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Sugar−Quantum Dot Conjugates for a Selective and Sensitive Detection of Lectins

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One-pot synthesized neoglycoconjugates with a reactive thiol group are introduced here for functionalization with carbohydrates for solubilization and stabilization of CdSe−ZnS quantum dots in aqueous solution. Three different sizes of quantum dots (QDs) with lactose, melibiose, and maltotriose on their surface have been utilized, for the first time, for lectin detection through agglutination assay. The sugar−QDs thus synthesized were characterized by transmission election microscopy (TEM), fluorescence, and absorption spectroscopy. Agglutination of sugar−QDs by three different lectins occurred through specific multivalent carbohydrate−lectin interactions and was studied extensively by monitoring the scattered light at 600 nm. This assay was very selective, which has been demonstrated by a more selective binding of soybean agglutinin (SBA) with melibiose−QD, as compared to lactose−QD, and specific deagglutination caused by α-D-galactose, while α-D-mannose did not show any effect. The detection sensitivity of the maltotriose−QD was tested with Concanavalin A (ConA), and as little as 100 nM of the lectin was detected using light scattering. The detection sensitivity of this protocol has been enhanced considerably by the fluorescence properties of QDs. This agglutination process seems to occur through formation of smaller soluble aggregates, which further associate to form larger aggregates.

INTRODUCTION

Lectins are proteins or glycoproteins of nonimmune origin that bind to mono/oligosaccharides with a high degree of stereospecificity. Since this carbohydrate recognition is very selective and often multivalent, lectins have been implicated in many important functions like cell−cell interactions, homing of leukocytes, host pathogen interactions, biosynthesis and quality control of glycoproteins, immune response, malignancy, and metastasis (*1*, *2*). Lectins also find applications in bioanalytical chemistry and molecular biology (*3*).

Agglutination refers to the process of clumping of cells, such as bacteria or red blood cells, in the presence of an antibody or a lectin (*4*). As this clumping reaction occurs rapidly, agglutination has become an important tool in microbiology and bioanalytical chemistry to identify blood groups, antigens, antibodies, prion proteins (*5*), bacteria such as *S. aureus* (*6*), and metal ions (*7*). Agglutination of erythrocytes has been the preliminary and rapid detection method for lectins since its discovery in the 19th century (*8*−*9*). Hemagglutination assay is qualitative, subjective, requires animal bleeding, and is not very sensitive. Various strategies have been adopted to develop an alternative method for agglutination assay. Although suitably derivatized carbohydrate lysozymes were able to detect nanograms of lectins, this strategy is not a direct method and could not be applied for the determination of the affinities of oligosaccharides (*10*). Carbohydrate-functionalized poly(*p*-phenylene ethynylene) (*11*−*13*) and commercially available Latex particles (*14*) have also been used for agglutination. A few reports of mono- or oligosaccharide functionalized gold (*15*−*16*) and semiconductor (*17*−*18*) nanomaterials have been explored for the study of carbohydrate-binding proteins. Penadés et al., very recently, have reported an excellent account of synthesis and application of various glyconanoparticles published so far in the literature (*19*).

Quantum dots (QDs) are semiconductor nanocrystals, which display very interesting optical properties such as single-wavelength excitation, size-dependent narrow emission, high luminescence, and low photobleaching. These properties make quantum dots very attractive candidates for biolabeling. However, insolubility in water has limited the utility of QDs in bioimaging, thus necessitating work on functionalization and solubilization to exploit QDs in imaging applications. Micellar (*20*), polymer encapsulation (*21*) and covalent coupling to thiol containing hydrophilic functional derivatives (*22*) are the two extreme strategies used frequently for the solubilization and functionalization of CdSe−ZnS QDs. Bioconjugated QDs have been utilized in various fields of in vivo bioimaging including early cancer detection (*23*), multiplexed optical coding (*24*), signal transduction (*25*), sensing (*26*), imaging (*27*), and in Western blot analysis (*28*). Some of the advantages of these nanomaterials in biodetection and biodiagnostics are (i) small size (1−100 nm) and large surface-to-volume-ratio, (ii) physical properties tailored readily according to the end application, (iii) unusual target binding properties, and (iv) structural robustness (*29*).

Here, we report a simple and easy-to-perform detection assay for lectins using a two-step-synthesized, water-soluble, carbohydrate conjugated core−shell CdSe−ZnS quantum dots. This non-erythrocyte agglutination strategy is versatile, reversible, selective, and highly sensitive to lectin detection.

EXPERIMENTAL PROCEDURES

^{1}H NMR spectra were recorded on a Bruker AMX-400 MHz spectrometer, and the mass spectra were recorded on Bruker Daltonics ESI mass spectrometer. The lectins, SBA and Jacalin, were isolated to homogeneity from soybean and jack beans, respectively. Concanavalin A and other chemicals were obtained from Sigma-Aldrich. All the agglutination assays involving

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ConA was prepared in pH 7.2 HEPES (0.1 M) buffer containing NaCl (0.15 M), Ca^{2+}, and Mg^{2+} (10 mM each); SBA and jacalin were prepared in pH 7.4 phosphate salined buffer (20 mM).

Synthesis of Core−Shell CdSe−ZnS Quantum Dots and Surface Modification with Pyridine. Core−shell CdSe−ZnS nanocrystals emitting green (QD-555), orange (QD-589), and red (QD-613) light were synthesized in one pot following a literature procedure (*30*) with slight modification (see Supporting Information).

The surface exchange of TOPO-capped QDs with pyridine was performed by heating a solution of CdSe−ZnS in chloroform with pyridine (three times the volume of chloroform) at 60 °C in an open vial for 3 h. The pyridine solution was precipitated with hexane and centrifuged. The obtained precipitate was redissolved in pyridine, and this stock solution was used for further reactions.

Preparation of Neoglycoconjugates of 2-Aminoethanethiol. For the synthesis of thiol-appended sugar molecules, reducing sugars were reductively aminated with 2-aminoethanethiol. Briefly, 2-aminoethanethiol hydrochloride (125 mg) was dissolved in water (100 μL) in a screw-capped vial (airtight), and pH was adjusted to ∼7.5 (by adding aqueous NaOH solution). To this, $NaCNBH_3$ (40 mg, 0.64 mmol) and a solution of sugar (0.04 mmol) in water (100 μL) were added, and the reaction mixture was heated at 90 °C with stirring. After 1 h, the reaction mixture was cooled to room temperature. The solution was concentrated in speedvac, and the obtained solid was extracted with absolute ethanol (three times) to remove excess starting material. The product was further purified in a Sephadex G-10 column (2 × 17.5 cm) by eluting with water. The products contained ∼5% of compound with a disulfide bond between the product and 2-aminoethane thiol. However, the presence of the disulfide compound is not expected to affect the conjugation with QDs, and hence, no further treatment of these compounds prior to the coupling with QDs was carried out. The compounds were characterized by ^{1}H NMR and ESI-MS.

***β*-D-Galactopyranosyl-(1→4)-(1-deoxy-D-glucityl)-(1→N)-cysteamine 1.** Yield 85%. ^{1}H NMR (400 MHz, D_2O): δ 4.39 (d, J = 7.8 Hz, 1H), 3.9 (m, 1H), 3.85−3.8 (m, 2H), 3.79−3.74 (m, 3H), 3.72−3.65 (m, 3H), 3.64−3.54 (m, 3H), 3.52 (m, 1H), 3.2−2.8 (br. m, 5H). The mass (*m/z*) values calculated for $C_{14}H_{29}NO_{10}S$: 404.16 $(M + 1)^+$, observed 404.1.

α-D-Galactopyranosyl-(1→4)-(1-deoxy-D-glucityl)-(1→N)-cysteamine 2. Yield 88%. ^{1}H NMR (400 MHz, D_2O): δ 4.8 (d, J = 2.76 Hz, 1H), 3.92−3.82 (m, 3H), 3.80−3.73 (m, 3H), 3.73−3.64 (m, 3H), 3.64−3.55 (m, 3H), 3.53 (br. d, J = 8.2, 1H), 3.2−2.8 (br. m, 5H). The mass (*m/z*) values calculated for $C_{14}H_{29}NO_{10}S$: 404.16 $(M + 1)^+$, observed 404.1.

α-D-Glucopyranosyl-(1→4)-α-D-Glucopyranosyl-(1→4)(1-deoxy-D-glucityl)-(1→N)-cysteamine 2. Yield 91%. ^{1}H NMR (400 MHz, D_2O): δ 5.27 (d, J = 3.4 Hz, 1H), 4.99 (d, J = 3.3 Hz, 1H), 4.13 (m, 1H), 3.87−3.84 (m, 3H), 3.75 (m. 2H), 3.72−3.66 (m, 4H), 3.64 (br. d, J = 4.1, 2H), 3.60 (br. s, 1H), 3.59−3.41 (m, 5H), 3.37−3.32 (m, 1H), 3.28 (br. t, J = 9.24 Hz, 1H), 3.15 (br. s, 2H), 2.94 (t, J = 6.34 Hz, 1H). The mass (*m/z*) values calculated for $C_{20}H_{39}NO_{15}S$: 566.21 $(M + 1)^+$, observed 566.2.

Surface Capping of CdSe−ZnS QDs with Neoglycoconjugates. Water solubilization and surface functionalization of CdSe−ZnS was achieved in a single step by covalently coupling QDs with thiol-derivatized sugar molecules. The excess of thiol-appended sugar derivative (**2**, 8 mg) was dissolved in doubled distilled water (50 μL) and DMSO (200 μL) in a microcentrifuge tube. To this solution was added a known concentration of pyridine-capped CdSe−ZnS (QD-589, 2.5 mg) in pyridine (200 μL). The thiol coupling with the ZnS shell of CdSe−ZnS was initiated by adding tetramethylammonium hydroxide (∼5 μL, pH ≈ 10.5) in methanol. The whole mixture was quickly vortexed and centrifuged. The obtained precipitate was resuspended in 50 μL of distilled water and centrifuged (15 000 rpm for 5 min) again. Resuspension and centrifugation were repeated three times to remove excess sugar derivatives. Finally, the precipitate was dissolved in water at pH ≈ 7 (by adding ∼3 μL of 10% AcOH/water) to get a clear solution. The concentration of carbohydrate-capped QD particles was estimated using the extinction coefficients of 0.736×10^5, 3.159×10^5, and 1.531×10^6 M^{-1} cm^{-1} at 350 nm for QD-555, QD-589, and QD-613, respectively (*31*).

For the preparation of a variable number of melibiose per QD particle, a solution of melibiose thiol derivative (20 mg) in 100 μL of water was prepared. From that stock solution, 2, 8, 30, and 40 μL were transferred to four different microcentrifuge tubes and dried. These samples were used for conjugation with QD-589 as described earlier.

Estimation of Concentration of Carbohydrates per Sugar−QD. The concentration of carbohydrate molecules coated on CdSe−ZnS was determined by the phenol-sulfuric acid method using a microtiter plate (*32*). The concentration of sugar molecules in each well was determined by the following procedure. Sugar−QD solution (50 μL) was added to the microtiter plate, followed by quick addition of concentrated sulfuric acid (150 μL) and freshly prepared aqueous phenol solution (5% w/v, 30 μL). The microtiter plate was carefully heated in a water bath at 90 °C. After 5 min, the plate was cooled at room temperature for another 5 min before measuring the absorbance at 490 nm. S−QD solution and sulfuric acid were used as a control. The concentration of sugar (nmol/well) was estimated by comparing the sample absorption with a standard curve. The number of sugar molecules per QD particle was calculated from the ratio of the concentration of sugar and the concentration of CdSe−ZnS QD particles.

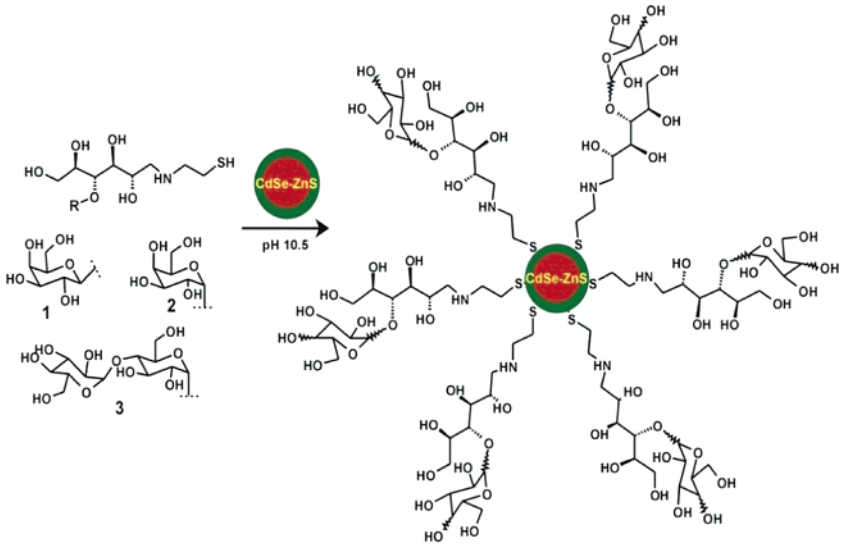
Kinetic Study of Agglutination of S−QDs by Lectins. When the sugar/sweet−QDs (S−QDs) were mixed with lectins, the clear solution slowly turned turbid, and after ∼2 h, the colloidal mass settled down at the bottom of the cuvette or microcentrifuge tube. Since this agglutination reaction occurred through the formation of aggregates of lectins binding to multivalent sugar-dots, we monitored the kinetics of agglutination by measuring the scattered light at 600 nm (in 1 cm cell). For example, a known concentration of lectin in the appropriate buffer was mixed with a solution of S−QD in the same buffer (total volume 120 μL), and the change in absorbance was monitored. An exponential growth curve was obtained as a function of time. The kinetic data were best fitted with $f = y_0 + a(1 - e^{-bx})$ equation, and the apparent rate constants were obtained. At high concentrations of QD and/or lectin, the apparent absorbance increases to a maximum and starts to decrease, which is due to the precipitation of agglutinated product.

Electron Microscope. TEM images were recorded in a TECHNAI F30 instrument with an acceleration voltage of 200 KV. The aqueous sample solutions were loaded in 200-mesh carbon-coated copper grids and stained with 2% uranyl acetate solution. The grids were dried completely before imaging.

RESULTS AND DISCUSSION

Synthesis of Neoglycoconjugates and Functionalization of QDs. The strategy adopted here for the preparation of carbohydrate-functionalized QDs was to first prepare sugar derivatives with reactive sulfhydryl groups and then couple them to CdSe−ZnS quantum dots. Hence, reducing sugars D-melibiose (Gal-1-α-4-Glc), D-lactose (Gal-1-β-4-Glc), and D-maltotriose (Glc-1-α-4-Glc-1-α-4-Glc) were reacted with an excess of 2-amino-

Scheme 1. Schematic Representation of Synthesis of Neoglycoconjugate CdSe−ZnS QD Constructs



ethanethiol hydrochloride salt at 90 °C, and subsequent in situ imine reduction by sodium cyanoborohydride furnished the corresponding carbohydrates with a thiol arm. This synthetic protocol consisted of two steps and therefore has an advantage over some of the other reported methods, where multiple-step, protection, and deprotection strategies were employed (*33*−*36*). Moreover, this neoglycoconjugation method can be applied to higher oligosaccharides as well. In these neoglycoconjugates, the reducing end of the sugar unit became acyclic and the nonreducible part remained cyclic (with retention of stereochemistry). We anticipated that these carbohydrate−thiol derivatives are appropriate molecules not only for the solubilization of quantum dots but also for the functionalization of the periphery in a single step. Furthermore, attachments of two or more sugar−thiol molecules to QDs resulted in the formation of a dendritic architecture with multiple functional carbohydrates on the surface (Scheme 1). The acyclic hydroxylated spacer chain would also reduce the diffusion of oxygen molecules, thereby protecting the CdSe−ZnS dots from surface oxidation. Thus, these multi-carbohydrate nanoparticles have tremendous potential in a number of applications in analytical chemistry.

The covalent coupling reaction of carbohydrate−thiol molecules with three different sizes (555-QD (OD = 3.5 nm), 589-QD (3.8 nm), and 613-QD (4.3 nm)) of CdSe−ZnS QDs was achieved at higher pH (see Experimental Procedures). The sugar−quantum dots were soluble in water at around neutral pH but not above pH 8. The water-soluble sugar−QDs were characterized by TEM, fluorescence microscopy, and absorption spectroscopy. Furthermore, these S−QDs emitted bright green (555-QD), orange (589-QD), and red (613-QD) colors upon excitation (at 400 nm). The number of sugar molecules present on the QD surface was estimated by the simple and very sensitive phenol-sulfuric acid method (see Experimental Procedures). To our expectation, these S−QDs were agglutinated by lectins rapidly and very selectively. The agglutinated product was reversibly deagglutinated/disaggregated by addition of selective carbohydrate molecules. This reversal of agglutination demonstrated that the interaction between the S−QDs and lectin is specific and reversible. The agglutinated products were characterized by TEM (Figure 1).

Effect of the Number of Sugar Molecules per Particle and Size of QDs on Agglutination. Our initial aim was to estimate the stability and agglutinability of various carbohydrate-capped CdSe−ZnS quantum dots. Hence, a series of S−QDs with varying number of melibiose−neoglycoconjugate (MB, **2**), approximately 2, 4, 6, 12, and 18 molecules per QD-589 particle, were prepared by appropriately changing the concentration of **2** (see Experimental Procedures). The ability of 589-MB-QDs to remain stable in solution (without aggregation) was monitored at room temperature and found to be <20, 47, 52, 72, and 180 h for QDs with 2, 4, 6, 12, and 18 molecules of **2** per particle, respectively. Thus, the stability of S−QDs increased upon increasing the number of sugar units. However, all the S−QD solutions were stable for months when kept in the dark at 4 °C. The agglutination efficiency of a variable number of melibiose-coated 589-QD was studied with an excess of Jacalin (533 μM)—an α-galactose specific lectin. A plot of variable numbers of melibiose-containing 589-MB−QDs as a function of the agglutination rate constant showed a linear behavior (Figure 2). Reversal of agglutination occurred when α-D-galactose (14 mM) was added to the agglutinated product of 589-MB−QDs and Jacalin (data not shown). These results suggested that, as the number of melibiose−neoglycoconjugate per QD increased, because of the multivalent nature, a greater number of lectins bound to S−QDs through specific carbohydrate-mediated interaction.

In order to study the effect of agglutination on the size of CdSe−ZnS QDs, 589-QD and 613-QD were surface-modified with **2**. The concentrations of melibiose conjugate and QD were adjusted to get an approximately equal number of average melibiose per unit area of particles of two different sizes of QDs. The mean numbers of melibiose moieties per unit area (percentage) of QD are 34.7 and 35 for 589-MB−QD and 613-MB−QD, respectively. The agglutination assay was performed, keeping concentrations of QD (6.4 μM) and Jacalin (107 μM) constant. The obtained data were fitted, and apparent rate constant values for orange and red emitting MB−QDs are 4.585

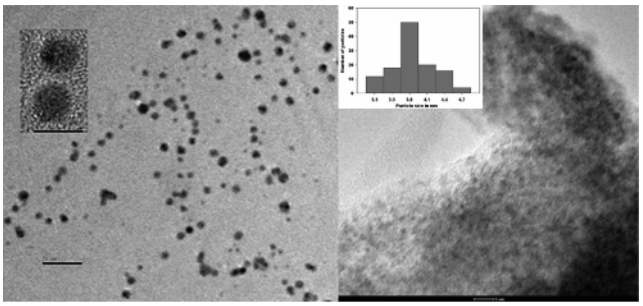


Figure 1. TEM images of melibiose−QD-589 conjugate (left, scale bar 20 nm), inset scale bar 5 nm, and its agglutinated product with jacalin (right, scale bar 5 nm; negatively strained with uranyl acetate). Inset graph shows the histogram of the TEM.

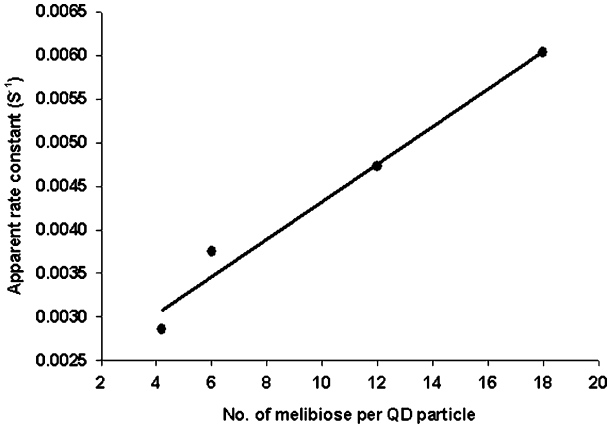


Figure 2. A plot of apparent rate constant of agglutination of 589-MB−QD by Jacalin (533 μM) as a function of number of melibiose molecules per QD particle.

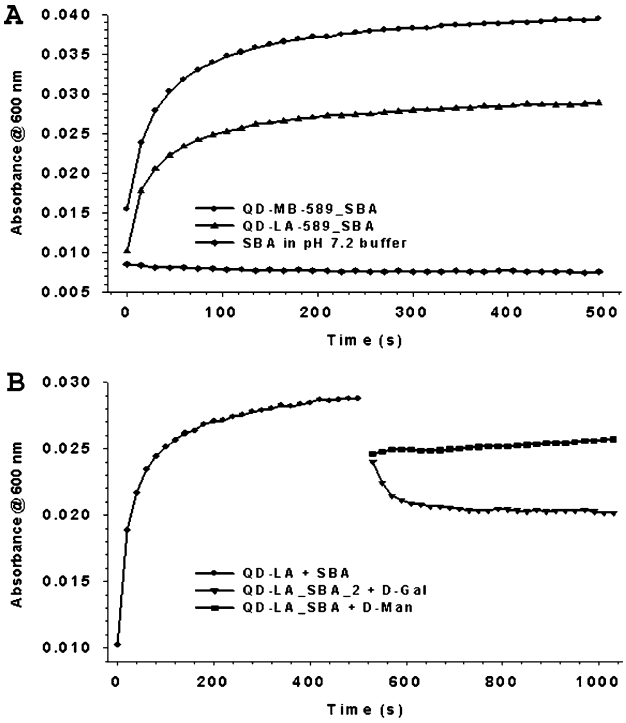


Figure 3. (A) Kinetics of agglutination of 589-MB−QD (5.9 μM particles) and 589-LA−QD (7.1 μM particles) by SBA (5.0 μM). (B) Deagglutination of the 589-LA−QD−SBA agglutinated product by α-D-Gal (100 mM) and α-D-Man (100 mM).

$\times 10^{-3}$ and 12.6×10^{-3} s^{-1}, respectively. Hence, an increase in the QD size appears to increase the apparent rate of agglutination, presumably because of the increase in the surface area and subsequently more accessible carbohydrate units.

Stereospecific Binding of S−QDs with Lectins. In order to assess the stereospecificity of the S−QD based agglutination assay, we studied the agglutinability of 589-MB−QD and 589-LA−QD (α-D-lactose−thiol−QD) with soybean agglutinin (25 μM). The binding constant of SBA with Me-α-Gal is slightly higher (1.2 times) compared to Me-β-Gal (*37*). The agglutination kinetics data of 589-LA−QD (6.8 μM, 6 LA/QD) and 589-MB−QD (6.8 μM, 6 MB/QD) with SBA indeed showed that MB−QD was able to agglutinate slightly more effectively than LA−QD (Figure 3A). The better agglutination observed for 589-LA−QD with SBA was ascertained when only α-D-galactose (7.7 mM) was able to deagglutinate, while α-D-mannose (7.7 mM) did not show any effect (Figure 3B). In order to further

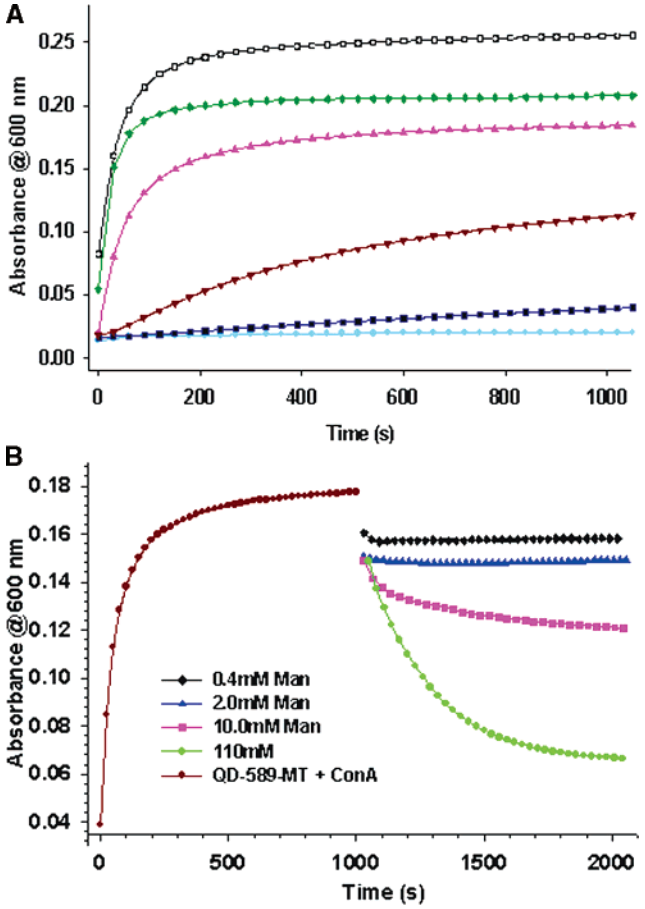


Figure 4. (A) Kinetic study of agglutination of 589-MT−QD (13.0 μM particles) by varying concentrations of ConA (top to bottom: 7.3, 3.6, 1.0, 0.5, 0.2, 0.1 μM). (B) Disaggregation study of agglutinated product of 589-MT−QD (13.0 μM particles) and ConA with variable concentrations of α-D-mannose.

confirm the specificity of the sugar−QDs, an agglutination experiment was performed between lacto−QD/melibio−DQ and ConA, and maltotrio−QD with Jacalin/SBA. In both cross combinations, we did not observe any agglutination. These studies suggest that even after conjugation to QDs the stereospecific binding and selectivity of the neoglycoconjugates toward lectins are retained.

Sensitivity of S−QD Based Agglutination Assay. In order to establish the detection sensitivity of the S−QD assay for agglutination, 589-MT−QD (D-maltotriose−thiol−QD) and a glucose-binding lectin Concanavalin A were studied. Here, the concentration of 589-MT−QD (13 μM, ~10.8 MT per particle) was kept constant, and the ConA concentration was varied from 7.3 μM to 100 nM. As the concentration of ConA decreased, the extent of agglutination also reduced, which is reflected in the intensity of the scattered light. Notably, we were able to follow the agglutination reaction between a very low concentration (100 nM) of ConA and 589-MT−QD by monitoring the change in absorbance at 600 nm (Figure 4A). We have also studied the inhibition or deagglutination by adding a variable concentration of α-D-mannose from 0.4 mM to 110 mM to the preformed agglutinated product of ConA 1.0 μM and MT-QD (13 μM). As the concentration of D-mannose was increased, the degree of deagglutination also increased (Figure 4B). These results again proved that agglutination is due to a very specific interaction between the lectin and the carbohydrates on the surface of QDs.

Sensitive Fluorescence Detection for Agglutination. Although studies discussed so far were primarily based on light

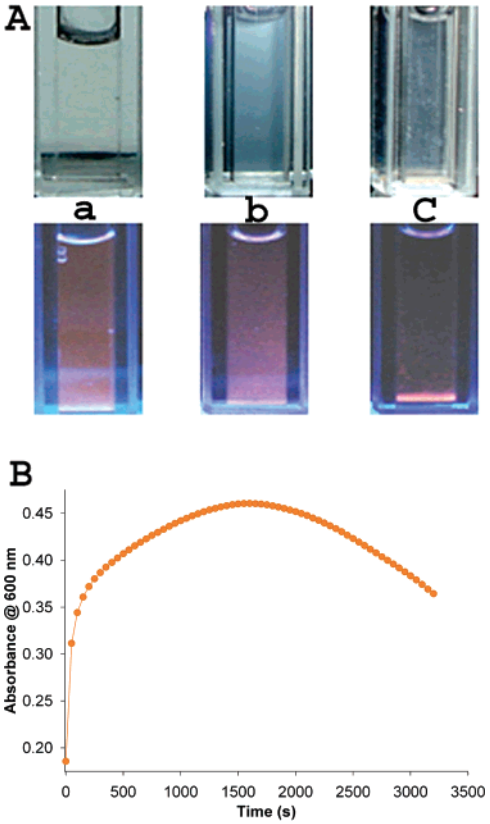


Figure 5. (A) Photographs of (a) 589-MT−QD alone, (b) 5 min after ConA addition (10 μM, pH 7.2 HEPES buffer), (c) 2 h after ConA addition, taken under white (top) and UV light (bottom). (B) Change in the absorbance of 589-MT−QD (0.103 μM particles) immediately after mixing with ConA (5 μM) as a function of time.

scattering data, the use of CdSe−ZnS quantum dots for sugar conjugation and agglutination assay can be rationalized as follows. First of all, the S−QDs are smaller in size. The larger surface-to-volume ratio allows the attachment of a greater number of functional groups. Second, the CdSe−ZnS QDs can easily be functionalized through a variety of chemical and noncovalent methods. In addition, the fluorescence property of QDs allow for a more sensitive detection of the agglutination. Thus, we tried to use fluorescence spectroscopy to study agglutination kinetics and to improve the detection limit of lectins. However, the data obtained from the fluorescence experiments could not be interpreted satisfactorily because of the simultaneous occurrence of various processes, like scattering and precipitation, and so forth, during the course of the experiment. Nevertheless, we went ahead and used a UV transilluminator for visual detection of lectins upon agglutination. In fact, agglutination of MT−QD by <100 nM of ConA was detected under UV irradiation, which was barely seen under normal light. This study thus confirms that sugar−QDs can be used for the detection of very small amounts of carbohydrate-binding proteins with high sensitivity. Furthermore, snapshots of the agglutination at various intervals may reveal insights into the process of agglutination which can be correlated with kinetic studies. The top row of Figure 5A shows the pictures of (a) QD-589−MT solution, (b) QD-589−MT and ConA (10 μM) 5 min after mixing, (c) and 2 h after mixing in a 200 μL fluorescence cuvette under white light, while the pictures at the bottom row are those of respective samples under UV illumination (see Supporting Information for emission spectra). It is quite evident from the above results that the detection of agglutinated products by quantum dot fluorescence has more advantages in terms of detection sensitivity and the ease with which the experiments are conducted.

On the basis of the kinetic studies (Figure 5B), the agglutination process of sugar−QDs by lectins can be hypothesized to occur in a stepwise manner as shown graphically (Figure 6). At first, soluble smaller S−QD−lectin aggregates are formed through specific interaction of carbohydrates and multivalent lectins, which lead to an increase in turbidity and consequently an increase in the scattering. Further association of the smaller aggregates into much larger aggregates induces the precipitation and subsequent decrease in the absorbance at longer time scales as observed experimentally.

CONCLUSIONS

The results reported here demonstrate that di- and oligosaccharides can be covalently attached via thiol functionality to CdSe−ZnS quantum dots without using protection and deprotection in two steps. The water-soluble, sugar-functionalized QDs were selectively agglutinated by lectins, which indicates that the synthesized neoglycoconjugates retain their lectin specificity. The selective deagglutination by specific sugars showed that this process is reversible and selective. The sensitivity of this nanomaterial-based assay was extended to nanomolar concentrations of the analyte—a lectin. Moreso, the value of this method has been enhanced further by using fluorescence properties of QDs. The present work not only has implications in the area of nanomaterials in analytical context but has a broad potential to study various carbohydrate−protein interactions, which are of considerable interest in both medicine and biology.

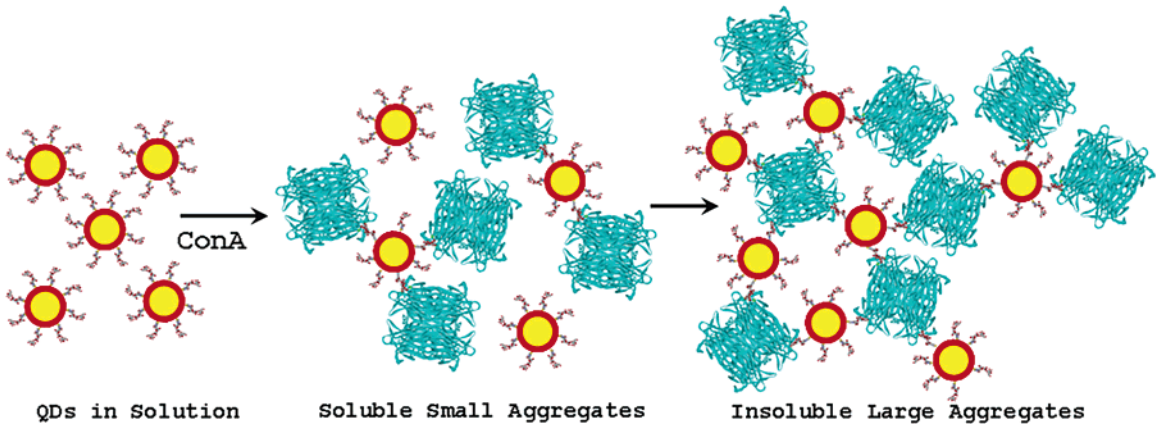


Figure 6. Graphical representation of agglutination of MT−QD-589 by ConA tetramer.

ACKNOWLEDGMENT

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Supporting Information Available: The contents included are (1) procedure for the synthesis of TOPO stabilized QDs, (2) emission spectra of three different sugar−QDs in water, (3) mass spectra of compounds **1**−**3**, (4) comparison of fluorescence spectra of 589-MT−QD in water and after mixing with ConA. This material is available free of charge via the Internet at http://pubs.acs.org/BC.

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