Size Effect of Polydiacetylene Vesicles Functionalized with Glycolipids on Their Colorimetric Detection Ability

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In this paper, the size effect of the polydiacetylene vesicles functionalized with glycolipids on their colorimetric detection ability has been studied. Polydiacetylene vesicles in which were incorporated glycolipids acted as a model system for the affinochromatic property. Visible color changes from blue to red could be observed to the naked eye owing to Con A binding to the sugar moiety and be detected quantitatively by the visible absorption spectrum. In the experiment, small and uniform vesicles were obtained after extrusion through membranes with different pore sizes. The morphology and mean size distribution of the extruded vesicles were studied by transmission electron microscopy (TEM) and dynamic light scattering (DLS), respectively. Our work shows that the smaller the vesicles are, the stronger is the effect, making the detection of Con A easier. The results may apply to the sensitivity enhancement of polydiacetylene biosensors for the recognition of other biomolecules.

1. Introduction

Molecular recognition events involving carbohydrates occur in a wide variety of important biological processes including the function of the immune system, interaction of viruses and bacteria,[1] and tissue growth.[2] Included in these phenomena are cell-to-cell communication events, which are driven by highly specific carbohydrate−protein interactions. These specific interactions occur through glycolipid, glycoprotein, and polysaccharide found on cell surfaces and lectins, proteins with carbohydrate-binding sites.[3]

Concanavalin A (Con A) is a lectin of the jack bean (*Canavalia ensiformis*). This protein is a widely used probe for studies of the cell surface and of the regulation of cell metabolism and cell division. It can specifically bind to saccharide-containing receptors, such as glucose, mannose, or *N*-acetylglucosamine (GlcNAc), although to a lesser extent. By means of the specific binding to saccharide, diseases and infections can be pharmacologically treated by the administration of molecules which mimic the recognition or adhesion process.[4]

Previously, carbohydrate structures have been formulated: as self-assembled monolayers (SAM) on 2D surfaces, both as single ligands[5−7] and more recently within molecular assemblies,[8−11] as dendrimers,[12] within phospholipid liposomes,[13] on the backbone of polymers,[14] and on the metal nanoparticles surface.[15−17]

In an attempt to design a simple, more direct method for detecting molecular recognition between lectins and their responsive binding saccharides, we chose to exploit the known chromatic properties of polydiacetylene vesicles. When the monomer of diacetylene was orderly arranged, it could be polymerized under UV irradiation and form a blue assembly. Polymeric diacetylene vesicles can present color changes from blue to red in response to a variety of external stimuli, such as pH,[18] organic solvent (solvatochromism),[19] temperature (thermochromism),[20] and mechanical stress (mechanochromism)[21] due to conformational changes in the conjugated backbone. Much research has revealed that polydiacetylene can be used as biosensors for detection of influenza virus,[22] *Escherichia coli*,[23] antibacterial peptides,[24] etc.

In this paper, a further development of polydiacetylene vesicles for colorimetric detection of Con A was reported. The saccharide-containing ligand neoglycolipid was inserted into diacetylene vesicles. There have been many reports that hydrophobic aglycon promotes the recognition of sugars by lectin.[25] With the addition of Con A, the vesicle solution transfers color from blue to red. This process is readily visible to the naked eye. Although much work has been carried out using the color-changeable polydiacetylene vesicle as a biosensor transducer, and very good results have been obtained, the influence of vesicle size and stability of such a vesicle has not been studied in details. In this paper, the size effect of the polyacetylene vesicles functionalized with glycolipids on their colorimetric detection ability has been studied. To examine the size effect on sensitivity detection, we used Con A as a recognition target and extruded the vesicle solution through nylon films with different pores to get vesicles of different sizes.

2. Experimental Section

2.1. Materials. 10,12-Pentacosadiynoic acid (PCDA) was purchased from Lancaster Co. in 98% purity, purified by dissolving in chloroform, and filtered to remove polymerized monomers before use; 10-tetradecyloxymethyl-3,6,9,12-tetraoxahexacosyl-2-acetamido-2-deoxy-β-D-glucopyranoside (PB 1124) was synthesized as already reported in the literature.[26] The preparation of PB 1097 and PB 915 (similar structures, but with different alkyl chain lengths) was also reported earlier.[27] Con A was purchased from Sigma with a purity of 98%. Tris

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buffer solution (pH 7.4, 0.1 mmol/L $CaCl_2$, 0.1 mmol/L $MnCl_2$) was used. Nylon films equipped with a pinhead were obtained from Poll Co. in the U.S.A. In all experiments, doubly distilled water was used.

2.2. Preparation of Polydiacetylene/Glycolipid Vesicles. PCDA and glycolipid were dissolved in chloroform at a concentration of 1 mmol/L. The various molar fractions were prepared by mixing the appropriate volume of stock solution of each component. A volume of 10 mL of lipid solution in chloroform was rotoevaporated to dryness, and the same amount of distilled water was added in order to obtain the lipid concentration of 1 mmol/L. The suspension was heated to 70 °C and sonicated in an ultrasonicator bath for about 15 min. As a result, a semitransparent or transparent vesicle solution was obtained. Prior to irradiation under light of 254 nm, a certain volume of the latter solution was extruded quickly through nylon membranes of various pore diameters by a syringe and kept at 4 °C overnight. Finally, we obtained nanometer-scale vesicles that were relatively uniform in size and shape.

2.3. UV−Vis Measurements. Con A solution was prepared using Tris buffer solution (pH 7.4, $[CaCl_2] = [MnCl_2] = 0.1$ mmol/L) to give a concentration of 1 mg/mL. The pH of the solution was 8.0 in all experiments. Briefly, the various volumes of Con A solution were injected into 1 mL of blue-colored vesicles solution with a syringe. After incubation for about 30 min or longer, the samples were recorded with a UV−vis spectrometer. In our experiment, the effect of a few divalent ions, such as Ca^{2+} and Mn^{2+}, on the chromatic behavior of polydiacetylene vesicles was very little, so we did not take them into account. All the samples were measured at 20 °C on a two-beam Hitachi U2800 UV−vis spectrometer, using a 1 cm optical path length.

A quantitative value for the extent of blue-to-red color change transition is given by the colorimetric response (CR), which is defined as follows: $CR = [(PB_0 - PB_v)/PB_0] \times 100\%$, where $PB = A_{blue}/(A_{blue} + A_{red})$, A is the absorbance at either the "blue" component in the UV−vis spectrum (≈640 nm) or the "red" component (≈540 nm), PB_0 is the red/blue ratio of the control sample (before induction of color change), and PB_v is the value obtained for the vesicle solution after addition of Con A.

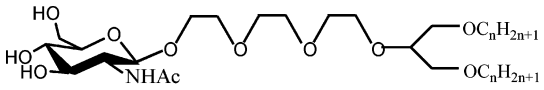
2.4. Transmission Electron Microscope (TEM). TEM images were completed using a JEOL TEM-2010 electron microscope (Japan) equipped with a CCD camera. The accelerating voltage is 200 KV. The vesicle solutions extruded through the nylon films with different pores were deposited onto a carbon-coated copper grid and stained with 2% uranium acetate solution.

2.5. Dynamic Light Scattering (DLS). The mean size distribution of the extruded and unextruded vesicles was determined by a BI-200SM dynamic light scattering analyzer (Brookhaven Instruments Co., U.S.A). Each experiment was repeated 3 times in order to acquire an average data.

3. Results and Discussion

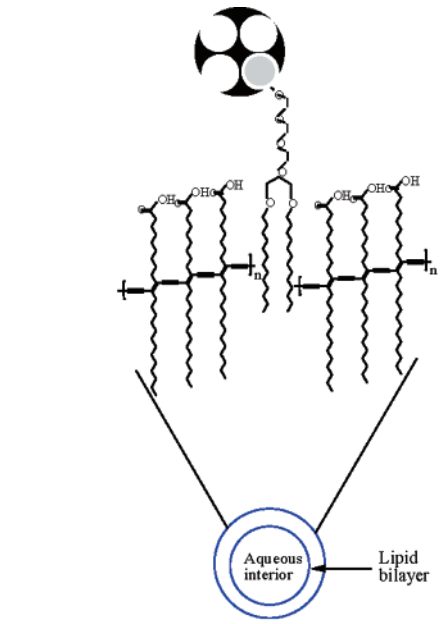
3.1. Molecular Recognition between PCDA−GlcNAc and Con A. Our initial investigations focused on two aspects: binding of the lectin Con A to glycolipid, as a model system for colorimetric detection and the effect of uniform and nanoscale vesicles on the assay of the lectin. To highlight the enhanced affinochromism using small-sized vesicles, we choose an *N*-acetylglucosamine-derivatized glycolipid (Chart 1) as the research object and Con A as the lectin. Although GlcNAc has a structure change at C-2 compared with that of glucose, the interaction of Con A with GlcNAc was well documented and was already reported.[28]

CHART 1: Structures of *N*-Acetamido-*β*-D-glucopyranoside Used in this Paper.



PB915(n=11); PB1124(n=14); PB1097(n=16)

[a] These glycolipids have different alkyl chain lengths.



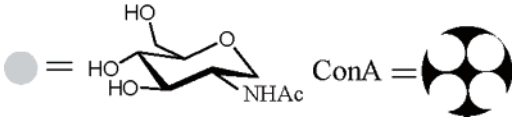


Figure 1. Schematic representation of Con A binding to PB1124 incorporated into a polymerized diacetylene liposome bilayer (blue circles). Various molar percentages of lipids are mixed to form into liposomes. Samples were prepared so as to give 1 mmol/L solutions in total lipids.

Figure 1 schematically represents the outer layer of a polymerized glycol−liposome bilayer. The glycolipid was mixed with PCDA at 5%, 10%, and 50% molar ratios, and then the mixture formed into liposomes. With the percentage of the glycolipid component above approximately 50%, polymerization was substantially inhibited. This is rationalized by the steric crowding of adjacent carbohydrate headgroups which prevent the proximal diacetylene from polymerizing. When tetramer Con A (pH > 7) was added to the glycolipid/polydiacetylene vesicles, as is shown in Figure 1, molecular recognition between carbohydrate and protein occurs via noncovalent interactions. The biochemical events lead to lipid chain disorder and tangling. Consequently, the effective conjugated length of the polydiacetylene backbone decreased. Conformational changes in the lipid chains affect the optical properties of the polymer backbone. Binding of Con A to the glycoliposomes gives rise to a blue-to-red color change that is visible to the naked eyes, as shown in Figure 2.

Figure 2 quantitatively indicates the degree of color change prior to (black line) and after (colorized lines) Con A incubation as a function of time by standard visible absorption spectros-

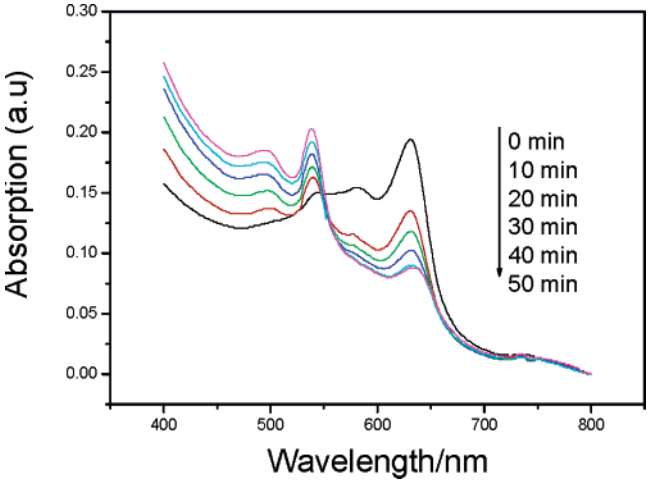


Figure 2. Time dependence of UV−vis absorption spectra of polymerized diacetylene vesicles (50% PB1124) after addition of Con A.

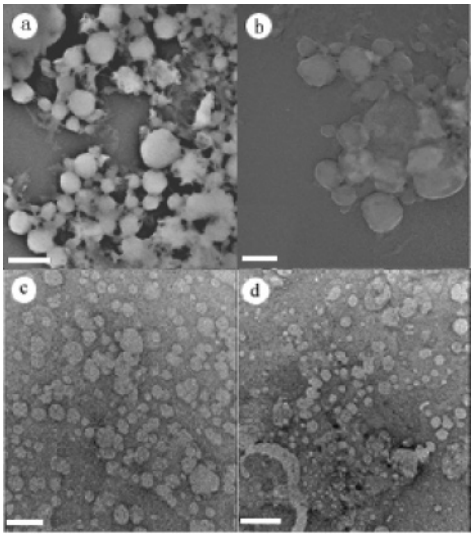


Figure 3. TEM photographs of PDA/glycolipid vesicles: (a) unextruded; (b) extruded through a 1 μm membrane; (c) extruded through a 0.45 μm membrane; (d) extruded through a 0.2 μm membrane. The bar indicates 1 μm in (a) to show a larger area and 200 nm in (b−d).

copy. The blue-colored vesicles have a strong absorption maximum at 640 nm and a weaker absorption at 540 nm. After addition of Con A, a dramatic change in the visible absorption spectrum took place. The maximum at 540 nm increased gradually with a concurrent decrease in the maximum at 640 nm, resulting in a red-colored vesicle solution. The visible absorption spectrum was recorded after exposure to Con A up to 50 min. Though the vesicle color began to change within a few minutes after addition of Con A, 50 min was found to be the average length of time required for the CR to reach a plateau value in a nonstirred solution, which was attributed to Con A binding to glycolipid via weak noncovalent interactions.

3.2. Transmission Electron Microscopy (TEM). Photographs in Figure 3 illustrate the decrease of vesicle diameters in accordance with decreasing pore sizes of membrane used for extrusion. Before extrusion, the morphology of vesicles was very regular and the size was in the range of 350−950 nm (Figure 3a). After extrusion through 1, 0.45, and 0.2 μm films, the vesicle diameters decrease to 126−230, 50−145, and 25−72 nm, respectively. Although the membrane pores are uniform in size, the obtained vesicles are polydispersed, which is similar to a previous report.[29] Observations show this to be due to steric hindrance of droplets forming simultaneously.

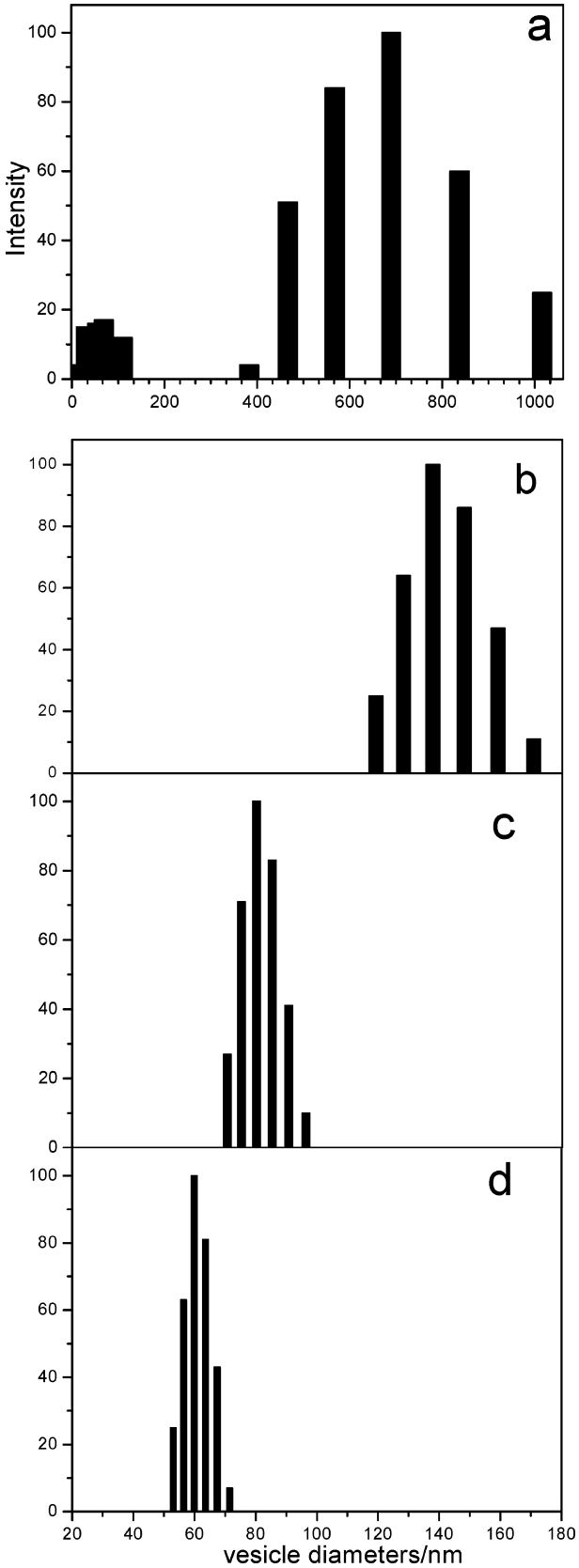


Figure 4. Size distribution determined by dynamic light scattering of PCDA/glycolipid vesicles sequentially extruded through nylon films: (a) unextruded; (b) extruded through a 1 μm membrane; (c) extruded through a 0.45 μm membrane; (d) extruded through a 0.2 μm membrane.

We also observe that there exist some sheets other than circular vesicles. This observation was already reported by

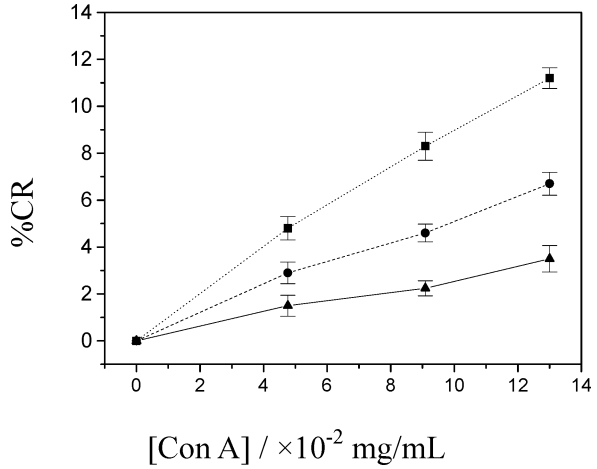


Figure 5. Effect of the glycolipid alkyl chain length on the colorimetric response: PB915 (▲), PB1097 (●), and PB1124 (■). In this experiment, the unextuded vesicles contained 5% glycolipid. Data were recorded after Con A incubation for 30 min.

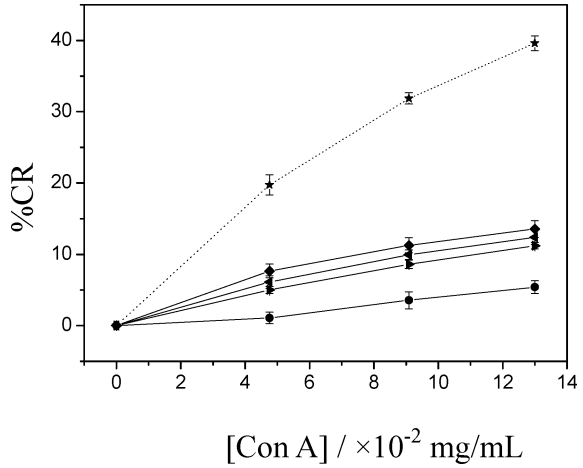


Figure 6. Effect of the amount of PB1124 on the colorimetric response in unextruded vesicles (solid lines). From bottom to top, the contents of glycolipid are 0 (●), 5% (right-pointing triangle), 10% (left-pointing triangle), and 50% (◆). The dashed line (★) denotes the CR value of the extruded vesicles (through a 0.2 μm film) composed of 50% PB1124. Data were recorded after Con A incubation for 30 min.

Peek[30] who mentioned that unpolymerized unilamellar vesicles and large multilamellar vesicles could change their morphologies when cooled to 4 °C, forming extended sheets and lipid tubules, respectively. But most sheets were eliminated upon extrusion through various membranes.

3.3. Dynamic Light Scattering (DLS). The laser dynamic light scattering analyzer is a versatile instrument for measuring size distribution. DLS presents the whole situation compared with the case of TEM, which only shows partial distribution. The mean diameter of vesicles prepared by the ultrasonic method is 517 nm, and the size distribution is wide, centralizing in the two ranges of 30−109 and 466−1015 nm (Figure 4a). When the vesicles are extruded through a 1 μm film (Figure 4b), the vesicle mean diameter decreases to 141 nm. The size distribution becomes relatively narrow, and the size of the majority of vesicles (90%) ranges between 128 and 160 nm. In the case of extrusion through a 0.45 μm membrane (Figure 4c), the mean size of the vesicles is 81 nm. Vesicles of the size range of 75−90 nm (88%) are the most abundant. The better dispersivity is obtained when a 0.2 μm membrane is used. The large proportion of vesicles (93%) comes in the size range of 53−67 nm (Figure 4d), and the mean size is down to 60 nm.

3.4. Molecular Recognition Arising from the Colorimetric Response. At first, we studied the effect of glycolipid with different alkyl chain lengths on the colorimetric response (Figure 5). In this experiment, the percentages of glycolipid were all 5%. It can be safe to say that the CR value is proportional to the alkyl chain length upon comparing glycolipid PB1124 with PB915. However, the CR decreases when PB1097, which has longer alkyl chains, is used as the ligand for Con A binding. Due to a longer alkyl chain, PB1097 was shown to exist in the gel phase at our experimental temperature, which is adverse to molecular recognition. Meanwhile, shorter alkyl chains do not perturb, to a great extent, the structure of PCDA vesicles, due to the relatively weak hydrophobic interaction with PCDA. Therefore, in the following discussion, we selected PB1124 as our study object.

The quantitative colorimetric data shown in Figure 6 indicate that the color changes are directly proportional to the quantities of Con A. However, the blue-to-red color changes are much more pronounced (higher CR) when Con A is added to the vesicle solutions that contain glycolipid. Furthermore, the CR value increases with the increasing percentage of PB1124 in the vesicles, which can be attributed to the binding of the

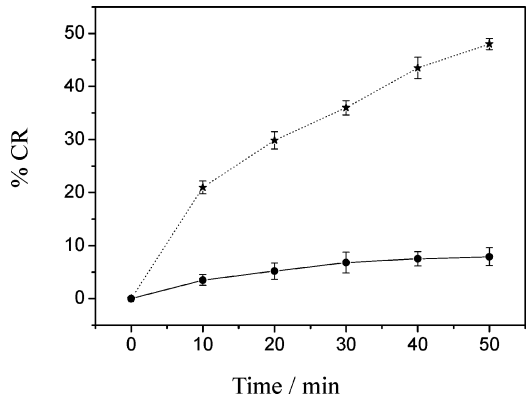


Figure 7. Control experiment carried out by addition of glucolipid [octadecyl-β-D-glucopyranoside] (●) to the extruded vesicle (through a 0.2 μm film) solution and without glucolipid (★).

TABLE 1: Comparison of Colorimetric Responses by Con A for Unextruded and Extruded Vesicles (through a 0.2 μm Film)[a]

vesicles	response time (min)	[Con A] ($\times 10^{-2}$ mg/mL)
before extrusion	24	10.5
after extrusion	7.5	3.5

[a] Molar ratio of PB1124/PCDA (5:5); measurements realized at 15% colorimetric response.

tetrameric protein (Con A) to glycolipid despite of the weaker affinity. The small background CR value change detected when Con A was added to pure PCDA vesicles is attributed to electrostatic interactions of Con A with charged headgroups of the polymerized lipid. Indeed, glycoliposome preparation in amino-terminated diacetylene lipid showed no color transition even at the highest concentration tested, 0.13 mg/mL (data not shown).[28] Therefore, the acidic groups are essential for strong binding activity. The result accords with the apparent requirement for an anionic group in Con A detection. It should be emphasized that the observation of significantly strong color transitions when PB1124/PCDA vesicles were employed, compared to that of pure PCDA, supports the proposal that the colorimetric effects are indeed biologically relevant, rather than due to electrostatic binding of Con A to the PCDA interface.

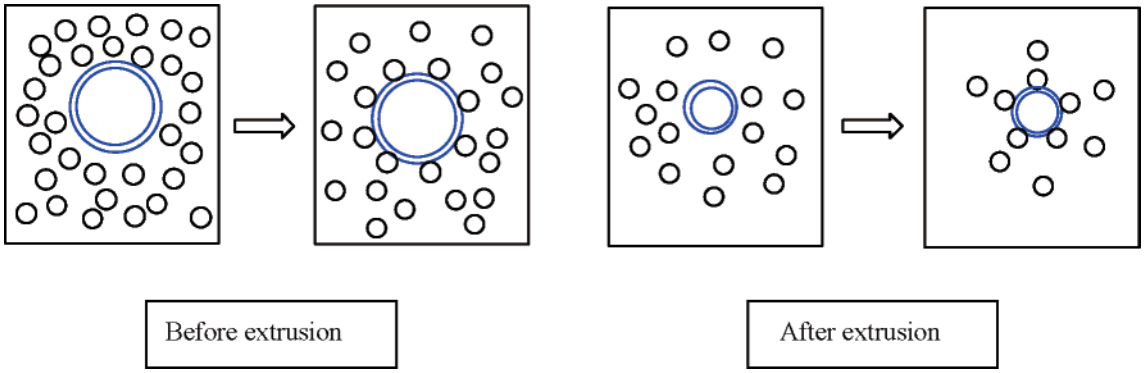


Figure 8. Sensitivity increase by compartmentalization of receptor sites. A smaller vesicle (after extrusion) can, in more dilute solution, reach the same relative signal as a larger vesicle. Small circle indicates a Con A molecule.

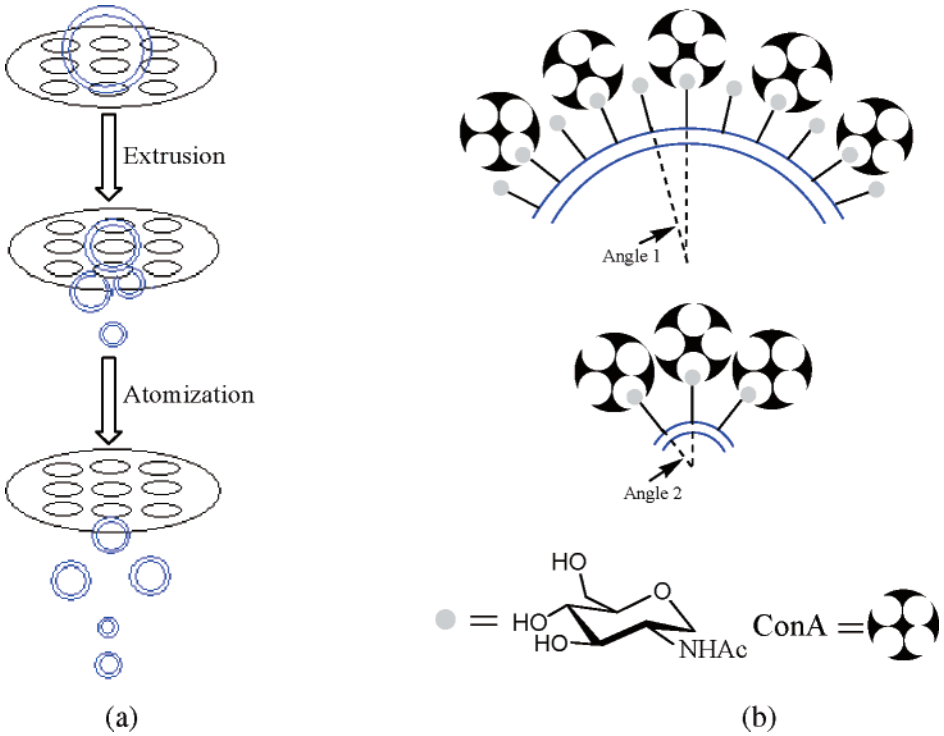


Figure 9. (a) Schematic representation of the extrusion process of vesicles (to simplify the picture, a polydiacetylene/glycolipid vesicle was indicated only with a blue circle). (b) Curvature effect on molecular recognition. Upper panel: fewer glycolipids were bound by Con A owing to the lack of lateral space. Lower panel: more glycolipids were bound due to larger curvature.

To confirm that the colorimetric response is induced by GlcNAc recognition by Con A, rather than electrostatic interactions, a control experiment was carried out in the presence of a glucolipid [octadecyl-β-D-glucopyranoside], which has a stronger affinity than GlcNAc for Con A, as shown in Figure 7. When 0.13 mg/mL of Con A and glucolipid mixture were added to the extruded vesicle solution composed of 50% PB1124, no color change was observed, and the time dependent CR change is similar to that of pure PCDA (solid line). The strong inhibitory effect of glucolipid with Con A shows that the colorimetric response is due to the molecular recognition of GlcNAc by Con A.

3.5. Sensitivity Increase by Extrusion of Vesicle Solution through a Micropore Membrane. It is worthwhile to note that when the vesicles composed of 50% PB1124 were extruded through a 0.2 μm pore nylon film, the degree of color transition dramatically increased (dashed line in Figure 6). Moreover, the extruded vesicles exhibit much faster response and higher CR value, as shown in Table 1. We can obviously see that the extruded vesicles need less amount of Con A than the unextruded ones and that the response time is largely shortened. The situation can be illustrated with Figure 8, where both larger or smaller vesicles with receptor sites (i.e., carbohydrate) on their surface are binding the analyte molecules (i.e., Con A) from the surrounding medium. If the vesicle is larger, more concentration of Con A molecules is needed to occupy the available binding sites on the vesicles (before extrusion) than that for a smaller vesicle, which can work also in more dilute solution (after extrusion). So, if the color change induced by the effector is the same in both cases, the smaller vesicles need less concentrated Con A solutions. An increase in sensitivity by diminishing the sensing particle size has already been described in other molecular recognition.[31]

Wang and Leblanc[32] have reported the steric hindrance effect of Con A binding to the PDA/MPDA monolayer at an air−water interface. They proposed that the maximum expansion of molecular area decreased as the molar ratio of MPDA increased due to the steric hindrance effect. This point was incarnated again in our studies, where a decrease in particle size was observed in the course of the extrusion. The size reduction mechanism was suggested to include the rupturing of vesicles and spontaneous rearrangement after membrane

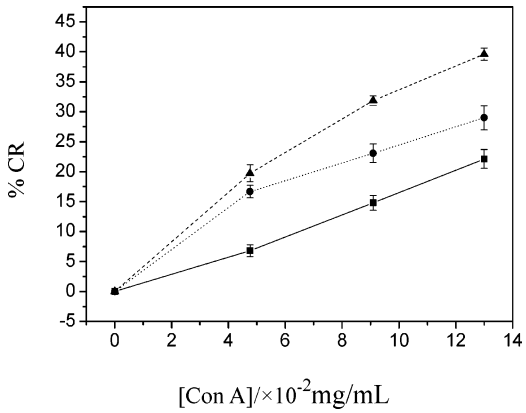


Figure 10. Influence of membrane pore sizes on the color transition; extruded through 1 μm (■), 0.45 μm (●), or 0.2 μm (▲) pore size membranes, respectively.

passage resulting in the formation of smaller vesicles,[33] which are illustrated in Figure 9. According to the X-ray diffraction studies,[34] the Con A protomer has a diameter of about 40 Å, which is 5 times bigger than that of the lipid molecules. At pH 7.4, Con A forms a tetramer, which has an even bigger size compared with that of the protomer. For larger vesicles, which have smaller curvature (as illustrated in Figure 9b, angle 1 is smaller than angle 2), the recognition sites of Con A cannot completely bind with carbohydrate because of the lack of lateral space on the side of glycolipid headgroups. On the contrary, in the case of smaller vesicles whose curvature is larger, the aforementioned adverse factor, i.e., the steric hindrance effect, is largely reduced. That is to say, the utilization ratio of binding sites to sugar moieties is elevated. An affinity increase can be obtained via decreasing the size, which induces vesicles to change color in a shorter time and with higher colorimetric response.

To further elucidate the problem, the effect of different pore sizes on the colorimetric response is examined, as shown in Figure 10. When decreasing the diameter of the membrane, we observed a decrease in vesicles size. The vesicles experienced rupturing and rearrangement to a higher extent. According to the above inference, the vesicles extruded through the smaller pore size film should have a much larger curvature. Bigger affinity resulted in a higher CR value in the smaller vesicles.

In the process of extrusion, the vesicles suffered from compression coming from every direction. This compression inevitably led to tiny looseness in the backbone of the vesicles. Therefore, the loose vesicles were in a substable state in which the PCDA network was liable to be perturbed upon exposure to environmental change such as molecular recognition.

Before concluding, it should be mentioned that even though better recognition of the smallest vesicles was attributed to curvature effects, other phenomena cannot be excluded at this stage of our work. Among them can be noted the formation of glycolipid clusters or rafts, changes in the PCDA/glycolipid ratio during extrusion, or glycolipid conformational changes or arrangements favorable to the lectin multivalency.

4. Conclusions

We have developed an interesting strategy for the enhancement of detection sensitivity of polydiacetylene vesicles by changing the vesicle sizes by means of extrusion technology. In a model system consisting of Con A as a representative recognition target and PCDA/glycolipid vesicles as a color-changeable sensor, we studied the effect of vesicle sizes and found that smaller and uniform vesicles could enhance colorimetric response remarkably. This effect was attributed to differences in particle curvatures induced by differences in membrane pore sizes; the large curvature of the small vesicles provides more space for Con A to combine and causes a more dense color change. Nevertheless, other phenomena cannot be excluded, and they are now under investigation. The use of extrusion technology to enhance the recognition ability of the color-changeable polydiacetylene vesicle represents a general, efficient route to the formation of such vesicles as a highly sensitive biosensor transducer for investigating the recognition of virus, antigen−antibody, lectins, and other biomolecules.

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Supporting Information Available: Visible absorption spectra of PCDA/PB1097 (5%), PCDA/PB915 (5%), PCDA/PB1124 (5%), PCDA/PB1124 (10%), PCDA/PB1124 (50%), and extruded PCDA/PB1124 (50%) vesicles and plots showing a control experiment carried out by the addition of glucolipid to the extruded vesicle. This material is available free of charge via the Internet at http://pubs.acs.org.

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