Facile Synthesis of Phosphatidyl Saccharides for Preparation of Anionic Nanoliposomes with Enhanced Stability

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Abstract

Physical stability during storage and against processing such as dehydration/rehydration are the cornerstone in designing delivery vehicles. In this work, mono-, di- and tri-saccharides were enzymatically conjugated to phosphatidyl group through a facile approach namely phospholipase D (PLD) mediated transphosphatidylation in a biphasic reaction system. The purified products were structurally identified and the connectivities of carbohydrate to phosphatidyl moiety precisely mapped by $^1$H, $^{31}$P, $^{13}$C NMR pulse sequences and LC-ESI-FTMS. The synthetic phosphatidyl saccharides were employed as the sole biomimetic component for preparation of nanoliposomes. It was found that the critical micelle concentration (CMC) of phosphatidyl saccharides increases as more bulky sugar moiety (mono- to tri-) is introduced. Phosphatidyl di-saccharide had the largest membrane curvature. In comparison to the zwitterionic phosphatidylcholine liposome, all phosphatidyl saccharides liposomes are anionic and demonstrated significantly enhanced stability during storage. According to the confocal laser scan microscopy (CLSM) and atom force microscopy (AFM) analyses, the nanoliposomes formed by the synthetic phosphatidyl saccharides also show excellent stability against dehydration/rehydration process in which most of the liposomal structures remained intact. The abundance hydroxyl groups in the saccharide moieties might provide sufficient H-bondings for stabilization. This work demonstrated the synthesized phosphatidyl saccharides are capable of functioning as enzymatically liable materials which can form stable nanoliposomes without addition of stabilizing excipients.

Introduction

Phospholipids-based liposomal vesicles have evolved as a promising tool for delivery of drug, genes and nutrients due to intensive investigations for decades [1-4]. Stability of liposomes has been a key issue which limit its application as most liposomes with phosphatidylcholine (PC) as primary components have low stability [4-6]. To circumvent this problem, different strategies have been attempted including development of polymeric liposomes [5-7], functionalization of liposome surface [8-10], synthesis of novel liposome constituting components [11,12], and addition of protectants [13-15]. Addition of protectants such as trehalose and proline has been shown to significantly enhance the stability of liposomes during storage and against dehydration/rehydration process [13-17]. These protectants were hypothesized to act as water replacement molecules which form hydrogen bonding with the phospholipids to maintain head group spacing and thus prevent fusion of liposomes [18,19].

Based on this concept, we hypothesize that conjugation of phosphatidyl and sugar moiety may integrate the function of phospholipids-based liposomes without addition of stabilizing excipients.
protectants as physical stability enhancer into phosphatidyl saccharides. In this work, we had developed a facile approach to conjugate mono-, di- and tri-saccharides to phosphatidyl group through phospholipase D mediated transphosphatidylolation in a biphasic reaction system and characterized phosphatidyl saccharide-based liposomes systemically with PC-liposomes as a comparison control. The synthetic phosphatidyl saccharides were found to be capable of forming nanoliposomes with enhanced physical stability against fusion and aggregation following storage, dehydration and rehydration procedures.

This work presented a good demonstration that there is a huge potential of using natural compounds as building blocks to develop low toxic, biodegradable and biocompatible excipients for a variety of applications.

Materials and Methods

Materials

Phospholipase D (Streptomyces sp.) was a gift from Nagase ChemteX Corporation, Japan. The activity of the PLD is 600,000 U/g (1 U is defined as the activity which liberates 1 µmol choline from L-α-phosphatidylcholine per minute). Phosphatidylcholine (PC), (Epikuron 200), was a gift from Cargill Inc. (Minneapolis, MN, USA). This PC originates from soybean and consists of at least 95% PC of which 3% is the lyso- form. All solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and of HPLC grade. Glucose, sucrose, raffinose and other chemicals were of analytical grade from Sigma-Aldrich Co. (St. Louis, MO, USA).

Enzymatic Synthesis of Phosphatidyl Saccharides

PLD-catalyzed transphosphatidylolation reaction between PC and saccharides was carried out using a biphasic reaction system in which 390 mg of PC was dissolved in 20 ml ethyl acetate and 60 U PLD was dissolved in 2 ml sodium acetate buffer (pH 5.6 adjusted by acetic acid). Phosphatidylcholine (PC), (Epikuron 200), was a gift from Cargill Inc. (Minneapolis, MN, USA). This PC originates from soybean and consists of at least 95% PC of which 3% is the lyso- form. All solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and of HPLC grade. Glucose, sucrose, raffinose and other chemicals were of analytical grade from Sigma-Aldrich Co. (St. Louis, MO, USA).

Quantitative Analysis of Synthesized Phosphatidyl Saccharides

The molar yield of phosphatidyl saccharides was determined by HPLC analysis. The HPLC was equipped with a silica gel column (5 µm, 4.6×250 mm, Thermo Fisher Scientific Inc.) and a Sedex (Alfortville, France) model 75 ELSD; the pressure of nebulizer gas (air) was maintained at 3.2 bar and the drift tube temperature was set at 40 °C. Three kinds of eluents namely chloroform (A), methanol (B), and 1% (V/V) triethylamine buffer (titrated at pH 3 with formic acid) (C) were used. The chromatographic separation was carried out using a linear gradient: 0-5 min A/B/C (87.5/12/0.5, v/v), 5-40 min A/B/C (87.5/12/0.5, v/v) to A/B/C (87/60/12, v/v), 40-45 min back to A/B/C (87.5/12/0.5, v/v), and 45-55 min re-equilibration. The flow rate of the eluent was 0.5 ml/min. The retention time of Ptd-Glu, Ptd-Suc and Ptd-Raff were 27.1 min, 28.2 min and 29.7 min, respectively. Phosphatidyl saccharides were quantified with standard curve of purified phosphatidyl saccharides, whose structure and purity have been confirmed by LC/MS and NMR analysis.

Purification of Phosphatidyl Saccharides from Reaction Mixture

The synthesized phosphatidyl saccharides were separated from extracted polar lipids according to a reported TLC method with slight modification [20]. Around 0.5 ml of the lipid extraction from reaction mixture was separated on thin layer chromatography plates (TLC silica gel 60, 10×20, Merck, Germany) using developing solvent of chloroform/methanol/ acetate acid/water (50:25:6:2). The Rf value for Ptd-Glu, Ptd- Suc and Ptd-Raff in TLC were 0.48, 0.26 and 0.09 respectively. The phosphatidyl saccharide band was scraped off and extracted by chloroform/methanol (1:1). The extract was dried after centrifugation (4000 rpm) for 20 min. The purity of extracted phosphatidyl saccharide was reconfirmed by TLC and HPLC with single plot and single peak, respectively. The structure of phosphatidyl saccharides was further identified by LC-ESI-FTMS and NMR.

Structure Conformation of Phosphatidyl Saccharides

The structures of the synthesized phosphatidyl saccharides (Ptd-Glucose, Ptd-Sucrose, and Ptd-Raffinose) were identified by NMR and LC/FTMS.

NMR investigations on Ptd-Glu was carried out at 14.1 T on a Bruker Avance III spectrometer in an inverse TXI (1H,31P,13C) 1.7 mm probe with a z-gradient at 300 K. In order to verify the phosphor-ether bond a 31P-1H HMBC pulse sequence was employed. The sequence was based on the standard Bruker parameter set HMBCGPNDE and optimized for 4 Hz couplings. The 64 indirect increments each with four scans were acquired and zero filled to 8192 times 128. Gradients in the pulse sequence were set in the ratio 20:30:10:16 in order to select for 31P-1H couplings. The 31P dimension was referenced externally to phosphatidylcholine in CDCl3:MeOD (2:1) at 0.8 ppm and the proton dimension was references to TMS at 0 ppm in CDCl3:MeOD (2:1). For assignment purposes HSQC, DQF-COSY, TOCSY and J-resolved spectra was also recorded all using standard parameters. For the Ptd-Suc and Ptd-Raff samples, cryogenic probes and RT probes where used. The following experiments, 13C-H HSQC, 13C-H HSQCTOSY and 1H-31P TOCSY was recorded in order to determine the phosphorys linkage all based on standard bruker pulse programs. The 1H-31P TOCSY utilized a 60 µs spinlock for 80 ms.

LC/FTMS includes chromatographic separation (Agilent 1100 System consisting of G1376A (Cap pump), G1377A (µWell-Plate Sampler), G1316A (Column Compartment) and
FTMS detection (LTQ Orbitrap MS, Thermo Scientific). The chromatographic separation was obtained by normal-phase conditions using a Kromasil Sil column, 150 mm x 0.5 mm id., 3.5 µm. The mobile phases were: Solvent A comprised of chloroform/MeOH/NH_4OH (25%) (800/200/5 vol.) and solvent B comprised of chloroform/MeOH/H_2O/NH_4OH (25%) (800/200/55/5 vol.). The flow rate was 16 µl/min. The gradient is 100% A (0-5 min), 100% A-100% B (5-10 min), 100% B (10-25 min), 100% B-100% A (25-32 min), and 100% A (32-40 min) for equilibration. The total cycle time was 40 min. The MS 335 nm was determined. I

### Preparation of Nanoliposome Suspension of Phospholipids by Thin Film Hydration Technique

Solid phospholipids (1 mg) were dissolved in 1 ml chloroform: methanol (1:1). For CLSM analysis, 0.1 ml of a 0.1 mM dichloromethane solution of DIL (1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) was added to the formulation. The solvent was evaporated by a stream of dry nitrogen until a dry lipid film was observed and followed by overnight vacuum drying to remove all solvent. Following overnight vacuum drying, the liposomes were suspended in Milli-Q water by vigorous vortexing for 30 mins and allowed to hydrate at room temperature for 3h.

### Characterization of Physical Properties

Physical property of phosphatidyl saccharides and PC nanoliposomes were evaluated by critical micelle concentration (CMC), dynamic light scattering (DLS), confocal laser scan microscope (CLSM), and atom force microscope (AFM).

#### Critical Micelle Concentration (CMC)

Measurement was done using the pyrene assay [21]. Fluorescence spectrometer (PerkinElmer LS55 luminescence spectrometer) was used in this study. Aggregate formation was examined over a wide range of phospholipid concentration (1x10^-7-1 mg/ml) by using pyrene in methanol as a fluorescent probe (final concentration 1x10^-6 M). Briefly, the ratio between the emission intensity at two wavelengths (I_373.5 and I_383 nm) upon excitation at 335 nm was determined. I_373.5/I_383 is around 1.0 in the absence of surfactant but increases to a plateau value of 1.42-1.53 in the presence of micelles, depending on the specific surfactant headgroups. The CMC was taken as the intersection of regression lines calculated from the linear portions of the graph.

#### Dynamic Light Scattering (DLS)

DLS was performed at 25 °C. The diameter and zeta potential of liposome were characterized by dynamic light scattering (DLS) using a Malvern Zetasizer Nano (Malvern Instruments Ltd., Worcestershire, UK). All measurements were performed at an angle of 173°. The size distributions and zeta potentials were analyzed by using the Malvern Dispersion Software (V5.10, www.zetasizer.com). Since size changes observation is easier with dispersion with low polydispersity index (PDI), the phospholipid suspensions from shelf life evaluation were passed through a 0.45 µm PTFE syringe filter (Whatman).

### Results and Discussion

#### Synthesis and Structural Mapping of Phosphatidyl Saccharides

One of the major challenges in synthesizing phosphatidyl saccharides is the selectivity because of presence of multiple hydroxyl groups in sugar ring. Taking advantage of high selectivity of biocatalysis, we employed PLD to catalyze the transphosphatidylolation of PC with saccharides (glucose, sucrose and raffinose) in a binary ethyl acetate/water system. This binary solvent system is able to dissolve both phosphatidylcholine and sugar, respectively [22]. 95 mol% of Ptd-Glu can be obtained at optimum condition. Substrates with bigger molecular size have higher steric hindrance and thus difficult to access the active site of enzyme. Therefore lower molar yields were obtained for Ptd-Suc (67 mol%) and Ptd-Raff (23 mol%). Figure 1 (a) shows the structures of the synthesized anionic phosphatidyl saccharides.

The chemical structures of the purified phosphatidyl saccharides were confirmed by LC/FTMS and NMR (Figure 2 for Ptd-Raff; Figures S1 and S2 in Supplementary Materials for Ptd-Glu and Ptd-Suc, respectively). LC/FTMS analysis showed all samples were of high purity and the molecular mass for all purified phosphatidyl saccharides were as predicted. Two abundant isomers can be found for all phosphatidyl saccharides which can be attributed to the differences in the fatty acid chains of the substrate PC ([18:2, 18:2], [16:0, 18:2]). For example, Ptd-Raff presented m/z 1181.62 [18:2, 18:2] and m/z 1157.62 [18:2, 16:0] (Figure 2 A). MS² spectra further
verified the structure of all the phosphatidyl saccharides as the typical fragments include loss of the glycan unit, fatty acids and C2H2O2 by glucan-ring cleavage. For example, fragments of 1019.57 or 1001.56 (fatty acids-glycan unit-C2H2O2) and 959.55 (fatty acids-glycan unit) were observed in Ptd-Raff (Figure 2 B-C). NMR Spectroscopy was used to assign the molecular moieties of the phosphatidyl saccharides. 31P-1H HMBC, 1H-31P TOCSY and 13C-1H HSQC NMR pulse sequences verified the connectivities of the carbohydrate functionality to the Ptd-backbone (Figure 2 D). For Ptd-Glu and Ptd-Raff, the phosphate ester of the Ptd-backbone binds to glucose-C6 and galactose-C6, respectively. A similar connectivity is dominant for Ptd-Suc, phosphorus was more selectively linked with glucose-C6 of sucrose, but less intense correlations (~20%), are also observed to fructose-C2 of sucrose (Figure S2D).

Physical Characteristics and Storage Stability of Phosphatidyl Saccharide-based Nanoliposomes

Commercial PC and the purified synthetic phosphatidyl saccharides were used to prepare nanoliposomes. The physical characteristics of nanoliposomes were evaluated (Table 1). Phosphatidyl saccharide-based nanoliposomes were smaller in size than the PC based ones. Saccharides have higher optimal headgroup area as compared to choline; therefore, phosphatidyl saccharides have smaller packing parameter which resulted in higher membrane curvature and smaller vesicle size [23]. In terms of surface charge, all the phosphatidyl saccharides-based nanoliposomes had negative surface charge (negative zeta potential) due to the negatively-charged phosphate group. Meanwhile, PC nanoliposomes had neutral charge due to the zwitterionic structure of PC. The anionic phosphatidyl saccharide nanoliposomes provided repulsive forces which prevent fusion and aggregation. A possible mechanism has been proposed as indicated in Figure 1(b).

Both phosphatidyl saccharides and PC nanoliposomes had similar critical micelle concentration (CMC). As CMC is strongly dependent on the alkyl chain length (hydrophobicity), the property change in headgroup despite insignificant still influences the CMC [24]. Increasing amount of sugar ring from glucose to raffinose (hydrophilic moiety) resulted in 1.1 to 1.3 times increments in CMC per glycan unit (Table 1).

Storage Stability of Phosphatidyl Saccharide-based Nanoliposomes

The nanoliposomes of phosphatidyl saccharides and PC loaded with hydrophobic fluorochrome marker 1,1-Dioctadecyl-3,3,3′,3′-tetramethyl indocarvicyanine perchlorate (DIL) were prepared and stored for 14 days at room temperature. For phosphatidyl saccharides nanoliposomes, no apparent precipitation and aggregation is observed after 14 days’ storage in comparison with the freshly prepared samples (Figure 3). Meanwhile, PC nanoliposomes aggregated and fused into bigger droplets which eventually precipitated (Figure 3). Materials with small particle size distribution of less than 10 nm can be observed in the aqueous suspension of PC which may represents the collapsed membrane pieces or micelle. However, only a little bit difference of the size distribution of Ptd-saccharide based nanoliposomes between the fresh sample and the sample after storing 14 days was observed (Figure 4). The stability of phosphatidyl saccharides nanoliposomes against aggregation and fusion during storage was further verified using Confocal Light Scattering Microscopy analysis (CLSM). The nanoliposomes loaded with hydrophobic fluorochrome marker DIL were fixed on agarose gel. After storage for 14 days, the DIL-labelled phosphatidyl saccharides nanoliposomes remained intact which can be shown by the unilamellar red-stained spherical morphology (Figure 4).
contrast, DIL-labelled PC nanoliposomes were characterized as red-stained elongated and irregular morphology which indicate aggregation and fusion of the nanoliposomes. One of the possible reasons for higher stability of phosphatidyl saccharide nanoliposomes against aggregation and fusion during storage is the OH groups of saccharide moiety. The OH-groups are capable of forming abundant hydrogen bond in the polar region of the membrane resulting in enhanced membrane mechanical stability. Surface charge also plays an important role. Liposomes formed by neutral sugar esters were found to aggregate during storage due to the low head group repulsive forces [25]. Thus, the anionic phosphatidyl saccharides

Figure 2. Structural elucidation of Ptd-Raff. A) Base-peak and extracted ion chromatograms and full FTMS spectrum of Ptd-Raff. The two major isomers are m/z 1181 [18:2, 18:2] and m/z 1157 [18:2, 16:0]. B) MS² spectra of the two major isomers. The primary fragments are explained in C). C) Chemical structure of Ptd-Raff. D-1) multiplicity edited $^{13}$C-$^1$H HSQC, D-2) multiplicity edited $^{13}$C-$^1$H HSQC-TOCSY and D-3) $^1$H-$^{31}$P TOCSY with $^{31}$P detection spectra of Ptd-Raff. Dotted lines shows correlation confirming small amount of phosphatidylcholine in the Ptd-raffinose sample (Ratio estimated by quant. $^{31}$P NMR to 80:20 Ptd-Raff/PC. The solid line in shows the major phosphor species in Ptd-raffinose conjugate on the galactose unit of raffinose.

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nanoliposomes provided repulsive forces between the headgroups to prevent aggregation and fusion of nanoliposomes (Figure 1b).

Stability of PhosphatidylSaccharide-based Nanoliposomes against Dehydration/Rehydration

Phosphatidyl saccharide nanoliposomes also demonstrated improved stability against dehydration and rehydration procedure. Following dehydration, most of the phosphatidyl saccharide nanoliposomes remained intact on the hydrophilic mica surface. Both Ptd-Glu and Ptd-Suc nanoliposomes had a height distribution within 18-80 nm and 30-130 nm, respectively. Ptd-Raff nanoliposomes had a height distribution of less than 20 nm. In comparison, nearly all of the PC nanoliposomes collapsed to form bilayer structure on the mica surface (height<10 nm) (Figure 5). Similar findings can be observed using the CLSM (Figure 6). The DIL-labelled phosphatidyl saccharide nanoliposomes remained intact with apparent red-stained spherical morphology following dehydration. Upon rehydration, the DIL-labelled phosphatidyl saccharide nanoliposomes can be recovered. Nevertheless, Ptd-Raff had reduced vesicle size upon rehydration procedure which is in agreement with the aforementioned AFM findings. Stark et al. [26] found stabilizing effects of protectants is strongly dependent on its water solubility. Protectants with higher water solubility have higher OH bonding capability. In present case, sucrose (2000g/L) has the highest water solubility followed by glucose (910g/L) and raffinose (143g/L). Thus, Ptd-Suc has the highest stabilizing effect followed by Ptd-Glu and Ptd-Raff. In comparison, the DIL-labelled PC nanoliposomes aggregated and fused (red-stained elongated and irregular morphology) upon dehydration. Rehydration procedure was not able to recover the liposomal structures of the DIL-labelled PC nanoliposomes.

Conclusions

In summary, a series of anionic phosphatidyl saccharides were successfully synthesized through enzymatic transphosphatidylation. Phosphatidyl saccharide nanoliposomes were smaller in size and had higher negative surface charge as compared to PC nanoliposomes. Presence of abundant OH groups in phosphatidyl saccharides renders them stabilizing effects. Anionic phosphatidyl saccharides nanoliposomes demonstrated enhanced physical stability against fusion and aggregation following storage, dehydration and rehydration procedures. The findings from this study can be used to overcome the physical instability of liposomes. And the synthetic phosphatidyl saccharides may provide an alternative anionic lipid for non-cytotoxic and serum stable delivery system [27]. In addition, sugar-coated nanoparticles as delivery system are becoming an area of interest since their reported cell internalization ability [28] and specific molecular recognition properties [29]. Thus, phosphatidyl saccharide nanoliposomes may have similar effects and can be used for
Figure 4. Characterization of nanoliposomes of PC, Ptd-Glu, Ptd-Suc and Ptd-Raff following prolonged storage by CLSM and DLS. (a-d) Morphology of freshly prepared nanoliposomes (size bar: 5 µm). (e-h) Morphology of nanoliposomes following storage for 14 days at room temperature (size bar: 5 µm). (i-l) Size dispersion of freshly prepared nanoliposomes and nanoliposomes stored for 14 days.

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targeted drug or functional ingredients delivery. More study on functional molecular encapsulation and cell internalization of the phosphatidyl saccharide liposomes will be further carried out.

Figure 5. AFM topographic images of the nanoliposomes of PC (a), Ptd-Glu (b), Ptd-Suc (c) and Ptd-Raff (d) on mica surface following dehydration procedure. The height distribution of these structures were depicted in (e).

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Figure 6. CLSM images of nanoliposomes following dehydration and rehydration procedure (size bar: 5 µm). (a-d) Morphology of freshly prepared nanoliposomes (e-h) Morphology of nanoliposomes following dehydration procedure (i-l) Morphology of nanoliposomes following rehydration procedure.

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Supporting Information

Figure S1. Structural elucidation of Ptd-Glu. (A) Base-peak and extracted ion chromatograms and full FTMS spectrum of Ptd-Glu. (B) MS² spectra of the two major isomers. The primary fragments are the loss of the glycan-unit, loss of C₇H₄O₂ from the glycan ring and loss of glycan and one fatty acid. (C) Structure of Ptd-Glu (D-1). ¹³C-H HSQC spectrum of Ptd-Glu, showing chemical shifts of the α- and β-conformer of the glucose unit (D-2). ¹³C-H HMBC spectrum of Ptd-Glu, showing correlations between phosphorous and the H₆ α, β protons.

Figure S2. Structural elucidation of Ptd-Suc. (A) Base-peak and extracted ion chromatograms and full FTMS spectrum of Ptd-Suc. (B) MS² spectra of the two major isomers. The primary fragments are the loss of the glycan-unit, loss of C₇H₄O₂ from the glycan ring and loss of glycans and one fatty acid (C) Structure of Ptd-Suc (D-1). multiplicity edited ¹³C-H HMSC, (D-2) multiplicity edited ¹³C-H HSQC-TOCSY and (D-3) ¹³C-H TOCSY with ¹⁳P detection spectra of Ptd-Suc. The solid line shows the major phosphor species in Ptd-Suc conjugate on 6-OH (C6) of glucose unit and only small amount of phosphor species conjugate on 2-OH (C2) of fructose unit. The phosphor peaks are in 72:20:8 ratio measured by quantitative ¹⁳P NMR. (TIF)

Author Contributions

Conceived and designed the experiments: SS LZC MD TT XX ZG. Performed the experiments: SS MF LL HMJ. Analyzed the data: SS LZC ZG. Contributed reagents/materials/analysis tools: MD HMJ. Wrote the manuscript: SS LZC LL HMJ KB MT XX ZG.

References

1. Hsu CYM, Ulluda H (2012) Cellular uptake pathways of lipid-modified cationic polymers in gene delivery to primary cells. Biomater 33: 7834-7848. doi:10.1016/j.biomaterials.2012.06.093.
2. Gratton SEA, Ropp PA, Pohhaisu PD, Luft JC, Madden VJ et al. (2008) The effect of particle design on cellular internalization pathways. Proc Natl Acad Sci U S A 105(33): 11513-11518. doi:10.1073/pnas.0801763105. PubMed: 18697944.
3. Torchilin VP (2008) Recent advances with liposomes as pharmaceutical carriers. Nat Rev Drug Discov 4: 145-160.
4. Sontaella C, Vierling P, Riess JG (1991) Highly Stable Lipids Derived from Perfluoralkylated Glycerophosphocholines. Angew Chem Int Ed Engl 30: 567-568.
5. Liu S, O’Brien DF (2002) Stable Polymeric Nanoballoons: Lyophilization and rehydration of Cross-linked Liposomes. J Am Chem Soc 124: 6037-6042. doi:10.1021/ja023507.
6. Schäfer J, Höbel S, Bakowsky U, Aigner A (2010) Liposome-polyethyleneimine complexes for enhanced DNA and siRNA delivery. Biomater 31: 6995-6990. doi:10.1016/j.biomaterials.2010.05.043.
7. Ma M, Chatterjee S, Zhang M, Bong D (2011) Stabilization of vesicular and supported membranes by glycolipid oxime polymers. Chem Commun 27: 2853-2855. doi:10.1039/c0cc05137c.
8. Ahmeda M, Jawanda M, Ishihara K, Narain R (2012) Impact of the nature, size and chain topologies of carbohydrates on binding and targetable cellular uptake of lipid-coated polysaccharide microcapsules. J Mater Chem 20: 2121–2127. doi:10.1039/b920469p.
9. Ingvarrison PT, Yang MS, Nielsen HM, Rantanen J, Foged C (2011) Stabilization of liposomes during drying. Exp Opin Drug Deliv 8(3): 375-388.
10. Qi W, Wang A, Yang Y, Du M, Bouchu MN et al. (2010) The lectin binding and targetable cellular uptake of lipid-coated polysaccharide microcapsules. J Mater Chem 20: 2121-2127. doi:10.1039/b920469p.
11. Perttu EK, Kohli AG, Szoka FC Jr (2012) Inverse-Phosphocholine Lipids: A Remix of a Common Phospholipid. J Am Chem Soc 134: 4485-4488. doi:10.1021/ja210989. PubMed: 23264493.
12. Huang Z, Szoka FC Jr (2008) Sterol-Modified Phospholipids: Cholesterol and Phospholipid Chimeras with Improved Biomembrane Properties. J Am Chem Soc 130: 15702–15712. doi:10.1021/ja8065557.
13. Van Hal DA, Bouwstra JA, vanRensen A, Jeremiasse E, deVringer T et al. (1996) Preparation and characterization of nonionic surfactant vesicles. J Colloid Interface Sci 178(1): 283-297. doi:10.1006/jcis.1996.0114.
14. Mady MM, Ghannam MM (2011) Stability of anionic liposomes in serum. Eur J Pharm Sci 44(5): 648-650. doi:10.2121/105575a013.
15. Latkague L, Ziane S, Chassande O, Patwa A, Daila MJ et al. (2011) Glycosylated nucleoside lipid promotes the liposome internalization in stem cells. Chem Commun 47(41): 12598-12600. doi:10.1039/c1cc13948g. PubMed: 21966673.
16. Chu CJ, Han DD, Cal CF, Tang X (2010) An overview of liposome lyophilization and its future potential. J Control Release 142(3): 299-311. doi:10.1016/j.jconrel.2009.10.024. PubMed: 19874861.
17. Koster KL, Webb MS, Bryant G, Lynch DV (1994) Interactions between Soluble Sugars and Pop (1-Palmitoyl-2-Oleoylphosphatidylcholine) during Dehydration - Vitrification of Sugars Alters the Phase-Behavior of the Phospholipid. BBA Biomembr 1193(1): 143-150. doi:10.1016/0005-2736(94)90343-3.
18. Maitani Y, Aso Y, Yamada A, Yoshikos (2008) Effect of sugars on storage stability of lyophilized liposome/DNA complexes with high transfection efficiency. Int J Pharm 356(1-2): 69-75. doi:10.1016/j.ijpharm.2007.12.033.
19. Austborn M, Schreier H, Brezesinski G, Fabian H, Meyer HW et al. (1994) The Protective Effect of Free and Membrane-Bound Cryoprotectants during Freezing and Freeze-Drying of Liposomes. J Control Release 30(2): 105-116. doi:10.1016/0168-3659(94)90257-7.
20. Miniasidis-Meimaroglou S, Kora L, Sinanoglu VJ (2008) Isolation and identification of phospholipid molecular species in a wild marine shrimp Peneaus kerathurus muscle and cephalothorax. Phys Chem Lipids 152(4): 104-112. doi:10.1016/j.chemphyslip.2008.01.003.
21. Ananthapadmanabhan KP, Goddard ED, Turno NJ, Kuo PL (1985) Fluorescence Probes for Critical Micelle Concentration. Langmuir 1(3): 352-355. doi:10.1021/la00063a015. PubMed: 21370917.
22. Song S, Cheong L-Z, Guo Z, Kristensen K, Glissius M et al. (2012) Cholesterol Depletion Drives the Phase Behavior of Liposomal Lipid–Glucose in Biphasic reaction system. Food Chem 135: 373-379. doi:10.1016/j.foodchem.2012.05.020. PubMed: 22868102.
23. Fisher K (2000) Formation of Self Assembling Structures: Bilayers, Micelles, Vesicles, Austin, US: Surface Phenomena Project Reports: the University of Texas at Austin.
24. Shinoda K, Yamanaka T, Kinoshita K (1959) Surface Chemical Properties in Aqueous Solutions of Nonionic Surfactants - Octyl Glycol Ether, Alpha-Octyl Glycerol Ether and Octyl Glucoside. J Phys Chem 63(5): 646-650. doi:10.1021/j150575a003.
25. Latkague L, Ziane S, Chassande O, Patwa A, Daila MJ et al. (2011) Glycosylated nucleoside lipid promotes the liposome internalization in stem cells. Chem Commun 47(41): 12598-12600. doi:10.1039/c1cc13948g. PubMed: 21966673.
29. Murthy BN, Zeille S, Nambiar M, Nussio MR, Gibson CT et al. (2012) Self assembly of bivalent glycolipids on single walled carbon nanotubes and their specific molecular recognition properties. Rsc Adv 2(4): 1329-1333. doi:10.1039/c2ra01192a.