Chemically-selective surface glyco-functionalization of liposomes through Staudinger ligation†

Hailong Zhang, Yong Ma and Xue-Long Sun*

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An efficient and chemoselective liposome surface glyco-functionalization method has been developed based on Staudinger ligation, in which the carbohydrate derivative carrying an azide spacer is conjugated onto the surface of preformed liposomes bearing a terminal triphosphine in PBS buffer (pH 7.4) at room temperature.

Liposomes, spherical closed self-assembled (phospho)lipids, have been extensively studied as models of cell membranes and carriers for drug/gene delivery applications.[1,2] Liposome surface functionalization facilitates an enormous potential application of liposomes.[3,4] Cell surface carbohydrates are attractive models for liposome surface modification with oligosaccharides due to their protein-rejecting ability, biodegradability, low toxicity and especially their cell targeting ability through specific binding interactions with receptors expressed at the targeted cell's surface. For example, monosialoganglioside (GM1) can enhance the circulation lifetimes of liposomes compared to that observed for PEG.[5] Sialyl lewis X decorated liposome has been demonstrated to target the delivery of drugs to endothelial cells based on the site-specific expression of E- and P-selectin in blood vessels during inflammation.[6] On the other hand, carbohydrate-decorated liposomes have been used as a multivalent platform of carbohydrates to inhibit carbohydrate-mediated cell adhesion. For example, sialic acid-decorated liposome shows strong inhibitory activity against influenza hemagglutinin and neuraminidase.[7]

Conventional methods to prepare surface-functionalized liposomes involve the initial synthesis of the key lipid–ligand conjugate, followed by the formulation of the liposome with all the lipid components. In this direct liposome formation method, some of the valuable ligands inevitably face the enclosed aqueous compartment and thus become unavailable for their intended interaction with target molecules. In particular, it is unrealistic if the targeting ligand is only available in minimum quantities. Furthermore, lipid–ligand conjugates normally have a limited solubility and stability in solvents, or are incompatible with the various stages of their manufacture. Alternatively, chemical modification methods, which in most cases involve the coupling of biomolecules to the surface of preformed vesicles carrying functionalized (phospho)lipid anchors, have been developed.[8,9] So far, variable success using amide[10] and thiol–maleimide coupling,[11] as well as imine[12] or hydrazone linking,[13] has been achieved. However, in many cases, there is a lack of specificity, resulting in the uncontrolled formation of covalent bonds between liposomes and the biomolecules of interest. Most recently, Cu(I)-catalyzed [3 + 2] cycloaddition, namely "click" chemistry, which can occur efficiently in aqueous media at room temperature and selectively between azides and alkynes,[14] has been investigated as a novel generic chemical tool for the facile *in situ* surface modification of liposomes.[15] However, the key limitation of click chemistry is the use of a Cu(I) catalyst, which results in residual copper in the targeted liposome preparations, which could be a potential concern. Normally, it is difficult to completely remove copper from the resultant liposome[15] and thus it will affect the liposome's subsequent biological applications.

Staudinger ligation, in which an azide and a triphosphine selectively react to form an amide, has been used for the chemically-selective modification of recombinant proteins under native conditions.[16] Recently, Staudinger ligation has been successfully performed in living animals without physiological harm, suggesting the potential for applications in cell surface functionalization.[17] In particular, the reaction is known to occur in high yield at room temperature in aqueous conditions and without any catalyst, and is compatible with the unprotected functional groups of a wide range of biomolecules. Herein, we report efficient and chemically-selective liposome surface functionalization through Staudinger ligation, with lactose as a model carbohydrate (Fig. 1). The high specificity and high yield, as well as the biocompatible reaction conditions, of the Staudinger ligation approach makes it an attractive alternative to all of the current protocols for liposome surface functionalization.

As a proof-of-concept, we have explored the possibility of preparing glycosylated liposomes by coupling unprotected

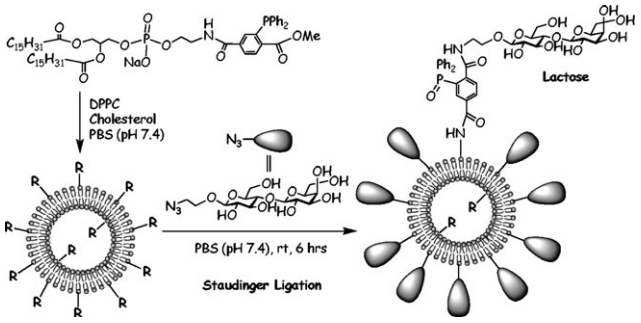
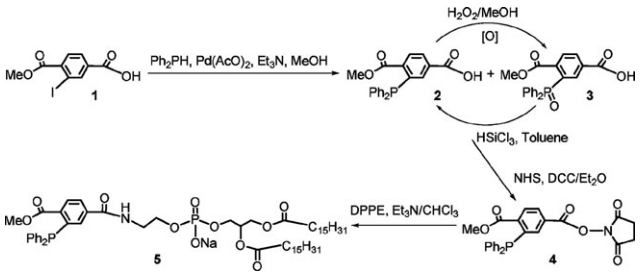


Fig. 1 A schematic illustration of liposome surface glyco-functionalization through Staudinger ligation.

Department of Chemistry, Cleveland State University, 2121 Euclid Ave, Cleveland, OH 44115, USA. E-mail: x.sun55@csuohio.edu; Fax: +1 216-687-9289; Tel: +1 216-687-3919

† Electronic supplementary information (ESI) available: Full experimental details, and ^{1}H and ^{31}P NMR spectra.



Scheme 1 Synthesis of anchor lipid **5**.

lactosyl derivatives carrying an ethyl spacer functionalized with an azide group to the surface of liposomes that incorporate synthetic anchor lipids bearing a terminal triphosphine *via* Staudinger ligation. Firstly, the terminal triphosphine carrying the anchor lipid, **5**, was synthesized by the amidation of commercially available DPPE with 3-diphenylphosphino-4-methoxycarbonylbenzoic acid NHS active ester **4**, which was prepared according to the procedure described by Ju *et al.*[18] (Scheme 1). However, it was found that an unwanted oxidation product, triphosphine oxide **3**, easily formed during the crystallization purification of triphosphine **2** when using aqueous methanol. Oxidized compound **3** was confirmed by comparing it to the oxidation product of triphosphine **2** with hydrogen peroxide in methanol, which gave a typical chemical shift at 34.2 ppm for a phosphine oxide, while phosphine **2** gave a chemical shift at −3.74 ppm in its ^{31}P NMR spectrum (Fig. 2A). Fortunately, phosphine oxide **3** could be converted back to phosphine **2** by reduction with trichlorosilane in toluene in high yield.[19] No oxidized product was formed during DPPE-triphosphine **5** synthesis and purification when organic solvents were used. With this oxidation in mind, the stability of intermediate triphosphine **2** and targeted lipid triphosphine conjugate **5** were monitored by ^{31}P NMR. As observed, the oxidation of phosphines **2** and **5** in organic solvents such as chloroform were very slow (Fig. 2B and 2C).

With anchor lipid **5** in hand, next, small unilamellar liposomes, composed of saturated phospholipids (DPPC) and cholesterol (2 : 1 molar ratio), and 5 mol% of anchor lipid **5**, were prepared by sequential extrusion through polycarbonate membranes with pore sizes of 600, 200 and 100 nm at 65 °C. This produced predominately small unilamellar vesicles with an average mean diameter of 120 ± 5 nm, as determined by dynamic light scattering (DLS). Next, the conjugation of the azide-containing lactose ligand[20] to the preformed liposomes was performed in PBS buffer (pH 7.4) at room temperature under an argon atmosphere for 6 h (Fig. 1). DLS was used to verify the

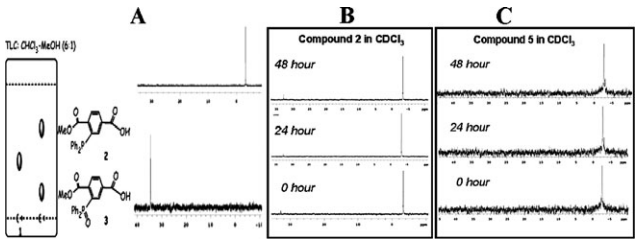


Fig. 2 TLC traces and ^{31}P NMR study of triphosphine derivatives.

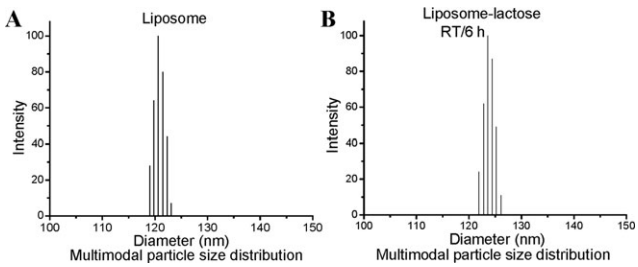


Fig. 3 Dynamic light scattering monitoring of the liposomes A: before conjugation and B: after conjugation.

integrity of the vesicles during and after the coupling reaction. As shown in Fig. 3, there was no significant change in the size of the vesicles during and after the conjugation reaction. Therefore, the reaction conditions described above do not alter the integrity of the liposomes.

Next, to test whether the experimental conditions used for the conjugation reaction could provoke some leakage of the liposomes, we exposed the same type of liposomes containing encapsulated self-quenching concentrations of 5,6-carboxyfluorescein (CF) to our standard conditions.[21] Based on our fluorescence quenching determinations (Fig. 4), we demonstrated that no apparent leakage was triggered by the conjugation reaction compared to liposomes incubated in the absence of the coupling reagents. Therefore, the conjugation conditions established here are harmless to liposome surface functionalization.

Furthermore, the grafted carbohydrate on the surface of the liposomes was quantified by the phenol–sulfuric acid method.[22] Briefly, a phenol solution was added to a solution of the liposomes with conjugated lactose and mixed. Next, concentrated H_2SO_4 was added directly to the solution. The mixture was then vortexed and allowed to stand for 30 min at room temperature. The optical density was then recorded at 490 nm. Considering that about 40% of the triphosphine anchor is oriented towards the interior of the vesicles, 80% of the out-oriented triphosphines were modified in this Staudinger ligation modification.

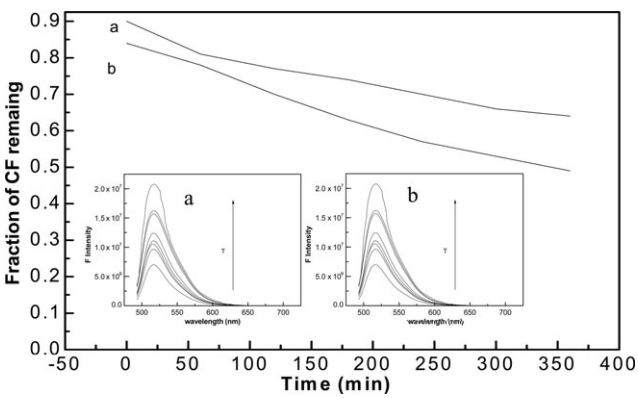


Fig. 4 The kinetics of 5,6-carboxyfluorescein release from liposomes a: in the presence of lactose-azide and b: in the absence of lactose-azide (control experiment). The inserts show the changes in the fluorescence intensity of the liposome reaction suspension and control during the conjugation and final decomposition with surfactant.

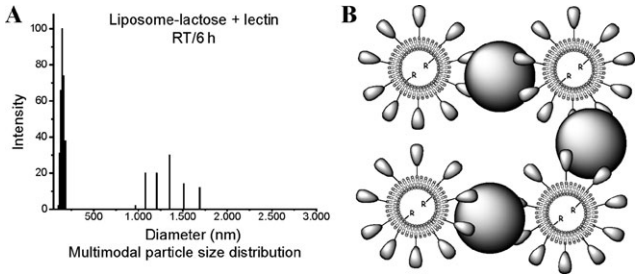


Fig. 5 A: DLS monitoring of the agglutination of glycosylated liposomes with lectin *via* B: multivalent interactions.

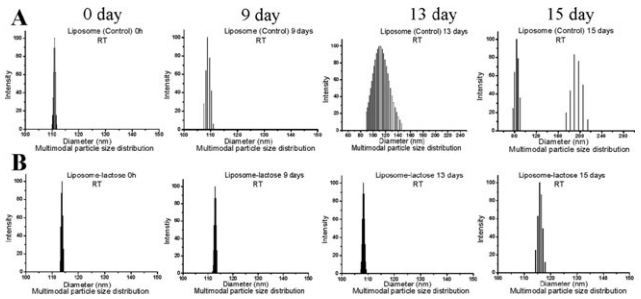


Fig. 6 A: The stability of liposomes without lactose and B: liposomes with lactose, as monitored by DLS.

To determine whether the grafted lactose residues were easily accessible at the surface of the liposomes, a lectin binding assay was conducted by incubating lactosylated liposomes in the presence of β-galactose binding lectin (*Arachis hypogae*, 120 kDa, Sigma) in PBS (pH 7.4). After 30 min, visible aggregation was apparent, and was monitored by a DLS experiment (Fig. 5A). In contrast, neither aggregation nor size change was observed with the control liposomes without lactose. Furthermore, the presence of free lactose (5.0 mM) prevented aggregate formation (not shown), confirming that the aggregation was due to a specific recognition of the lactose residues on the surface of the liposomes by lectin *via* multivalent interactions (Fig. 5B).

The stability of liposomes over time is an important concern in drug/gene delivery applications. It is known that GM1 could enhance the circulation lifetimes of liposomes compared to that observed for PEG.[5] In this study, the stability of the lactosylated liposomes was evaluated by comparing them with liposomes without lactose at room temperature, as monitored by DLS. As shown in Fig. 6, both types of liposomes showed good stability during an 8 d period. However, the liposomes without lactose began to collapse and aggregate from day 9 (Fig. 6A), while there was no apparent size change for the lactosylated liposomes (Fig. 6B). This result demonstrates that the presence of lactose on the liposome surface provides a steric barrier that prevents liposome aggregation.

In conclusion, we have developed an efficient and chemoselective conjugation method for liposome surface glycofunctionalization based on Staudinger ligation. The reaction could be performed under mild conditions in aqueous buffers without catalyst, in high yields and with reasonable reaction times. The reaction conditions developed in this work did not alter the integrity of the bilayers in terms of liposome size and leakiness, and provided perfectly functional vesicles. This versatile approach, which is particularly suitable for the ligation of water soluble molecules and which can accommodate many chemical functions, is anticipated to be useful for the coupling of many other ligands to liposomes.

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