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Continuous Production of Immunoliposomes using a Microvalve-controlled Microfluidic Device (μFD)

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Immunoliposomes (antibody-conjugated liposomes) are highly useful as both a drug carrier in drug delivery and as a reporting probe in immunodiagnostics. However, antibody conjugation is lengthy and cumbersome, because this includes several steps such as derivatization of the antibody, conjugation of the derivatized antibody to liposomes, and separation of the unbound antibodies from immunoliposomes. Recently, liposome preparation steps have simplified by using microfluidic devices (μFDs) where liposomes are formed when a stream of lipids in solvent is hydrodynamically focused between two oblique buffer streams in a microchannel. Herein, we report a simple method for the production of immunoliposomes (rabbit IgG-conjugated liposomes) using microvalve-controlled μFD. The presence of antibody on the liposome was verified by observing the binding of immunoliposomes to rabbit IgG on the surface. The results suggest that immunoliposomes can be easily prepared through sequential mixing of antibody, conjugation reagents, preformed liposomes using microvalve-controlled μFD.

Key Words : Liposomes, Immunoliposomes, Microfluidic device, SRB

Introduction

Liposomes can be described as spherical vesicles consisting of one or more phospholipid bilayers surrounding an aqueous cavity. Their main application is drug delivery for targeting particular types of cells, such as cancer cells.[1-3] To increase their targetability, liposomes should be conjugated with an antibody.[4] Antibody-conjugated liposomes, known as 'immunoliposomes,' are highly useful as both a drug carrier in drug delivery[4] and as a reporting probe in immunoassays.[5] However, antibody conjugation is lengthy and cumbersome, because it includes several steps such as derivatization of the antibody, conjugation of the derivatized antibody to liposomes, and separation of the unbound antibodies from immunoliposomes.[4-6] Recently, microfluidic devices (μFDs) have been used to continuously produce liposomes. Liposomes are formed when a stream of lipids in solvent is hydrodynamically focused between two oblique buffer streams in a microchannel.[7] Although liposome preparation is simplified by omitting the solvent evaporation step to make lipid thin films using μFD, the antibody conjugation step for preparing immunoliposomes has yet to be simplified by μFD. Previously, we showed that complicated chemical reactions in nanoliter volume can be performed easily using a microvalve-controlled μFD.[8] In this study, we present a simple method for the continuous production of immunoliposomes using microvalve-controlled μFD. The characteristics of immunoliposomes such as the size and stability were investigated. The targetability of immunoliposomes was verified by investigating their binding to the surface where anti-IgG molecules were periodically immobilized by microcontact printing.[9]

Experimental

Design and Fabrication of Microvalve-controlled μFD. The μFD was designed to have two modules for the preparation of liposomes (i) and immunoliposomes (ii), as shown in Figure 1. The μFD consists of a microchannel and control layers, and was fabricated by multilayer soft lithography.[10] Each layer was made of polydimethyl siloxane (PDMS, Dow-Corning) from Si molds by soft lithography.[11,12] Then, the two layers were aligned to each other and cured at 80 °C for 1 h to form bonding between the layers.[10] Once the μFD was fabricated, tubing (Tygon® Tubing, USA) for nitrogen gas supply was connected to the μFD, and pneumatic valves were controlled using LABVIEW.

Preparation of Lipid Thin Film. 7.2 μmol of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE, Avanti Polar Lipids) and 14.3 μmol of *N*-succinimidyl-*s*-acetylthioacetate (SATA, Pierce) were mixed in 1 mL of 0.7% triethylamine at 45 °C under N_2.[13] 2 mL of chloroform was added to the mixture and evaporated under vacuum at 45 °C to remove triethylamine. This step was repeated two times. 40.3 μmol of DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, Avanti Polar Lipids), 4.2 μmol of DPPG (1,2-dipalmitoyl-*sn*-glycero-3-[phosphor-rac-(1-glycerol)], Avanti Polar Lipids), and 40.9 μmol of cholesterol (Sigma-Aldrich) were first mixed in 3 mL of chloroform in a round flask, and 0.5 mL of methanol was later added to the mixture. One half of a solution containing 1 mL of prepared DPPE-ATA and 3 mL of isopropyl ether were added to the lipid mixture, and

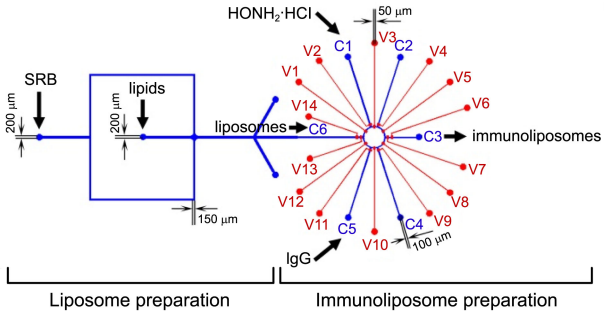

Figure 1. Design of the microfluidic device.

the mixture was kept at 45 °C for 1 min while sonicating under N_2. The solvent in the mixture was then evaporated at 45 °C, and lipid film was formed at the bottom of the flask.

On Chip Formation of Liposomes. 1 mL of isopropyl alcohol (IPA) was added into a flask containing lipid thin film.[13] Lipid/IPA was flown into the μFD through a lipid inlet (Figure 1) at 2 μL/min, while 0.02 M HEPES buffer (pH 7.5) containing 100 mM SRB (sulforhodamine B, Sigma-Aldrich) was flown into the μFD through SRB inlet (Figure 1) at 20 μL/min. Then, large unilamellar liposomes (LUVs) encapsulating SRB were formed in the T-junction where the SRB meets the lipid/IPA. LUVs were moved to the immunoliposome preparation module through a C6 channel (Figure 1).

On Chip Conjugation of Antibody to ATA-Liposomes through Actuation of Microvalves. Before being loaded into the immunoliposome preparation module in the μFD, goat anti-rabbit IgG-FITC (fluorescein isothiocyanate) was derivatized with maleimide groups by sulfo-KMUS (*N*-[κ-maleimidoundecanoyloxy] sulfosuccinimide ester, Pierce).[14] In detail, one milligram of goat anti-rabbit IgG-FITC was dissolved in 1 mL of 0.05 M phosphate buffered saline (PBS) containing 1 mM of EDTA (ethylenediaminetetra acetic acid), and 0.01% NaN_3 (pH 7.8). A sulfo-KMUS solution was prepared by dissolving (6 mg) in 0.3 mL of solvent mixture of DMSO/MeOH (2:1, v/v). Then, 2.25 μL of sulfo-KMUS solution was added to 1 mL of IgG solution and incubated on a shaker at room temperature for 3 h. Then, the derivatized IgG was loaded into the μFD through C5 channel (Figure 1). 0.5 M hydroxylamine hydrochloride (Sigma-Aldrich) in 0.1 M HEPES buffer (pH 7.8) containing 25 mM EDTA was prepared. The loading and mixing of chemical reagents in the μFD were done as described elsewhere.[8] To remove acetylthioacetate group from ATA-liposomes using hydroxylamine, ATA-liposomes and hydroxylamine solution were loaded into the center chamber of the immunoliposome preparation module at 5 μL/min for 10 s through C6 and C1, respectively, while the valves (V3, V6, V7 and V12) were closed (Figure 1). ATP-liposomes and hydroxylamine in the chamber were mixed through on/off control of the valves (V5, V8 and V12) every second for 10 min. The derivatized FITC-conjugated goat anti-rabbit IgG was loaded into the chamber through C5 while all the valves except V9-V11 were closed. For mixing, the valves (V2, V4, V6, V9, V11, and V13) were closed while actuating the valves (V1, V5, V8, V10, and V12) on and off every second for 10 min. These processes were repeated for continuous production of immunoliposomes. Immunoliposomes from C3 were collected in a 1.5-mL tube and reacted with 100 mM ethylmaleimide in 0.02 M PBS to remove unreacted SH-group from liposomes. All the bioconjugation processes performed on the μFD are depicted in Figure 2.

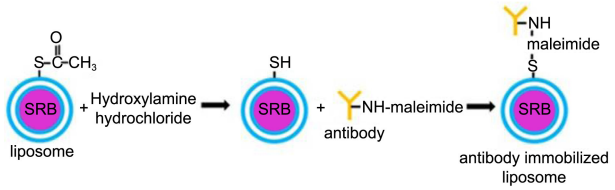

Figure 2. A schematic for antibody conjugation to ATA-liposomes.

Detection of Rabbit IgG on Surface Printed with Goat Anti-Rabbit IgG by Immunoliposome-Based Sandwich Immunoassay. Goat anti-rabbit IgG molecules were printed on glass slides using microcontact printing (μCP).[9,15] A PDMS elastomeric stamp containing 20-μm grating patterns was fabricated by soft lithography. The stamp was cleaned by sonicating in 50% EtOH for 10 min, rinsed five times with D.I. water, and dried gently by a stream of N_2 gas.[15] The stamps were then inked by immersion into a solution containing goat anti-rabbit IgG at 50 μg/mL for 30 min, and rinsed five times. The inked anti-rabbit IgG was printed onto a freshly cleaned glass slide by placing the stamp in direct contact with the slide for 30 min and washing the slide glass five times with PBS. Later, the glass slide was blocked by a solution containing BSA at 50 μg/mL for 30 min and rinsed five times with PBS. The glass slide printed with goat anti-rabbit IgG were incubated with 200 μL of rabbit IgG (100 μg/mL) for 30 min and rinsed five times with PBS. The glass slides were then incubated in 1 mL of immunoliposomes solution (1×10^7 immunoliposomes/mL in PBS) for 5 min and washed with PBS five times.[14] As a control experi-

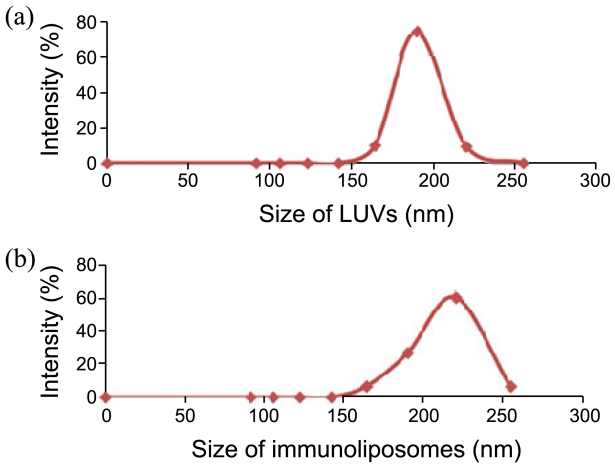

Figure 3. Size distribution of liposomes (a) and immunoliposomes (b). Their sizes were measured by dynamic light scattering (DLS) by Zetasizer Nano ZS (Malvern).

ment, the glass slides with goat anti-rabbit IgG were incubated with immunoliposomes without prior incubation with rabbit IgG. Fluorescence on both slides was observed by confocal laser scanning microscopy (LSM510, Zeiss, Germany).

Results and Discussion

The characteristics of LUVs produced from the μFD are shown in Figure 3 and Table 1. The diameters of both LUVs and immunoliposomes mostly range from 150 to 250 nm, indicating that antibody conjugation to LUVs did not cause aggregation of liposomes, which was often observed in a previous report.[6] Immunoliposomes were stable for at least 8 weeks (Table 1). Neither leakage nor aggregation was observed during the storage period. The presence of antibody (FITC-conjugated goat anti-rabbit IgG) on immunoliposomes were verified by observing their binding to rabbit IgG bound to the surface previously printed with goat anti-rabbit IgG (Figure 4(c) and 4(d)). Red fluorescence on the patterned surface also indicates that the immunoliposomes on the surface were bound to the contact-printed surface. Green fluorescence on the patterned surface confirms the presence of FITC-conjugated goat anti-rabbit IgG on the liposomes (Figure 4(d)). In contrast, neither green (Figure 4(a)) nor red (Figure 4(b)) fluorescence was observed on the surface printed with goat anti-rabbit IgG only when immunoliposomes were added. The results indicate that immunoliposomes do not show any nonspecific adsorption on the printed surface. Based on these results, it is suggested that immunoliposomes have good features as a reporting probe, such as good binding avidity to a target and stability, and that they can be used for immunoassays.

Table 1. Characteristics of immunoliposomes

Mean diameter (nm)	~185 (184.7642)
Volume of one liposome	3.3×10^{-12}
Liposome conc., (liposomes/mL)	2.4×10^{12}
SRB conc., preparation (mM)	100
SRB content (molecules/liposome)	2×10^{5}
Stability (weeks)	~8

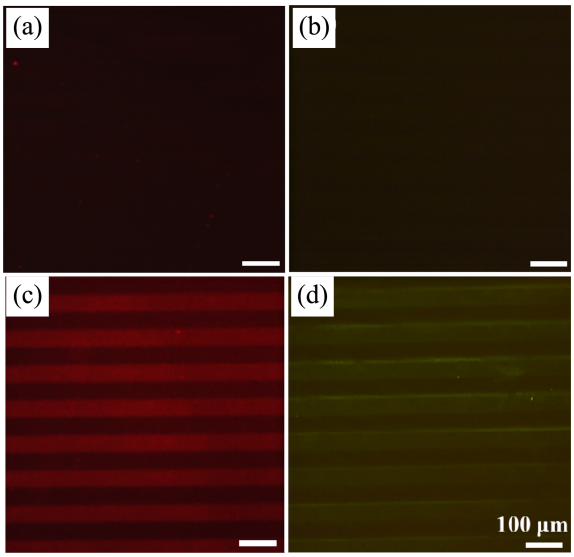

Figure 4. Binding of immunoliposomes to the target on the glass slides. (a) No red fluorescence (SRB) on the slide printed with goat anti-rabbit IgG but no prior incubation with rabbit IgG on the surface. (b) No green fluorescence (FITC) on the slide (a). (c) Red fluorescence on the slide printed with goat anti-rabbrit IgG and prior incubation with rabbit IgG. (d) Green fluorescence on the slide (c). All the glass slides were contact-printed with goat anti-rabbit IgG by μCP. Before fluorescence observation, the slides were incubated with immunoliposomes which were prepared through conjugation of FITC-conjugated goat anti-rabbit IgG to liposomes encapsulating SRB (red fluorescence).

Our microfluidic-based method is advantageous over conventional methods for the preparation of immunoliposomes.[6,14,16] It takes only a few hours and requires microliter volumes of reagents, while the conventional methods take at least two days and require at least several milliliters of reagents. Due to the automated loading and mixing steps through the actuation of microvalves on the μFD, both the liposome and immunoliposome preparation steps can be automated. Furthermore, immunoliposomes can be continuously produced on the μFD, and the amounts of immunoliposomes can be scaled up by producing them over a long period. This suggests that our immunoliposome preparation method is highly suitable for the easy production of immunoliposomes for the diagnostic industry.

Conclusion

Our method for the preparation of immunoliposomes is highly suitable for industrial settings in which the economic production of a large amount of immunoliposomes is needed.

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