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Giant unilamellar vesicles containing Rhodamine 6G as a marker for immunoassay of bovine serum albumin and lipocalin-2

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ABSTRACT

Functionalized giant unilamellar vesicles (GUVs) containing a fluorescence dye Rhodamine 6G is proposed as a marker in sandwich-type immunoassay for bovine serum albumin (BSA) and lipocalin-2 (LCN2). The GUVs were prepared by the electroformation method and functionalized with anti-BSA antibody and anti-LCN2 antibody, respectively. The purification of antibody-modified GUVs was achieved by conventional centrifugation and a washing step in a flow system. To antigen on an antibody slip, antibody-modified GUVs were added as a marker and incubated. After wash-out of excess reagents and lysis of the bound GUVs with Triton X-100, the fluorescence image was captured. The fluorometric immunoassays for BSA and LCN2 exhibited lower detection limits of 4 and 80 fg ml^{-1}, respectively.

Vesicular bilayer membranes such as uni- and multilamellar liposomes, often functionalized with receptors, have found a variety of applications in biosensing studies [1–4]. Encapsulation of a dye into the inner aqueous phase of liposomes, the lysis of the liposomes with a detergent, and successive measurements of spectroscopic signal from the released dye are the common procedures in the so-called sandwich-type liposome immunoassay. The larger the amount of dyes encapsulated, the larger the spectroscopic signal after the lysis will be as far as the signal transduction step is appropriately designed.

Giant unilamellar vesicles (GUVs) of 3–100 μm in diameter can be formed by several protocols [5], including electroformation on either platinum wires [6–8] or indium tin oxide-coated glasses [9], swelling in solution [10,11], solvent exchange [12], and other methods [13–16]. The electroformation in high concentration of sugars is most common because of its easiness of GUV preparation. Because GUVs can be imaged using a variety of optical microscopic approaches, the manipulation of GUVs is relatively easy. In addition, because of their large volume that allows encapsulating a large amount of dyes, GUVs will be attractive as a marker in immunoassay. Rough calculation indicates that GUVs of 10 μm in diameter prepared in the presence of a 1-mmol L^{-1} marker encapsulate approximately 10^8 molecules. This amount is much larger than that (200 molecules) for large unilamellar vesicles (LUVs; 100 nm in diameter). However, to our knowledge, GUVs have not been used for designing sandwich-type liposome immunoassay (abbreviated as SLI), seemingly because the recovery of functionalized GUVs from the reaction mixtures is difficult.

In general, the recovery of antibody-modified liposomes requires the removal of coupling agents, unreacted antibody, and unreacted liposomes. For this purpose, antibody is often linked to liposomes immobilized on a solid support, that is, heterogeneous modification [2,3]. This approach has the advantage that both unreacted antibody and a coupling reagent are easy to be washed out, but the immobilization step is unsuitable for recovering antibody-modified liposomes. Alternatively, in homogeneous modification, antibody-modified liposomes are isolated by size exclusion chromatography [17–19]. However, the size exclusion

Abbreviations used: GUV, giant unilamellar vesicle; LUV, large unilamellar vesicle; SLI, sandwich-type liposome immunoassay; BSA, bovine serum albumin; LCN2, lipocalin-2; DPhPC, 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; B-cap-PE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(cap biotinyl); EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; Chol, cholesterol; DMSO, dimethyl sulfoxide; NHS, *N*-hydroxysuccinimide; ELISA, enzyme-linked immunosorbent assay; R6G, Rhodamine 6G; MTS, 3-mercaptopropyltrimethoxysilane; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)-propionate; NEM, *N*-ethylmaleimide; VPP, Vesicle Prep Pro; ITO, indium tin oxide; S/N, signal/noise; HSA, human serum albumin.

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chromatography is capable of separating only liposomes smaller than 300–500 nm [20,21]. The density gradient centrifugation [17,22,23] and ultracentrifugation [24] are also used for recovering antibody-modified unilamellar liposomes, but these methods necessitate specialized instruments and training. In addition, mechanically stress is likely to cause antibody aggregation [25]. Consequently, lower speed centrifugation is simple and will be a more preferable choice for recovering antibody-modified GUVs.

In this article, we propose the use of GUVs containing a fluorescence dye Rhodamine 6G as a marker in SLI. The GUVs, which are prepared by the electroformation method, are modified with anti-BSA (bovine serum albumin) and anti-LCN2 (lipocalin-2), respectively, in a homogeneous protocol. The unreacted GUVs are removed by centrifugation under almost the same conditions as those used for multilamellar liposomes, followed by a washing step in a flow system. The proposed assay is applied for detecting lipocalin-2, which is a neutrophil gelatinase-associated lipocalin [26,27], in highly diluted human serum. The quantification of lipocalin-2 in human serum and/or urine is important for biomarker of various diseases [26,28–31].

Materials and methods

Materials

1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC, 10 mg ml^{-1} chloroform solution), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, 10 mg ml^{-1} chloroform solution), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(cap biotinyl) (sodium salt) (powder, B-cap-PE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was obtained from Dojindo Laboratories (Kumamoto, Japan). Cholesterol (Chol), D(−)-sorbitol, D-(+)-glucose, dimethyl sulfoxide (DMSO, dehydrated), and *N*-hydroxysuccinimide (NHS) were obtained from Wako Chemicals. Chol was recrystallized three times from methanol. Anti-BSA antibody (polyclonal, rabbit) (anti-BSA) was obtained from Funakoshi (Tokyo, Japan). Albumin from bovine serum (BSA, >97%), lipocalin-2 (human recombinant), human serum (male AB), transferrin human, haptoglobin from pooled human plasma (lyophilized powder), and sucrose were obtained from Sigma–Aldrich Chemical (St. Louis, MO, USA). A human lipocalin-2/NGAL enzyme-linked immunosorbent assay (ELISA) kit was obtained from R&D Systems (Minneapolis, MN, USA). Anti-lipocalin-2 antibody (monoclonal) was obtained from R&D Systems. Rhodamine 6G (R6G) was obtained from Tokyo Kasei (Tokyo, Japan). 3-Mercaptopropyltrimethoxysilane (MTS, >99.9%) was obtained from Shin–Etsu Chemical (Tokyo, Japan). *N*-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) and *N*-ethylmaleimide (NEM) were obtained from Thermo Scientific (Rockford, IL, USA). All other chemicals used were of analytical reagent grade. Milli-Q water (Millipore reagent water system, Bedford, MA, USA) was used throughout the experiments. Micro cover glasses (diameter 15 mm, thickness 0.12–0.17 mm) were obtained from Matsunami Glass Industries (Tokyo, Japan).

Apparatus

All fluorometric images of liposomes on cover slips were obtained with a FluorImager 595 (Molecular Dynamics, Sunnyvale, CA, USA). Vesicle Prep Pro (VPP; Nanion Technologies, Germany) was used for electroformation of GUVs. A Denki Kagaku Keiki (Tokyo, Japan) glass electrode pH meter (model IOL30) was used for pH measurements.

Preparation of R6G-encapuslating GUVs

GUVs were prepared from a lipid mixture consisting of 4.2 mg DPhPC, 0.22 mg cholesterol, and 0.021 mg DOPE in chloroform at a molar ratio of 9:1:0.050 by the electroformation method. Here, 20 μl of the lipid mixture in chloroform was put on an indium tin oxide (ITO)-coated slide glass and air-dried to a lipid film. After setting a silicon O-ring on it, 0.30 ml of 1 mol L^{-1} sorbitol containing 1.0 mM R6G was added. Then, an electric field (5 Hz, ac 3 V) was applied through two ITO-coated slide glasses for 2 h with VPP. The GUV suspension (abbreviated as R6G–GUVs) was recovered and stored in a microcentrifuge tube (1.5 ml).

Preparation of immuno-GUVs

The functionalization of GUVs with protein (anti-BSA or anti-LCN2) was carried out as follows (see Fig. S1a in online supplementary material). A 10-μl portion of 16.5 μg ml^{-1} anti-BSA containing 22 mM NHS and 55 mM EDC in 1 mol L^{-1} sorbitol was mixed with 0.10 ml of R6G–GUVs and incubated at room temperature for 15 min. The suspension in a plastic centrifugation tube (1.5 ml) was centrifuged at 14,000 *g* for 30 min at 20 °C. After centrifugation, a 50-μl portion of the suspension from the top surface was taken out with a micropipette and its fraction was discarded. Then, the remaining part of the suspension (i.e., 50 μl) was collected with a micropipette into a plastic centrifuge tube. The collected GUVs hereafter are abbreviated as anti-BSA–R6G–GUVs. The anti-BSA–R6G–GUVs were stored at 4 °C until use.

Preparation of GUVs modified with an anti-LCN2 was performed in the same manner as described for anti-BSA–R6G–GUVs. The immuno-GUVs hereafter are abbreviated as anti-LCN2–R6G–GUVs.

Preparation of anti-BSA or anti-LCN2 modified slips

An antibody slip used for immobilizing antibody was prepared as follows (see Fig. S1b in supplementary material). First, cover slips were cleaned in 1 M NaOH for 3 h and washed thoroughly with Milli-Q water. The one-side surface of the cover slip was treated with 0.10 ml of 50% (v/v) MTS in toluene for 60 min at room temperature. The MTS-modified cover slip was washed thoroughly with toluene and dried at room temperature. On the other hand, a 0.10-ml portion of an anti-BSA antibody (not fragmented) or an anti-LCN2 antibody (not fragmented) solution (1 mg ml^{-1}) was mixed with 1.25 μl of 20 mmol L^{-1} SPDP in DMSO (anhydrous) and incubated at room temperature for 1 h. Unreacted SPDP was removed with a dye removal column (Thermo Scientific) and replaced by 0.15 mol L^{-1} NaCl solution containing 0.1 mol L^{-1} NaH_2PO_4/NaOH buffer (pH 7.2) (a PBS buffer). Then, 0.10 ml of the activated antibody (0.1 mg ml^{-1}) was placed on an MTS-modified slip and incubated for 60 min at 4 °C. After washing with Milli-Q water, the slip was incubated with 53 mg ml^{-1} NEM in a PBS buffer in order to block unreacted sulfhydryl sites. The antibody slip was washed with 6 ml of Milli-Q water and stored in a PBS buffer until use.

Procedure for immunoassay

The procedure described here is that for the assay of BSA. However, the assay of lipocalin-2 was performed in the same manner as described for BSA except that anti-LCN2–R6G–GUVs were diluted 300 times with 10 mmol L^{-1} NaH_2PO_4 containing 10 mmol L^{-1} NaCl buffer (pH 7.4, a PB buffer) before its use.