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SIZE CHARACTERIZATION OF CORE-SHELL POLY(L-LACTIDE) MICROSPHERES BY FLOW/HYPERLAYER FIELD-FLOW FRACTIONATION

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ABSTRACT

Flow/hyperlayer field flow fractionation (Fl/Hy FFF) has been applied for the rapid separation and size characterization of core-shell type poly(l-lactide) (PLLA) microspheres which are widely studied as a potential drug carrier due to its biodegradability. Examined microspheres are prepared by varying the entrapped amount of the retinoic acid as an anti-cancer therapeutic and varying PEG-PLLA copolymers. It is demonstrated that the separation of PLLA microspheres can be achieved within 5 min. by Fl/Hy FFF and the particle size distribution (PSD) is readily obtained from the experimental fractograms. From the size distribution of PLLA particles obtained by Fl/Fl FFF, it is shown that PSD increases with the amount of drug loaded but it does not seriously change with the addition of PEG-PLLA copolymers.

INTRODUCTION

Biodegradable polymers are of great interest in the biomedical applications since these polymers can be utilized as a drug delivery material in the form of micro(nano)spheres containing drugs or genetic materials inside the particle shell and they can be degraded in the body.[1~5] Advantages of using biodegradable polymeric particles are the versatility of modifying polymer properties for the controlled release of drug and the applicability in injectable formulations, oral formulations, and bioadhesive systems.[1] In utilizing the polymeric microspheres as a drug delivery system, there are many factors related to the characteristics of drug release. The type of polymers, the type and amount of drug, the insertion of copolymers as a stabilizer, and the preparation methods are the interrelated factors in designing controlled release of microspheres and the combination of them often influences the size of prepared particles.[1,2]

Since the particle size of drug delivery material is related to the uptake or transport rate of drugs when they are injected,[3] it is one of the important parameters in pharmaceutical applications. Characterization of pharmaceutical microspheres is commonly carried out by electron microscopy.[2,3] Microscopic measurement normally gives an accurate data but requires a lot of efforts with time when an accurate size distribution needs to be provided. Light scattering techniques are conveniently used for pharmaceutical particles.[6~9] It is widely used for nanoparticles since micron sized particles have a relatively low diffusivity for light scattering measurements. However, light scattering techniques provides an average diameter of particles instead of size distribution.

Flow field-flow fractionation (FlFFF), one of the sub-techniques of FFF family, is a separation technique applicable to the separation and size characterization of proteins, polysaccharides, and synthetic polymers as well as particulate materials such as cells, liposomes, environmental particles, and etc.[10~15] Separation in FlFFF takes place in a thin rectangular channel constructed by clamping two plastic blocks which have permeable ceramic frits on both sides and the spacer material cut as ribbon like shape is inserted between these frits. In FlFFF, a sheet of semipermeable membrane is placed at one side of channel walls in order to keep sample materials from penetrating the accumulation wall. While a stream of flow is introduced through the channel inlet for the migration of sample materials towards the end of the FlFFF channel, a second stream of flow (crossflow) is applied through the one side of the channel walls that consists of ceramic frit. The crossflow is transferred from the one side of frit to the other and this is the perpendicular direction to the migration flow (channel flow). The crossflow acts as a driving force to push sample components toward the bottom wall (accumulation wall).

The basic principle of particle retention in an FlFFF channel is based on the diffusion of sample materials against the applied field (crossflow) which plays a role in selective placement of sample materials at different flow velocity

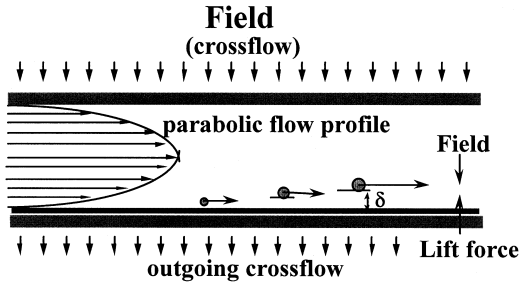

Figure 1. Diagram of side view of flow FFF channel with the particle migration phenomena under the external field that is counterbalanced with lift force.

regions of parabolic flow streamlines.[10] Since particles of small diameter diffuse faster than those of large diameter, they protrude into the faster streamline of parabolic flow in a thin channel and thus they elute earlier than the large ones. Therefore, sample components migrate down the channel with the order of increasing Stoke's diameter and this retention profile is referred as the normal mode of FFF retention. Thus, prediction of particle retention is theoretically obtained if experimental conditions are known. This relationship enables one to calculate particle size from the experimental retention time of sample particles.

At this regime, the Brownian diffusion of particles dominates and it governs the retention of particles of size up to approximately 1 μm. Above this limit, particle retention is affected by the particle size itself since the diffusion of large particles (larger than 1μm normally) is relatively small or negligible and it does not play an important role in counterbalancing the external field. In this case, large particles are driven very closely to the channel wall by the field and the height of particle's center becomes a decisive factor in determining the average migration velocity of particles in which large particles occupy the faster flow streamline than the small ones. Therefore, large particles elute earlier than the small ones and the elution order at this regime is opposite to that of normal FFF.

However, it is known by experiments that large particles migrate down the channel at certain positions elevated from the wall due to the existence of hydrodynamic lift forces[16] which push particles away from the wall as shown in Figure 1 for flow FFF. This is described as hyperlayer mode of operation and is denoted as flow/hyperlayer FFF (Fl/Hy FFF) when it is applied to flow FFF. Contrary to the theoretical clearness of the retention in normal mode, the retention in hyperlayer mode can not be predicted by theory since the lift forces are not completely understood yet. Therefore, particle size calculation in Fl/Hy FFF requires an empirical relationship between the particle diameter d and the retention time t_r which is satisfied by a calibration procedure as:[17,18]

$$\log t_r = -S_d \log d + \log t_{r1} \quad (1)$$

where S_d is the diameter-based selectivity and t_{r1} is the extrapolated retention time of particles of unit diameter. In Fl/Hy FFF, a calibration is normally carried out with polystyrene standards, and the calculation of particle diameter (hydrodynamic diameter in exact) and the size distribution of any other particulate materials can be easily obtained.[17,18]

The objective of this study is to demonstrate the capability of particle separation in Fl/Hy FFF and to apply the size characterization of core-shell type pharmaceutical microspheres, which are widely studied as drug delivery materials. Examined microspheres are poly(l-lactide) (PLLA) particles of which the particle shell is prepared with or without the presence of copolymers [PEG(polyethylene glycol)–PLLA]. In general, polylactides are being widely used for the carrier system in the form of microspheres and nanospheres due to its biodegradability.[1,4,5] PLLA particles examined in this work contain the retinoic acid being used as an anti-cancer therapeutics. They were prepared by varying the amount of copolymer addition and the amount of the entrapped drug. In this work, it is observed by Fl/Hy FFF the dependency of the size distribution of PLLA particles upon particle preparations. Since particle elution in Fl/Hy FFF is independent of particle density, core-shell particles like PLLA microspheres are sufficiently resolved without the need of the density compensation[19] from PS standards as required in sedimentation FFF.

EXPERIMENTAL

For the preparation of microspheres, poly(l-lactide) (MW 110K) was purchased from Boehringer Ingelheim (Ingelheim, Germany), all-trans-retinoic acid from Sigma Chemical Co. (St. Louis, Mo, USA), polyvinyl alcohol (PVA, 98% hydrolyzed, MW 13~23K) from Aldrich Chemical Co. (Milwaukee, WI, USA). PEG-PLLA copolymers were directly prepared in lab and the number average molecular weights are measured as 5K for PEG and 25K for the copolymers. Microspheres are prepared from oil in water emulsion with sonicator by the solvent evaporation method. A fixed amount of PLLA (250 mg), variable amount of PEG-PLLA (2~20 mg), and retinoic acid (5~20 mg) were dissolved in 5 mL of dichloromethane and this mixture was injected into 40 mL of 2% polyvinyl alcohol (PVA) solution.

The mixture was emulsified by using a Ultra-Turrax homogenizer from IKA-Labortechnik (Staufen, Germany) for 10 min. The emulsified mixture was then stirred for 2 hrs. to allow the solvent to evaporate and the collected microspheres were washed with distilled water several times to eliminate PVA and finally freeze dried for 24 hrs. The types of microspheres prepared are listed in Table 1 with the varied amount of copolymers and the retinoic acid.

Table 1

The List of Microspheres with the Polymer Composition (PLLA and PEG-PLLA Copolymers) and the Amount of Loaded Drug Used in This Work

No.	PLLA/PEG-PLLA/Retinoic Acid (mg)
Sample 1	250/0/5
Sample 2	250/0/10
Sample 3	250/10/5
Sample 4	250/20/5
Sample 5	250/20/20

Flow Field-Flow Fractionation

The flow FFF system utilized here is a model F-1000 Universal Fractionator from FFFractionation, LLC (Salt Lake City, UT, USA). The system consists of two HPLC pumps for the delivery of carrier liquid: a model M930 solvent delivery pump from Young-Lin Co. (Seoul, Korea) and a model cc-100-s Eldex pump from Rainin Instrument Co. Inc. (Woburn, MA, USA). Monitoring of eluted sample materials is achieved at 254 nm by a model M720 UV detector and the detector signal is transferred to a pc by using an Autochro data acquisition software both from Young-Lin Co. The flow FFF channel has a tip-to-tip length of 27.2 cm, a breadth of 2.0 cm, and thickness of a Mylar spacer of 127 μm. A sheet of membrane used for the accumulation wall at the channel bottom is YM-30, a regenerated cellulose from Amicon (Beverly, MA, USA).

The carrier solution used in this work is ultrapure water (purified by reverse osmosis and deionized) containing 0.05% SDS for particle dispersion and 0.02% NaN_3 for bactericide. Carrier solution is filtered through the membrane filter (0.4 μm pore size) before use. Particle samples are injected to the flow FFF channel via a Rheodyne 7125 loop injector from Rheodyne (Cotati, CA, USA) with the injection amount of 5 μL (approximately 30 ~ 36 μg) of the sample suspension. The standard particles used for calibration are polystyrene (PS) latex beads having nominal diameters of 9.975, 6.995, 4.991, 4.000, 3.004, and 2.013 μm (hereafter called as 10, 7, 5, 4, 3, and 2 μm) from Duke Scientific (Palo Alto, CA, USA).

Eluted microspheres from the detector are collected at short time interval (12 seconds) by using a Dynamax FC-2 fraction collector from Rainin Instrument Co. Inc for the confirmation of particle size at each fraction by a Leo 420 scanning electron microscope from Leo Electron Co. (Cambridge, UK).

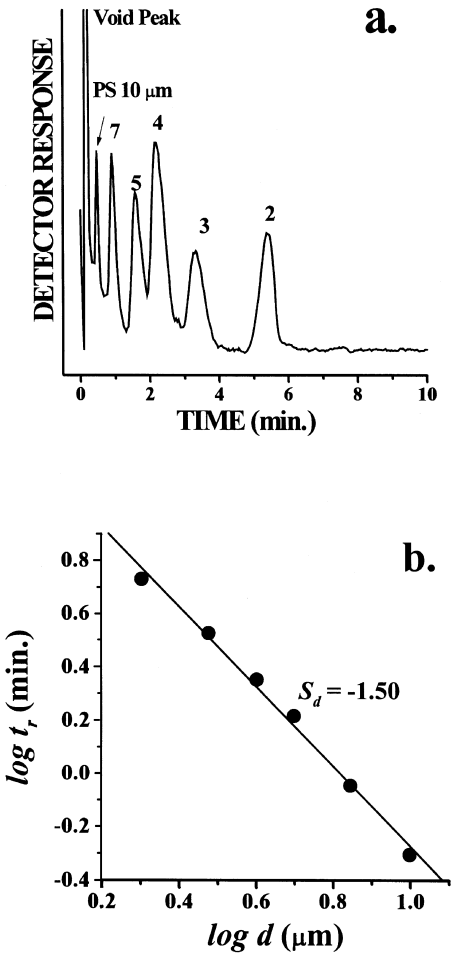

Figure 2. (a) Separation of PS latex standards by Fl/Hy FFF and (b) the plot of *log* t_r vs. *log d* by least squares fit. Experimental data are represented with filled circles.

Microscopic measurements are made with collected particles filtered on polycarbonate membrane of 13 mm in diameter which is later placed on aluminum stub by using the graphite colloid adhesives. Followed by drying the microspheres, they are treated with gold sputtering and are examined by EM at 20 kV with magnifications of 500 times. Photographic images are transferred to pc and saved as image files.

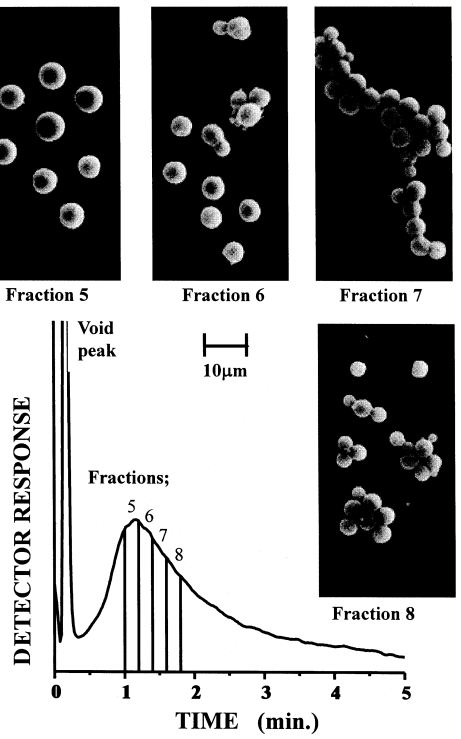

Figure 3. Fractogram of PLLA microspheres (sample 3) by Fl/Hy FFF along with electron micrographs of each collected fraction. Fractions are collected at the time interval marked in the fractogram. Run condition is the same as used in Figure 2.

RESULTS AND DISCUSSION

Separation of supramicron sized particles by Fl/Hy FFF is normally achieved at a high speed and a typical fractogram is shown with polystyrene latex mixtures in Figure 2a. The run condition used in Figure 2a is 4.91 mL/min. for the channel flow rate and 4.80 mL/min. for the crossflow rate. Figure 2a demonstrates a resolving capability of Fl/Hy FFF system for the size range of 2~10 μm in diameter within 6 min. The linear relationship between *log* t_r and *log d* of the standard particles is shown in Figure 2b by the least squares fit of the experimental retention time of PS beads. The correlation coefficient of the fit is 0.997 and the slope of the curve S_d is 1.50 with t_{r1} of 16.90 min (the extrapolated time of unit diameter particle). The run condition used in Figure 2 is selected for the size characterization of PLLA microsphere samples since it accommodates the entire size range of PLLA micropheres used in this study with a high speed separation.

Table 2

Comparison of Average Diameter of Collected Fractions by SEM and Fl/HyFFF for PLLA Microsphere Samples

a) Sample 3

Fraction No.	Time (min)	Average Particle Size (•m) FFF Calculation	SEM
5	1.0 - 1.2	6.57 - 5.82	5.92 ± 0.23
6	1.2 - 1.4	5.82 - 5.25	5.43 ± 0.31
7	1.4 - 1.6	5.25 - 4.80	5.01 ± 0.12
8	1.6 - 1.8	4.80 - 4.44	4.40 ± 0.21

b) Sample 4

4	0.6 - 0.8	8.45 - 6.97	7.40 ± 0.43
5	0.8 - 1.0	6.97 - 6.01	6.40 ± 0.32
7	1.2 - 1.4	5.33 - 4.81	5.00 ± 0.21

Figure 3 shows the elution profile of a PLLA particle sample (Sample No. 3 in Table 1) obtained by Fl/Hy FFF along with electron micrographs of narrow particle fractions collected at the end of the FFF run. Particle elution in hyperlayer run of flow FFF proceeds with large particles first as expected by the standards run. Fractogram of sample 3 appears as broad due to the result of consecutive migration of different diameter particles and, thus, it is expected that the particle size should be broad in its distribution. Collected particles in each fraction appear to be nearly uniform in their sizes from micrographs and it implies that particles are well fractionated by the size. Average diameter of particles at each fraction is measured for more than 50 particles and the data is compared with the calculated value from the calibration by using eq. 1. These are listed in Table 2a.

By comparing the average diameter with the values calculated from the Fl/Hy FFF, it falls within the diameter range of each fraction. This implies the PLLA particles used in this study behave well in Fl/Hy FFF. The particular PLLA sample contains retinoic acid in the core of particles and its shell is stabilized with the addition of PEG-PLLA copolymers.

Another PLLA particle sample (Sample No. 4) prepared with the increased amount of the copolymers is resolved in Fl/Hy FFF and the fractions are examined by EM in Figure 4. Fractogram of sample 4 appears to be similar to that of sample 3. Micrographs of collected fractions show that size based separation is well accomplished except that very small particles appear to stick

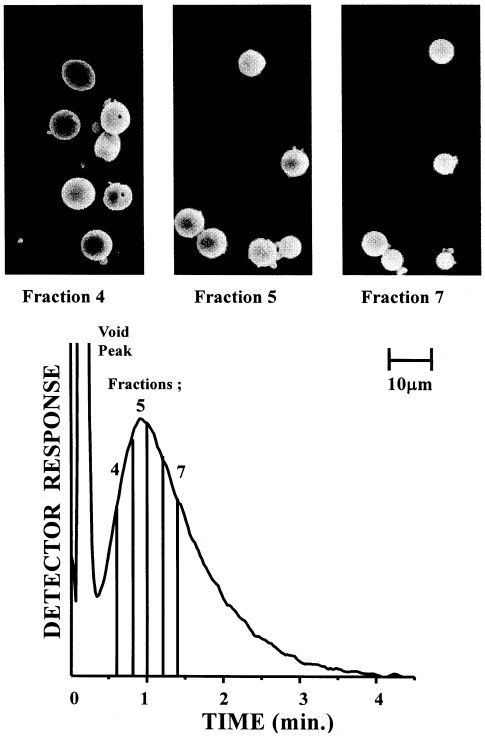

Figure 4. Fractogram of PLLA microspheres (sample 4) by Fl/Hy FFF along with electron micrographs of each collected fraction. Run condition is the same as used in Figure 3.

to large particles as they are aggregated. Average diameters measured at each fraction are listed in Table 2b and they also match with FFF calculation well. Particle size distribution of PLLA particles can be obtained by converting the experimental fractogram into diameter scale by using the calibration parameters. Size Distributions will be discussed later.

The other three PLLA samples are fractionated by Fl/Hy FFF at the same run condition used at the above and the fractograms are superimposed in Figure 5. Depending on the type of preparation, eluted peak shifts to a shorter time scale when the amount of retinoic acid is increased. For the systematic examination of the influence of the preparations on the particle size distribution (PSD), all the fractograms are converted to size distributions by an FFF software with the use of the calibration parameters previously established. Figure 6 shows the superimposed PSD's according to the influence of drug amount (a) and of copolymer amount (b) during preparation on PSD.

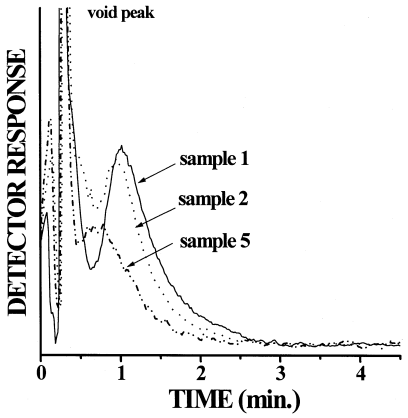

Figure 5. Superimposed fractograms of samples 1, 2, and 5 obtained by Fl/Hy FFF.

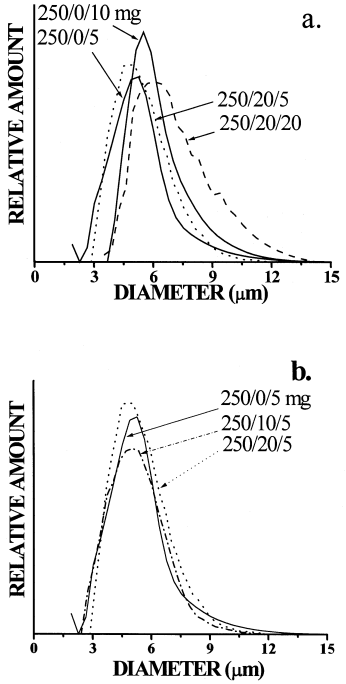

Figure 6. Particle size distributions compared by the particle preparations. Influence of (a) the amount of retinoic acid and (b) the amount of copolymers on PSD.

Figure 6a shows how the particle size distribution is affected by increasing the amount of retinoic acid with or without the presence of copolymers during particle preparation. When the amount of retinoic acid is doubled (sample 2 from sample 1) without adding the copolymer (two solid lines), particle size distribution shifts to a large diameter scale. A similar phenomenon is found with samples 4 and 5 and the shift is obviously large at four times the increase of drug amount. It is likely that particle size distribution shifts to a large diameter scale when the retinoic acid is increased. On the other hand, it is expected that copolymer addition does not seem to alter the particle sizes by comparing PSD's of samples 1 and 4 in Figure 6a. This is shown clearly in Figure 6b. by superimposing PSD's of three different preparations (samples 1, 3,and 4). Addition of PEG-PLLA copolymers to PLLA particle formation with a fixed amount of the retinoic acid does not seem to change the size distribution profile significantly.

In this study, flow/hyperlayer FFF has been applied to the size characterization of core-shell particles like PLLA microspheres. Since Fl/Hy FFF separation does not rely on particle mass but on the hydrodynamic radius, it provides a great advantage of resolving such core-shell particles without the difficulty in obtaining density value by other means. By utilizing Fl/Hy FFF, it is demonstrated that the variation in particle sizes from the different preparations can be monitored at a high speed.

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