Proteoliposome for Tumor-Targeting by Postinsertion of BAM-Homing Molecule Complex

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ABSTRACT

The objectives were to fabricate the nano-scale, stable liposome, of which the surface was decorated with proteineous and active homing molecules for tumor-specific targeting, and to demonstrate its targetability by imaging technique. Sterically stable, ligand-targeted liposomes were prepared by a postinsertion method using BAM (biological anchor for membrane) as a linker. We used inert phosphatidylcholine as a liposomal phospholipid, and BAM-homing molecule complex was simply postinserted after a unilamellar liposome (ca. 200 nm in diameter) was made. Epidermal growth factor was used as the homing molecule to target EGF receptor expressed on tumor surface. The selective targeting was confirmed by fluorescence microscopy. To our knowledge, it is the first report of employing the simple postinsertion method to obtain proteoliposome of which the surface was decorated with both PEG moiety and a protein homing molecule. The method is expected to serve as a platform technology for liposome-based, protein-targeting drug delivery system.

Keywords: proteoliposome, epidermal growth factor, biocompatible anchor molecule, active cell targeting, biofunctionalization

1 INTRODUCTION

Among various nanoparticles used as drug delivery systems, liposomes are the most well studied materials because of their amphiphilic and biocompatible characteristics [1,2]. Liposome is composed of a vesicular lipid bilayer surrounding a large inner aqueous phase. Their therapeutic advantages include the targeting ability to deliver a large amount of encapsulated drug to specific sites, resulting in improved drug pharmacokinetics and efficacy and reduced drug toxicity [3]. One of the drawbacks of a liposomal system is that it can be easily cleared by the reticular endothelial system (RES) resulting in a reduced targeting yield [4]. This can be overcome by modifying the liposome surface with flexible hydrophilic polymers such as polyethylene glycol (PEG) to produce so called ‘stealth’ liposomes [5]. This PEGylated liposome was usually prepared by using phosphatidylethanolamine (PE) or PEG-cholesterol derivatives and the common method to prepare it (i.e., simple mixing with other phospholipid components) reduced the inner space resulting in lower drug loading capacity [6].

Active targeting usually uses guidance ligand molecules called ‘homing molecules’ that have high affinity for cell surface receptors or epitopes. Thus, conjugation of a proteineous homing molecule to liposome surface, i.e., targeting proteoliposome, would be interesting for targeted delivery. To prepare targeting liposome composed of relatively inert phospholipid such as PC, a ‘post-insertion’ method for liposome surface modification was employed. It could attach the homing molecule only on the outer surface maintaining the liposome inner space [7]. However, a relatively high temperature (up to 60°C) was required for post-insertion which could reduce the activity of the ligand biomolecules, although a thermostable molecule such as affibody was post-inserted at 55°C and still maintained the antigen-binding activity [7]. Recently, a post-insertion method operating at the ambient temperature was reported for stable insertion into various liposomal surfaces [8]; however, it characterized only the post-insertion linker and did not present surface modification efficiency or liposomal targeting efficiency.

In this study, we report the preparation of proteoliposome of which the surface was biofunctionalized by using a commercially available linker, biocompatible anchor molecule (BAM). First, the insertion efficiency and stability of BAM-protein complex into a relatively inert liposomal bilayer (100% DPPC) was compared with those of the more reactive liposome (90% DPPC with 10% DPPE). Second, in vitro targeting and delivery performance of the proteoliposome to a breast cancer cell line was experimentally demonstrated.

2 EXPERIMENTAL PROCEDURES

2.1 Materials and liposome preparation

Both dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylethanolamine (DPPE) were purchased from Avanti Polar Lipid, Inc., USA, and cholesterol (Chol) and dicetylphosphate (DCP) were from Sigma Aldrich, USA. All other chemicals used such as chloroform were of a reagent grade. For liposome preparation, DPPC, cholesterol, and DCP were dissolved in a 64:30:6 molar ratio in 5 mL of chloroform and mixed in a 50 mL round bottom flask. The mixture was evaporated under vacuum in a rotary evaporator to produce a thin lipid film. To thoroughly remove the residual chloroform, the lipid film was stored under vacuum overnight. Six mL of 10 mM PBS (pH 7.4) were added to the lipid film, which was then warmed at 45°C for 10 min and finally peeled off the flask. The mixture was then subjected to 5 cycles of freezing (−70°C) and thawing (45°C). Finally, the liposome solution was extruded 15 times through a 200 nm polycarbonate membrane using a mini-extruder (Avanti Polar Lipids, USA).

BAM® (Sunbright OE-080CS, NOF, Japan) was used as a post-insertion linker. BAM is commonly used to immobilize floating cells on a solid surface [9]. Figure 1(A) shows its molecular schematics: It consists of an oleyl group as a hydrophobic membrane anchor at one end, an N-hydroxysuccinimide (NHS) group for protein conjugation at the other end, and they are connected by a linear PEG chain (ca. 3,350 Da) for increased solubility and stealth effect. Because of its lipophilic interaction ability, the oleyl chain of BAM is expected to interact with the hydrophobic domains inside the phospholipid bilayers so as to be inserted into the liposomal bilayer.

2.2 Encapsulation of Cy5 and biofunctionalization with EGF-BAM conjugate

To image and trace the liposomal targeting, Cy5-NHS ester was encapsulated inside liposome. When rehydrating the lipid film, Cy5-NHS ester (2 mg) was added to 6 mL of 10 mM PBS (pH 7.4). The Cy5-encapsulated liposomes were separated from free or unreacted Cy5-NHS ester by gel permeation chromatography (Sephadex G-25, GE Healthcare, USA) using 10 mM PBS (pH 7.4) as an elution buffer.

2.3 Preparation of proteoliposome

For the proteoliposome, epidermal growth factor (EGF) was used as a homing molecule to target the liposome to breast cancer cells since 20-30% of breast cancer cell lines over-express human epidermal growth factor receptor (EGFR) on their surfaces [12,13]. Recombinant human EGF, a stable protein known to undergo receptor-mediated endocytosis [12,13], was gifted from Daewoong Pharmaceutical Co Ltd., Seoul, Korea. To biofunctionalize the liposome surface with EGF, three sequential steps were performed as follows: (1) BAM was first dissolved in 10 mM PBS (pH 7.4) to yield 1 mg/mL, (2) to form a BAM-EGF complex, 1 mg/mL of EGF solution was incubated with 1 mg/mL of BAM solution for 3 hr at room temperature, and (3) for the post-insertion to the liposome surface, the BAM-EGF complex was incubated with the liposome for 2 hr at room temperature.

2.4 In-vitro active targeting of biofunctionalized proteoliposomes

MDA-MB-231 mammary gland breast cancer cell line (Korean Cell Line Bank #30026) that over-expresses EGFR on its surface was used as the target cell line. MCF-7 mammary gland breast cancer cell line (Korean Cell Line Bank #30022), which does not express EGFR on its surface, was used as the negative control cell line. Both cell lines were propagated in DMEM supplemented with 10% FBS (fetal bovine serum), 100 IU/mL penicillin and 100 IU/mL streptomycin (GIBCO, Belgium). The cells were incubated at 37°C in humidified air containing 5% CO2. Ca. 5 × 10^4 of MCF-7 and MDA-MB-231 cells were prepared on a cover slip in a 24 well-plate 1 day beforehand. The medium was suctioned and the cells were washed twice with 2 mL of 10 mM cold PBS for 5 min. Cy5-encapsulated liposomes of which the surface was modified with BAM-EGF complex (100 µg/mL) were incubated with MCF-7 and MDA-MB-231 cells for 1 hr at 37°C. Two mL of 3.7% paraformaldehyde were added for fixation and allowed to react for 15 min. The cover slip was washed 5 times with 10 mM PBS (pH 7.4) for 5 min. The interaction between Cy5-encapsulated liposome and the cell was monitored by the confocal laser scanning microscopy. The excitation wavelength was 488 nm, and the emission wavelength was 580-620 nm.

2.5 Preparation of doxorubicin-encapsulated liposome

After confirming the targeting performance of the EGF-BAM inserted proteoliposome, its subsequent intracellular delivery performance was investigated by encapsulating doxorubicin inside the proteoliposome. Doxorubicin-HCl (6.5 mg) (Fluka, USA) was dissolved in methanol (11 mL) in a round-bottom flask, and the methanol was evaporated. Then, 21 mg of DPPC and 7.5 mg cholesterol in 5 mL chloroform were added to the dried doxorubicin and the mixture was stirred to achieve a homogeneous solution, followed by chloroform evaporation. This doxorubicin-HCl concentration was reported to be sufficiently lethal to the cells [14]. The flask was then placed into a vacuum desiccator for at least 12 hr to ensure complete solvent removal. The dried lipid film was resuspended with 6 mL of 10 mM PBS (pH 7.4). The mixture was subjected to 5 cycles of freeze-thawing, and extruded 15 times through the 200 nm polycarbonate filter using the mini-extruder. The BAM-EGF complex was inserted into a doxorubicin-encapsulated liposome as described above. The free or unencapsulated doxorubicin was removed by the gel permeation chromatography (Sephadex G-25, GE Healthcare, USA) with 10 mM PBS (pH 7.4).

SYTOX® Green (Invitrogen, USA) was used as a dye to visualize the cytotoxicity. The mixture of 0.1 µg/mL doxorubicin-encapsulated liposomes and 2.5 µM of SYTOX® Green was incubated with the cell sample for 5 min at 37°C. SYTOX® Green (Invitrogen, USA) was used as a dye to visualize the cytotoxicity. The mixture of 0.1 µg/mL doxorubicin-encapsulated liposomes and 2.5 µM of SYTOX® Green was incubated with the cell sample for 5 min at 37°C.
Green were incubated with MCF-7 and MDA-MB-231 cells for 1 hr at 37°C. Two mL of 3.7% paraformaldehyde were added and allowed to react for 15 min. The cover slip was washed 5 times with 10 mM PBS (pH 7.4) for 5 min. CLSM was used to check the toxicity of doxorubicin-encapsulated liposomes toward EGFR over-expressing breast cancer cells. The excitation wavelength was 488 nm and the emission wavelength was 515-525 nm, and 580-600 nm, respectively.

3 RESULTS AND DISCUSSION

Figure 1 shows the schematics of a proteoliposome of which the surface is modified with BAM-EGF complex for targeting and the core is encapsulated with Cy5 dye for fluorescence tracing. For the proteoliposome to have targeting ability, the conjugated EGF should maintain its binding activity toward EGFR after the post-insertion step. To demonstrate the active targeting to EGFR expressed on the cell surface, the proteoliposomes biofunctionalized with BAM-EGF complex were incubated with MDA-MB-231 (EGFR-positive cell) or MCF-7 cells (EGFR-negative cell). As shown in Figure 2, the BAM-EGF inserted liposome was selectively targeted to the MDA-MB-231 cells, but not to the MCF-7 cells. After 1 hr incubation, most liposomes were bound to the MDA-MB-231 cell surface on which EGFR was expressed.

To further demonstrate that the proteoliposome could be actually delivered into a cell, cytotoxic doxorubicin was encapsulated inside the same biofunctionalized liposome. Doxorubicin is a DNA interacting drug widely used in cancer chemotherapy. As a cell viability probe, SYTOX Green, a nucleic acid dye, was used. It was used to image only dead cells, since it could easily penetrate plasma membranes of weak or damaged integrity yet could not cross the membranes of healthy live cells. As shown in Figure 3(b) and (e), doxorubicin was taken up and distributed only in the MDA-MB-231 cells and not into MCF-7 cells. The SYTOX green fluorescence was detected also only in the MDA-MB-231 cells, which indicated that most of the MDA-MB-231 cells were not viable (Figure 3(f)). In contrast, Figure 3(c) shows no intracellular green fluorescence, meaning that the EGF-BAM complex liposome was not targeted to MCF-7 cells.

The surface modification efficiency and stability of BAM inserted into 100% DPPC liposome were compared with those of a more conventional liposome surface, i.e., 90% DPPC/10% DPPE. PE that contains a primary amine as a reactive group in amine coupling method was traditionally used as a phospholipid component of liposomes to be surface modified by amine coupling method. In addition, it was reported that the PE composition of 10% was commonly used to prepare the liposomes for surface activation [8,15]. Our experimental data clearly indicated the BAM-mediated modification was much superior to the traditional amine coupling to PE.

The 24 hr stability of the inserted BAM was much higher than amine coupling modification. Since the bilayer of 90% DPPC/10% DPPE liposome was supposedly more rigid than 100% DPPC liposome, this result can be compared with a previous report, in which the insertion stability of tresylated PEG-sterol anchor was dependent on the liposome fluidity [8]; rigid liposome showed about 40% stability of that of flexible liposome. In addition, while the previous post-insertion methods required incubation at 55-60°C [6,7], the BAM-mediated post-insertion can be accomplished at room temperature with good stability. Thus, it can serve as a more effective method to biofunctionalize the liposome surface with temperature-sensitive proteins.

For the proteoliposome to be targeting, the conjugated homing protein molecule should maintain its binding activity after the post-insertion step. We demonstrated it by monitoring the targeting ability of the post-inserted EGF toward the EGFR on the cell surfaces. The fluorescence tracing clearly showed the proteoliposome biofunctionalized with BAM-EGF complex could be selectively targeted to the EGFR-expressing cells. However, it took longer time (about 1 hr) for the BAM-EGF biofunctionalized liposomes to bind to the MDA-MB-231 cell surface than the liposomes modified with anti-EGFR antibody (about 30 min) [16]. It is probably because the PEG moiety of the BAM not only induced the protective stealth effect but also decreased the targeting yield.

Since intracellular delivery is another important function of a proteoliposome, we encapsulated DNA interacting doxorubicin inside the proteoliposome and monitored its cytotoxic effect by using a nucleic acid dye, SYTOX Green.

![Figure 1. Schematic diagram of proteoliposome biofunctionalized with BAM-EGF complex. Cy5 was encapsulated inside the liposome for fluorescence tracing. EGF was conjugated at the end of the BAM by reacting with NHS group. The PEG moiety connects the EGF and oleyl groups. BAM-EGF conjugate was inserted only into the outside of the liposome.](image)
employing the post-insertion method to obtain proteoliposome biofunctionalized with EGF showed distinct cytotoxicity only to the MDA-MB-231 cells. It clearly indicates that the EGF maintained its binding bioactivity after the post-insertion. To our knowledge, it is the first report of employing the post-insertion method to obtain proteoliposome of which the surface was biofunctionalized with both PEG moiety and a protein homing molecule.

4 CONCLUSION

Sterically stable, ligand-targeted liposomes were prepared by post-insertion modification using BAM as a linker. By measuring the fluorescence density of Cy5 bound to the liposome surface, we showed that the modification efficiency of BAM-mediated biofunctionalization as well as the stability of the inserted BAM was clearly higher than those of the conventional amine coupling method. By using fluorescence marker, we confirmed the selective and active targeting of the BAM-EGF-modified liposome to the EGFR over-expressing MDA-MB-231 cells. Furthermore, the doxorubicin-loaded proteoliposome biofunctionalized with EGF showed distinct cytotoxicity only to the MDA-MB-231 cells. It clearly indicated that the EGF maintained its binding bioactivity after the post-insertion. To our knowledge, it is the first report of employing the post-insertion method to obtain proteoliposome of which the surface was biofunctionalized with both PEG moiety and a protein homing molecule.

5 REFERENCES