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Research Paper

Highly Efficient Encapsulation of Anionic Small Molecules in Asymmetric Liposome Particles

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Abstract Anionic small molecules are hard to penetrate the cell membranes because of their negative charges. Encapsulation of small molecules into liposome particles can provide target specific delivery of them. In our previous study, siRNA could be efficiently encapsulated into liposome particles using an asymmetric preparation method of liposomes. In this study, the same method was applied for encapsulation of small anionic fluorescent chemicals such as calcein and indocyanine green (ICG). More than 90% fluorescent chemicals were encapsulated in the asymmetric liposome particles (ALPs). No intracellular fluorescent signal was observed in the tumor cells treated with the unmodified calcein/ALPs and ICG/ALPs, whereas the surface modification with a cell-penetrating polyarginine peptide (R8 or R12) allows cellular uptake of the ALPs. The results demonstrate that the ALPs encapsulating small anionic drugs will be useful for target-specific delivery after modification of target-specific ligands.

Keywords: Asymmetric liposome particle, Encapsulation of anionic small molecules, Target specific drug delivery

I. Introduction

Hydrophilic molecules with anionic charge(s) are limited to use directly as drugs because of low permeability of cell membranes [1,2], and therefore their hydrophobic prodrug derivatives have been developed to increase intracellular uptake [3]. However, many of the anionic molecules are hard to be applicable for development of prodrugs.

Liposomes have been currently used for small drug delivery to sustain drug release, reduce drug side effects, and increase drug stability [1,2,4]. Liposomal drugs have some additional advantages, such as general tumor targeting using the enhanced permeability and retention (EPR) and target specific delivery after surface modifications with target specific ligands [5]. Furthermore, the surface PEGylation of liposomes is possible to minimize rapid uptake by *in vivo* phagocytic systems as well as to improve safety and efficacy of drugs [6].

Liposomes are composed of lipid bilayers, and therefore enable to encapsulate hydrophilic molecules their internal aqueous spaces. However, the small internal cavity of the liposome usually provides low efficiency of encapsulation of the molecules. Several methods have been reported to increase hydrophilic drug encapsulation [7]. The reverse phase evaporation method can be applied for encapsulation of various hydrophilic molecules [8,9]. The method initially prepares one-phase inverted micelles in the organic solvent and evaporation of the organic solvent generates liposomes with encapsulation of a large amount of the hydrophilic molecules. However, the uniform lipid system is hard to achieve an encapsulation level of more than 70%. The freeze-thawed method has been reported to allow 90% encapsulation yield cations such as sodium and magnesium ions [10], but is hard to generalize the hydrophilic organic molecules important for drug development.

Previously, we developed a novel method for preparation of asymmetric liposome particles (ALPs) which allow over 90% siRNA encapsulation [11]. To produce the ALPs, we initially prepared two different inverted micelles, the inner one composed of the inner cationic lipids encapsulating siRNA and the outer one composed of neutral and PEGylated lipids, in organic solvents. In this study, the method was applied for encapsulation of hydrophilic and anionic chemicals that are hard to penetrate cell membranes. The methods also allowed over 90% encapsulation of the chemicals. The unmodified ALPs were unable to internalize into cells, but the polyarginine modification induced the cellular uptake of ALPs encapsulating the chemicals.

II. Experiment

1. Materials

The lipids for this study, which were purchased from Avanti Polar Lipids, Inc. (USA), were 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-*sn*-

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glycero-3-phosphoethanolamine (DOPE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (mPEG-PE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (miPEG-PE), 1,2-dioleoyl-3-dimethylammonium-propane (DODAP), and cholesterol (CH). The polyarginine peptides, R8 (RRRRRRRRGGWC) and R12 (RRRR RRRR RRRRGGC), were synthesized using Fmoc-chemistry. The anionic fluorescent chemicals, calcein and indocyanine green (ICG), were purchased from Sigma-Aldrich (USA).

2. ALP preparation and surface modification

The ALPs were prepared by a modified reverse phase evaporation method using two different inverted micelles as reported previously [11]. Briefly, the inner and outer inverted micelles were separately prepared in two different glass tubes. The outer lipid mixture composed of DSPC, DOPE, mPEG-PE and miPEG-PE and CH with a molar ratio of 3:3:1.8:0.2:4 (1.7 μmole total) was suspended in 120 μl ethanol and 200 μl HBS (20 mM HEPES and 150 mM sodium chloride; pH 7.5). The inner lipid mixture composed of DODAP and DOPE with a molar ratio of 9:1 (1.5 mmole total) were suspended in 150 μl of 150 mM sodium citrate buffer (pH 4) containing 0 to 0.1 mg of calcein or ICG. To prepare the outer and inner inverted micelles, 600 μl and 400 μl diethyl ether to the outer and inner lipid suspensions, respectively, and sonicated the mixture using a cup-horn probe of a Sonics ultrasonic processor (Sonics & Materials, USA). The inverted micelles were mixed, and then removed the diethyl by nitrogen blowing and dialysis with a 10 K membrane in HBS at 4°C to remove the remaining organic solvents. The final volume was adjusted to 500 μl. The particle sizes and zeta potentials of the ALPs were analyzed with an ELS-Z zeta-potential and particle size analyzer (Photal Otsuka Electronics, Japan). The encapsulation yield was analyzed by measuring fluorescent intensities of either before or after dialyzed samples treated with 1/10 volume of 10% nonidet P-40 (NP-40) using the SpectraMax M2 microplate reader (Molecular Devices, USA). The fluorescent intensities of the ICG/ALPs depending on the ICG concentrations were also monitored using a homemade fluorometer under 780 nm excitation and 830 nm emission in the presence or absence of 1% NP-40.

To conjugate the cell penetrating peptide, R8 or R12, to ALPs, 5 times molar excess peptide than miPEG-PE was mixed with ALPs, and the mixture was incubated for 3 h at room temperature. The unreacted maleimide groups of ALPs were neutralized by adding a hundredth volume of 200 mM 2-mercaptoethanol, and the unbound peptide and 2-mercaptoethanol were removed by dialysis with a 50 K membrane in HBS.

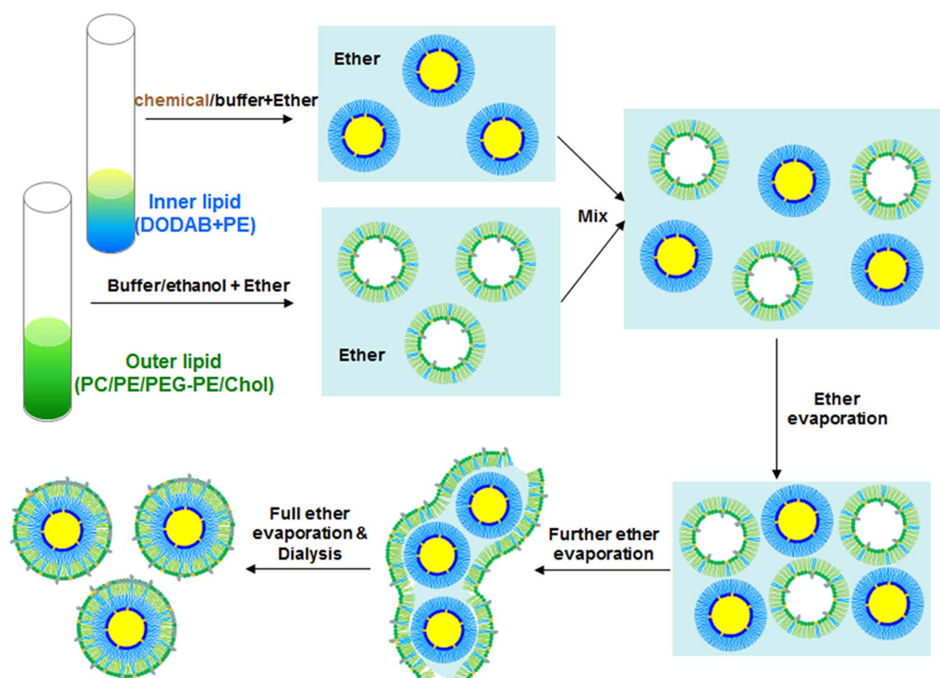
3. Cellular uptake assay of ALPs

The non-small cell lung cancer (NSCLC) cells, A549, NCI-H322 and NCI-H460, were cultured in micro-slide eight-well microscopy chambers (ibidi, Germany) in DMEM medium containing 10% FBS (DMEM-FBS). The attached cells were washed with DMEM-FBS, and then treated with the ALPs modified by R12 or R8 peptides in DMEM-FBS. After 1 day of incubation in the media, the cells were rinsed twice in PBS and fixed with 4% formaldehyde in PBS at room temperature for 15 min. After the subsequent DAPI treatment, the fluorescent images of 4',6-diamidino-2-phenylindole (DAPI), calcein and ICG in cells were monitored with a confocal microscope (Carl Zeiss LSM 510 META, Germany).

III. Results and Discussion

The reverse-phase evaporation method is reported to induce high encapsulation of a variety of molecules in liposome particles [8], but it is difficult to achieve an encapsulation level of more than 70% using the uniform lipid system. Previously, we developed a novel method for preparation of ALPs using two different inverted micelles in order to encapsulate siRNAs [11]. The strategy proved more than 90% encapsulation of siRNAs in ALPs as well as anionic and PEGylated liposome surfaces protecting nonspecific penetration into the cells. Since siRNAs are hydrophilic and anionic molecules, the strategy was expected to be directly applicable for encapsulation of small hydrophilic molecules with negative charges.

Scheme 1 shows the overall procedures for preparation of the ALPs encapsulating the anionic chemicals. The water-soluble and negatively charged fluorescent dyes, calcein and ICG, were selected as model chemicals because the cellular uptake of the chemicals easily detected using the fluorescent images. The inner lipid mixture, DODAP/DOPE (9:1 molar ratio; total 1.5 μmol) with small head groups, was used for preparation of the inner inverted micelle. The inner lipids were hydrated with a 150 mM citrate buffer (pH 4.0) containing the fluorescent chemicals in order to provide a strongly positive charge of DODAP. Since DODAP and DOPE have the small head groups, the inner inverted micelle composed of the lipids is considered to be stable. In contrast, the compositions of the outer lipids were DSPE/DOPE/mPEG-PE/miPEG-PE/CH (3:3:1.8:0.2:4 molar ratio; total 1.6 1.5 μmol). The overall head size composed of the lipids is larger than their tail size, and therefore the outer inverted micelle is expected to be relatively unstable. These stability-difference is considered to provide the higher encapsulating yields for the chemicals. After mixing the inner and outer inverted micelles, the ALPs were produced by organic solvent evaporation. The possible mechanism of the ALP formation is that the



Scheme 1. Scheme of the ALP preparation. The inner and outer lipid films were hydrated with sodium citrate (pH 4.0) containing either calcein or indocyanine green (ICG) and HBS/ethanol, respectively, and Diethylether was then added to the hydrated lipid solutions. The inner and outer inverted mixtures were prepared by extensive sonication, and both inverted micelles were mixed and evaporated by nitrogen blowing to prepare the ALPs encapsulating fluorescent chemicals. During diethylether evaporation, the outer inverted micelles are considered to be quickly disrupted due to the large head groups, whereas the inner inverted micelles are still stable due to the small head group. The resulting ALPs were equilibrated with HBS by dialysis. The yellow color inside the liposome represents the fluorescent chemical.

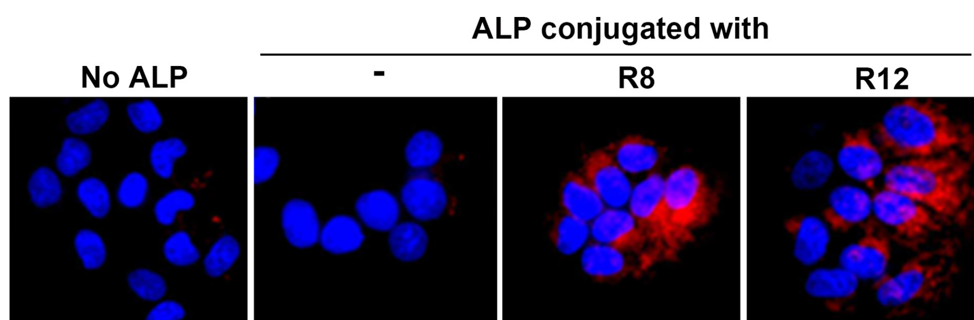


Figure 1. (A) Concentration-dependent fluorescence intensity of ICG encapsulated in ALPs. The numbers indicate the ICG concentrations (μg/ml), and the maximum intensity was observed at 10 μg/ml ICG in ALPs, whereas almost no intensity was observed at 200 μg/ml. (B) Increase of the fluorescence intensity by treatment of 1% NP-40. Treatment of NP-40 (b and d) to the ICG/ALPs with 60 μg/ml (a) and 200 μg/ml ICG (c), respectively, was significantly enhanced the fluorescence intensity. The (e) tube contains HBS only. The fluorescence intensity was monitored at 780 nm excitation light and 830 nm emission light.

unstable outer inverted micelles are readily destroyed during organic solvent evaporation and the resulting outer lipids subsequently surround the stable inner lipids encapsulating the chemicals as proposed in Scheme 1.

The unencapsulated chemicals were removed by dialysis at 4°C. To measure the encapsulation efficiency of the chemical in ALPs, either before or after dialyzed ALPs were adjusted their volume, disrupted by treatment of 1/10 volume of 10% nonidet-P40, and then measured fluorescent intensities. The encapsulation yields were calculated using the following equation, (fluorescent intensity after dialysis)/(fluorescent intensity before dialysis) $\times$ 100 (%). The encapsulation yields of the ALPs were more than 90% as similar to siRNA encapsulation [11], demonstrating that the present method is much more effective for the anionic chemical encapsulation than that using the uniformed inverted micelles. Interestingly, the ICG/ALPs with approximate 200 μg/ml ICG displayed almost no fluorescent signal under 780 nm excitation and 830 nm emission, whereas those with approximate 10 μg/ml ICG displayed the highest fluorescent signals (Fig. 1A). The ICG molecules have been reported to form aggregates quenching the fluorescent signals at the higher concentration [12], and therefore no fluorescent signal at 200 μg/ml demonstrates that the ICG aggregates are also formed inside of ALPs at the condition. The disruption of the

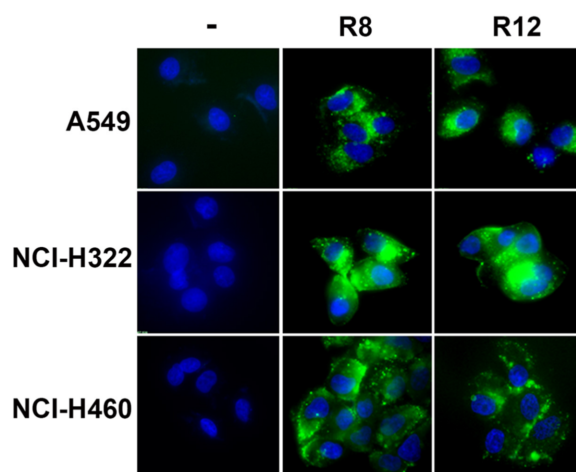


Figure 2. Cellular uptake of calcein/ALPs with and without modification of the polyarginine peptide R8 or R12. The non-small cell lung cancer cell lines, A 549, NCI-H322, and NCI-H 460, were incubated with calcein/APLs with and without R8 or R12 modification on their surfaces. The non-conjugated ALPs (–) cannot penetrate into any of the cells, whereas the peptide modified calcein/ALPs efficiently internalize into the cells. The nuclei of the cells were stained with DAPI. The blue and green colors represent the fluorescent signals for DAPI and calcein, respectively.

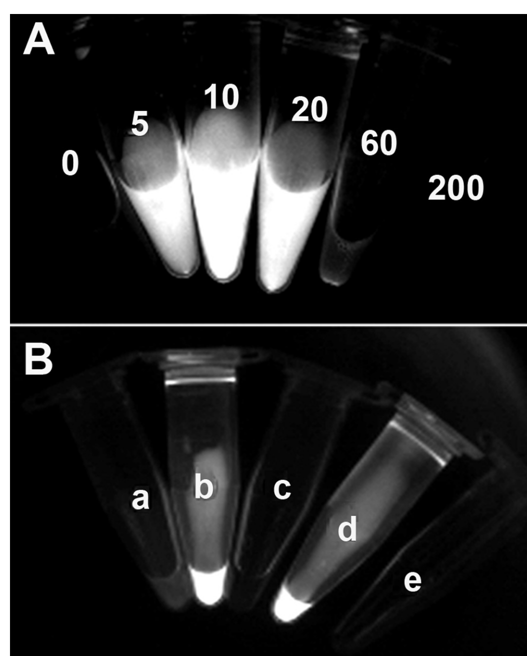


Figure 3. Cellular uptake of ICG/ALPs with and without modification of the polyarginine peptide R8 or R12. The NCI-H322 cells were incubated with ICG/APLs with and without R8 or R12 modification on their surfaces. The non-conjugated ALPs (–) show background level uptake into the cells, whereas the peptide modifications of the ICG/ALPs greatly enhance their cellular uptake. The nuclei of the cells were stained with DAPI. The blue and red colors represent the fluorescent signals for DAPI and ICG, respectively.

ALPs containing the ICG aggregates using 1% NP-40 greatly enhanced the signals due to increase of the ICG solubilization and increase of surrounding hydrophobicity (Fig. 1B).

The liposomes with positively charged surfaces are hard to totally block the nonspecific uptake in cells, even with PEGylated lipids [13]. However, our previous study demonstrated that the siRNA/ALPs displayed no cellular uptake without surface modification [11]. The calcein/ ALPs without surface modification also showed almost no uptake into any NSCLC cells without surface modification under 37°C incubation (Fig. 2). Although the weak signals were observed in the NCI-H322 cells treated with the unmodified ICG/ALPs, the untreated cells also displayed the similar signals. The calcein/ALPs and ICG/ALPs were judged to have slightly negative charges on their surfaces as similar to the siRNA/ALPs reported previously [11], and the property in addition to the surface PEGylation is considered to protect the cellular uptake of the ALPs. The polyarginine modification of ALPs has been reported to induce effective liposome delivery into cells by receptor mediated endocytosis through negatively charged heparin sulfate membrane proteoglycans [14,15]. In this study, the calcein/ALPs and ICG/ALPs were modified by either R8 or R12 peptide through the maleimide group of miPEG-PE on the ALPs. The polyarginine modifcation of the calcein/ALPs effectively was found to induce the liposome internalization in the NSCLC cells. Although some endocytotic vesicles were observed in the cells, the most of calcein signals was dominantly monitored in cytoplasm. The results demonstrate that if the ALPs encapsulate drugs and their surfaces are modified by the target specific ligands, the drugs may be readily released from the endocytotic vesicles into cytoplasm in the target cells. The polyarginine-modified ICG/ALPs were also effectively internalized into the NCI-H322 cells (Fig. 3). Taken together, the present method will be useful for developing target-specific delivery carriers of anionic drugs after being modified their surfaces with target-specific ligands.

In conclusion, the ALPs developed in this study are able to encapsulate approximately 90% anionic small chemicals without nonspecific uptake. The surface modifications of the ALPs with the cell penetrating peptides successfully induce the ALP internalization. These properties suggest that the ALPs encapsulating anionic drugs will be possible for target specific delivery of the drugs to the certain organ after modifying their surfaces with the target specific ligands. In addition, the asymmetric method for preparing liposomes may be also applicable for high efficient encapsulation of a variety of valuable hydrophilic drugs by optimizing the lipid compositions.

Acknowledgments

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