CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200902423

Sugar-Decorated Sugar Vesicles: Lectin–Carbohydrate Recognition at the Surface of Cyclodextrin Vesicles

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Abstract: An artificial glycocalix self-assembles when unilamellar bilayer vesicles of amphiphilic β-cyclodextrins are decorated with maltose and lactose by host–guest interactions. To this end, maltose and lactose were conjugated with adamantane through a tetra(ethyleneglycol) spacer. Both carbohydrate–adamantane conjugates strongly bind to β-cyclodextrin ($K_a \approx 4 \times 10^4\,M^{-1}$). The maltose-decorated vesicles readily agglutinate (aggregate) in the presence of the lectin concanavalin A, whereas the lactose-decorated vesicles agglutinate in the presence of peanut agglutinin. The orthogonal multivalent interaction in the ternary system of host vesicles, guest carbohydrates, and lectins was investigated by using isothermal titration calorimetry, dynamic light scattering, UV/Vis spectroscopy, and cryogenic transmission electron microscopy. It was shown that agglutination is reversible, and the noncovalent interaction can be suppressed and eliminated by the addition of competitive inhibitors, such as D-glucose or β-cyclodextrin. Also, it was shown that agglutination depends on the surface coverage of carbohydrates on the vesicles.

Keywords: cyclodextrins · lectins · molecular recognition · self-assembly · vesicles

Introduction

The extracellular surfaces of most prokaryotic and eukaryotic cells display a dense layer of complex carbohydrates, which is commonly known as "glycocalix". Carbohydrates in the glycocalix act as ligands for proteins in cell–cell and cell–matrix interactions. For example, the human blood types (A, B, AB, and O) originate from the presence of different oligosaccharides at the erythrocyte surface. Proteins that bind to carbohydrates in the glycocalix are generally called lectins.[1] The association constant, K_a, for a 1:1 carbohydrate–lectin complex is $\approx 10^3$–$10^4\,M^{-1}$. Most lectins have multiple binding sites for carbohydrates, and the multivalent interactions of lectins and carbohydrates mediate the recognition and adhesion of cells. However, the binding of antibodies to foreign cells and viruses, as well as the infection of cells with viruses, are also essentially mediated by protein–carbohydrate interactions at the cell surface. Carbohydrate–lectin interactions also constitute a promising strategy for drug delivery in which either the lectins or the carbohydrates can function as "address labels" to target specific cells.[2]

Liposomes and vesicles are versatile membrane-mimetic systems. In particular, the dynamic multivalent molecular recognition of their outer membrane surface has become an increasingly popular topic in supramolecular chemistry in recent years.[3] The interaction of natural glycolipids and synthetic membrane-bound carbohydrates ("neoglycolipids") with various lectins have been studied by several groups.[4] Because most lectins possess multiple carbohydrate-binding sites, the interaction of lectins with glycolipid and neoglycolipid vesicles results in aggregation of the vesicles, which can be easily detected as an increase in the optical density of the vesicle solution. This type of experiment is known as an "agglutination assay". In parallel, there has also been considerable effort towards the design of so-called carbohydrate clusters, including polymers and nanoparticles, that display a large number of carbohydrates at their surface and thus mimic the glycocalix of cells and its affinity for lectins.[5]

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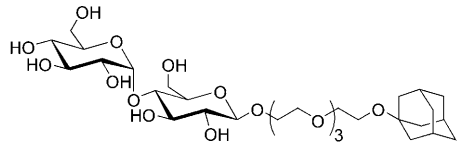
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.200902423.

Amongst others, these carbohydrate clusters have been based on macrocycles such as calixarenes and cyclodextrins.[6] If such cyclodextrins are additionally equipped with hydrophobic groups, they can form vesicles, micelles, or nanoparticles that display a high density of carbohydrates on their surface and thus aggregate in the presence of lectins.[7]

However, vesicles can also be decorated with functional molecules through noncovalent interactions.[3] We have shown that cyclodextrins modified with long hydrophobic alkyl tails and short hydrophilic oligo(ethyleneglycol) head groups form unilamellar bilayer vesicles in aqueous solution.[8] The cyclodextrin cavities at the surface of these vesicles are available for the complexation of hydrophobic guests, such as adamantyl and *tert*-butylbenzoyl derivatives.[9] We have found that the host–guest interaction can be enhanced by multivalency in the case of guest polymers[10] or guest molecules that associate at the vesicle surface through orthogonal noncovalent interactions, such as metal–ligand coordination[11] or hydrogen bonding.[12] We have also shown that orthogonal multivalent interactions at the cyclodextrin vesicle surface can result in receptor clustering,[13] vesicle clustering,[11] and vesicle–nanotube transitions.[12]



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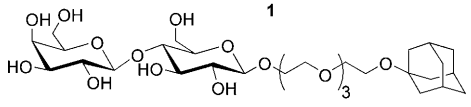

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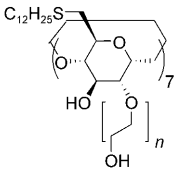

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Herein we describe the hierarchical self-assembly of an artificial glycocalix that occurs when unilamellar bilayer vesicles of amphiphilic β-cyclodextrins are decorated with maltose and lactose through host–guest interactions. To this end, we prepared bifunctional conjugates **1** and **2**, which can bind to amphiphilic β-cyclodextrin **3** with their adamantane group and to lectins (concanavalin A (ConA) in the case of maltose **1** and peanut agglutinin (PNA) in the case of lactose **2**) with their carbohydrate group. In this artificial glycocalix, three carbohydrates (β-cyclodextrin, maltose, and lactose) simultaneously engage in highly specific orthogonal interactions (Figure 1). Therefore, this artificial glycocalix is even more sophisticated than the glycocalix designed by

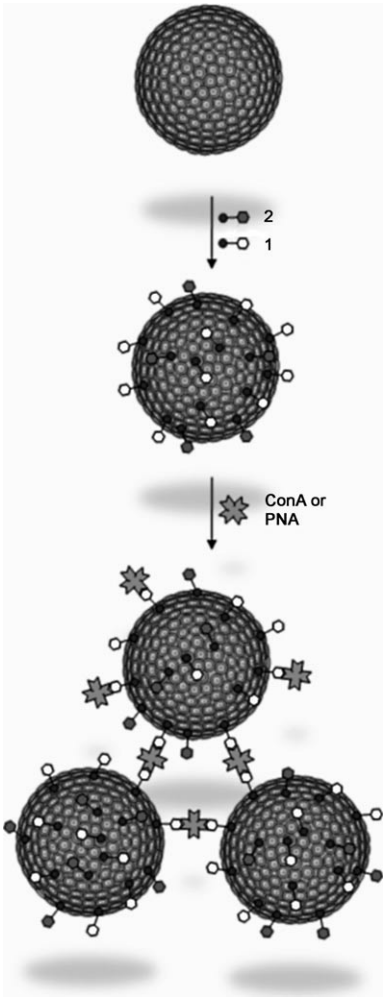

Figure 1. Schematic representation of cyclodextrin vesicle agglutination induced by the lectin ConA in the presence of maltose–adamantane conjugate **1** (white/black linker). Similarly, agglutination of cyclodextrin vesicles can be induced by PNA in the presence of lactose–adamantane conjugate **2** (gray/black linker).

Kim et al., which was based on cucurbituril vesicles decorated with carbohydrates.[14] The orthogonal multivalent interaction in the ternary system of host vesicles, guest carbohydrates, and lectin was investigated by using isothermal titration calorimetry (ITC), dynamic light scattering (DLS), UV/Vis spectroscopy, and cryogenic transmission electron microscopy (cryo-TEM).

Results and Discussion

The synthesis of maltose–adamantane conjugate **1** and lactose–adamantane conjugate **2** was carried out by using trichloroimidate glycosidation chemistry. Details of the synthesis are reported in the Supporting Information. The spectroscopic and analytical data for **1** and **2** are consistent with their molecular structure. Amphiphilic β-cyclodextrin **3** was synthesized as described in the literature.[9,15]

The adamantane unit on conjugates **1** and **2** is known to be an excellent inclusion guest for β-cyclodextrin (K_a = 32000 M^{-1} for adamantylcarboxylate) and for β-cyclodextrin vesicles (K_a = 7000 M^{-1} for adamantylcarboxylate).[9] In general, guests bind to the cyclodextrin vesicles somewhat more weakly than to cyclodextrins in solution due to the presence of the oligo(ethyleneglycol) residues on the vesicle surface.[9] Conjugates **1** and **2** were tested for their complexation behavior towards β-cyclodextrin by using ITC. A 10 mM solution of the adamantane conjugate (**1** or **2**) was titrated into a 1.0 mM solution of β-cyclodextrin. The results of these titrations are presented in Figure 2 and Table 1.

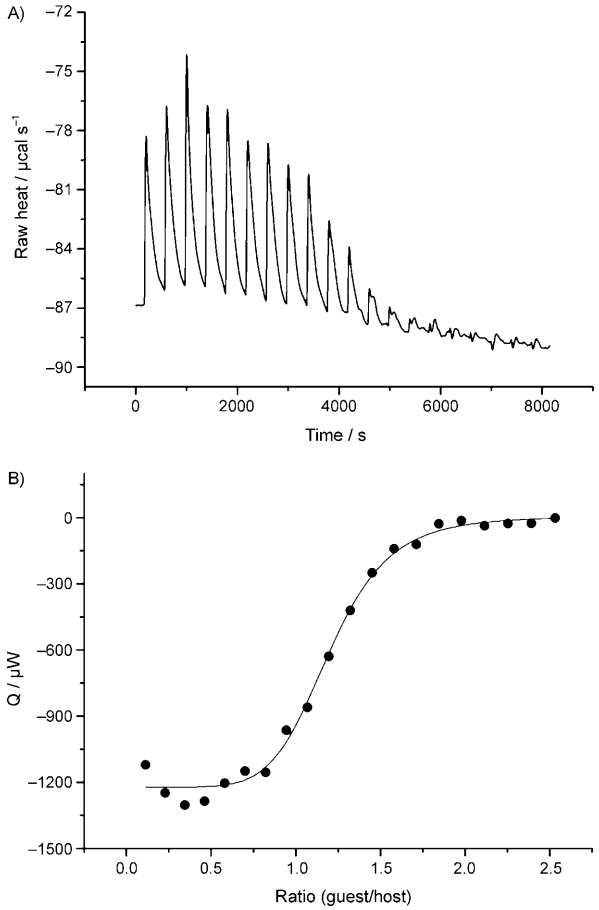

Figure 2. ITC of maltose **1** (10 mM) with β-CD (1.0 mM). A) Injection peaks (raw data vs. time). B) Integration of the injection peaks (heat vs. guest/host ratio).

Table 1. Thermodynamic parameters for the interaction of **1** and **2** with β-cyclodextrin.

Guest	K_a [M^{-1}]	n	ΔH [kJ mol^{-1}]	ΔS [J K^{-1} mol^{-1}]	ΔG [kJ mol^{-1}]
1	4.41×10^4	1.12	−11.8	49.0	−26.3
2	4.02×10^4	1.29	−11.8	48.1	−26.0

The thermodynamic parameters for the titration are close to identical for **1** and **2** and are characteristic for the formation of an inclusion complex of **1** and **2** with β-cyclodextrin. The association constants $K_a = 4.4\times10^4$ M^{-1} for **1** and $K_a = 4.0\times10^4$ M^{-1} for **2** are typical for nonionic adamantane derivatives and β-cyclodextrin. The stoichiometry, n, is slightly higher than 1, which indicates that a 1:1 inclusion complex with some secondary interaction with the tetra(ethyleneglycol) spacer is formed. The complexation is essentially driven by a large positive entropy change. The quality of the titration is somewhat affected by a broadening of the injection peaks that can be explained by the amphiphilic character of **1** and **2**, which might have the ability to form micelles in aqueous media.

Having established that conjugates **1** and **2** form strong inclusion complexes with β-cyclodextrin, we investigated the properties of vesicles of cyclodextrin **3** decorated with **1** and **2**. To this end, a 0.1 mM solution of **1** (or **2**) was added to a 0.2 mM solution of vesicles of **3**. Considering the high association constant, and assuming that **1** and **2** can not (or only very slowly) permeate the vesicle membrane, we reasoned that at these concentrations most of the cyclodextrin cavities on the outside of the vesicles would be occupied with **1** (or **2**), so that the carbohydrates would be displayed in high density at the vesicle surface, but relatively little would be present in solution. Due to the tetra(ethyleneglycol) spacer, the carbohydrates on the vesicle surface should still be available to interact with lectins. For this investigation, we selected known lectins concanavalin A (ConA) and peanut agglutinin (PNA). Both ConA and PNA form tetramers at neutral pH and each lectin has four carbohydrate-binding sites (see the Supporting Information). ConA binds exclusively to glucose, mannose, and their derivatives (including maltose) and shows no significant affinity towards other carbohydrates (such as galactose and lactose). Because ConA binding between the vesicles is mediated by a multivalent instead of a monovalent interaction, it was our hypothesis that the addition of ConA to cyclodextrin vesicles decorated with maltose **1** (not lactose **2**) results in agglutination (aggregation) of the vesicles (Figure 1). On the other hand, PNA binds exclusively to galactose and lactose and their derivatives and shows no affinity towards other carbohydrates (such as glucose and maltose). Because PNA binding between the vesicles is also mediated by a multivalent interaction, the addition of PNA to cyclodextrin vesicles decorated with lactose **2** (not maltose **1**) results in agglutination of the vesicles (Figure 1).

Optical density measurements at 400 nm (Figure 3A) show that spontaneous agglutination of the vesicles occurs in the ternary system of conjugate **1**, β-cyclodextrin vesicles, and ConA. In every other case (lactose **2** instead of maltose **1**, PNA instead of ConA, no conjugate, no lectin, no β-cyclodextrin vesicles), no agglutination is observed. These experiments demonstrate that agglutination is indeed the result of a high density of maltose **1** on the vesicle surface. These experiments also confirm the selectivity of ConA towards maltose. The results of the optical density measurement were corroborated by DLS measurements before and after agglutination (Figure 4). Again, only in the presence of conjugate **1**, β-cyclodextrin vesicles, and ConA does sponta-

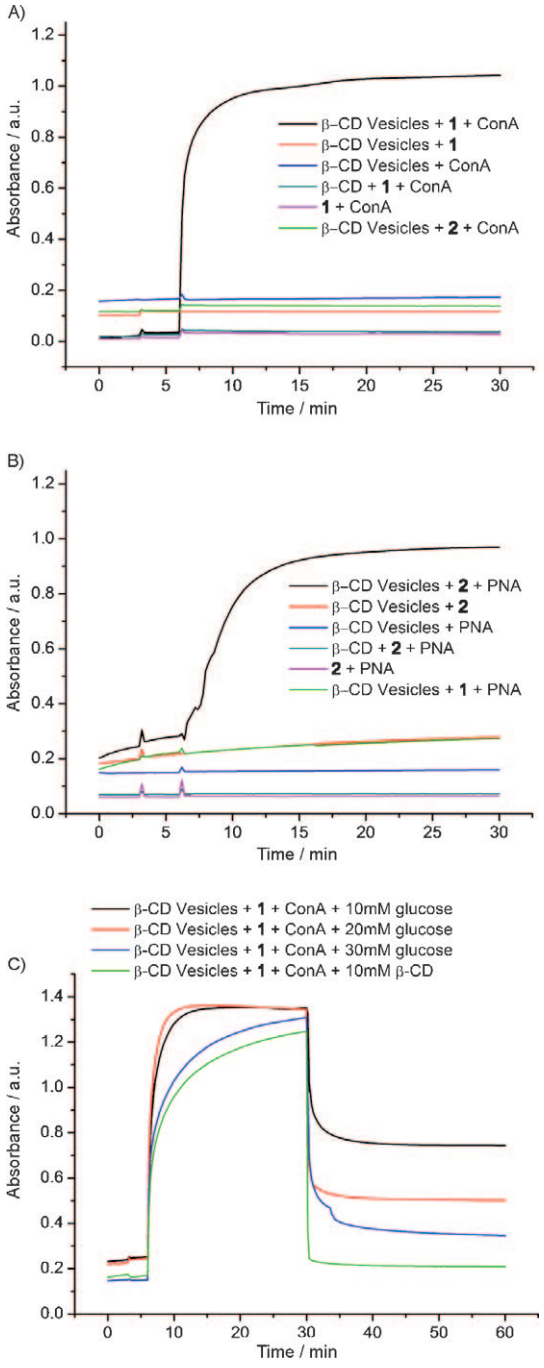

Figure 3. Optical density measurements at $\lambda = 400$ nm. Conditions: [β-cyclodextrin **3**] = 0.2 mM; [maltose **1**] = [lactose **2**] = 0.1 mM; [ConA] = [PNA] = 0.1 mg mL^{-1}; [β-cyclodextrin] = 0.1 mM, 20 mM HEPES buffer (1.0 mM $MnCl_2$ and 1.0 mM $CaCl_2$) at 23 °C.

neous agglutination of the vesicles occur. According to DLS, the average particle size in the vesicle solution increases from about 100 nm to more than 320 nm. In every other case, no aggregation was observed.

Optical density measurements (Figure 3B) also show that spontaneous agglutination of the vesicles occurs in the ternary system of conjugate **2**, β-cyclodextrin vesicles, and PNA. In every other case (maltose **1** instead of lactose **2**,

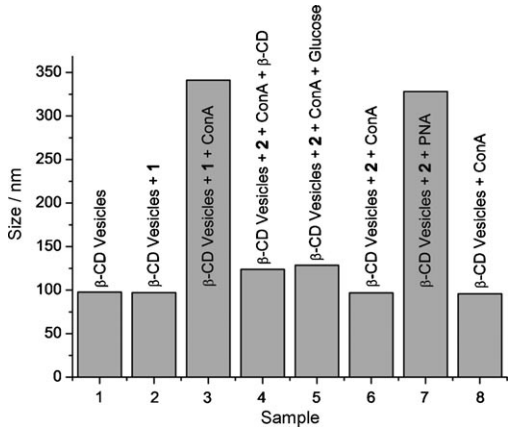

Figure 4. DLS measurements. Conditions: [β-cyclodextrin **3**] = 0.2 mM; [maltose **1**] = [lactose **2**] = 0.1 mM; [ConA] = [PNA] = 0.1 mg mL^{-1}; [D-glucose] = 30 mM; [β-cyclodextrin] = 10 mM, 20 mM HEPES buffer (1.0 mM $MnCl_2$ and 1.0 mM $CaCl_2$) at 23 °C.

ConA instead of PNA, no conjugate, no lectin, no β-cyclodextrin vesicles) no agglutination was observed. These experiments demonstrate that agglutination is the result of a high density of lactose **2** on the vesicle surface. These experiments also confirm the selectivity of the PNA towards lactose. The results of the optical density measurement were corroborated by DLS measurements before and after agglutination with PNA (Figure 4).

To investigate the role of the surface coverage of carbohydrates on the vesicle surface, we also measured the agglutination of cyclodextrin vesicles that were decorated with a mixture of maltose **1** and lactose **2**. The overall carbohydrate concentration was kept at 0.1 mM, but the ratio of maltose **1** and lactose **2** was varied from 100 % maltose and 0 % lactose to 0 % maltose and 100 % lactose. Because both conjugates have similar affinities for cyclodextrin (Table 1), and because the cyclodextrin vesicle concentration was kept constant (0.2 mM), it can be assumed that the surface coverage of carbohydrates on the vesicle is identical to the composition of the mixture added to the vesicle solution. It can be seen from Figure 5 and from additional data in the Supporting Information that the agglutination of the cyclodextrin vesicles by ConA is strongly suppressed when the surface coverage of maltose **1** is less than 50 % (i.e., the coverage of inert lactose is >50 %). On the other hand, the agglutination of the cyclodextrin vesicles by PNA is strongly suppressed when the surface coverage of lactose **2** is less than 75 % (i.e., the coverage of inert maltose is >25 %). These observations confirm that carbohydrates **1** and **2** must be displayed in a rather high surface density to bind the lectins in a multivalent fashion. However, it should be emphasized that the maximum surface density of the carbohydrate guests is limited by the spacing of the rather large β-cyclodextrin host. The average spacing of the cyclodextrins at the vesicle surface is approximately 2.2 nm[9] and the average spacing of carbohydrate guests at the surface at 100 % coverage should be the same. A decrease in surface coverage leads to an increase in average spacing of the carbohydrates.

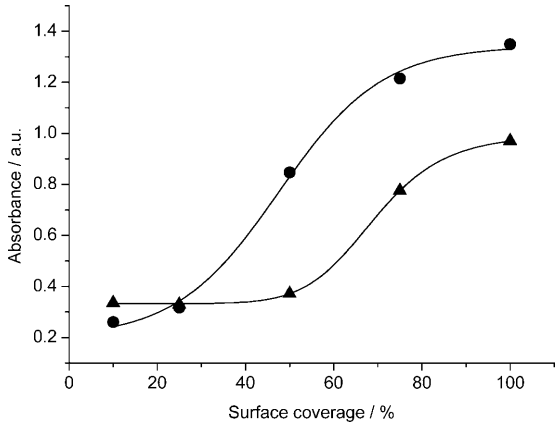

Figure 5. Maximum optical density at $\lambda = 400$ nm as a function of carbohydrate surface coverage for maltose **1**+ConA (●) and lactose **2**+PNA (▲). Conditions: [β-cyclodextrin **3**] = 0.2 mM; [maltose **1**+lactose **2**] = 0.1 mM; [ConA] = [PNA] = 0.1 mg mL^{-1}; 20 mM HEPES buffer (1.0 mM $MnCl_2$ and 1.0 mM $CaCl_2$) at 23 °C.

Once the average spacing exceeds the distance of the carbohydrate-binding sites in the lectin, multivalent binding is no longer possible and agglutination is suppressed. According to a recent study, the effective binding-site separation for ConA binding to a glycosylated bilayer membrane is 3.6 to 4.3 nm.[4m] Indeed, this critical distance is reached at a surface coverage of around 50% of the cyclodextrin vesicles. To the best of our knowledge, there is no comparable measurement of the effective binding-site separation for PNA. From our data, we conclude that the effective binding-site separation of PNA must be significantly smaller than for ConA because multivalent binding is already strongly suppressed when the surface coverage is lower than 75%.

Furthermore, we investigated the reversibility of the aggregation process in the ternary system of cyclodextrin vesicles, maltose **1**, and ConA. If indeed the agglutination of the vesicle is dependent on the formation of a ternary complex, the system must respond to the addition of competitive binders (inhibitors), such as β-cyclodextrin and D-glucose. It can be seen from the optical density measurements in Figure 3C that an excess of β-CD and an excess of D-glucose (each added after 30 min) leads to a rapid disappearance of the vesicle clusters. Interestingly, it is necessary to use a concentration of D-glucose (30 mM) three times higher than β-cyclodextrin (10 mM) to disperse the vesicle clusters. This difference might indicate that the multivalent binding of the carbohydrate to the lectin is stronger and less dynamic than the hydrophobic interaction between the adamantane unit and the β-cyclodextrin cavity. DLS measurements of cyclodextrin vesicles that are first agglutinated with ConA and subsequently exposed to excess D-glucose or β-cyclodextrin (Figure 4) confirm that free vesicles are readily re-obtained. These experiments show that the agglutination of cyclodextrin vesicles decorated with **1** in the presence of ConA is the result of the formation of two orthogonal interactions that can each be selectively suppressed and inhibited by the addition of a competitive binder.

Cryo-TEM imaging was used to verify the results obtained by optical density measurements and DLS. A selection of images is shown in Figure 6. It is evident from Figure 6A (and C) that the cyclodextrin vesicles are unilamellar

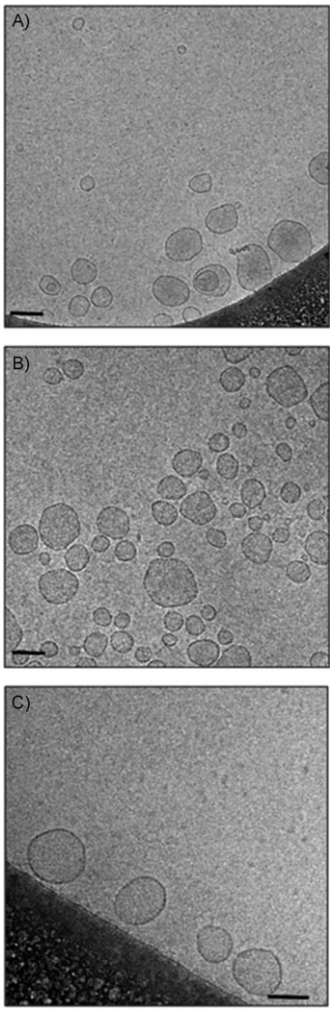

Figure 6. Cryo-TEM pictures of A) cyclodextrin vesicles and B) cyclodextrin vesicles agglutinated with maltose **1** and ConA. C) Dispersal of agglutinated cyclodextrin vesicles upon the addition of 10 mM β-cyclodextrin. Scale bar represents 100 nm. Conditions: [β-cyclodextrin **3**] = 1 mM; [maltose **1**] = 0.4 mM; [ConA] = 0.3 mg mL^{-1}.

spheres that do not approach closely due to steric repulsion from the oligo(ethyleneglycol) residues at their surface. However, in the case of agglutination with maltose **1** and ConA (Figure 6B), it can be seen that the minimum distance between the vesicles substantially decreases and vesicle clustering is observed. The vesicles deform to establish extended areas of contact between the membranes. The distance between single vesicles is about 6 nm throughout the contact areas. Agglutination does not affect an increase or decrease in the size of the vesicles. In other words, the cryo-TEM images do not show any indication of fusion of the vesicles, which is in-line with the biological role of lectins (i.e., lectins mediate adhesion not fusion of membranes). Upon addition

of excess β-cyclodextrin, the vesicles readily dissociate and regain their spherical shape (Figure 6C).

Conclusion

An artificial glycocalix can self-assemble from simple components. In this glycocalix, three carbohydrates are involved in orthogonal noncovalent interactions: β-Cyclodextrin operates as a receptor for adamantane, maltose is a ligand for ConA, and lactose is a ligand for PNA. The addition of lectins induces agglutination (aggregation) of the vesicles without disrupting the vesicle bilayer. Agglutination requires a high carbohydrate density at the vesicle surface. Furthermore, vesicle agglutination is reversed by the addition of competing binders for either of the noncovalent binding sites (cyclodextrin and lectin). We conclude that the artificial glycocalix at the vesicle surface is a dynamic self-assembled system in which several orthogonal noncovalent interactions operate simultaneously. Our work will further the understanding of molecular recognition at cell surfaces, and additionally it will provide new strategies for the hierarchical self-assembly of dynamic soft materials.

Experimental Section

Materials: All chemicals and lectins used in this study were purchased from Acros Organics (Schwerte, Germany) or Sigma–Aldrich Chemie (Taufkirchen, Germany) and were used without further purification. β-Cyclodextrin was kindly donated by Wacker Chemie (Burghausen, Germany). All solvents were dried according to conventional methods before use.

Synthesis: The syntheses of maltose–adamantane conjugate **1** and lactose–adamantane conjugate **2** were carried out by coupling of trichloroimidates of the peracetylated disaccharides maltose and lactose[16] with adamantyl-[(ethoxyethoxy)ethoxy]ethanol.[17] After deprotection with $NaOCH_3$, the target products where obtained. Details of the synthesis are reported in the Supporting Information. The spectroscopic and analytical data for **1** and **2** are consistent with their molecular structure. The synthesis of cyclodextrin **3** was performed as described in the literature.[9,15] All reactions were carried out in oven-dried glassware under an inert gas atmosphere. Analytical TLC was performed on Merck silica gel 60 F_{254} plates. All compounds were visualized by dipping in basic permanganate solution. Column chromatography was carried out by using Kieselgel 60 (230–400 mesh). ^1H and ^{13}C NMR spectroscopic measurements were carried out by using Bruker ARX 300 MHz or Varian 500 MHz INOVA spectrometers. Chemical shifts were referenced to internal standards $CDCl_3$ ($\delta = 7.26$ ppm for ^1H and 77.0 ppm for ^{13}C) or TMS ($\delta = 0.00$ ppm for ^1H and ^{13}C). HRMS was performed by using a Bruker MicroTof instrument.

Methods: Isothermal titration calorimetry (ITC) was performed by using a Nano-Isothermal Titration Calorimeter III (model CSC 5300; Calorimetry Sciences Corporation, London, Utah, USA). ITC measurements were performed in milli-Q water. A 10 mM solution of **1** or **2** was titrated into a 1 mM solution of β-cyclodextrin host. Twenty injections (11 μL) were performed with an interval of 400 s. The stirring rate was 300 rpm.

Unilamellar β-cyclodextrin vesicles with an average diameter of 100 nm were obtained by extrusion of a buffer solution with multilamellar vesicles through a 100 nm polycarbonate membrane in a Liposofast manual extruder.[9,18]

Optical density measurements were carried out in 1 mL disposable cuvettes kept at 23 °C at $\lambda = 400$ nm by using an Uvikon 923 double beam photospectrometer. [β-cyclodextrin **3**] = 0.2 mM, [**1**] = [**2**] = 0.1 mM, [ConA] = [PNA] = 0.1 mg mL^{-1}, [β-cyclodextrin] = 0.1 mM. HEPES buffer (20 mM, pH 7.5) with 1.0 mM $CaCl_2$ and $MnCl_2$ was used for the agglutination assays. Freshly prepared vesicle solutions were used for each measurement.

Dynamic light scattering (DLS) measurements were performed by using a Malvern Instruments Nano-ZS instrument by using low-volume disposable cuvettes kept at 23 °C. [β-cyclodextrin **3**] = 0.2 mM, [**1**] = [**2**] = 0.1 mM, [ConA] = [PNA] = 0.1 mg mL^{-1}, [D-glucose] = 30 mM, [β-cyclodextrin] = 10 mM. A 20 mM HEPES buffer with 1.0 mM $CaCl_2$ and $MnCl_2$ additive was used.

Samples for cryogenic-transmission electron microscopy (cryo-TEM) were prepared by deposition of a few μL of vesicle solution on holey carbon-coated grids (Quantifoil 3.5/1, Quantifoil Micro Tools, Jena, Germany). After blotting the excess liquid, the grids were vitrified in liquid ethane and transferred to a Philips CM 120 cryo-electron microscope equipped with a Gatan model 626 cryo-stage, operating at 120 kV. Micrographs were recorded under low-dose conditions with a slow-scan CCD camera.

Acknowledgements

We are grateful to Ms. Anne Hentschel for her contributions to the glycoside synthesis and the agglutination assays. β-Cyclodextrin was kindly donated by Wacker Chemie. The Deutsche Forschungsgemeinschaft is acknowledged for financial support of this work (grant Ra 1732/1-1).

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Received: September 1, 2009
Published online: January 22, 2010

