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Homogeneous and reproducible liposome preparation relying on reassembly in microchannel laminar flow

Kenichi Yamashita[a], Maria Portia B. Nagata[a], Masaya Miyazaki[b], Hiroyuki Nakamura[a], Hideaki Maeda[b,*]

[a] Measurement Solution Research Center, National Institute of Advanced Industrial Science and Technology (AIST), 807-1, Shuku-machi, Tosu, Saga 841-0052, Japan
[b] Graduate School of Engineering Sciences, Kyushu University, 6-1, Kasuga-Kouen, Kasuga, Fukuoka 816-8580, Japan

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ABSTRACT

We investigated a method for size homogenization of liposomes using microchannel laminar flow. This microchannel method combined with sonication produced the desired homogeneous liposome populations with size controlled by reassembly of liposomes in laminar flow, using simple operations that offer good reproducibility and organic-solvent free procedures. The liposome solution, which was prepared using the traditional method of film hydration, was loaded into a syringe. This liposome solution was sonicated while being transported into the capillary tubing using syringe injection. In both non-sonicated and sonicated batchwise preparations, liposomes displayed non-homogeneous and non-reproducible size profiles. On the other hand, homogeneous liposomes were obtained with good reproducibility using our microchannel method combined with sonication.

1. Introduction

Liposomes are widely used in biological, pharmaceutical, and industrial applications [1]. Liposomes are able to encapsulate various hydrophilic substances such as DNA vectors, drugs, and therapeutic agents, and deliver them to cells in *in vivo* applications [2–7]. Such applications, known as drug delivery systems (DDSs) require homogeneous liposome populations of controlled size to achieve accurate targeting [8]. Generally, larger liposomes are trapped by the liver; smaller liposomes are excreted because of their weaving path through the renal filtering system [9]. Traditional liposome preparations commonly use batchwise processing for film hydration [10] and alcohol injection [11,12]. Unfortunately, such preparation methods often cause polydispersity in size and lamellarity. For obtaining desired homogeneous liposome populations of controlled size, additional post-processing steps such as membrane extrusion [13,14], detergent dialysis [15], filtering [15] and sonication [16] are often used. In particular, sonication is also useful for obtaining smaller and unilamellar liposomes [11]. Moreover, microfluidic methods for obtaining the desired homogeneous liposome populations of controlled size are used, for example, by employing a crossflow [12] and multiple laminar flow [17,18]. Also, fine size W/O emulsion could be prepared by size homogenization via the microfluidic methods [19–21], and homogenized liposomes could be obtainable from these W/O emulsions [22,23]. However, these methods require the use of organic solvents, which is particularly undesirable when intended for clinical applications. Preparation of lipid films on internal channel walls and stripping by microfluidic share stress methods instead of conventional rigorous agitation has also been reported [24]. On the other hand, liposomes have a restricted lifetime because of leakage of their internal components. Therefore, main efforts are being directed to the development of liposomal preparation methods for DDS applications.

Our previous study specifically addressed the fundamental behavior of solutes in microchannel laminar flow. In a batchwise system, solvent molecules interact isotropically with solute molecules. In contrast to a batchwise system, interaction of solutes with solvent in a microchannel laminar flow is non-isotropic, suggesting that such a behavior is characteristic of solutes in a laminar condition. We have confirmed that the shear stress of a laminar condition causes orientational and/or conformational changes of solutes through direct observation [25,26] and linear dichroism (LD) spectra measurements [27]. We have reported characteristic reactivity changes in a microchannel laminar flow – such as a shift in the thermal stability of DNA duplex [28–30], efficient hybridization [26], and the reaction rate change of enzymatic reaction [31,32] – by the microfluidic orientational and conformational

* Corresponding author at: Measurement Solution Research Center, National Institute of Advanced Industrial Science and Technology (AIST), 807-1, Shuku-machi, Tosu, Saga 841-0052, Japan. Tel.: +81 942 81 3676; fax: +81 942 81 3657.
E-mail address: maeda-h@aist.go.jp (H. Maeda).

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change of solutes. Moreover, based on physicochemical analysis, we have reported that "entropy" is a key factor in the microfluidic reactivity change described above [27–30]. That is, these results indicate that microfluidic laminar flow offers the ordered state to reaction field.

Herein, we have examined an application of the characteristics of laminar flow in terms of liposome self-assembly. Our approach, using a simple operation that relies on laminar flow for the reassembly of liposomes, yielded homogeneous liposome populations of controlled size.

2. Experimental methods

2.1. Liposome preparation

Dioleoyl phosphatidyl ethanolamine (DOPE; NOF Corp., Japan) and L-3-phosphatidic acid sodium salt from egg yolk lecithin (PA; Doosan Serdary Research Laboratories, Korea) dissolved in dry chloroform in a molar ratio of 7:10. The chloroform solvent was evaporated at room temperature to leave a dry lipid film on the bottom of a scintillation vial, which was then placed into a vacuum desiccator for at least 4 h to ensure complete solvent removal. As a hydration buffer, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) buffered solution (pH 7.2, 10 mM) was used. The hydration was conducted at 70 °C. The final lipid concentration was 3.4 mg/ml.

2.2. Microchannel and batchwise sonication

Batchwise sonication portrayed in Fig. 1B was conducted for 2 min using a polycarbonate vessel. The liposome solution was injected into the Teflon tubing (330 μm internal diameter, 1/16 in. external diameter, 92 cm length) under sonication at the flow rate of 39 μl/min (=46 cm/min) in the experiment depicted in Fig. 1C and the liposome solution was collected in an ice bath. The net retention time for the microchannel flow with sonication was 2 min. In experiments presented in Table 1, tubings of the indicated sizes were used for each experiment. Other conditions are also shown in Table 1. All sonication processes were conducted at 37 kHz, 600 W, and 60 °C.

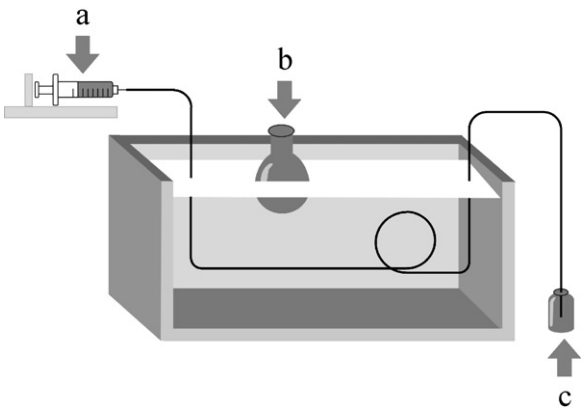

Fig. 1. Schematics showing the experimental setup used for liposome size homogenization method. The labels (a), (b) and (c) correspond to the results shown in Fig. 2.

2.3. DLS measurement

Size distribution profiles are obtained using histographic analysis of dynamic light scattering (DLS) measurements (nanoZS; Malvern Instruments Ltd., UK). Results of multiple experiments are shown in Fig. 1. Mean diameters and polydispersity index (PDI) values in Table 1 are obtained using cumulant analysis of DLS measurements. All DLS measurements were conducted at 30 °C.

3. Results and discussion

In this study, to obtain the homogeneous liposome populations of controlled size, we addressed the reassembly in laminar flow of liposome prepared by film hydration, a traditional liposome preparation method. Batchwise sonication is commonly used for obtaining size-homogeneous and unilamellar liposomes as an additional post-processing step. However, such treatment often causes size polydispersity.

Fig. 1 presents a schematic representation of the experimental setup. The liposome solution, which was prepared using the traditional method of film hydration, was loaded into a syringe. Size profiles of this non-sonicated liposome solution are presented in Fig. 2a. This liposome solution was sonicated while being introduced into the capillary tubing by syringe injection. The size profiles

Table 1
Size and dispersity of liposomes prepared under various conditions.[a]

	Tubing material (external diameter)	Tubing internal diameter	Tubing length	Flow rate	Reynolds number	Sonication time	Mean diameter/nm[b]	PDI[b]
1	Teflon (1/16 in.)	330 μm	92 cm	39 μl/min (=46 cm/min)	5.3	2 min	129 ± 2.0	0.20 ± 0.0031
2	Teflon (1/16 in.)	330 μm	160 cm	68 μl/min (=80 cm/min)	9.2	2 min	133 ± 5.7	0.23 ± 0.012
3	Teflon (1/16 in.)	250 μm	80 cm	19.5 μl/min (=40 cm/min)	3.5	2 min	157 ± 3.6	0.29 ± 0.0091
4	Teflon (1/16 in.)	250 μm	160 cm	39 μl/min (=80 cm/min)	7.0	2 min	148 ± 2.1	0.23 ± 0.0036
5	Teflon (1/16 in.)	170 μm	160 cm	18 μl/min (=80 cm/min)	4.8	2 min	182 ± 3.6	0.36 ± 0.036
6	Teflon (1/16 in.)	170 μm	346 cm	39 μl/min (=173 cm/min)	10.3	2 min	193 ± 3.1	0.30 ± 0.013
7	Teflon (1/16 in.)	250 μm	160 cm	19.5 μl/min (=40 cm/min)	3.5	4 min	133 ± 6.7	0.24 ± 0.014
8	Glass (350 μm)	200 μm	160 cm	12.5 μl/min (=40 cm/min)	2.8	4 min	533 ± 6.2	0.40 ± 0.068

[a] All measurements were performed using the same liposome and conditions as those described for Fig. 1.
[b] Mean diameters and polydispersity index (PDI) values were obtained using cumulant analysis of DLS measurement; they are presented as the avarage ± standard deviation.

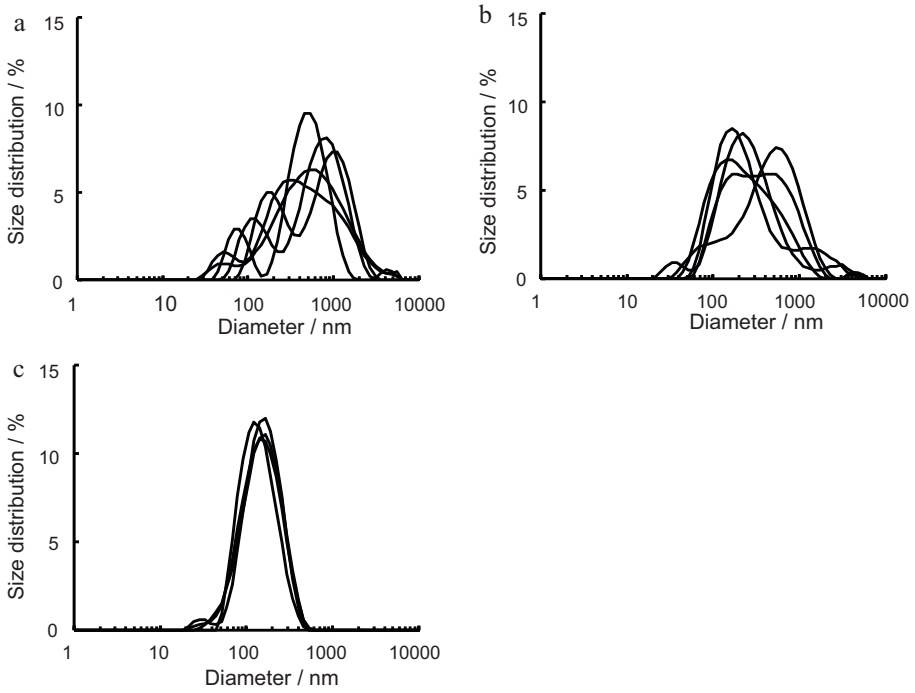

Fig. 2. Size distribution profiles of: (a) non-sonicated liposome prepared by film hydration; (b) batchwise sonicated liposome; and (c) microchannel sonicated liposome.

of liposome after such treatment are presented in Fig. 2c; Fig. 2b presents the size profiles of liposome prepared batchwise with sonication at a similar retention time to that shown in Fig. 2c. In both non-sonicated and sonicated batchwise preparations, liposomes demonstrated non-homogeneous and non-reproducible size profiles. On the other hand, homogeneous liposomes with good reproducibility were obtained using the microchannel method combined with sonication.

We consider the larger liposomes to be unstable under the laminar flow condition because such liposomes are exposed to shear stress. In the laminar flow, the shear stress might deform liposomes, but liposomes return to a spheroidal shape for minimizing the surface energy: only smaller liposomes can exist in the laminar flow. In the case of batchwise sonication, larger liposomes divide into smaller liposomes simultaneously as smaller liposomes fuse into larger liposomes. In contrast, under microchannel sonication method, larger liposomes divide into smaller liposomes, but smaller liposomes do not fuse into larger liposomes. In fact, as shown in the homogenous size distribution in Fig. 2c, the average liposome size is nearly the same size as those of the smaller sizes found in the heterogenous size distribution in Fig. 2a and b. In addition, control experiments at much slower flow rate were carried out, and the results are shown in Supplementary Information. The results on experiments with much lower flow rate failed to demonstrate reproducibility and homogenization of liposome size. These results are similar to those of the batchwise sonication treatment. These results support the mechanism of liposome size homogenization described above.

We also examined the influence of various preparation conditions on the liposome size distribution. Table 1 presents the mean diameter and PDI values of liposomes obtained for each preparation condition. In the above mentioned size homogenization mechanism, it is predicted that smaller liposomes are obtained under the conditions of faster flow rate and smaller channel because such conditions could offer stronger shear stress. However, the significant differences have not yet been confirmed in the flow rates and channel sizes conditions employed in this study. This is because excessively fast or slow flow rates, and excessively large or small channels are not applicable in obtaining the proper sonication time of this experiment. In addition, due to constraints on lipid molecular geometry and the surface energy considerations in the membrane, it is obtainable a limited particle size of liposomes. The calculated Reynolds number becomes large under faster flow rate and larger channel, therefore the Reynolds number magnitude does not relate to the strength of shear stress of microchannel laminar flow. In fact, the relation between prepared liposome size and the Reynolds number for each condition was not confirmed. On the other hand, we were able to regulate the liposome size by adjusting the ultrasonic transmission efficiency. A comparison of results for liposomes prepared with the same channel internal surface area (**1**, **4**, **6**) revealed that liposomes prepared using 170 μm internal diameter tubing – which has the thickest walls – produced slightly polydispersed liposomes compared to tubings of other diameters. Such a trend was also observed in the comparison of results on liposomes prepared under the same linear flow rate condition (**2**, **4**, **5**). In addition, the sizes of liposomes were grouped according to the channel diameter. Results confirmed that liposomes prepared with the same channel diameter but with a different flow rate ((**1**, **2**), (**3**, **4**) and (**5**, **6**)) demonstrated liposomes of almost identical size, therefore indicating the importance of tubing wall thickness in controlling liposome size and dispersivity. On the other hand, almost identical dispersity and about a 10% difference in size were confirmed between liposomes prepared under sonication times of 2 min and 4 min in 250 μm channel diameter conditions (**4**, **7**). These results indicate that the channel wall regulates the transmission of ultrasonic into the channel interior. Moreover, much larger liposomes were obtained using glass tubing (**8**) than with teflon tubing (**1–7**). This size difference resulted from the difference of acoustic impedance between materials, which is explainable by the fact that ultrasonic treatment is more difficult with glass tubes because of the much larger acoustic impedance of glass than those of water and plastic materials.

Moreover, we confirmed that our microfluidic sonication method is applicable to liposome preparations of other compositions: DOPE:PA = 7:5 (molar ratio); DOPE:PA = 7:20; non-hydrogenated egg phosphatidylcholine:cholesterol = 7:3;

and DOPE:cholesteryl hemisuccinate = 9:2. All results of these liposomes prepared using our microchannel sonication method are presented in Supplementary Information. The reproducible size homogenization in all kinds of liposome has been confirmed, but the average size for homogenized liposome was different for each kind of liposome.

4. Conclusions

In conclusion, using the microchannel method combined with sonication, we obtained the desired homogeneous liposome populations with control by reassembly of liposomes in laminar flow, along with simple operation, good reproducibility, and organic-solvent-free procedures. But its homogenized liposome size is influenced by the lipids properties. We believe that such a simple procedure can suggest and facilitate applications for on-demand liposome-mediated delivery of point-of-care personalized therapeutics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cej.2010.09.007.

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