplets. In Fig. 8, this study evaluated the effect of PLGA concentration in good solvent on the particle size of PLGA nanospheres under the optimum operating conditions (i.e. stirring rate: 1000 rpm, feed rate of good solvent: 20 mL/min, poor solvent ratio: 73 vol%, the temperature of poor solvent: 40 °C, PVA concentration in poor solvent: 1 wt%, and ethanol concentration in good solvent: 38 vol%). As seen, the mean particle size of PLGA nanospheres decreased dramatically as PLGA concentration in the good solvent reduced from (e) to (a) under the optimum operating conditions. Although the decrease of PLGA concentration caused lower precipitation rate in the droplets and more droplet coalescence as mentioned above, the droplets were more constricted and led to the formation of smaller PLGA nanospheres overall. This was because PLGA molecule precipitated in the emulsion droplet had a certain constant density at very low PLGA concentration.

The TEM photographs of the nanospheres in Fig. 8 were also used to determine the TEM observation diameter. The photograph showed that several nanospheres smaller than 100 nm were agglomerated together. It was believed that PLGA nanospheres prepared by ESD method always had some degree of agglomeration in water. This agglomeration phenomena as a function of ESD operating parameters will further be investigated by TEM method in the future.

3.2. Pressure filtration property of PLGA nanospheres and bacteria challenge tests of filtered PLGA nanospheres

The filtration fraction of PLGA nanospheres could be calculated by comparing PLGA concentrations in the suspension before and after the filtration operation. Using the technique to measure the PLGA concentration in the suspension as mentioned earlier, the filtration fraction of PLGA nanospheres with a mean particle diameter of 102–163 nm was determined to be 100–98%, which were far more than the one (less than 10%) prepared by standard operating conditions as reported in the past (Tsujimoto et al., 2004, 2005; Yamamoto et al., 2004; Tsukada et al., 2006). The decrease in the particle size from 200–300 nm to 102–163 nm did significantly increase the amount of PLGA nanospheres passing through the membrane filter and resulted in a very high-filtration fraction. Fig. 9 showed the particle size distributions of the PLGA nanospheres prepared with 33 mg/mL of PLGA concentration in good solvent under optimum operating conditions. Its mean particle diameter was 163 nm as measured by dynamic light scattering method, which was about 2.9 times larger than the TEM observation diameter (i.e. 56 nm). Although the maximum particle size measured by dynamic light scattering method was over 300 nm, its pressure filtration fraction was 98% through the polyethersulfone membrane filter with a pore size of 0.2 μm. The TEM observation diameter appeared more relevant to the test result of pressure filtration.

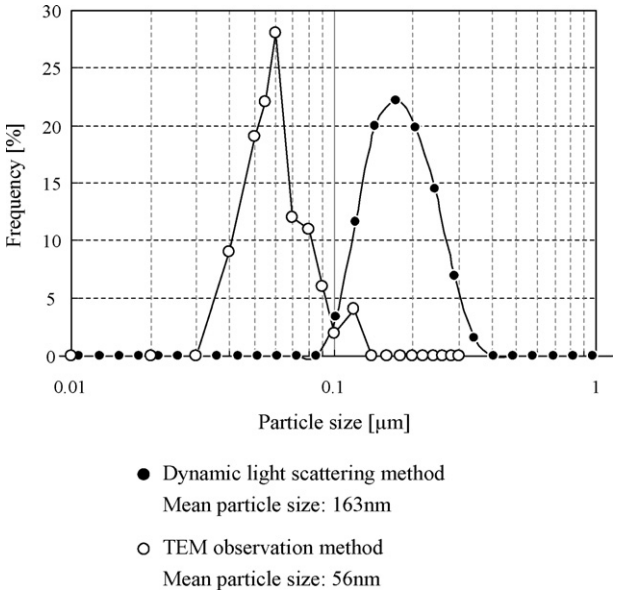

Fig. 9. Particle size distributions of PLGA nanospheres prepared with 33 mg/mL of PLGA concentration in good solvent under optimum operating conditions.

As to the bacteria challenge tests, no biological indicator was found in the incubated culture medium after applying the filtrate for all samples prepared in Fig. 8. The test was validated by the following procedures: (1) the membrane filter before and after use passed a bubble point test; (2) no bacteria existed in the filtrate obtained by a control test using sterile RO water; and (3) the biological indicator formed many colony of itself after being cultured for 24 h if the culture solution containing the biological indicator was filtrated using a membrane filter with 0.45 μm pore size. The test results found it is possible to obtain sterile PLGA nanospheres suspension efficiently by pressure filtration.

4. Conclusion

By adjusting the operating parameters of ESD method, this study demonstrated that PLGA nanospheres could be prepared to pass through a membrane filter used for sterilization.

Optimum operating conditions obtained in this study could produce PLGA nanospheres with a mean particle diameter of 102–163 nm. Although the produced PLGA nanospheres had some degree of agglomeration under TEM observation, their filtration fraction was 100–98% and they could also pass the bacteria challenge test. These results demonstrated that sterile PLGA nanospheres could be efficiently produced with proper ESD preparation conditions.

References

Allémann, E., Gurny, R., Doelker, E., 1992. Preparation of aqueous polymeric nanodispersions by a reversible salting-out process, influence of process parameters on particle size. Int. J. Pharm. 87, 247–253.

Bodmeier, R., Cohen, H., 1990. Indomethacin polymeric nanosuspension prepared by microfluidization. J. Control Release 12, 223–233.

Fessi, H., Puisieux, F., Devissaguet, J.P., Ammoury, N., Benita, S., 1989. Nanocapsule formation by interfacial polymer deposition following solvent displacement. Int. J. Pharm. 55, R1–R4.

Igarashi, R., Takenaga, M., Takeuchi, J., Kitagawa, A., Matsumoto, K., Mizushima, Y., 2001. Marked hypotensive and blood flow-increasing effects of a new lipo-PGE1 (lipo-AS013) due to vascular wall targeting. J. Control Release 71, 157–164.

Ishida, T., Kiwada, H., 2004. Accelerated blood clearance of PEGylated liposomes after repeated injection. Drug Deliv. Syst. 19, 495–510.

Kawashima, Y., Yamamoto, H., Takeuchi, H., Hino, T., Niwa, T., 1998. Properties of a peptide containing DL-lactide/glycolide copolymer nanospheres prepared by novel emulsion solvent diffusion methods. Eurp. J. Pharm. Biopharm. 45, 41–48.

Kataoka, K., Harada, A., Nagasaki, Y., 2001. Block copolymer micelles for drug delivery: design, characterization and biological significance. Adv. Drug Deliv. Rev. 47, 113–131.

Kawamura, K., 2006. GMP and development of pharmaceuticals in Japan from view point of an industry scientist. PDA J. GMP Valid. Jpn. 8, 2–17.

Kawashima, Y., 2006. Design of poly(lactic-co-glycolic acid) (PLGA) nanosphere for developing to DDS. J. Pharm. Sci. Technol., Jpn. 66, 224–238.

Konan, Y., Gurny, R., Allémann, E., 2002. Preparation and characterization of sterile and freeze-dried sub-200 nm nanoparticles. Int. J. Pharm. 233, 239–252.

Murakami, H., Kobayashi, M., Takeuchi, H., Kawashima, Y., 1999. Preparation of PLGA nanoparticles by modified spontaneous emulsification solvent diffusion method. Int. J. Pharm. 187, 143–152.

Murakami, H., Kobayashi, M., Takeuchi, H., Kawashima, Y., 2000. Further application of a modified spontaneous emulsification solvent diffusion method to various types of PLGA and PLA polymers for preparation of nanoparticles. Powder Technol. 107, 137–143.

Okada, H., Doken, Y., Ogawa, Y., Toguchi, H., 1994. Preparation of three-month depot injectable microspheres of leuprorelin acetate using biodegradable polymers. Pharm. Res. 11, 1143–1147.

Toguchi, H., Ogawa, Y., Okada, H., Yamamoto, M., 1991. Once-a-month injectable microcapsules of leuprorelin acetate. YAKUGAKU ZASSHI 111, 397–409.

Tsujimoto, H., Hara, K., Huang, C.C., Yokoyama, T., Yamamoto, H., Takeuchi, H., Kawashima, Y., Akagi, K., Miwa, N., 2004. Percutaneous absorption study of biodegradable PLGA nano-spheres via human skin biopsies. J. Soc. Powder Technol., Jpn. 41, 867–875.

Tsujimoto, H., Hara, K., Kawashima, Y., 2005. Evaluation of glycaemia control in beagle dogs by the administration of insulin encapsulated PLGA nano-composite preparations. J. Soc. Powder Technol., Jpn. 42, 765–772.

Tsukada, Y., Tsujimoto, H., Hara, K., Sakaguchi, M., Aoki, M., Morishita, R., Kawashima, Y., 2006. The developments of NFκB decoy oligodeoxynucleotides loaded PLGA nanosphere and the applications for atopic dermatitis. In: 2nd International Technical Forum Inspiring Powder Technology, November 9, Japan, pp. 45–46.

Yamamoto, H., Kurashima, H., Katagiri, D., Yang, M., Takeuchi, H., Kawashima, H., Yokoyama, T., Tsujimoto, H., 2004. Poly(lactic-co-glycolic acid) nanosphere composite prepared with Mechanofusion® dry powder composition system for improving pulmonary insulin delivery with dry powder inhalation. J. Pharm. Sci. 64, 245–253.