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Short communication

Carbohydrate-functionalized surfactant vesicles for controlling the density of glycan arrays

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ABSTRACT

We report on the development of a method for rapidly characterizing the glycan binding properties of lectins. Catanionic surfactant vesicles, prepared from cationic and anionic surfactants, spontaneously formed in water and remained stable at room temperature for months. By varying the amount of glycoconjugate added during preparation, glycans were incorporated onto the outer surface of the vesicles in a controlled range of densities. The carbohydrate-functionalized vesicles were applied to commercially available, nitrocellulose-coated slides to generate glycan arrays. As proof of concept, the binding of two lectins, concanavalin A and peanut agglutinin, to the arrays was quantified using a biotin-avidin fluorescence sandwich assay. This facile method of preparing a glycan array by using vesicles to control the glycan density can be expanded to provide a platform for characterizing unknown lectins.

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1. Introduction

Carbohydrate–protein interactions have long been known to play essential roles in biological processes including intercellular signaling, molecular recognition, immune response, and the progression of diseases [1–4]. The development of analytical methods to evaluate carbohydrate interactions has been difficult in part because of the enormous structural complexity and diversity of carbohydrates [5,6]. In addition, carbohydrate-binding proteins generally form weak monovalent carbohydrate interactions with low selectivity. Therefore, carbohydrate-binding proteins typically possess multiple binding sites which allow them to bind two or more carbohydrate ligands simultaneously. The multivalent complexes that form are highly selective with an overall strong interaction between the protein and the bound carbohydrates, or glycans [7–9]. To successfully investigate the glycan interactions that form these complexes, tools must be capable of monitoring binding events that vary with the spacing, or density, of carbohydrates in a particular region. One strategy is to use lectins, carbohydrate-binding proteins with highly specific binding functionalities, for carbohydrate analysis.

Glycan arrays provide a high-throughput platform for the investigation of carbohydrate–lectin binding and require minimal amounts of materials. A common method for fabricating glycan arrays involves spotting the glycans onto a surface. Different spacing distances between the carbohydrates are formed by printing a range of carbohydrate concentrations. Variations of this method have been reported and improved upon by several groups [10–13], including the Consortium for Functional Glycomics which developed a mammalian glycan array of 611 glycans for screening purposes [14]. Difficulties can arise in achieving optimal spacing of the carbohydrates, which is thought to be unique to each lectin and required for maximal binding to occur, due to heterogeneity between printed spots as well as within a single spot. To improve overall spot homogeneity, a technique for preparing glycan arrays utilizing microcontact printing has recently been reported [15,16]. This involves covalent immobilization of the carbohydrates onto a surface via a Diels-Alder chemical reaction. Gildersleeve and coworkers have developed an alternative process for fabricating a glycan array whereby neoglycoproteins are attached to a surface [17,18]. Using that method, biologically relevant variations in carbohydrate spacing were successfully reported; however, the ease of synthesizing the neoglycoproteins is unclear. The development of an easily fabricated array capable of measuring lectin binding dependence on carbohydrate density would greatly expand the number and type of carbohydrate–lectin systems characterized.

Our approach to measuring carbohydrate–lectin interactions was to use easily-prepared, surfactant vesicles to fabricate a glycan array. The spontaneous formation of stable, catanionic vesicles from mixing together cationic and anionic surfactants in water was published by Zasadzinski and coworkers in the late 1980s [19].

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The theory behind vesicle formation and the source of their stability has been well documented [20–22]. More recently, it was demonstrated that the vesicles can be produced with carbohydrate moieties on their surface [23–25]. The current study reports on the characterization of carbohydrate-functionalized vesicles and their use in the development of a glycan array that displays sugars at various densities for the investigation of lectin binding.

2. Experimental

2.1. Reagents

All chemicals were used as received and purchased from Sigma[1] (St. Louis, MO) unless otherwise stated. Cetyltrimethylammonium tosylate (CTAT) was purified by ethanol-acetone recrystallization. HEPES-buffered saline (10 mmol/L HEPES, 150 mmol/L NaCl, 1.0 mmol/L $CaCl_2$, 1.0 mmol/L $MnCl_2$) and all aqueous solutions used ultrapure (18 MΩ cm) water (Thermo Scientific Barnstead Nanopure, Dubuque, IA). Biotinylated lectins concanavalin A (Con A) and peanut agglutinin (PNA) were purchased as lyophilized powders and prepared at 100 μg/mL (Vector Laboratories, Burlingame, CA). Fluorescently labeled NeutrAvidin® (100 μg/mL, Oregon Green 488, Invitrogen, Carlsbad, CA) solutions were prepared in saline buffer.

2.2. Vesicle preparation and characterization

Vesicles were prepared by stirring two ionic surfactants, sodium dodecylbenzene sulfonate (SDBS) and CTAT, together with 0.000, 0.005, 0.050, 0.100, 0.200, or 0.300 mole fraction of either *n*-dodecyl-β-D-glucopyranoside (C_{12}OGlu) or *n*-dodecyl-β-D-maltoside (C_{12}OMalt) in water for 1 h. Samples that did not contain glycoconjugates were prepared in a 3:1 mole ratio of SDBS to CTAT (70:30, w/w) at a 1 wt% total surfactant concentration. For vesicle samples containing C_{12}OMalt or C_{12}OGlu, the total surfactant concentration was reduced accordingly (dependent on particular mole fraction of glycoconjugate added), while the 3:1 mole ratio of the two surfactants was held constant. Following an equilibration period of at least 48 h, vesicles were passed through a syringe filter (0.45 μm) to remove any impurities and stored at room temperature.

Dynamic light scattering (DLS) and zeta potential information for the vesicles in solution was acquired with a Malvern Zetasizer (Nano ZS, Westborough, MA). A 173° detection scattering angle was used to obtain autocorrelation data, which was analyzed by the cumulant method to obtain the diffusion coefficient. The hydrodynamic size of the vesicles was calculated using the Stokes–Einstein equation, and the polydispersity index (PDI), or normalized variance, of the particle size was determined assuming a Gaussian distribution. Five measurement cycles, each consisting of ten 10 s scans (total measurement time 500 s), were averaged to obtain the mean diameter (Z-ave) reported for each sample. The zeta potential of the vesicles was calculated from electrophoretic mobility measurements made using M3-PALS technology (Malvern, Westborough, MA).

2.3. Colorimetric detection of glycans

Size exclusion chromatography (SEC) was used to separate glycoconjugate-functionalized vesicles from any free glycans present in solution [26]. Vesicle solutions were eluted in 1.0 mL aliquots through a disposable PD-10 desalting column (GE Healthcare Life Sciences, Piscataway, NJ) hand-packed with Sephadex G-100. The fractions that contained vesicles were identified with DLS. A well-established colorimetric assay was used to determine the relative glycan content in each fraction [27]. Briefly, 0.53 mol/L phenol (125 μL) and concentrated H_2SO_4 (625 μL) were added to 250 μL of each fraction. Fractions that contained sugar appeared yellow–orange in color. After vortexing, the samples were allowed to cool for at least 1 h at room temperature. Ethanol (250 μL) and water (300 μL) were added to each sample which was then vortexed and allowed to cool for 10 min before the absorbance was measured at 490 to 495 nm (Lambda Bio 20 UV–vis spectrometer, PerkinElmer, Waltham, MA). To obtain reproducible results for the amount of glycans originally added during vesicle preparation versus the amount of glycans detected, the two vesicle-containing fractions (3 and 4) from a particular SEC run were combined and treated as one sample for the colorimetric assay. Combined vesicle samples (fractions 3 and 4) were used for array fabrication (detailed in Section 2.4) to ensure only vesicles with incorporated glycoconjugates were applied to the arrays.

2.4. Glycan array fabrication and fluorescence measurements

Silicon gaskets (16-well, Grace Bio-Labs, Bend, OR) were assembled onto 16-pad nitrocellulose-coated glass slides (ONCYTE® Nova™, Grace Bio-Labs). The bottom of each well measured 6.5 mm × 6.5 mm. The Grace Bio-Labs ProPlate™ Multi-Array system with Delrin snap clips was used to convert the slides into a well-plate platform that is recognized by instruments designed to read standard-sized microtiter plates. After the vesicle solutions (100 μL/well) were applied, the wells were covered with seal strips and allowed to incubate for 1 h at room temperature. Slides were rinsed 3 times with HEPES-buffered saline, and biotinylated lectin solutions were applied for 1 h (100 μL/well). Unbound lectins were washed from the wells with saline prior to incubation with 100 μL/well of NeutrAvidin® for 1 h. Wells were thoroughly rinsed, incubated with saline (50 μL/well), and sealed until fluorescence measurements were made later that day. Just prior to the measurements, an additional 100 μL of saline was added to each well.

The fluorescence emission spectrum of each well was recorded (510 to 600 nm) using a Varian Cary Eclipse Fluorescence Spectrophotometer fitted with a microplate reader accessory (now Agilent Technologies, Santa Clara, CA). The mean intensity from a nitrocellulose slide containing saline (150 μL/well) was subtracted from fluorescence measurements to account for the average background fluorescence. Origin Software (OriginLab Corporation, Northampton, MA) or Prism 5.0 (GraphPad Software, La Jolla, CA) was used for all graphs and statistical analyses. Inkscape, an open-source vector graphics editor, was used for the illustrations in Fig. 4.

3. Results and discussion

3.1. Factors affecting the incorporation rate of glycoconjugates with surfactant vesicles

When the two surfactants, SDBS and CTAT, are mixed in certain ratios, catanionic vesicles with bilayer membranes spontaneously form in water. These vesicles are unilamellar, relatively monodisperse, and stable at room temperature for an extremely long period of time [19,21]. An initial report on encapsulating glucose inside anionic SDBS/CTAT (3:1 mole ratio) vesicles demonstrated that both the incorporation rate and retention time for the sugar molecule are quite low [23]. During preliminary experiments, we explored the incorporation of glycoconjugates with varying

[1] Certain commercial equipment, instruments, or materials are identified in this document. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor intended to imply that the products identified are necessarily the best available for the purpose.

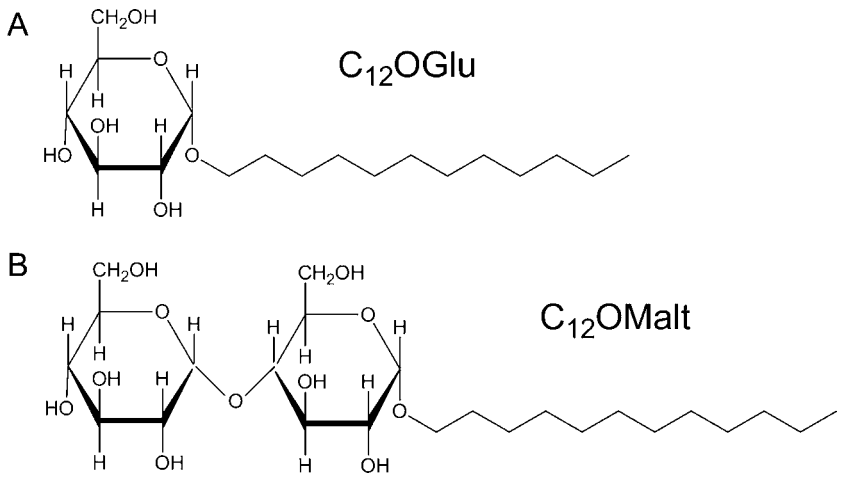

Fig. 1. Chemical structures of glycoconjugates used to functionalize the surface of the surfactant vesicles (A) *n*-dodecyl-*β*-D-glucopyranoside (C_{12}OGlu) and (B) *n*-dodecyl-*β*-D-maltoside (C_{12}OMalt).

hydrocarbon lengths into the vesicle bilayer (data not shown). We found that vesicles mixed with glycoconjugates composed of hydrocarbon chains with 12 carbon atoms had significantly higher incorporation rates than those vesicles mixed with glycoconjugates composed of hydrocarbon chains with 8 carbon atoms (≈100% versus ≈10%). This finding is in agreement with previous work done in the DeShong lab where they reported the incorporation rate of C_8-glucose into surfactant vesicles to be 18% [24]. Therefore, vesicles were modified with either C_{12}OGlu (Fig. 1A) or C_{12}OMalt (Fig. 1B), both of which are glycoconjugates with hydrocarbon chains composed of 12 carbon atoms. Our data further supports the theory that the surfactant vesicle bilayer membrane can be modified with glycans by hydrophobic insertion of the hydrocarbon chain portion of a glycoconjugate.

3.2. Reproducibility of glycoconjugate-modified vesicle solutions

Prior to using the carbohydrate-functionalized surfactant vesicles to fabricate a glycan array, characterization experiments were done to determine the reproducibility of vesicle size and glycoconjugate incorporation for each solution. Vesicles were prepared with 0.000 (plain vesicles), 0.005, 0.050, 0.100, 0.200, or 0.300 mole fraction of either the C_{12}OGlu or C_{12}OMalt glycoconjugate as detailed in Section 2.2. Previously, it was reported that precipitation of C_{12}OGlu was observed during preparation of surfactant vesicles with glycan mole fractions greater than 0.300, so we focused our study on the characterization of vesicles with 0.300 mole fraction or less of glycoconjugate [25].

The hydrodynamic size of the vesicles was determined using DLS and is reported in Table 1. The mean size of the vesicles in all the glycoconjugate solutions was between 117 nm and 141 nm in diameter which indicates that vesicles formed with ≤0.300 mole fraction glycoconjugates are similar in diameter to plain vesicles (≈130 nm). The PDI, or normalized variance, of the C_{12}OGlu and C_{12}OMalt vesicle solutions suggests vesicles of monodisperse size population are formed (average PDI = 0.258) [28]. Zeta potential measurements for the C_{12}OGlu vesicles ranged from −85 mV to −100 mV, while the range for the C_{12}OMalt vesicles was slightly larger (−75 mV to −105 mV). In general, the zeta potential decreased in magnitude as the glycoconjugate mole fraction amount increased for vesicles made with either glycoconjugate. This trend is expected since the amount of anionic SDBS used to prepare the vesicles decreased as the glycoconjugate mole fraction increased. Similar hydrodynamic size results have been previously reported for C_{12}OGlu-modified surfactant vesicles [25]; however, to our knowledge this is the first report of C_{12}OMalt incorporation with surfactant vesicles. This demonstrates that monodisperse vesicles of similar size form in the presence of various glycoconjugates, and suggests the vesicles could be used to form a complex array displaying more than one type of glycan for characterizing the binding of unknown lectins.

SEC was used to separate the carbohydrate-modified vesicles from any unincorporated glycoconjugate in solution. The resulting 14 fractions were analyzed with the colorimetric assay described in Section 2.3 to determine which fractions contained glycans and with DLS to determine which fractions contained vesicles. By plotting the relative absorbance and scattering intensity for each fraction, it was determined that essentially 100% of the glycoconjugates incorporated into the vesicles for all mole fraction amounts of C_{12}OGlu and up to 0.200 mole fraction amounts of C_{12}OMalt. In addition, all the vesicles eluted out of the column in fractions 3 and 4. Representative data for 0.200 mole fraction C_{12}OGlu and 0.100 mole fraction C_{12}OMalt are shown in Fig. 2A and B. The relative amount of sugar in the vesicle-containing fractions was determined by measuring the absorbance of a sample (fractions 3 and 4 combined) and was found to increase linearly with the mole fraction amount of glycoconjugate originally added during vesicle preparation (Fig. 3A and B). The maximum incorporation of sugar

Table 1
Hydrodynamic diameter and polydispersity index (PDI), or normalized variance, of glycoconjugate-functionalized surfactant vesicles (mean ± SD, $n = 3$).

Mole fraction of glycoconjugate	C_{12}OGlu		C_{12}OMalt	
	Diameter (nm)	PDI	Diameter (nm)	PDI
0.005	124 ± 1	0.250 ± 0.002	123 ± 5	0.252 ± 0.009
0.050	136 ± 4	0.254 ± 0.003	126 ± 1	0.268 ± 0.002
0.100	132 ± 1	0.330 ± 0.012	117 ± 2	0.230 ± 0.010
0.200	132 ± 1	0.274 ± 0.005	118 ± 3	0.210 ± 0.012
0.300	132 ± 8	0.271 ± 0.006	141 ± 1	0.237 ± 0.002

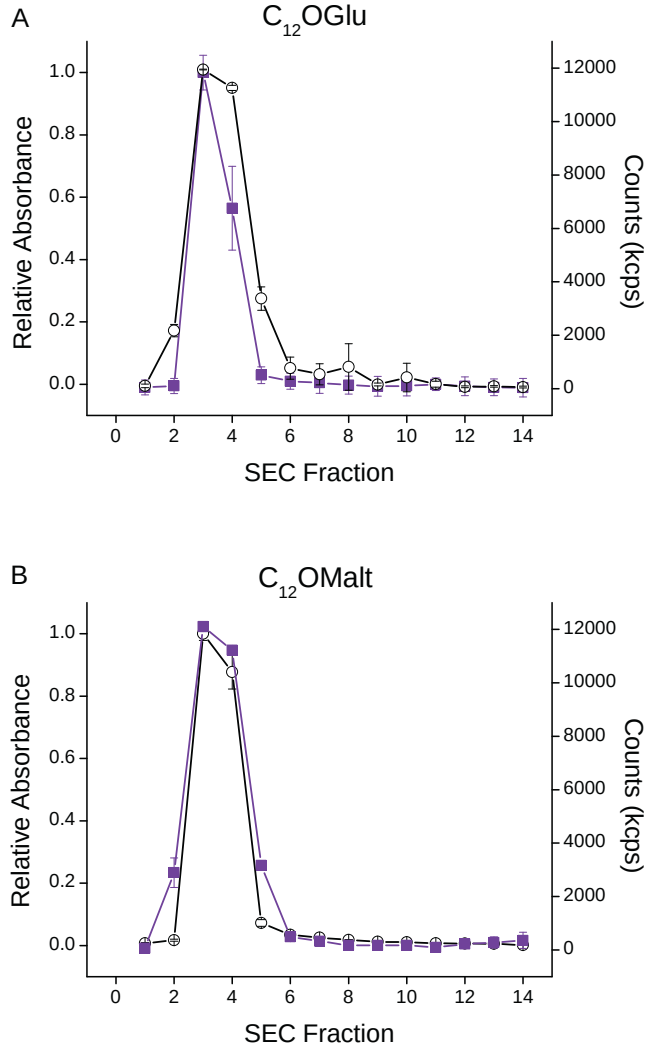

Fig. 2. Glycoconjugate incorporation into the surfactant vesicles. (A) Representative plot of colorimetric assay (purple solid squares) and dynamic light scattering (DLS, black open circles) results plotted versus size exclusion chromatography (SEC) fraction number for 0.200 mole fraction of C_{12}OGlu. (B) Representative plot of colorimetric assay (purple solid squares) and DLS (black open circles) results plotted versus SEC fraction number for 0.100 mole fraction of C_{12}OMalt. Uncertainty bars are SD ($n = 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

into the vesicles was found to be lower for the C_{12}OMalt glycoconjugate versus the C_{12}OGlu glycoconjugate. For mole fractions greater than 0.200, the excess C_{12}OMalt remained free in solution and did not incorporate into the vesicles (Supplemental Fig. 1). When considering steric hindrance effects, this is a logical finding as the C_{12}OMalt glycoconjugate contains two glucose units per hydrocarbon chain in comparison to C_{12}OGlu which is composed of one glucose unit per hydrocarbon chain. These results demonstrate that the amount of sugar incorporated into the vesicles can be controlled by the amount of glycoconjugate added during preparation.

3.3. Fabrication of glycan array platform

By attaching the vesicles to a surface, a glycan array can be created whereby the number of glycans exhibited on the array surface is controlled through the mole fraction of glycoconjugate originally used during vesicle preparation. Previous studies have demonstrated that dextran, a polymer composed of glucose, can be immobilized onto nitrocellulose-coated slides, and the immunological properties of the dextran are maintained [29]. Therefore, we explored using commercially available nitrocellulose-coated glass slides as the platform for the array surface. The carbohydrate-modified vesicles were simply deposited via micropipette injection into wells without any chemical conjugation steps. The nitrocellulose-coated slide surface formed the bottom of each well. Because the glycans are attached to vesicles that are kept hydrated in solution, problems inherent with printing solutions of small molecules on substrates, such as non-uniformity between spots and unwanted density gradients appearing within each spot as they dry on a surface, are potentially circumvented with this method of fabrication for the array [30,31]. Preliminary fluorescent imaging of the surface suggests the negatively charged vesicles are arranged on the nitrocellulose platform in a uniform layer (Supplemental Fig. 2). We speculate the vesicles form a monolayer due to the charge repulsion between individual vesicles and are continuing investigation to confirm this theory.

Following vesicle deposition onto the nitrocellulose-coated slides, lectin binding to the glycan array was monitored using a biotin-avidin fluorescence sandwich assay. Biotinylated lectins were applied to the glycan array as depicted in Fig. 4. After several wash steps (see Section 2.4 for details), fluorescently labeled NeutrAvidin® was applied. NeutrAvidin® and biotin exhibit a strong affinity for each other with a dissociation constant, K_d, on the order of 10^{-15} mol/L [32]. This is comparable to the K_d of streptavidin and biotin, which form one of the strongest noncovalent interactions found in nature [33–35]. NeutrAvidin® is a deglycosylated form of avidin, thereby reducing its potential for nonspecific interactions with the lectins. These interactions could give rise to fluorescence signals that would correspond to lectins that are not directly bound to the carbohydrate-modified vesicles. Thus, binding of a particular lectin to the glycan array was determined by measuring the fluorescence intensity of labeled NeutrAvidin® bound to the biotinylated lectin.

3.4. Analysis of lectin binding to the glycan array

The performance of the glycan array was evaluated using Con A and PNA. These plant lectins are well-studied model systems and have been extensively used in the literature to investigate multivalent lectin binding [36]. Biotinylated Con A, a lectin that readily binds to glucose, was applied to arrays of vesicles functionalized with either 0.000 (plain vesicles), 0.005, or 0.050 mole fraction C_{12}OGlu glycoconjugate. The fluorescence intensity detected from fluorescently labeled NeutrAvidin® bound to the arrays is shown in Fig. 5. When multiple wells are considered, the mean fluorescence intensity was significantly higher for C_{12}OGlu-functionalized vesicles versus plain vesicles (one-way analysis of variance (ANOVA); $p < 0.0001$, $n = 8$ to 16). The binding of Con A to arrays of C_{12}OGlu-functionalized vesicles was significantly altered by changing the density of C_{12}OGlu displayed on each vesicle surface (post hoc Tukey pairwise comparisons; $p < 0.001$ for the 0.005 mole fraction versus 0.050 mole fraction C_{12}OGlu vesicles). Multivalent complexes require proper spacing of the sugars for binding to occur. Therefore, sugar density can be either too low or too high to achieve maximum binding. The reduction of Con A binding on increasing the C_{12}OGlu mole fraction from 0.005 to 0.050 illustrates the importance of developing arrays with controlled glycan densities to optimize lectin binding. In control experiments with PNA (data not shown), a lectin that does not bind to glucose, the mean fluorescence intensity for the glycan arrays was significantly less than that of corresponding arrays with Con A ($p < 0.05$). These data indicate that Con A does not significantly bind to plain surfactant vesicles and the carbohydrate-modified vesicles adhere to the nitrocellulose-coated slide. In addition, the arrays were capable

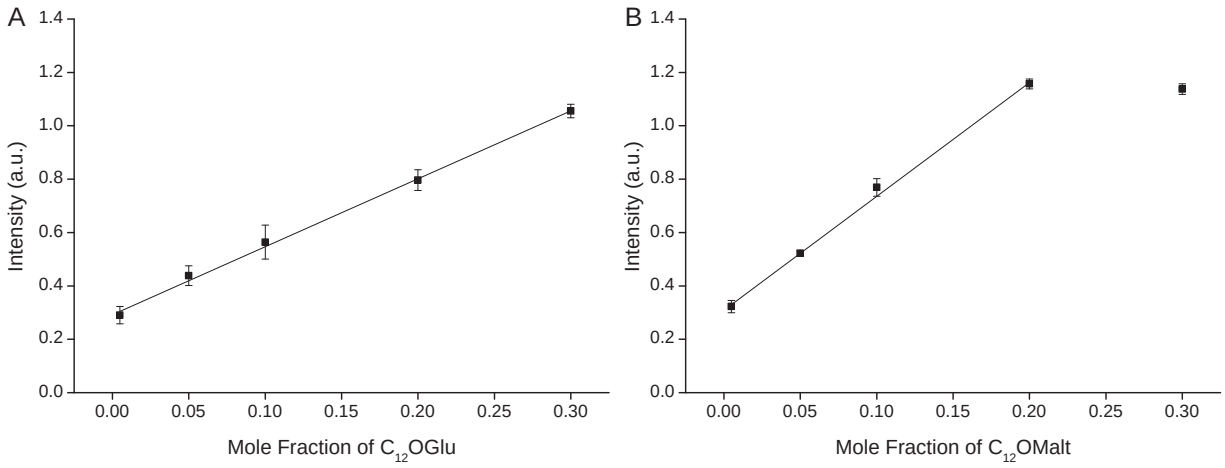

Fig. 3. Relationship between the amount of glycan detected in the vesicles and the mole fraction of glycoconjugate used in vesicle preparation. (A) C_{12}OGlu-functionalized vesicles exhibit a linear relationship between the amount of glycan incorporated into the vesicles and the amount of glycoconjugate used in vesicle preparation up to 0.300 mole fraction. (B) C_{12}OMalt-functionalized vesicles exhibit a linear relationship up to 0.200 mole fraction glycoconjugate. Glycoconjugate in excess of this does not incorporate into the vesicles; therefore, the measured intensity for 0.300 mole fraction is not included in the linear fit. Uncertainty bars are SD for both A and B ($n = 3$, $r^2 > 0.99$).

of quantifying different amounts of Con A that bound when the glycoconjugate density on the surface of the vesicles was changed.

While fluorescence measurements of PNA applied to the C_{12}OGlu arrays were statistically lower than for Con A, the results do point to a level of interaction between the vesicle arrays and PNA. Since glucose is not a sugar that PNA readily binds, this result is due to other factors. We speculate that the PNA lectin could be nonspecifically interacting with the glycoconjugates, and future optimization of the washing steps would eliminate this issue. In addition, further development of the array fabrication procedure could include using a slide blocker, such as nonfat milk, casein, or gelatin, to decrease possible nonspecific binding interactions between the nitrocellulose and biotinylated lectins or NeutrAvidin® [37,38].

It has been experimentally demonstrated that Con A binds to maltose as well as glucose [39]. Therefore, the binding of Con A to arrays composed of 0.005 mole fraction C_{12}OMalt vesicles and arrays of 0.005 mole fraction C_{12}OGlu vesicles was compared (Fig. 6). A statistically significant difference was observed between the amount of Con A that bound to C_{12}OMalt-functionalized vesicles versus C_{12}OGlu-functionalized vesicles (Student's t-test; $p < 0.05$, $n = 8$). This demonstrates that the glycan arrays can be further expanded to include vesicles functionalized with a variety of glycoconjugates. Based on the lectin binding measurements, the feasibility of displaying glycans on the surface of the vesicles at biologically relevant densities is confirmed. This facile method is a viable way to fabricate a glycan array for investigating lectin binding.

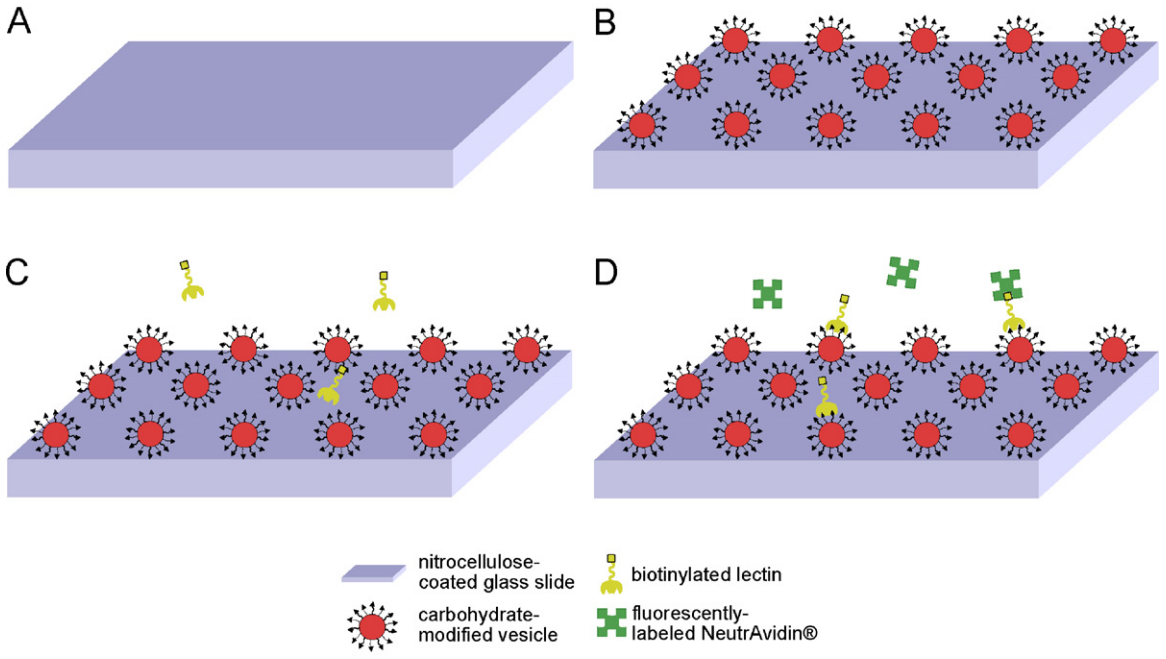

Fig. 4. Schematic diagram depicting the fabrication steps for a glycan array composed of carbohydrate-functionalized surfactant vesicles on a nitrocellulose surface. (A) Nitrocellulose-coated glass slide, (B) carbohydrate-modified vesicle application, (C) biotinylated lectin application and (D) fluorescently labeled NeutrAvidin® application.

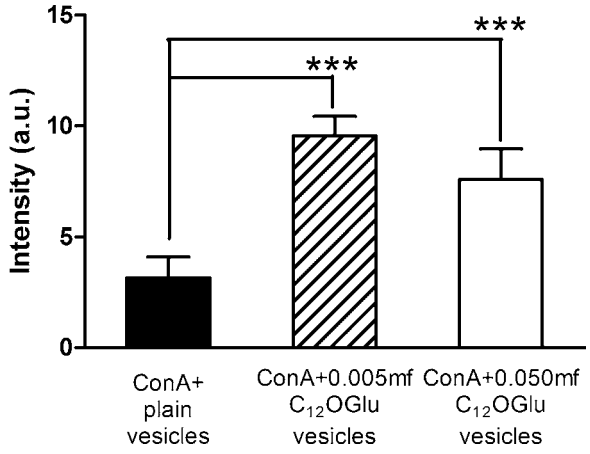

Fig. 5. Fluorescence detection corresponding to the relative amount of Con A lectin bound to arrays composed of vesicles with either 0.000, 0.005, or 0.050 mole fraction (mf) C_{12}OGlu glycoconjugate (mean ± SD; one-way ANOVA; $p < 0.0001$ (***), post hoc Tukey pairwise comparisons; $p < 0.0001$ (***) for the 0.005 mf and 0.050 mf C_{12}OGlu vesicles versus plain vesicles, $p < 0.001$ (**) for the 0.005 mf versus 0.050 mf C_{12}OGlu vesicles, $n = 8$ to 16).

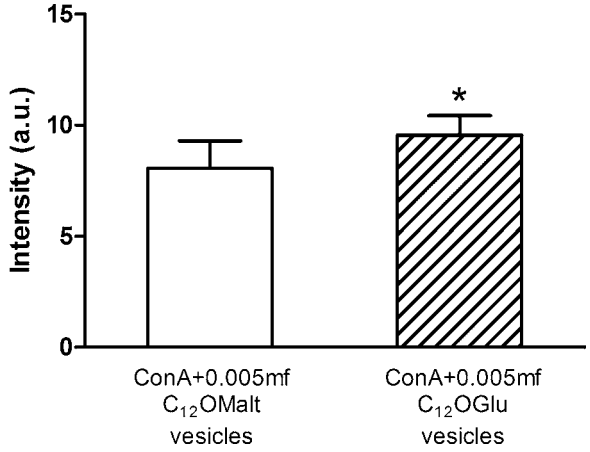

Fig. 6. Fluorescence detection corresponding to the relative amount of Con A lectin bound to arrays composed of vesicles with 0.005 mole fraction (mf) of either C_{12}OMalt or C_{12}OGlu glycoconjugate (mean ± SD; Student's *t*-test; $p < 0.05$ (*), $n = 8$).

4. Conclusion

Catanionic surfactant vesicles have been prepared and characterized with glycoconjugates incorporated into their bilayer surface in a controlled range of densities. These carbohydrate-modified vesicles are composed of SDBS, CTAT, and either C_{12}OGlu or C_{12}OMalt and were used to form glycan arrays. After the vesicles were applied to nitrocellulose-coated slides, the binding of lectins to the arrays was detected using a biotin-avidin fluorescence assay. Results demonstrated that the carbohydrate density on the glycan array surface was controlled by varying the amount of glycoconjugate incorporated onto each vesicle surface. In addition, the vesicle array displayed glycans at biologically relevant densities for lectin binding to occur. These data support the continued development of this method for the use of characterizing the binding properties of unknown lectins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2012.01.036.

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