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Size characterization of liposomes by flow field-flow fractionation and photon correlation spectroscopy
Effect of ionic strength and pH of carrier solutions

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Abstract

The effect of ionic strength and pH of carrier solutions on the separation of liposomes by flow field-flow fractionation (flow FFF) has been studied for the determination of accurate vesicle size distribution of liposomes. Retention behaviors of liposomes (PC/PG/cholesterol) are observed in typical buffer solutions (PBS and Tris–HCl) of various ionic strengths as carrier liquids in flow FFF. The average diameters of collected fractions at each flow FFF run are measured by photon correlation spectroscopy (PCS) for the comparison with FFF calculations at corresponding time interval of collected fractions. A reasonable separation of liposomes is observed at $I=0.016$ M for both buffer solutions. Retention of liposomes is found to be elongated at ionic strengths higher than an optimum condition found experimentally, but it is shortened at a lower ionic strength due to the electrostatic interaction between the channel wall and the liposomes. Finally, size distributions of liposomes are provided comparing the liposome preparations by flow FFF.

Keywords: Field-flow fractionation; Flow field-flow fractionation; Ionic strength; pH effects; Liposomes

1. Introduction

Liposomes, or lipid vesicles, are currently of interest as model systems for the study of biological membranes and as a number of pharmaceutical applications including potential drug delivery systems [1–4]. Among the various physicochemical properties of liposomes as a drug delivery material, size of the vesicle, with its contents, is an important factor in the liposome properties in vivo [5–7]. Techniques that are currently used to characterize the vesicle size are electron microscopy (EM) [4,6,8], dynamic light scattering [2,5,9], and size exclusion chromatography (SEC) [10]. Electron microscopy provides an impression of size, number, and form of lamellae, but it requires the laborious evaluation of the data with costly equipment. Dynamic light scattering has become a regularly used and convenient technique in liposome size measurement, but precise and accurate results are obtained only for vesicles having a narrow size distribution.

Flow field-flow fractionation (FlFFF) is an elution technique in which particles and macromolecules are separated by flow (separation flow) in aqueous media. This is done by the application of field force

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generated by the transverse movement of carrier liquid (so-called cross flow) across the channel [11–13]. It is capable of fractionating particulates according to their size and providing an accurate size and size distribution of polydisperse particle samples from an observed retention profile. Since the separation in flow FFF takes place in a ribbon-like empty channel space, chances of interaction between particle and stationary phase that are often encountered in SEC can be minimized. When particles or vesicles injected into flow FFF channel are driven toward the bottom of the channel wall (accumulation wall) by the cross flow, they reach equilibrium positions away from the wall due to their diffusive transports against the wall. Brownian motion of particles leads them to be differentially distributed against the wall according to their size: the large particles having a small diffusion coefficient are driven closer to the accumulation wall than the smaller ones. Thus small particles which float further from the wall are displaced by the fast flow stream of the parabolic flow profile and are eluted earlier than the large ones. Fig. 1a illustrates a simple diagram of a rectangular-shaped FFF channel and the enlarged side view of particle migration according to the size. A typical separation of polystyrene standards by FlFFF is shown in Fig. 1b obtained at a channel flow-rate of 6.45 ml/min and a cross flow-rate of 1.06 ml/min. Fig. 1b demonstrates a complete baseline separation of standard mixtures of nearly 10-fold diameter range by FlFFF. The selected run condition used in Fig. 1b will be used to separate liposome samples for most cases in this work since it is appropriate to resolve those which are extruded by membranes having pore size less than 0.4 μm.

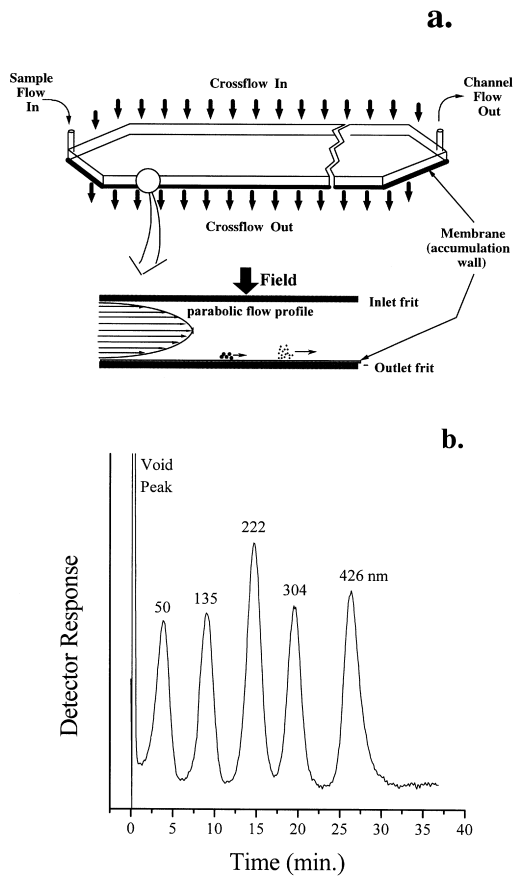

Fig. 1. (a) Schematic diagram of flow FFF channel with the enlarged view of particle separation under the external field and (b) an example of flow FFF separation of polystyrene latex standards obtained at a channel flow-rate of 6.45 ml/min and at a crossflow-rate of 1.06 ml/min.

In flow FFF, retention time, t_r, of a particle or vesicle can be predicted by the theory described elsewhere [12,13]. From this relationship, Stokes diameter, d_s, of vesicle is calculated from the observed retention time by using a simplified expression for well-retained samples

$$d_s = \frac{2kT}{\pi\eta w^2}\frac{\dot{V}}{\dot{V}_c}t_r \quad (1)$$

where kT is thermal energy, η the carrier viscosity, w the channel thickness, $\dot{V}$ the volumetric flow-rate of channel flow, and $\dot{V}_c$ the crossflow-rate. By using Eq. (1), vesicle diameter can be readily calculated from experimental retention time, t_r, with the known experimental parameters.

Utilization of FFF techniques in size determination of liposomes has been done with sedimentation FFF (SdFFF) in studying sonicated small unilamellar vesicles and in monitoring size variation during the formation of fused vesicles [15–17]. While SdFFF provides mass distribution of liposomes, flow FFF can give a direct measure of liposome size since separation of flow FFF is based on the difference in the hydrodynamic radius of particles. Especially, flow FFF can be preferentially used for obtaining the direct diameter distribution of multilamellar vesicles

which are treated as heavy ones compared to unilamellar vesicles of an identical diameter in SdFFF. An earlier report shows the possibility of utilizing flow FFF in the size characterization of sonicated liposomes [13]. In the present work, the influence of ionic strength and pH of carrier solutions on the separation of liposome vesicles is studied by flow FFF for the accurate determination of vesicle size distribution of liposomes. The liposome samples are prepared in our laboratory by a combination of reversed-phase evaporation and sequential extrusion through polycarbonate membranes. Retention behaviors of liposomes that are strengthened by the addition of cholestrol during the liposome preparation are examined by varying ionic strengths and pH of carrier solutions. The average diameter of each narrow fraction collected at the end of flow FFF run is measured by photon correlation spectroscopy (PCS) and the measured diameter is compared with the value calculated by FFF theory using Eq. (1) for the selection of an optimum run condition by flow FFF. Finally, it is demonstrated that the vesicle size distribution of liposomes can be readily obtained from the experimental fractogram obtained by FlFFF using theoretical calculations.

2. Experimental

2.1. Sample preparation

Liposomes were prepared in our laboratory by the reversed-phase evaporation and extrusion procedure [8]. A mixture of 180 μmol of phosphatidylcholine–phosphatidylglycerol–cholesterol (1:4:5 molar ratio) in chloroform solution was placed in a 250-ml round-bottomed flask. After removal of the organic solvent by rotary evaporation, the lipid mixture was redissolved with diethyl ether which was predistilled in the presence of $NaHSO_3$. Three milliliters of aqueous electrolyte solution were added to the mixture and the flask was purged with He. Four different electrolyte solutions were individually used for the liposome preparations: Tris–HCl buffer (pH 7.8), PBS buffer, 0.02% NaN_3 solution, and 0.0236 *M* lactose solution with NaCl. The ionic strengths and pH conditions of the above solutions are listed in Table 1. The mixture was sonicated in a bath type sonicator for 5 min at 20°C under He. Then the organic solvent was completely removed under vacuum on a rotary evaporator until the mixture became a gel-like suspension. This suspension was diluted to 6 μmol/ml with the same electrolyte solution used at each preparation. Diluted suspension was extruded through the polycarbonate membrane from Poretics (Livermore, CA, USA) in a Millipore 25-mm filtration unit from Millipore Corp. (Bedford, MA, USA), which has a polyester drain disc. Extrusions were made with a 0.4-μm membrane followed by 0.2- and 0.1-μm membranes. Extrusions at every pore size level were repeated 5 times. Extruded liposome samples were stored in the refrigerator.

2.2. Flow field-flow fractionation

The flow FFF system used is nearly identical to a Model F1000 Universal FFFractionator of FFFractionation, LLC (Salt Lake City, UT, USA). The channel space is cut from a 254-μm thick Mylar sheet, of 2.0 cm in breadth and 27.2 cm from tip-to-tip length. Membranes used for the accumulation wall at the channel bottom are YM-10, a regenerated cellulose from Amicon (Beverly, MA, USA). The effective channel thickness is calculated as 265 μm by correlating the FFF theory with the elution times of five polystyrene latex standards from Duke Scientific (Palo Alto, CA, USA).

The carrier solution used for the separation of polystyrene latex standards is ultrapure water (purified by reverse osmosis and deionized) containing 0.05% SDS for particle dispersion and 0.02% NaN_3 for bactericide. Carrier liquids are delivered to the flow FFF channel through channel inlet and crossflow inlet by using two HPLC pumps; a Dynamax Model SD-200 and an Eldex cc-100-s Model both from Rainin Instrument Co. Inc. (Woburn, MA, USA). For the separation of liposomes by flow FFF, the electrolyte solutions which are utilized for the liposome preparations, are directly used but the concentrations are varied. All samples are injected into the channel via a Rheodyne 7125 loop injector from Rheodyne (Cotati, CA, USA) with the injection amount of 5 μl (approximately 34 μg/μl) of the liposome suspension. Eluted samples are monitored by a Model M720 UV detector from Young-In Scientific (Seoul, South

Korea) at a wavelength of 254 nm. The detector signals are then recorded by Autochro data acquisition software from Young-In Scientific and are converted to vesicle size distribution curves using Eq. (1) with laboratory software. Liposomes eluted at the end of the detector are collected into narrow fractions within a time interval of 1 min by using a Dynamax FC-2 fraction collector from Rainin.

2.3. Photon correlation spectroscopy

Mean vesicle diameter of each narrow fraction collected from the FFF run is measured by a Model BI-9000AT instrument from Brookhaven Instruments Corp. (Holtsville, NY, USA) using an Ar ion laser at a wavelength of 488 nm. Measurements of the fractionated liposomes are repeated about 3–5 times for each fraction and the results show a reasonable repeatability. The measured values of each fraction are compared with diameters calculated at each time scale of fraction intervals from FFF theory.

3. Results and discussion

Liposome samples prepared in various aqueous media are run by using the corresponding solution as a carrier liquid in FlFFF. This provides the same aqueous surrounding for liposomes in FlFFF separation. Fig. 2 shows the fractograms of two liposome samples obtained by different carrier solutions. Fig. 2a represents the variation of retention profiles of a liposome sample prepared in lactose–NaCl solution subjected to the other carrier solutions used in the liposome preparation. The solutions used in Fig. 2 are listed in Table 1 with the ionic strength of each preparation. When 3.08 m*M* NaN_3 solution and lactose–NaCl solution are used as a carrier solution, respectively, retention behaviors of liposomes appear to be quite similar to each other. However, a broad and more retained peak is obtained when 0.1 *M* Tris–HCl buffer solution is used as a carrier liquid. There is a big difference in the retention of phospholipid vesicles according to the carrier solutions used by flow FFF. Similar phenomena are observed with the liposome sample prepared in Tris–HCl buffer solution in Fig. 2b. Compared to Fig. 2a, retention profiles seem to shift toward the shorter

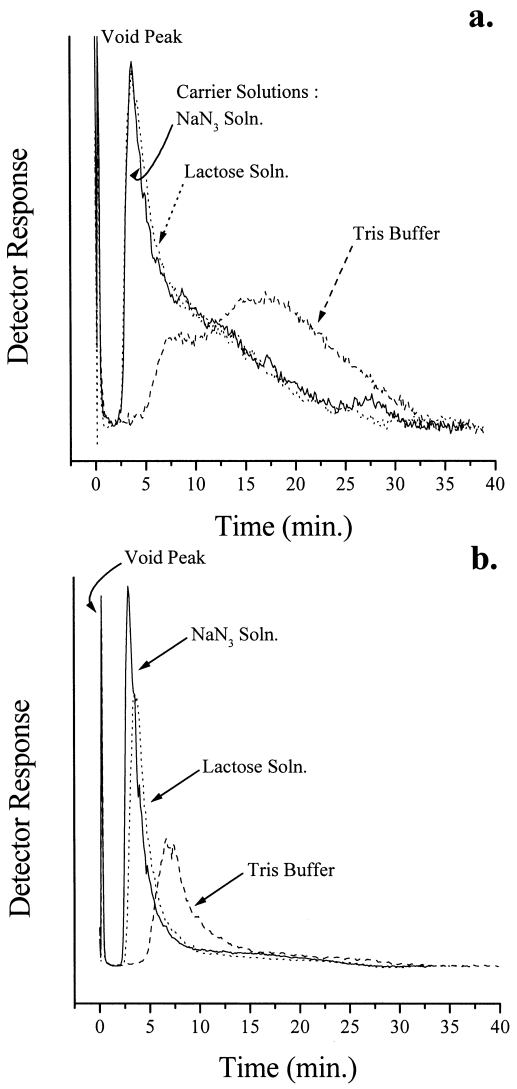

Fig. 2. Elution profiles of liposomes in different carrier solutions. Liposome samples are prepared in (a) lactose–NaCl solution and (b) Tris–HCl buffer solution. The experiments are obtained at a channel flow-rate of 6.44 ml/min and at a crossflow-rate of 1.15 ml/min. Injected amount is approximately 170 μg for each sample.

retention time scale with a relatively narrow distribution. By comparing the ionic strength of carrier solutions (listed in Table 1), Tris–HCl solution has much higher ionic concentration than the other two solutions used in Fig. 2. However, the variations in retention profiles that appear to be dependent on carrier solutions may suggest that there is an in-

Table 1
Type of solutions used for the preparation of liposomes

Type	Contents	pH	I (M)
Tris–HCl	0.1 M Tris–HCl, 0.093 M NaCl	7.80	0.10
PBS	0.64 mM Na_2HPO_4, 0.14 mM NaH_2PO_4, 13.7 mM NaCl	7.40	0.16
Lac–NaCl	0.0236 mM lactose, 4.62 mM NaCl		0.0046
NaN_3	3.08 mM NaN_3		0.00308

fluence of carrier composition on the retention of liposome vesicles. According to the earlier study on the influence of ionic strength of carrier solutions on particle retention in flow FFF, it is known that the ionic strength of carrier liquid plays a decisive role in particle migration at the vicinity of the accumulation wall [14]. Therefore, one can expect that vesicles at certain carrier solutions are not sufficiently driven to their equilibrium positions confined by flow FFF theory and are eluted at elevated positions due to the strong electrostatic interactions between the channel wall (practically a membrane surface) and the charged particles like lipid vesicles. For the case of polystyrene particles, such electrostatic influence on particle retention in flow FFF has been observed to be significant under low-ionic strength conditions ($I<1.0\times10^{-3}$ M) [14]. However, at a finite range of ionic concentration ($10^{-3}<10^{-2}$ M) it is known that particles migrate well in a flow FFF channel along the theoretical trajectories. In contrast, at a high ionic strength particles are retained longer than expected by theory. This is due to the decrease in the electric double layer which results in the closer approach of particles to the channel.

In order to estimate the departures of particle retention from the theory, fractions of eluted vesicles are collected at 1-min intervals during each flow FFF run and are subjected to the photon correlation spectroscopy for the measurement of average vesicle diameter of each liposome fraction. Since PCS provides an accurate size of monodisperse particle sample, vesicle size of each fraction measured by PCS can be treated as the average diameter of the fraction. The average diameter is compared with the calculated value based on FFF theory in Eq. (1). Fig. 3 shows the fractograms of liposome samples prepared (a) in NaN_3 solution and (b) in Tris–HCl buffer, and the runs are obtained by using a carrier

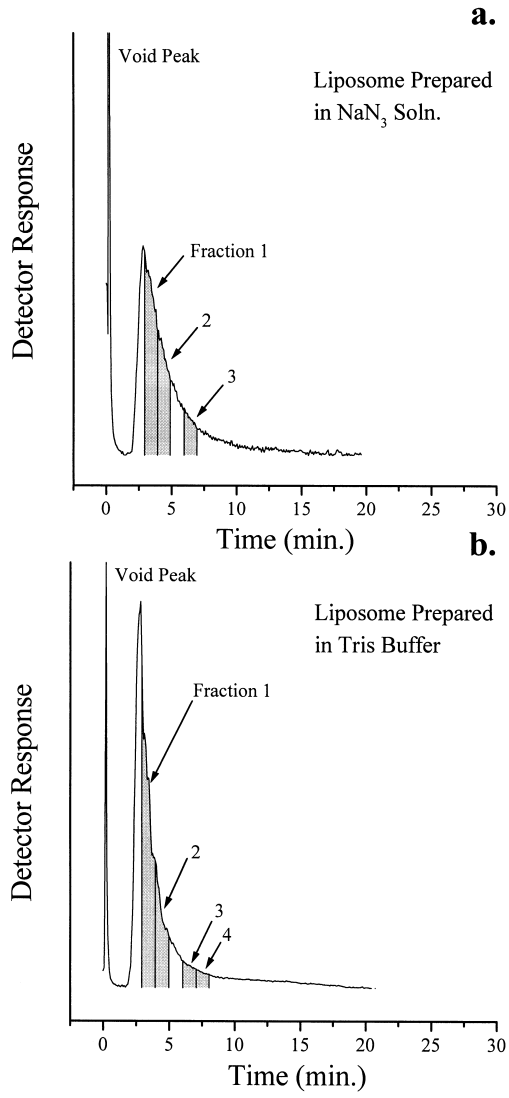

Fig. 3. Fractograms along with time intervals for the fraction collection of liposome sample run at NaN_3 solution in flow FFF. The average diameter of each fraction measured by PCS is compared with FFF data in Table 2. Run condition and the sample amount injected are the same as used in Fig. 2.

solution containing only NaN_3. These liposomes are prepared by extruding with 0.4 μm polycarbonate membrane (all the later liposome samples are extruded with 0.1 μm). PCS diameters of each fraction collected at 1-min intervals are listed in Table 2 along with the FFF diameter calculated by using Eq. (1) at the average time of each fraction. For the confirmation of the PCS system used in this work, diameters of the above polystyrene standards are measured. The relative error in the PCS measurements of five polystyrene standards is approximately 3–9% from the nominal diameters of polystyrene latices. When the two liposome samples, prepared in different solutions, are run in the flow FFF under the same carrier solution used in Fig. 3, it appears that liposomes are not resolved well in terms of size fractionation by comparing the PCS measurement with the FFF. Even though the retained peaks are separated from the void peak completely, it shows that vesicles pass along the channel without following the designated retention profiles. This indicates that separation of the current liposome sample under NaN_3 carrier solution is not appropriate at all in flow FFF. Since the ionic strength of the NaN_3 solution falls within the optimum condition known for the separation of typical PS standards, liposome vesicles are expected to elute normally in flow FFF unless there is a strong influence from the surface charge of particles. Apparently, vesicles seem to lift further away from the channel wall due to the repulsion between the vesicle surface and the channel wall under the current ionic circumstance. It is likely that charged vesicles like liposomes seem to have stronger interactions with the wall at such an optimum ionic strength regime previously described than typical latex standards, which have no permanent ionic charges exerted on the particle surface (besides the double-layer charge on the surface).

For the examination of the influence of ionic strength, tests are further accomplished with the change of ionic strengths of carrier liquid in flow FFF. Fig. 4 shows the superimposed fractograms of the liposome samples (sample (a), prepared in PBS, and sample (b), prepared in Tris–HCl solution, and both extruded by 0.1 μm pore) obtained at the same flow-rate condition as used in Fig. 3, but the ionic strengths are varied from $I=0.160–0.008$ M by diluting the PBS solution. When the ionic strength decreases, liposome sample (a) appears to be retained briefly with a narrow distribution as shown in Fig. 4a. Liposome sample (b) prepared in Tris–HCl solution does not show a significant change in retention profiles in Fig. 4b but it shows a similar trend, as observed in Fig. 4a. During these runs, liposome fractions are collected at the end of each run at 1-min intervals and are subjected to PCS for the measurement of average diameter of each fraction. In Table 3, the measured diameters of collected fractions are compared with the calculated diameter values at each corresponding time interval from FFF theory. Table 3a lists the data for Fig. 4a, and Table 3b those for Fig. 4b. By comparing the PCS data with the values calculated from the FFF calculation, it is found that measured diameters of each fraction run at $I=0.16$ M are smaller than what would be expected from the flow FFF theory. An explanation for this is that the decrease of electrical double layer under a strong ionic condition enables the vesicles to

Table 2
Comparison of average diameters of each fraction measured by PCS and the value calculated by FFF theory from the retention time

Fraction no.	Time interval (min)	Diameter by flow FFF (nm)	Av. diameter by PCS (nm)
(a) Liposome prepared in NaN_3 solution			
1	2.93–3.93	45.3–61.1	142.9
2	3.93–4.93	61.1–76.9	135.8
3	5.93–6.93	92.7–108.5	134.7
(b) Liposome prepared in Tris-HCl buffer solution			
1	2.93–3.93	45.3–61.1	164.7
2	3.93–4.93	61.1–76.9	163.7
3	4.93–5.93	76.9–92.7	167.4
4	6.93–7.93	108.5–124.3	155.0

The corresponding runs are shown in Fig. 3. A carrier liquid used for flow FFF run is 0.02% NaN_3 solution ($I=3.08$ mM).

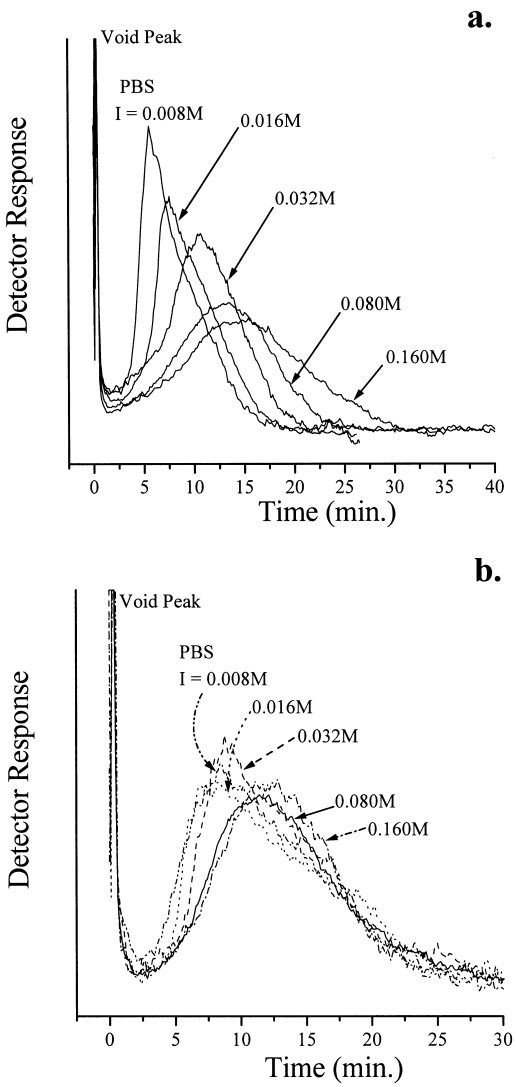

Fig. 4. Influence of ionic strength of carrier solution on the separation of liposome sample prepared in (a) PBS and (b) Tris–HCl by flow FFF obtained at the same run condition as used in Fig. 1. The carrier solution used throughout the runs is PBS by diluting the mother solution ($I=0.16$ M). Fractions are collected and confirmed by PCS, the results are listed in Table 3. Amount injected is approximately 170 μg for each sample.

approach the channel surface closely, and it results in the longer retention of vesicles. This is similar to the result observed with typical polymeric latex standards in flow FFF [14] and in SdFFF [18,19]. When the ionic strength decreases, the measured diameters by PCS appear larger at the same time fraction, which indicates that retention of liposomes becomes close to the theoretical expectations in flow FFF. When it is run at $I=0.016$ M, average diameters at most fractions appear to match the FFF values, with the exception of early fractions. The deviation in the diameter measurement at early fractions is thought to be induced by an incomplete relaxation. Some vesicles located at equilibrium position may be perturbed by the incoming fast flow streamlines when the channel flow is suddenly resumed after the relaxation process and, thus, perturbed vesicles migrate all at once at the early stage. This perturbation may arise from an overloading of sample injection since a certain amount of vesicles need to be injected to the channel for the PCS measurement of each fraction which is diluted during the flow FFF run. Except for the above fractions, the remainder of the liposomes appear to be retained well according to their sizes which are quite close to the FFF calculations. When the ionic strength decreases further, vesicle samples appear to elute much earlier than expected by theory. PCS measurements are repeated 3–5 times for each fraction, and the deviations during the repeated measurements are about 2–4% from the average value. Similar results are obtained with the sample (b) and they are listed in Table 3b. Since sample (b) (prepared in a different aqueous medium) is resolved with the same carrier solution (dil. PBS, $I=0.016$ M) used in Fig. 4a, it is noted that the ionic strength is more significant in the retention of liposomes in flow FFF rather than compositional variation. It is also found that the particular liposome samples used in this study behave well in flow FFF when the ionic strength of PBS solution is around $I=0.016$ M.

The carrier solutions (dil. PBS) used so far are fixed at pH 7.4 which is close to the biological fluid system. However, for the evaluation of pH effect on the liposome retention in flow FFF, dil. PBS solutions of various pH are prepared at a fixed ionic strength of $I=0.016$ M and are used as carrier solutions. The selected ionic strength ($I=0.016$ M) has been shown to be a reliable condition for the current liposome samples from the results so far. Fig. 5 shows the fractograms of the liposome sample (prepared in PBS solution and extruded by 0.1 μm) obtained at four different pH values (pH 8.6, 7.40, 6.50, 5.05). Retention of liposomes at pH 8.60

Table 3
Comparison of average diameter of collected fractions measured by PCS and flow FFF for the liposome samples run by flow FFF at different ionic strengths shown in Fig. 4

Fraction no.	Time (min)	FFF diameter (nm)	PCS measurement (nm)				
			$I=0.16$ *M*	$I=0.08$ *M*	$I=0.03$ *M*	$I=0.016$ *M*	$I=0.008$ *M*
(a) Liposome prepared in PBS (extruded with 0.1 μm)							
6	6.0–7.0	89.4–104.6				126.3	134.9
7	7.0–8.0	104.6–119.8			120.6	139.5	140.0
8	8.0–9.0	119.8–135.1		128.1		144.0	
9	9.0–10.0	135.1–150.3	133.1	135.3	141.4	151.9	160.2
11	11.0–12.0	165.6–180.8	143.5	146.3	158.4	168.5	180.7
13	13.0–14.0	196.1–211.3	154.7	160.0	171.7	195.4	
15	15.0–16.0	226.5–241.8	163.9	176.8	193.6		
18	18.0–19.0	272.3–287.5	183.7	205.1			
20	20.0–21.0	302.7–318.0	193.4				
(b) Liposome prepared in Tris–HCl (extruded with 0.1 μm)							
6	6.0–7.0	89.4–104.6				137.7	
7	7.0–8.0	104.6–119.8		129.2			
8	8.0–9.0	119.8–135.1	136.9		165.4	152.9	
9	9.0–10.0	135.1–150.3		146.8			
10	10.0–11.0	150.3–165.6	140.1		173.0	164.3	
11	11.0–12.0	165.6–180.8		163.0			
12	12.0–13.0	180.8–196.1	153.7		199.5	178.1	
13	13.0–14.0	196.1–211.3		175.4			
14	14.0–15.0	211.3–226.5	177.2				
15	15.0–16.0	226.5–241.8		189.9			
16	16.0–17.0	241.8–257.0	194.7				

Carrier liquids used for the entire runs are PBS and dil. PBS solutions.

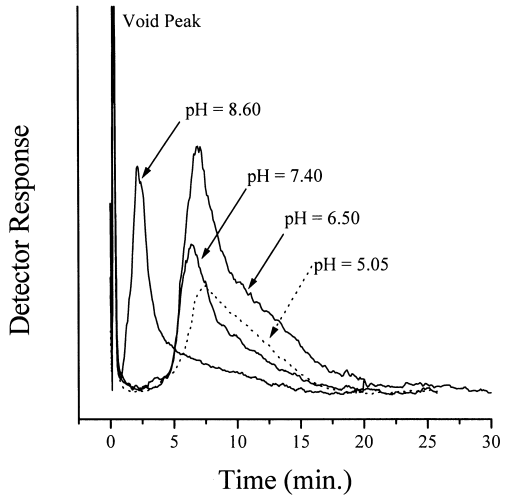

Fig. 5. Effect of pH of carrier solution on the retention of liposomes in flow FFF. The ionic strength of carrier solution is nearly the same as $I=0.016$ *M*. The run condition is the same as used in Fig. 4.

appears to be shifted to a shorter time scale than the other peaks due to the increased charge interactions between the vesicles and channel surface, thus leading to an early elution of vesicles. In cases of acidic conditions at pH 6.5 and 5.5, there seems to be no significant change in the retention time scale compared to the run at pH 7.4, but it is evident that peaks are larger at the early part of the elution. It could be similar to the perturbation observed at the previous measurement. The fractions of the eluted liposomes are confirmed by PCS and are listed in Table 4. By examining the data, it is found that the diameters after fraction number 9 agree with the theory, and the perturbations appearing at early fractions appear intense at acidic conditions. Among the given pH conditions, it is shown that the unexpected perturbations observed are less serious under pH 7.4 conditions.

Based on these experimental investigations, liposome size distributions are examined as follows. As a first step, retention profiles of the same liposome

Table 4
Average diameters of collected fractions measured by PCS and flow FFF for the liposome samples run by flow FFF at carrier solutions of different pH shown in Fig. 5

Fraction no.	Time (min)	FFF diameter (nm)	PCS measurement (nm)		
			pH 7.4	pH 6.5	pH 5.5
5	5.0–6.0	73.6–88.9		138.4	
6	6.0–7.0	89.4–104.6	126.3		146.0
7	7.0–8.0	104.6–119.8	139.5	149.6	
8	8.0–9.0	119.8–135.1	144.0		150.8
9	9.0–10.0	135.1–150.3	151.9	156.3	
10	10.0–11.0	150.3–165.6			158.0
11	11.0–12.0	165.6–180.8	168.5	175.3	
12	12.0–13.0	180.8–196.1			172.9
13	13.0–14.0	196.1–211.3	195.4	198.2	
14	14.0–15.0	211.3–226.5			194.5

Carrier liquid for the entire runs is dil. PBS solution ($I=0.016$ M).

sample are compared by running them with the two different carrier solutions (dil. PBS and dil. Tris–HCl buffer). Fig. 6 shows the fractograms of the liposome samples prepared in (a) PBS and (b) Tris–HCl that are run with both solutions as a carrier liquid individually. The ionic strength of all carrier solutions is fixed at 0.016 M, the pH is adjusted at 7.4 for dil. PBS and at 7.8 for dil. Tris–HCl. By looking at the fractograms in Fig. 6, retention of liposomes at two different buffers but the same ionic strength shows almost the same distribution. However, the aqueous media used for the liposome preparations reflect a large difference in the retention profile. Though the ionic strengths of the original solutions used for the liposome preparation are somewhat different from each other ($I=0.10$ M for Tris–HCl, $I=0.16$ M for PBS preparation in Table 1), they are in the same order of magnitude. The difference in the retention profiles can be a result of the influence of chemical composition of aqueous solutions during the liposome preparations. The resulting size distribution of vesicles is calculated from the experimental fractogram by using Eq. (1) and both are superimposed in Fig. 7. The liposome prepared in PBS solution shows a narrower distribution than the sample in Tris–HCl solution. Though the size distribution curves at the lower size limit shown in Fig. 7 are not absolutely accurate due to the perturbation from the large particles at the beginning of separation, flow FFF demonstrates its capability of monitoring the size distribution of liposomes according to the difference in preparations.

4. Conclusion

Flow FFF has been applied to the size characterization of liposomes by evaluating factors that might affect the separation of liposomes. The ionic strength of the carrier solution in the flow FFF operation is shown to be important in the efficient separation of phospholipid bilayer vesicles from the diameter measurement of collected fractions by photon correlation spectroscopy. One remarkable finding is that liposomes (PC(1)/PG(4)/cholesterol(5)) currently used in this work require a carrier solution having a relatively high ionic strength compared to the typical run conditions widely used for the separation of most particulate materials in flow FFF. While flow FFF requires specific run conditions for charged vesicles like liposomes, it is shown that the vesicle size distribution can be readily obtained from a flow FFF fractogram once the proper ionic conditions are provided.

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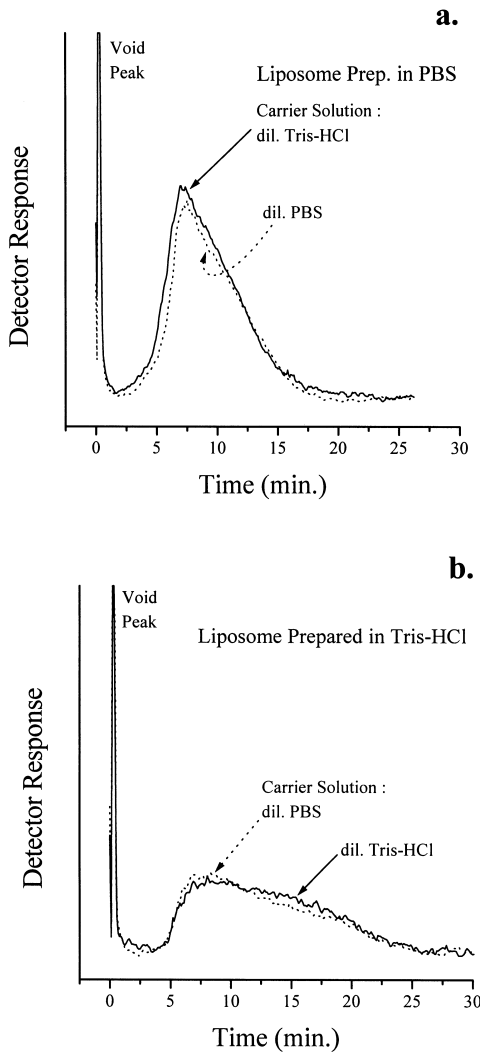

Fig. 6. Comparison of the retention behaviors of liposome samples run at two different carrier solutions (dil. PBS and dil. Tris–HCl) having an identical ionic strength $I=0.016$ *M*. Samples are prepared in (a) PBS and (b) Tris–HCl solution.

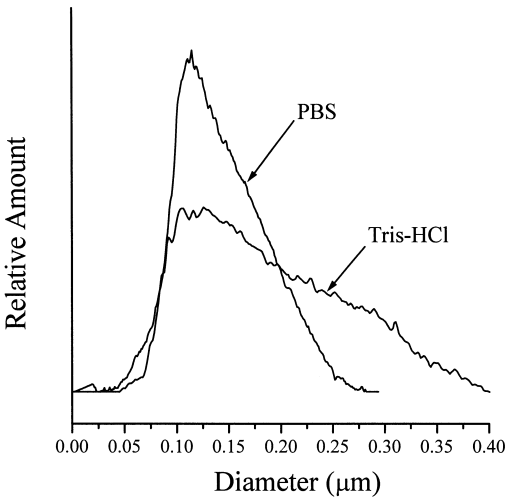

Fig. 7. Liposome size distributions for the two liposomes preparations run at each diluted solution ($I=0.016$ *M*), respectively.

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