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ORIGINAL RESEARCH

Microfluidic Post-Insertion Method for the Efficient Preparation of PEGylated Liposomes Using High Functionality and Quality Lipids

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Introduction: Targeted liposomes using ligand peptides have been applied to deliver therapeutic agents to the target sites. The post-insertion method is commonly used because targeted liposomes can be prepared by simple mixing of ligand peptide-lipid and liposomes. A large-scale preparation method is required for the clinical application of ligand-peptide-modified liposomes. Large-scale preparation involves an increase in volume and a change in the preparation conditions. Therefore, the physicochemical properties of liposomes may change owing to large alterations in the preparation conditions. To address this issue, we focused on a microfluidic device and developed a novel ligand peptide modification method, the microfluidic post-insertion method.

Methods: We used integrin αvβ3-targeted GRGDS (RGD) and cyclic RGDfK (cRGD)-modified high functionality and quality (HFQ) lipids, which we had previously developed. First, the preparation conditions of the total flow rate in the microfluidic device for modifying HFQ lipids to polyethylene glycol (PEG)-modified (PEGylated) liposomes were optimized by evaluating the physicochemical properties of the liposomes. The targeting ability of integrin αvβ3-expressing colon 26 murine colorectal carcinoma cells was evaluated by comparing the cellular association properties of the liposomes prepared by the conventional post-insertion method.

Results: When the RGD-HFQ lipid was modified into PEGylated liposomes by varying the total flow rate (1, 6, and 12 mL/min) of the microfluidic device, as the total flow rate increased, the polydispersity index also increased, whereas the particle size did not change. Furthermore, the RGD- and cRGD-modified PEGylated liposomes prepared at a total flow rate of 1 mL/min showed high cellular association properties equivalent to those prepared by the conventional post-insertion method.

Conclusion: Microfluidic post-insertion method of HFQ lipids might be useful for clinical application and large-scale preparation of targeted liposomes.

Keywords: liposomes, targeting, post-insertion method, polyethylene glycol, microfluidic

Introduction

Liposomes have been used as carriers for the efficient delivery of therapeutic drugs and nucleic acids.[1] Polyethylene glycol (PEG)-modified (PEGylated) liposomal doxorubicin (Doxil®) is widely used clinically for cancer therapy. It enables liposomes to prolong blood circulation and accumulate at the tumor site due to reducing recognition of the reticuloendothelial system (RES).[2] Moreover, targeted liposomes with peptides, antibodies, or small-molecule ligands have been recently developed for more selective accumulation of the liposomes at the tumor sites.[3]

For the incorporation of PEG lipids or ligands onto the surface of the nanoparticles, the post-insertion method (bulk mixing) is commonly used,[4] wherein lipids are added to pre-formed nanoparticles in an aqueous solvent. This method enables the modification of therapeutic drug-loaded nanoparticles by ligands without unnecessary interactions between

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the therapeutic drugs and the ligands. Moreover, by comparing the physicochemical properties of the nanoparticles before and after its modification by ligands, it is possible to evaluate whether there is an unintended change in the physicochemical properties owing to the modification by ligands. Therefore, it becomes easy to ensure the quality of the ligand-modified nanoparticles. Furthermore, the dispersibility of ligand-modified lipids in aqueous solvents is important, because the bulk mixing requires the ligand-modified lipid to be dispersed in an aqueous solvent. However, this depends on the overall structural properties of the lipid (whether it is hydrophobic or hydrophilic). In addition, for the clinical application of targeted nanoparticles, a high-quality ligand-modified lipid is required because conventional ligand-modified lipids using PEG as a spacer (ligand-PEG-lipids) have a broad range of molecular weights.

We reported high functionality and quality (HFQ) lipids for the preparation of targeted PEGylated liposomes.[5,6] HFQ lipids have a serine-glycine repeat $(SG)_5$ spacer and have a single molecular weight for efficient ligand presentation on the surface of liposomes and high quality as a functional lipid. In addition, HFQ lipids were designed to meet the criteria for dispersibility in water (0.2 mM and above to enable ligand modification using the post-insertion method (bulk mixing)).[7–9] In the bulk mixing method, lipid composition of the nanoparticles, mixing temperature, and duration may affect the physicochemical properties.[10,11] For the large-scale preparation of targeted liposomes, it is necessary to change the preparation conditions, including the conditions mentioned above, and change the volume. Therefore, a highly reproducible bulk mixing method is required to prepare targeted liposomes for its clinical applications.

The microfluidic-based preparation of nanoparticles, such as liposomes and lipid nanoparticles, has been studied as a novel method for the production of nanoparticles.[12] Microfluidic devices enable fast, highly reproducible, seamless, and scale-up preparation process of nanoparticles by well-controlled mixing of the lipids and drugs, such as anticancer drugs and RNA.[13] Micromixer on the microfluidic channels allows complete mixing of the aqueous phase containing the drug or RNA and the organic phase containing the lipids.[14] This process facilitates the formation of nanoparticles.

Considering these observations, we hypothesized that the conventional microfluidic mixing of an organic and aqueous phase can be applied to the mixing of an aqueous and aqueous phase post-insertion as well. Preparation using a microfluidic device facilitates seamless scale-up while maintaining the quality of ligand-modified liposomes. In our microfluidic post-insertion method, HFQ lipid micelles and PEGylated liposomes in the aqueous phase were injected into the channels, and ligand peptides were modified into PEGylated liposomes by thorough stirring with a micromixer. We selected for HFQ lipids that used integrin αvβ3-targeted GRGDS (RGD-$(SG)_5$-lipid) and cyclic RGDfK (cRGD-$(SG)_5$-lipid) as ligand peptides. Compared to conventional PEG lipids, HFQ lipids form unstable micelles because they have shorter spacer lengths than those of PEG lipids and only a poorly hydrated layer owing to the hydroxyl group of the serine residue at the spacer. Therefore, we hypothesized that they would rapidly disintegrate in the flow path and could be rapidly modified into the lipid membrane of PEGylated liposomes. First, we evaluated whether the RGD ligand peptide could be modified into PEGylated liposomes using bulk mixing for 1 min. Then, we optimized flow conditions in the mixing of RGD-$(SG)_5$-lipid micelles and PEGylated liposomes in the microfluidic channel by investigating the physicochemical properties of liposomes and comparing the cellular association between RGD-$(SG)_5$/PEGylated liposomes prepared by bulk mixing for 1 h. Finally, we evaluated whether the microfluidic post-insertion method could be applied to cRGD-$(SG)_5$-lipid.

Materials and Methods

Synthesis of RGD- and Cyclic RGD-Modified HFQ Lipids

RGD-$(SG)_5$-lipid and cRGD-$(SG)_5$-lipid were synthesized and characterized by a previously described method.[6,9] The sequence of RGD and cRGD are shown in the introduction.

Preparation of PEGylated Liposomes by Thin-Film Hydration Method

PEGylated liposomes were composed of 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) (Avanti, Alabaster, AL, USA), cholesterol, and 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE) (55:35:10 molar ratio) and prepared by the same method as previously reported.[6] For fluorescence-labeled liposomes, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (rhodamine-

DOPE) (Avanti, Alabaster, AL, USA) was incorporated at 0.5 mol% of total lipids. The zeta potential and particle size of the liposomes were determined using a Zetasizer Pro or Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK).

Modification of PEGylated Liposomes by Bulk Mixing

6 mol% RGD-$(SG)_5$-lipid micelles or 9 mol% cRGD-$(SG)_5$-lipid micelles were incubated with PEGylated liposomes while shaking at 150 rpm and 60 °C on a bioshaker (Taitec, Saitama, Japan) for 1 h or 1 min. Each liposome sample was dispersed in 1× phosphate-buffered saline (PBS) (pH 7.4) by adding 10× PBS and then filtered using a 0.45-μm filter.

Modification of PEGylated Liposomes by Microfluidic Post-Insertion Method

6 mol% RGD-$(SG)_5$-lipid micelles or 9 mol% cRGD-$(SG)_5$-lipid micelles and PEGylated liposomes were injected into the microfluidic chip on a NanoAssemblr Benchtop (Precision NanoSystems Inc., Vancouver, BC, Canada) at 60 °C. The preparation conditions were a flow rate ratio of 1:1 and a total flow rate of 1 mL/min. The prepared liposomes were then immediately placed on ice for 10 min. Each liposome sample was dispersed in 1× PBS (pH 7.4) by adding 10× PBS and then filtered using a 0.45-μm filter.

Measurement of the Modification Efficiencies

Ligand insertion efficiency onto the surface of the liposomes was calculated by a previously reported method.[6]

Cell Culture

Colon 26 (murine colorectal carcinoma) cells from the Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan) were cultured as previously described.[6,9]

Flow Cytometry Analysis

The cells were cultured at a density of 5.0×10^4 cells/cm^2. After 24 h, the cells were treated with rhodamine-labeled liposomes (100 μM) in serum-free medium and then were processed as previously described.[6] The fluorescence intensities of the samples were measured using a BD LSR Fortessa flow cytometry (BD Biosciences, San Jose, CA, USA).

Confocal Laser Microscopy Analysis

The cells were cultured on a glass-bottomed 35-mm dish at a density of 5.0×10^4 cells/cm^2. After 24 h, the cells were treated with rhodamine-labeled liposomes (100 μM) in serum-free medium for 3 h and then were processed as previously described.[6] The samples were analyzed using Carl Zeiss LSM800 (Carl Zeiss Microimaging GmbH, Jena, Germany).

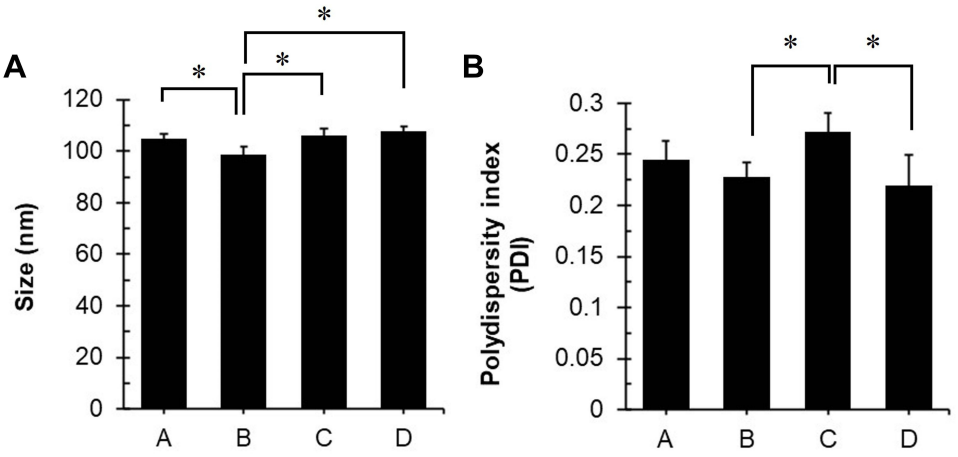
Statistical Analysis

Analysis of variance was used to analyze the statistically significant differences between the groups. Tukey-Kramer test was used for multiple comparisons between all groups. Differences were considered statistically significant at $p<0.05$.

Results

Characteristics of RGD-Modified Liposomes Prepared by Bulk Mixing for 1 h

First, we compared different incubation methods as shown in the table (Figure 1) to evaluate the effects of mixing conditions of bulk mixing for 1 h. As shown in Figure 1A, the particle size of RGD-$(SG)_5$/PEGylated liposomes mixed in a 50-mL tube (method B in the table) was significantly smaller than that in a 5-mL tube (method A, C and D in the table). When PEGylated liposomes were mixed with RGD-$(SG)_5$-lipid micelles in a ratio of 7:1 (v/v) (method C in the table), the size distribution (polydispersity index (PDI)) significantly increased compared to that mixed in a ratio of 1:1 (method B and D in the table) (Figure 1B).



	Tube size	Shaking	Mixing ratio (v/v) (Liposomes/RGD-$(SG)_5$-lipid micelles)
A	5 mL	+	1:1
B	50 mL	+	1:1
C	5 mL	+	7:1
D	5 mL	-	1:1

Figure 1 Comparison of the physicochemical properties of RGD-$(SG)_5$/PEGylated liposomes prepared by bulk mixing in different conditions. ((**A**) particle size, (**B**) polydispersity index (PDI)). PEGylated liposomes were incubated with RGD-$(SG)_5$-lipid micelles for 1 h in tubes of different size or mixing ratio. The X-axis corresponds to the incubation method in the table (shown as alphabet).
Notes: Data represent the mean ± standard deviation (SD) of six experiments. *$p<0.05$.

Investigation of RGD Modification Using Bulk Mixing for 1 Min

In our previous study, RGD-$(SG)_5$/PEGylated liposomes were prepared by mixing RGD-$(SG)_5$-lipid micelles and PEGylated liposomes for 1 h using bulk mixing and showed high cellular association ability to integrin αvβ3-expressing colon 26 cells.[6] In contrast, in the microfluidic post-insertion method, the ligand peptide was modified onto the surface of the liposomes by mixing the micelles and liposomes for a short period of time. Therefore, we mixed RGD-$(SG)_5$-lipid micelles and PEGylated liposomes for as short as 1 min in bulk mixing and evaluated the extent to which the RGD ligand was modified on the liposomal surface. As shown in Table 1 and S1, RGD-$(SG)_5$/PEGylated liposomes prepared by bulk mixing for 1 min and RGD-$(SG)_5$/PEGylated liposomes prepared by bulk mixing for 1 h exhibited larger particle size and higher PDI than those of PEGylated liposomes. Moreover, RGD-$(SG)_5$/PEGylated liposomes prepared by bulk mixing for 1 min had high cellular association properties, which were equivalent to those prepared by bulk mixing for 1 h (Figure 2). From these results, we determined that 1 min was sufficient for modifying the RGD ligand onto the liposomal surface and then proceeded to evaluate the preparation using the microfluidic post-insertion method.

Table 1 Physicochemical Properties of RGD-Modified Liposomes Prepared by Short-Time Mixing

	Particle Size (nm)	ζ-Potential (mV)	Polydispersity Index
PEGylated liposomes	85.5 ± 2.2	1.2 ± 3.3	0.176 ± 0.019
RGD-$(SG)_5$/PEGylated liposomes (1 min of bulk mixing)	99.9 ± 1.0	2.0 ± 1.2	0.190 ± 0.021
RGD-$(SG)_5$/PEGylated liposomes (Microfluidic post-insertion method)	101.2 ± 2.2	2.1 ± 0.4	0.175 ± 0.009

Note: Data are the mean ± standard deviation (SD) (n=3).

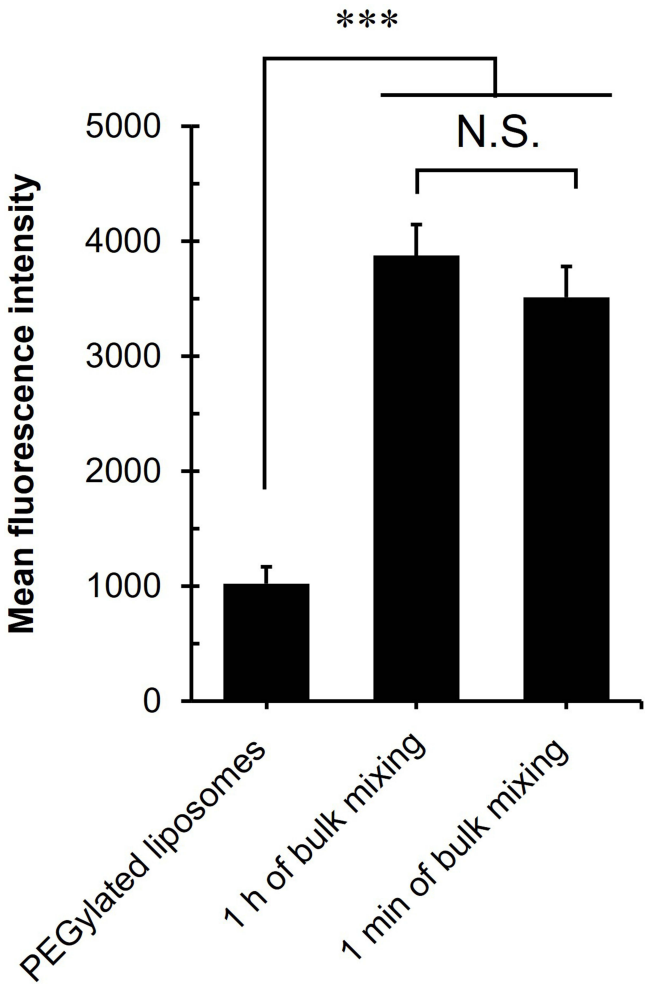


Figure 2 Cellular association of RGD-$(SG)_5$/PEGylated liposomes prepared by bulk mixing for 1 min analyzed by flow cytometry. Colon 26 cells were treated with rhodamine-labeled liposomes (100 μM) for 3 h.
Notes: Data represent the mean ± SD of triplicate experiments. ***$p<0.001$.

Effect of Microfluidic Mixing Conditions on the Characteristics of RGD-Modified Liposomes

We evaluated the influence of the total flow rate and the flow rate ratio on the physicochemical properties of RGD-$(SG)_5$/PEGylated liposomes prepared by microfluidic post-insertion method. The total flow rate was varied from 1 to 12 mL/min at the flow rate ratio of 1:1. As shown in Figure 3, RGD-$(SG)_5$/PEGylated liposomes prepared by microfluidic post-insertion method had a slightly larger particle size than that of PEGylated liposomes, similar to that of liposomes prepared by bulk mixing for 1 h. The total flow rate had no effect on liposome size while the PDI tended to increase depending on the total flow rate. Moreover, the flow rate ratio was varied from 0.5:1 to 2:1 (Liposomes: RGD-$(SG)_5$-lipid micelles) at the total flow rate of 1 mL/min. The particle size and the PDI tended to increase slightly when the flow rate ratio was 2:1 and 0.5:1, respectively (Figure S1). Based on these results, we selected a total flow rate of 1 mL/min and a flow rate ratio of 1:1 for further experiments when HFQ lipids were modified into PEGylated liposomes. In addition, when we compared the modification efficiencies, those of microfluidic post-insertion method, 1 h of bulk mixing, and 1 min of bulk mixing were 85.3% ± 9.6%, 86.9% ± 11.6%, and 88.7% ± 10.5%, respectively.

Cellular Association of RGD-Modified Liposomes Prepared by Microfluidic Post-Insertion Method

To evaluate the targeting ability of RGD-$(SG)_5$/PEGylated liposomes prepared by microfluidic post-insertion method, cellular association experiments were performed on colon 26 cells by flow cytometry. RGD-$(SG)_5$/PEGylated liposomes

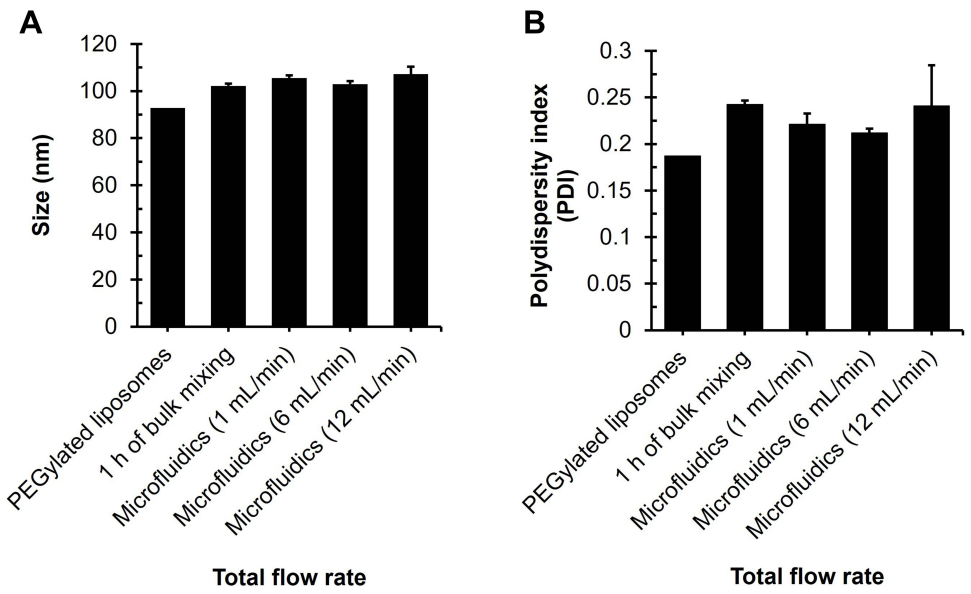


Figure 3 Effect of total flow rate on the physicochemical properties of RGD-$(SG)_5$/PEGylated liposomes. ((**A**) particle size, (**B**) polydispersity index (PDI)).
Notes: Data represent the mean ± SD of triplicate experiments.

prepared by microfluidic post-insertion method at any flow rate showed a similar cellular association to that of bulk mixing for 1 h (Figure 4). The cellular association of liposomes was evaluated over time at 30 min, 1 h, 2 h and 3 h. Compared to PEGylated liposomes, RGD-$(SG)_5$/PEGylated liposomes prepared by microfluidic post-insertion method (1 mL/min) exhibited higher cellular association at all time points, and the cellular association properties increased with time (Figure S2). To confirm the flow cytometry results, we performed a qualitative evaluation using confocal laser scanning microscopy. RGD-$(SG)_5$/PEGylated liposomes prepared by bulk mixing for 1 h and the microfluidic post-insertion method showed similar and stronger fluorescence than that shown by PEGylated liposomes (Figure 5).

Application of the Microfluidic Post-Insertion Method to Cyclic RGD Ligand Modification

Through these studies, we detected the optimal conditions for targeting PEGylated liposomes using HFQ lipid with GRGDS as the peptide ligand. Peptide cyclization generally augments binding affinity, selectivity, and enzymatic stability of linear peptides.[15] Cyclic RGD has high affinity and selectivity for Integrin αvβ3.[16] In our previous study, we had developed the cyclic RGDfK-modified HFQ lipid (cRGD-$(SG)_5$-lipid). However, since cyclic RGD has a bulky structure, we hypothesized that it may be difficult to modify the liposome surface by only bringing the liposomes in contact with cyclic RGD-modified lipids for a short period of time in the microfluidic post-insertion method. Thus, using this lipid, we evaluated whether the microfluidic post-insertion method could be applied to cyclic RGDfK ligand. As shown in Table 2 and S2, cRGD-$(SG)_5$ /PEGylated liposomes prepared by microfluidic post-insertion method were similar in size to those prepared by bulk mixing for 1 h and 1 min, whereas PDI was slightly higher and zeta potential was slightly larger. In addition, they showed a high cellular association, which was equivalent to that of liposomes prepared by bulk mixing for 1 h and 1 min (Figure 6).

Discussion

For the clinical applications of targeted liposomes, it is essential to establish a method that ensures their quality and can be prepared on a large scale. However, large-scale production using bulk mixing involves volume increase and a change in the mixing conditions.

Destabilization of the lipid membrane by higher temperature and addition of ethanol is effective for the rapid insertion of PEG lipid micelles into nanoparticles.[17–20] However, these conventional methods might affect the physicochemical properties of nanoparticles. We have previously reported that ligand-peptide-modified liposomes can be prepared by bulk

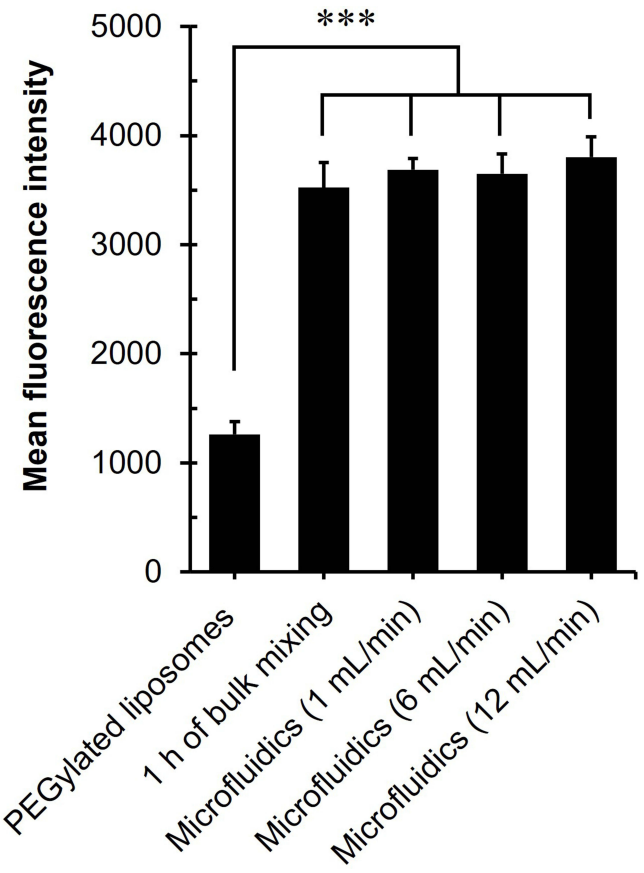


Figure 4 Cellular association between RGD-$(SG)_5$/PEGylated liposomes prepared by the microfluidic post-insertion method analyzed by flow cytometry. Colon 26 cells were treated with rhodamine-labeled liposomes (100 μM) for 3 h.
Notes: Data represent the mean ± SD of triplicate experiments. ***$p<0.001$.

mixing for 1 h using HFQ lipids with $(SG)_5$ as spacer.[5,6,8,9] Our HFQ lipids might form unstable micelles compared to those observed with conventional PEG lipids. Therefore, we hypothesized that HFQ lipids would be rapidly inserted into PEGylated liposomes without any additional destabilization of the liposomal membrane. Furthermore, in the preparation of nanoparticles using microfluidic devices, rapid and controlled mixing of aqueous and organic solvent phases enable highly reproducible and rapid preparation. This facilitates the preparation of large-scale high-quality nanoparticles by parallelization or continuous preparation without changing the preparation conditions. In our study, using HFQ lipids, we hypothesized that targeted liposomes with high quality and functionality for clinical application could be prepared in a short time by applying this microfluidic device to the conventional bulk mixing method.

Temperature, incubation period, and lipid composition of liposomes can affect their physicochemical properties. Little information is available about mixing conditions, such as tube size and mixing ratio by bulk mixing. Therefore, we investigated whether the tube size and mixing ratio affected the properties of liposomes when bulk mixing RGD-$(SG)_5$-lipid micelles and PEGylated liposomes for 1 h. The RGD-$(SG)_5$/PEGylated liposomes showed smaller particle size when mixed in larger tube and larger PDI when mixed at remarkably different ratios (Figure 1A and B). These results suggested that the mixing conditions of bulk mixing affect the physicochemical properties of RGD-$(SG)_5$/PEGylated liposomes and can be a crucial factor in the scale-up preparation process.

In the microfluidic post-insertion method, the mixing time between HFQ lipid micelles and liposomes is short. Thus, it is necessary to investigate the extent to which ligand modification can be achieved when bulk mixing for a short time. The post-insertion efficiency of PEG lipid depends on the length of the incubation time and it reached the maximum value after 1 h at 60 °C.[21] However, in our study, RGD-$(SG)_5$/PEGylated liposomes prepared by bulk mixing for 1 min showed equivalently high cellular association ability compared to that prepared for 1 h (Figure 2), which was consistent

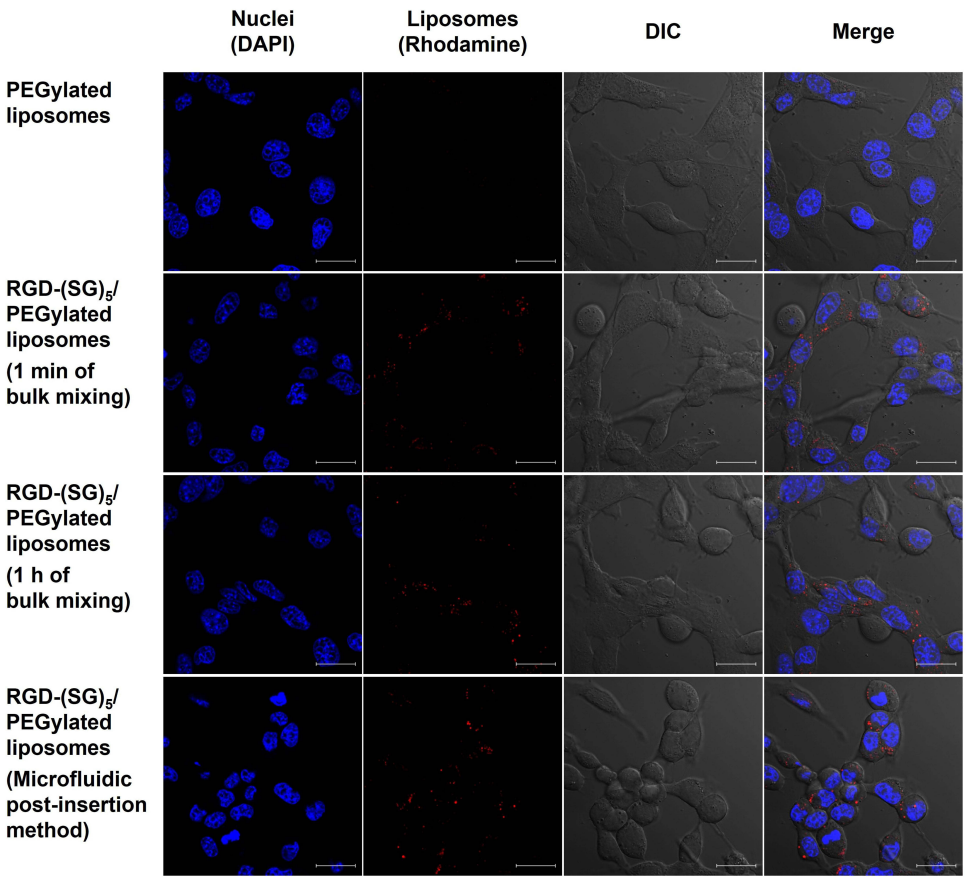


Figure 5 Confocal microscopy images in colon 26 cells of PEGylated liposomes, RGD-$(SG)_5$/ PEGylated liposomes prepared by bulk mixing for 1 min, RGD-$(SG)_5$/PEGylated liposomes prepared by bulk mixing for 1 h, and RGD−$(SG)_5$/PEGylated liposomes prepared by the microfluidic post-insertion method. The cells were treated with rhodamine-labeled liposomes (100 μM) for 3 h. Nuclei were stained with DAPI (blue). Liposomes are indicated by red fluorescence (rhodamine). Scale bar=20 μm.

with the data showing equivalent modification efficiencies of 88.7% ± 10.5% and 86.9% ± 11.6%, respectively. Moreover, RGD-$(SG)_5$/PEGylated liposomes prepared by bulk mixing for 1 min showed a larger particle size and higher PDI than those of PEGylated liposomes, which was similar to those prepared for 1 h (Tables 1 and S1). The physicochemical properties and cellular association data of RGD-$(SG)_5$/PEGylated liposomes prepared by bulk mixing for 1 h showed the same trend agreed with previous reports.[6] These result supports our hypothesis that RGD-$(SG)_5$-lipids form unstable micelles because they have a poorly hydrated layer compared to that of conventional PEG lipids and can be modified by contact with liposomes for a short period of time. Another possible reason for this result is that the $(SG)_5$ spacer of RGD-$(SG)_5$-lipid is linear, resulting in a less sterically hindered structure than that of conventional PEG lipids.[5,6] In addition, it could be related to the fact that RGD-$(SG)_5$-lipid micelles tend to interact with liposomes because the net charge of RGD-$(SG)_5$-lipid micelles is cationic.

Table 2 Physicochemical Properties of cRGD-Modified Liposomes Prepared by Short-Time Mixing

	Particle Size (nm)	ζ-Potential (mV)	Polydispersity Index
PEGylated liposomes	84.3 ± 1.6	1.3 ± 1.3	0.176 ± 0.014
cRGD-$(SG)_5$/PEGylated liposomes (1 min of bulk mixing)	90.3 ± 5.4	1.0 ± 0.7	0.150 ± 0.028
cRGD-$(SG)_5$/PEGylated liposomes (Microfluidic post-insertion method)	89.3 ± 4.1	1.7 ± 0.4	0.180 ± 0.014

Note: Data are the mean ± standard deviation (SD) (n=3).

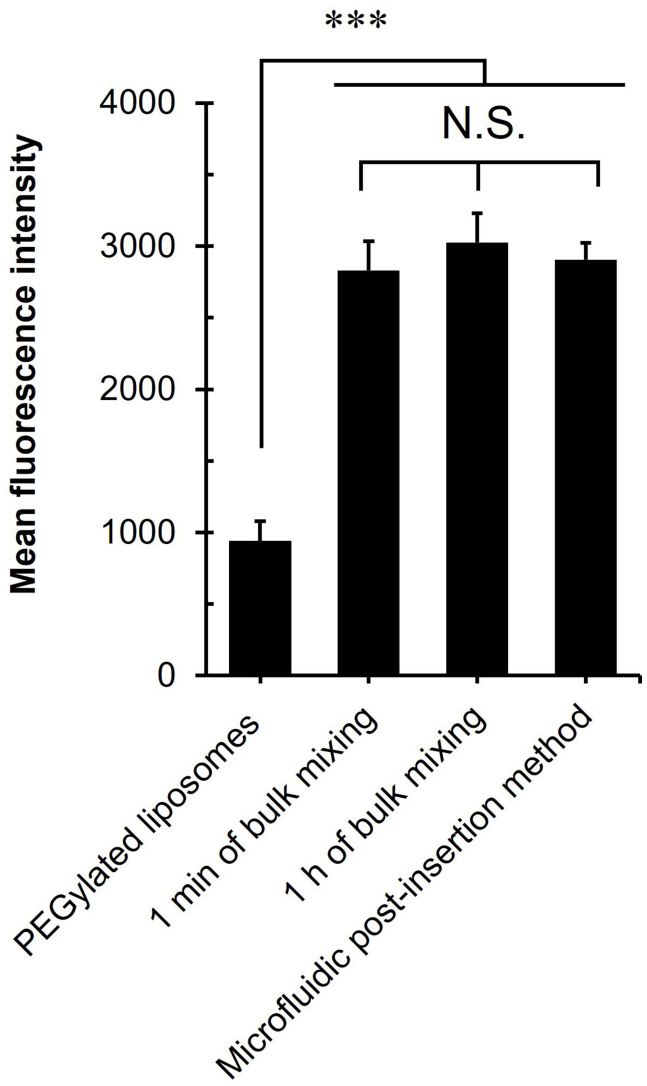


Figure 6 Cellular association of cRGD-$(SG)_5$/PEGylated liposomes prepared by the microfluidic post-insertion method analyzed by flow cytometry. Colon 26 cells were treated with rhodamine-labeled liposomes (100 μM) for 3 h.
Notes: Data represent the mean ± SD of triplicate experiments. ***$p<0.001$.

No reports are available on the mixing conditions in the microfluidic post-insertion method; therefore, the effect of flow conditions of the microfluidic device post-insertion is unclear. Thus, we investigated the optimal flow conditions for modifying RGD-$(SG)_5$-lipid micelles into PEGylated liposomes (Figure 3). Evaluation of the total flow rate showed that increasing the total flow rate had almost no effect on the particle size; however, it increased the PDI. In addition, evaluation of the flow rate ratio showed that the particle size and PDI tended to increase slightly when the mixing ratio of liposomes and RGD-$(SG)_5$-lipid micelles were different (Figure S1). Liposomes prepared by microfluidic post-insertion method showed a size of about 100 nm, which was similar in size to RGD-$(SG)_5$/PEGylated liposomes prepared by bulk mixing for 1 min (Table 1). In a previous study, PDI decreased as the total flow rate increased in the preparation of nanoparticles by the conventional self-assembly method using a microfluidic device.[13] On the other hand, these results suggest that slower mixing in microfluidic channel leads to homogeneous insertion of RGD-$(SG)_5$-lipid micelles into the liposomal membrane because the contact between RGD-$(SG)_5$-lipid micelles and PEGylated liposomes is necessary for ligand modification in the microfluidic post-insertion method.

Evaluation of cellular association in colon 26 cells showed that the cellular association properties of RGD-$(SG)_5$ /PEGylated liposomes prepared by the microfluidic post-insertion method of 1 mL/min exhibited cellular association

properties equivalent to those obtained using bulk mixing for 1 h (Figure 4), which was consistent with the results of quantitative analysis by confocal microscope (Figure 5). These results agree with the data showing equivalent modification rates of 85.3% ± 9.6% and 86.9% ± 11.6%, respectively. Furthermore, the cellular association properties of liposomes prepared by the microfluidic post-insertion method (1 mL/min) increased over time (Figure S2). In our previous study, the cellular association of RGD-$(SG)_5$/PEGylated liposomes prepared by bulk mixing for 1 h was inhibited for 30 min when liposomes were added to the cells with the free RGD peptide.[6] These data suggest that the microfluidic post-insertion method can be used to modify RGD-$(SG)_5$-lipid into PEGylated liposomes and the liposomes prepared by the microfluidic post-insertion method are taken up into cells via integrin αvβ3. In contrast, RGD-$(SG)_5$ /PEGylated liposomes prepared by the microfluidic post-insertion method showed differences in PDI between the total flow rates; however, no difference was detected in the cellular association properties (Figures 3 and 4). Although the effect of particle size on cellular uptake has been extensively studied, the effect of PDI is not yet fully understood. Accordingly, it might be necessary to evaluate the effect of the total flow rate from a different perspective for the evaluation of physicochemical or cellular association properties of the liposomes in the future.

We recently reported the targeting of PEGylated liposomes by modifying HFQ lipid with cyclic RGDfK (cRGD) as a ligand peptide using bulk mixing for 1 h.[9] In our study, we evaluated whether short-time preparation by the microfluidic post-insertion method can be applied to the modification of functional lipids with cyclic ligand peptides into PEGylated liposomes. cRGD-$(SG)_5$/PEGylated liposomes prepared by the microfluidic post-insertion method showed almost the same particle size and PDI as those of PEGylated liposomes (Table 2), suggesting that cRGD was modified into PEGylated liposomes using the microfluidic post-insertion method without substantially changing the physicochemical properties of PEGylated liposomes. Moreover, the cellular association ability of cRGD-$(SG)_5$/PEGylated liposomes prepared by the microfluidic post-insertion method was equivalent to that of liposomes prepared by bulk mixing for 1 h or 1 min. (Figure 6), which was consistent with the results of RGD-$(SG)_5$/PEGylated liposomes (Figures 2 and 4). This result suggests that the cRGD ligand may be inserted into the liposomal surface for a short time using the microfluidic post-insertion method.

Lipid nanoparticles (LNPs) and exosomes with a lipid bilayer membrane, which is similar to that of liposomes, have recently attracted attention as carriers for the highly efficient delivery of nucleic acid.[22–28] In the preparation of targeted LNPs, the post-insertion method can avoid unnecessary interactions between ligands and molecules, such as siRNA or mRNA. In addition, since exosomes cannot be reconstituted, the post-insertion method is suitable for functionalizing biological nanoparticles, such as exosomes. Consequently, the ligand modification method using this microfluidic post-insertion method might be useful for the mass production of targeted formulations of LNPs, exosomes, and liposomes.

Conclusion

In summary, we successfully developed a microfluidic post-insertion method for PEGylated liposomes using RGD- and cRGD-modified HFQ lipids. In this method, a short time contact between HFQ lipid micelles and PEGylated liposomes enables modification on the surface of liposomes even for 1 min. However, it may be necessary to confirm this method for other ligands, including antibodies and proteins. Future studies should evaluate whether targeted liposomes can be prepared at the manufacturing scale used in clinical applications and whether PEG lipids can also be modified using the microfluidic post-insertion method. Our findings from this study would provide valuable information for the large-scale production of targeted liposomes with high quality and functionality for clinical applications.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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