

Polymeric vesicles mimicking *glycocalyx* (PV-*Gx*) for studying carbohydrate–protein interactions in solution†

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Received 28th February 2012, Accepted 25th March 2012

DOI: 10.1039/c2py20110k

Glycocalyx, the carbohydrate coat on cell surfaces, has been proved to be particularly important in a variety of biological events. In this work, polymeric vesicle mimicking of *glycocalyx* (PV-*Gx*), as a simplified model system, is achieved *via* our NCCM (non-covalently connected micelles) strategy. Briefly, a thermal responsive poly(*N*-isopropylacrylamide) with phenylboronic acid (BA) end (BA-PNIPAM) and two novel hydrophilic glycopolymers, **PGal** and **PGlc** are prepared by RAFT polymerization, where the latter are prepared from *N*-linked β -pyranoside monomers containing respective units of galactoside (**Gal**) and glucoside (**Glc**). Upon heating PNIPAM-BA and **PGal** (**PGlc**) self-assemble into vesicles **V-PGal** (**V-PGlc**), driven by the dynamic covalent bond between sugars and BA. Both **V-PGal** and **V-PGlc** could serve as our target artificial *glycocalyx* (PV-*Gx*) because they have sugar-coated surfaces and disperse stably in water. Dynamic light scattering (DLS) has been employed to monitor the binding process between the sugars on PV-*Gx* and three lectins *i.e.* *Arachis hypogaea* (PNA), *Erythrina cristagalli* (ECA) and Concanavalin A (Con A), concluding that the PV-*Gx* shows clear specificities in the sugar-protein interactions: **V-PGal** interacts well with PNA and ECA to form aggregates but not with ConA, while **V-PGlc** interacts with none of the lectins. The results clearly prove that the PV-*Gx* constructed from the polymers with well-defined sugar units is a new and promising platform for the study of carbohydrate-protein interactions in solution.

Introduction

Carbohydrates, including polysaccharides and glycoconjugates, are ubiquitous as “cell coats” on the surface of various cells from bacteria to animals as well as human beings. Such carbohydrate coats are referred to as *glycocalyxes*.¹ Molecular recognition between the sugar moieties of glycoconjugates, including glycolipids and glycoproteins, and extracellular proteins has been found to be particularly important for many biological events, such as innate immunity, cellular communication and pathogen invasion.² Some research groups have made great contributions in demonstrating the specificity of this interaction,³ in which different techniques, such as microarray,⁴ surface plasmon resonance (SPR),⁵ enzyme-linked immunosorbent assay (ELISA), isothermal titration calorimetry (ITC)⁶ *etc.*, have been effectively used. However, until now, most research on the carbohydrate-protein interactions has been performed with

individual oligo-saccharides or glycopolymers, which differ from “*glycocalyx*” as the heavy layer of glycoconjugates with high molecular weight and higher density at the cell surface. Thus, the exploration of the carbohydrate-protein interaction on vesicles with a carbohydrate surface in solution is expected to yield a much closer approximation of the living systems. To this goal, polymeric vesicles mimicking *glycocalyx* (PV-*Gx*) have been designed, fabricated and investigated in this paper.

In the past decade, our group has developed a new strategy for constructing polymeric micelles from homo-polymer pairs,⁷ of which the core and shell were non-covalently connected (Non-Covalently Connected Micelles, NCCM). Here, inter-polymer hydrogen bonds,⁸ inclusion complexation⁹ and metal–ligand interactions¹⁰ were employed as the driving forces for NCCM by us and other research groups. Not limited to micelles, vesicles can also be prepared.¹¹ In this work, the NCCM strategy is employed for preparing polymeric vesicles from two polymers, *i.e.* temperature-sensitive poly(*N*-isopropyl acrylamide) with a phenylboronic acid (BA) end (BA-PNIPAM) and a hydrophilic glycopolymer, with carbohydrates as pendant groups. The two polymers self-assemble into vesicles in water governed by the dynamic covalent bond¹² between BA and the carbohydrate when temperature increases above the lower critical solution temperature (LCST) of BA-PNIPAM. The vesicles here are regarded as a simplified model of *glycocalyx*, *i.e.* PV-*Gx* in

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† Electronic supplementary information (ESI) available: Synthesis and characterization of **PGal** and **PGlc** including ¹H NMR, ¹³C NMR and GPC results, as well as MALDI-TOF, details of DLS and SLS results, UV-vis, FLS, FTIR are all available in supporting information. See DOI: 10.1039/c2py20110k

aqueous solution, since there is a high density of sugars on the vesicle surface. Compared to the previously reported sugar-stabilized inorganic nanoparticles,¹³ PV-Gx is a hollow sphere with a rather large diameter, which makes itself an attractive candidate for the study of the specificity of carbohydrate-protein interactions on cell surfaces. Since the hydrophobic polymer and the carbohydrates here are connected by non-covalent bonds, other synthetic glycopolymers, biologically-extracted oligosaccharides or polysaccharides can be introduced for the fabrication of PV-Gx using this method without extra chemical handles.

Although several techniques can detect carbohydrate-protein interactions, some limitations including using fluorescence-labelled proteins, performing detections on solid-liquid interfaces and lacking *in situ* monitoring, may still hinder our knowledge of the real “sweet” binding process on cell surface. Dynamic light scattering (DLS), a classical characterization technique for synthetic polymers and biomacromolecules,¹⁴ is a powerful technique for detecting the size and shape of single chains,¹⁵ their aggregates, assemblies, and networks,¹⁶ as well as the aggregation of charged colloidal particles.¹⁷ In this paper, DLS is found to be effective for the study of carbohydrate-protein interactions on vesicle surfaces in solution by monitoring the aggregation of the vesicles. The technique shows high sensitivity and simplicity as it avoids the use of fluorescence-labelled proteins, which is necessary in most of the existing techniques.

Results and discussion

PV-Gx design and polymer preparation

In designing our PV-Gx, two kinds of original water-soluble polymers, *i.e.* BA-PNIPAM and glycopolymer are designed and prepared. The two polymers are connected together *via* the dynamic bond between BA and the sugar groups. It is well known that PNIPAM is hydrophilic below its LCST but becomes hydrophobic leading to aggregation in water above the LCST. In this process it is expected that PNIPAM and the glycopolymer may self-assemble into NCCM or vesicles with the glycopolymer on the surface. This makes the assemblies stably dispersed in water, which benefits our subsequent investigation, as it is crucial for biological assays. Moreover, vesicles prepared by this method are at rather low concentration, which ensures further biological investigations on samples with limited amount of materials.

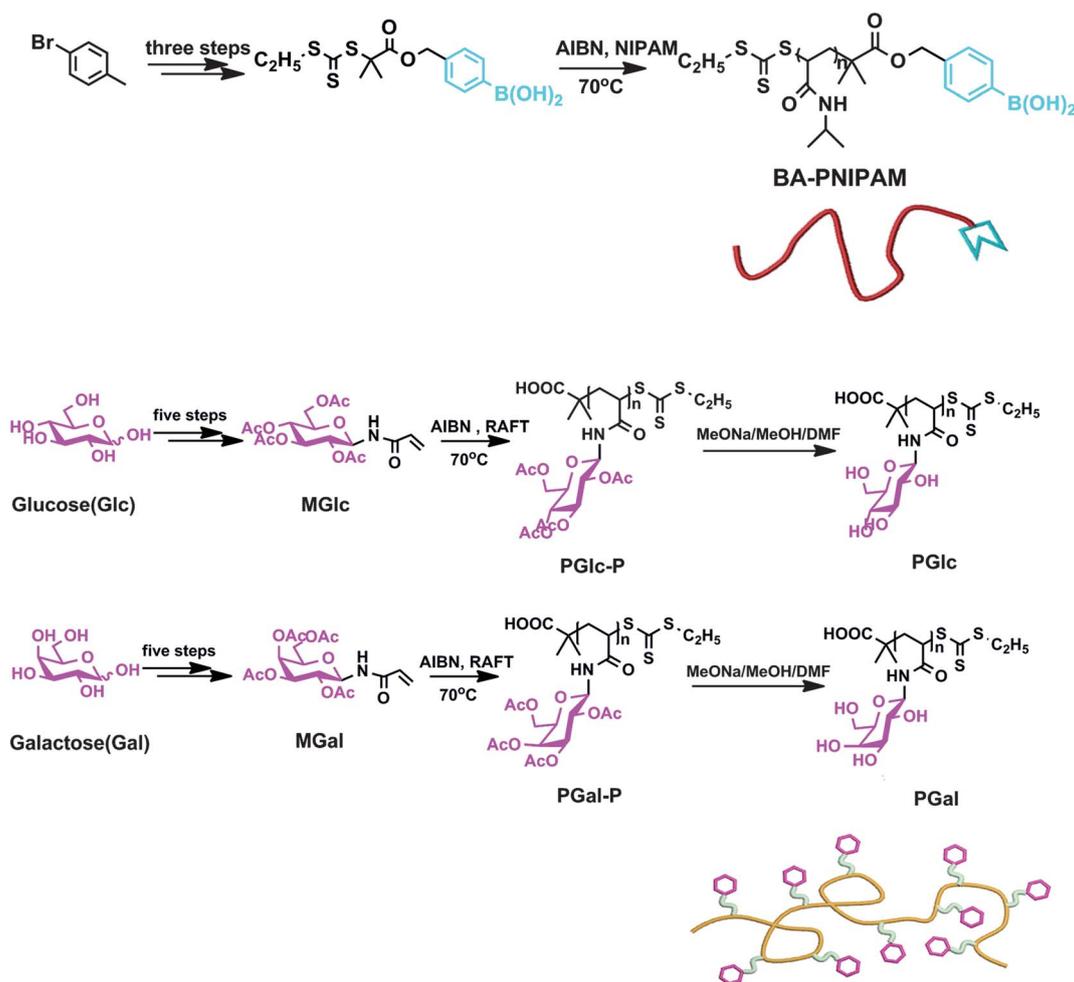
BA-PNIPAM (Scheme 1) with a narrow molecular weight distribution used in this paper ($M_w = 7 \times 10^3$, PDI = 1.10), was prepared by RAFT polymerization (Reversible Addition-Fragmentation chain Transfer polymerization) (Scheme S1, GPC result in Fig. S1) using the chain transfer agent reported by us very recently.¹⁸

Synthetic glycopolymers, recently used for probing the mechanism of multivalent binding between carbohydrates and proteins, are chosen because the carbohydrate-repeating units are introduced in a controllable manner. Generally, there are two strategies to synthesize glycopolymers. Bertozzi *et al.*^{19a} and Haddleton *et al.*^{19b} performed efficient post-polymerization modification using oxime formation and a click reaction, respectively. Other groups directly polymerized glyco-monomers.²⁰ For the purpose of studying carbohydrate-protein

interactions, two facts are worth mentioning: 1) in some cases, glyco-monomers were in α/β - mixtures, or even without anomeric linkage; 2) acetate was widely used as a general protecting group, which could be removed mildly after polymerization. In recent years, to polymerize sugar monomers in a controllable manner, pyranosyl acrylates were introduced,²¹ which were nevertheless labile during the deprotection of acetate, and subsequently reduced the total amount of sugars on each polymer chain. More importantly, based on the knowledge of glycobiology, the specificity of the interactions between carbohydrates and proteins is greatly controlled by the anomeric linkage of different sugars.²² Thus, a clarified anomeric linkage of glyco-monomers is crucial to test the bioactivity of the corresponding glycopolymers. Therefore, in this work, we aim at preparing well-defined and reliable glycopolymers with explicit anomeric linkage *via* polymerization of adequate well defined glyco-monomers. For this goal, instead of acrylate, acryl amide linked to the anomeric carbon of sugars is introduced for glyco-monomers, which is stable during acetate removal. Starting from natural monosaccharides galactose (**Gal**) and glucose (**Glc**), two *N*-linked β -pyranoside monomers, **MGal** and **MGlc** (Scheme 1), were synthesized *via* five steps with overall yield of 35 and 40%, respectively (Scheme S2†, synthetic details and characterizations including ¹H and ¹³C NMR as well as MALDI-TOF MS). The corresponding polymers, **PGal** ($M_w = 1.4 \times 10^4$, PDI = 1.18) and **PGlc** ($M_w = 1.4 \times 10^4$, PDI = 1.20) were prepared by RAFT polymerization followed by deprotection (GPC, ¹H NMR and FT-IR characterizations as well as dn/dc data are in Fig. S2–S6†). It is worth mentioning that although it takes five steps to obtain each monomer, the chemistry is reliable, with a high yield and only the β -pyranosidic bond is formed, which is essential to our further investigation.

Dynamic covalent bond and the “graft-like” complex

With the polymers in hand, we first attempted to prove the formation of the dynamic covalent bond of BA and the carbohydrates on polymer chains. It is known that reversible boron-oxygen cyclic ester bonds can be formed between BA and sugars, as well as other diols in basic solution.²³ In order to characterize this interaction between BA-PNIPAM and **PGal**, a well-known fluorescent dye, Alizarin Red S (ARS) was used as a probe.^{23c} As shown in Fig. 1, ARS is inherently non-fluorescent but gives strong fluorescence when it is mixed with BA-PNIPAM due to its binding as a catechol with BA in alkaline conditions (pH 9). However, as **PGal** was gradually added, the fluorescence intensity of ARS progressively decreased, accompanied by an obvious red shift of the peak (from 578 nm to 614 nm). This proves that the sugar units of **PGal** show a stronger binding ability to BA-PNIPAM than ARS does. Meanwhile, in this process, the colour of the solution changed from orange to purple (the original colour of ARS itself), as observed by the naked eye (Fig. 1, UV-vis spectroscopy in Fig. S7†). Similar phenomena were also detected by adding **PGlc** to the solution of BA-PNIPAM and the dye, which is shown in Fig. S8 and S9†. In addition, the pH-controlled reversibility of the bond between **PGlc** and BA-PNIPAM was demonstrated by fluorescence intensity changes (Fig. S10†).



Scheme 1 Synthetic routes of BA-PNIPAM and glycopolymers, including **PGlc** and **PGal**.

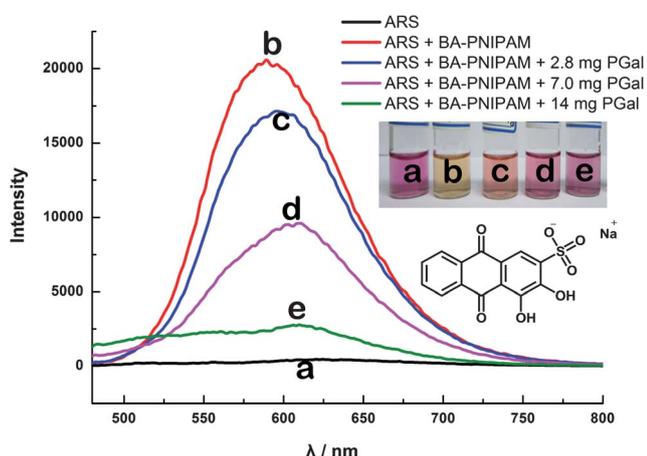


Fig. 1 Fluorescence intensity of ARS (10^{-4} M) with BA-PNIPAM (10^{-3} M) in the absence and presence of **PGal** in 2 mL aqueous solution (pH 9.0). Inset: chemical structure of ARS and photos of the corresponding samples.

As a result of the formation of the BA-sugar dynamic bonding interaction, BA-PNIPAM and glycopolymer formed an interpolymer complex, which was investigated by DLS.²⁴

As shown in Fig. 2a, when BA-PNIPAM is mixed with **PGal** at different molar ratios, the relaxation curve shifts to a longer time than those of both of the component polymers, which indicates formation of the complexes with a larger molecular size. This opinion is supported by the results shown in Fig. 2b which displays the dependence of scattered light intensity on the mass ratio ($m(\text{PGal})/m(\text{PGal} + \text{BA-PNIPAM})$) at a given total weight concentration of **PGal** and BA-PNIPAM at 20 °C. Over the whole composition range, the scattered light intensity of the mixture shows a large positive derivation from the additive values. Considering the interaction site in BA-PNIPAM is solely at the chain end, the resultant soluble molecular conjugates would have “graft-like” complex structure (Fig. 2c).²⁵ The maximum light scattering intensity is observed when the mass ratio of **PGal** and BA-PNIPAM reaches 1 : 2, showing the best apparent complexation ratio. In this condition, the calculated molar ratio of free sugars vs. sugars binding to BA is 11 : 1, implying that a large proportion of the sugars on each polymer chain remain free, which is required by the desired PV-*Gx* structure. Similar results are observed for **PGlc** and BA-PNIPAM, confirming the dynamic covalent bond and complex formation between the two polymers (Fig. S11†).

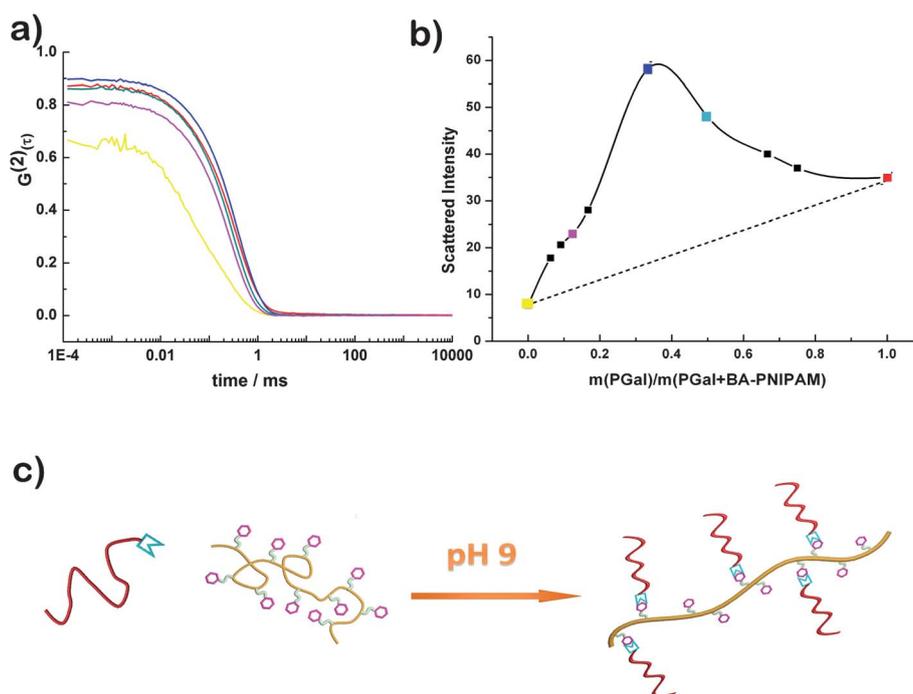


Fig. 2 a) Auto correlation function obtained by DLS analysis ($\theta = 90^\circ$) of BA-PNIPAM, PGal and their mixtures. b) Dependence of the scattered intensity on the mass ratio $m(\text{PGal})/m(\text{PGal} + \text{BA-PNIPAM})$, where the total weight concentration of polymers was fixed to 1 mg mL^{-1} (coloured data were extracted from the corresponding curve in (a); while the curves corresponding to the black dots were not shown). (c) Diagram showing “graft-like” complex formed by free BA-PNIPAM and glycopolymer.

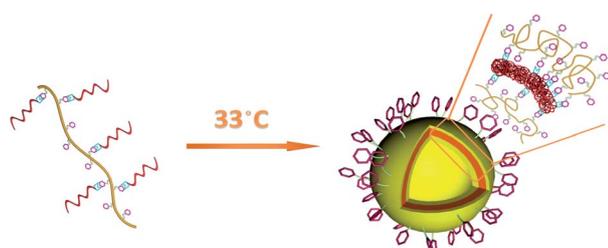


Fig. 3 Diagram showing the “graft-like” complex of BA-PNIPAM and glycopolymer and the thermal-induced assemblies, PV-Gx (V-PGal and V-PGlc). For clarity, glycopolymers at the inner surface of the vesicle are not shown.

Formation of PV-Gx

PNIPAM is a well-known temperature sensitive polymer showing a sharp transition from coil to globule at its LCST around 32°C , which would induce self-assembly of the “graft-like” complex of BA-PNIPAM and PGal or PGlc into nanoparticles. In our experiment, typically, after BA-PNIPAM (0.05 mg mL^{-1}) was mixed with PGal (0.05 mg mL^{-1}) at a lower temperature, forming the “graft-like” complex, the sample was then heated to and kept at 33°C . Uniform nanoparticles with hydrodynamic radius $\langle R_h \rangle < 62 \text{ nm}$ and narrow size distribution ($\text{PDI} = 0.098$) were formed. (Fig S12[†]) The nanoparticles were found to have a hollow structure as their $\langle R_g \rangle / \langle R_h \rangle$ value was 0.97 ($\langle R_g \rangle$, radius of gyration), which is very close to the

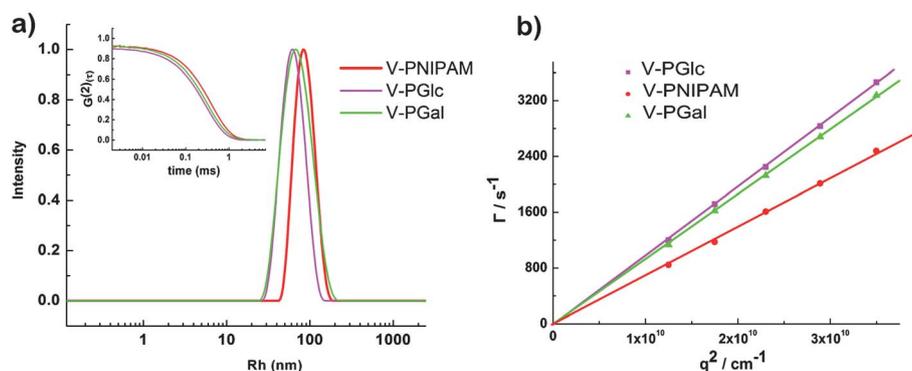


Fig. 4 DLS results of V-PGlc, V-PGal and V-PNIPAM: (a) hydrodynamic radius (R_h) distribution at a scattering angle of 90° (inset) auto correlation functions, and (b) dependence of Γ on q^2 .

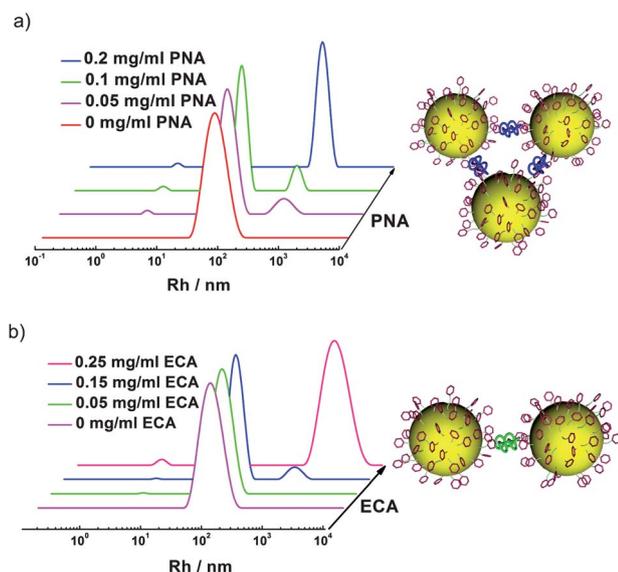


Fig. 5 DLS profiles of vesicle aggregation induced by lectins, when (a) PNA and (b) ECA was titrated into the solution of **V-PGal**, respectively (concentration of lectins for each curve is shown in the figure).

characteristic value of hollow spheres (1.0). This hollow structure was confirmed by transmission electron microscopy (TEM) observations (Fig S13†) and named **V-PGlc**. Similarly, increasing the temperature to 33 °C induced **PGlc** and BA-PNIPAM to form vesicles **V-PGlc** ($\langle R_h \rangle$ 68 nm, PDI = 0.102 and $\langle R_g \rangle / \langle R_h \rangle = 1.05$). Considering that at this condition PNIPAM chains become hydrophobic and glycopolymers remain hydrophilic, we suggest that the resultant vesicles have PNIPAM as the central layer sandwiched by the glycopolymer layers, which enables the sugar units to cover the surfaces. Therefore, both **V-PGal** and **V-PGlc** are our target vesicles mimicking *glycocalyx* (PV-Gx) (Fig. 3). Meanwhile, at a high pH (e.g. pH 9)—the standard condition to form cyclic boronate ester with sugars—BA itself forms tetrahedral boronate with a hydroxide anion, which is also hydrophilic. As the temperature was increased from 20 to 33 °C, BA-PNIPAM (0.1 mg mL⁻¹) alone formed vesicles (**V-PNIPAM**, $\langle R_h \rangle$ 82 nm and PDI = 0.073, $\langle R_g \rangle / \langle R_h \rangle > 0.99$) with the tetrahedral boronates on their surface. The vesicles will be used in the following experiments as a control. $\langle R_h \rangle$ of

V-PNIPAM is larger than that of **V-PGal** and **V-PGlc**, because there are fewer hydrophilic portions in **V-PNIPAM**. DLS analysis also indicates that all the vesicle samples exhibit only one relaxation time and consequently a monomial distribution (Fig. 4a). In addition, the linear variation of the relaxation frequency (Γ) versus the squared scattering vector q^2 passing through the origin is the hallmark of a translational diffusive process (Fig. 4b).

Monitoring carbohydrate-protein interactions

Proteins that have a specific binding ability to carbohydrates are called lectins. In this paper, three different lectins, *i.e.* *Arachis hypogaea* (PNA), *Erythrina cristagalli* (ECA), and Concanavalin A (Con A), are utilized to demonstrate the specificity of carbohydrate-protein interactions on our PV-Gxs by DLS. Lectins may have multiple carbohydrate-binding sites inducing aggregation of PV-Gxs, which could be sensitively detected by DLS. In our experiment shown in Fig. 5a, DLS measurements were performed for solutions of **V-PGal** (0.1 mg mL⁻¹, same for other PV-Gxs) with different amounts of added PNA solution. Every collection of scattered light data lasted at least 500 s and was repeated for each measurement, when the solution was kept inside the light scattering chamber at 33 °C for 1 h before addition of the next portion of PNA. The free **V-PGal** showed a large peak of $\langle R_h \rangle$ around 62 nm. When PNA reached 0.05 mg mL⁻¹, a peak with a larger $\langle R_h \rangle$ around 640 nm appeared, which was obviously attributed to the aggregates of **V-PGal** connected by PNA. $\langle R_h \rangle$ of the aggregated vesicles increased further to 1120 nm at a PNA concentration of 0.2 mg mL⁻¹, where the free **V-PGal** completely disappeared. Similar phenomenon of virus aggregation induced by dendron *via* electronic interaction was recently reported by Kostianen, Cornelissen and Nolte.²⁶ For the case of ECA, as shown in Fig. 5b, a similar process to PNA was observed, but the peak belonging to the aggregated vesicles appeared at a much higher concentration of 0.15 mg mL⁻¹ than that for PNA (0.05 mg mL⁻¹). This is understandable because PNA is a homotetramer—each subunit has one carbohydrate-binding site²⁷—while ECA is a dimer in which each protomer contains one functional carbohydrate-binding site. Although Con A itself has similar size to PNA and ECA as shown in DLS (Fig. S14†), the size distribution of **V-PGal** remains unchanged during the whole titration process of Con A (Fig. 6a), as it only

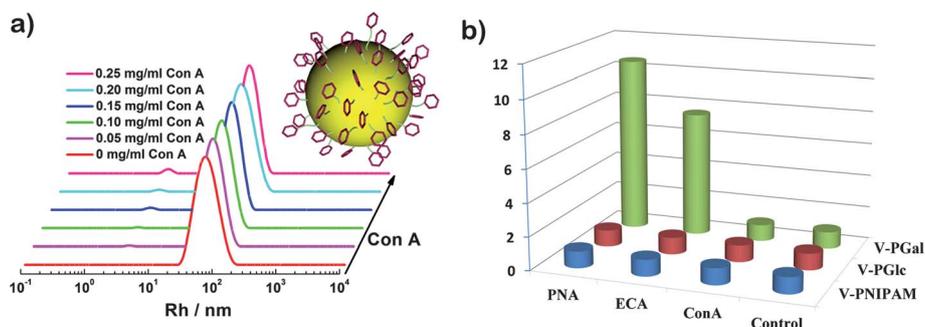


Fig. 6 a) DLS profiles for the titrations of **V-PGal** with Con A (concentration of lectins for each curve is shown in the figure). b) $\langle R_h \rangle / \langle R_{h0} \rangle$ after titration of different lectins (0.2 mg mL⁻¹) to different PV-Gx and **V-PNIPAM** (Control: vesicles were kept without any lectin titration at the same condition).

selectively binds to α -mannopyranoside and α -glucopyranoside.⁶ Furthermore, for the case of **V-PGlc**, the size distribution remains stable when the same amount of the lectin is added. This is because none of the three lectins have the specificity to β -glucopyranoside (Fig. S15–S17†). All the results can be summarised by comparing the ratio of $\langle R_h \rangle / \langle R_{h0} \rangle$ (Fig. 6b), where $\langle R_h \rangle$ and $\langle R_{h0} \rangle$ are the hydrodynamic radius of PV-Gx in the presence and absence of lectins (0.2 mg mL⁻¹), respectively. A dramatic increase of $\langle R_h \rangle / \langle R_{h0} \rangle$ is found in the pairs of PNA/**V-Gal** and ECA/**V-Gal**, while the pairs of ConA/**V-Gal**, and all the lectins with **V-Glc**, as well as the control samples using V-PNIPAM and lectins (Fig. S18–S20†), exhibit unchanged $\langle R_h \rangle$. These results are in good agreement with the reported specificity of protein-sugar interactions, which were achieved using low-molecular-weight sugars and the results of the turbidity test which used disaccharide-decorated vesicles by inclusion complexation.²⁸ We noticed that in the literature, light scattering in the studies of particle aggregation resulted in far more information than $\langle R_h \rangle$ changes, as demonstrated by Borkovec *et al.*¹⁷ This technique is expected to present a deep insight into the binding process between proteins and *glycocalyx* in future studies. In short, our result clearly proved that the PV-Gx self-assembled from polymers with well-defined sugar units has constructed a new and promising platform for the study of carbohydrate-protein interactions and that there is a wide range of unknown binding pairs to explore.

Conclusions

Using the reversible boron-oxygen ester bond and our novel glycopolymer containing β -glucopyranoside and β -galactopyranoside, we successfully prepared non-covalently connected vesicles with sugars as surface layers (PV-Gx). The specificity of the interactions between carbohydrates of the PV-Gx and lectins has been studied *in situ* by monitoring the aggregation process of the vesicles in aqueous solution by DLS. These results are in good agreement with the reported specificity of protein-sugar interactions, which were achieved using low-molecular-weight sugars as well as that from the turbidity test using disaccharide-decorated vesicles.²⁸ Current results also show that the method is fast, reliable and reproducible, and does not require the fluorescence-labelling of proteins. Investigations of further binding pairs of carbohydrate-protein and further data explication of light scattering are currently underway in our laboratory.

Acknowledgements

National Natural Science Foundation of China (No. 20834004 and 20904005) and Ministry of Science and Technology of China (2009CB930402 and 2011CB932503) are acknowledged for their financial supports. G.C. thanks the State Key Laboratory of Bioorganic and Natural Products Chemistry, CAS (No. 10420). Dr Junfang Li (Shanghai Institute of Organic Chemistry, CAS) is acknowledged for his helpful discussions about DLS.

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