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Design and fabrication of multivalent Gal-containing quantum dots and study of its interactions with asialoglycoprotein receptor (ASGP-R)

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ABSTRACT

Multivalent lactose (Lac-QDs)- and galactose (Gal-QDs)- coated CdSeS-ZnS core-shell quantum dots (QDs) were prepared. The formula of the glyco-QDs was determined by nuclear magnetic resonance (NMR) and inductively coupled plasma-optical emission spectrometry (ICP-OES). The uptake of the Gal-containing glyco-QDs by HepG2 cells was investigated. Flow cytometry (FCM) and fluorescence microscopy analysis indicated that the uptake is receptor mediated and selective. The prepared multivalent glyco-QDs could be used to mimic the oligosaccharides in the study of hepatic endocytosis. Furthermore, this type of glyco-QDs can be used as a useful fluorescent probe in cell imaging and analysis of carbohydrate–protein interactions.

1. Introduction

The asialoglycoprotein receptor (ASGP-R) on hepatocytes plays a critical role in the clearance of desialylated proteins from the serum.[1,2] It is a C-type (Ca^{2+}-dependent) lectin and is responsible for galactose (Gal) or *N*-acetylgalactosamine (GalNAc) terminal glycoproteins from the circulation by receptor-mediated endocytosis.[3] Due to its high specificity, predominant expression on hepatocytes, and important roles in receptor-mediated endocytosis, the ASGP-R has been validated as a potential target for drug and gene delivery to the liver.[4]

In addition to its preference for sugars (GalNAc ≫ Gal), the binding to the ASGP-R strongly depends on the valency of the ligand (tetraantennary > triantennary ≫ biantennary ≫ monoantennary galactosides).[5] As a result, most efforts for the synthesis of high affinity ligands were focused on multivalent ligands, containing primarily GalNAc but also Gal or lactose (Lac) as terminal recognition elements.[6] But some research efforts were also directed toward the discovery of potent monovalent ligands.[7] The synthesis of the multivalent oligosaccharides needs a series of conversion of the protecting groups and purification by chromatography. Furthermore, the bioassay is often through fluorescence labeling, but the organic dyes (e.g., Alexa Fluor® 488 and FITC) used can be easily bleached and are not suitable for long time observation.

Recently, the use of oligosaccharide quantum dots (glyco-QDs) to mimic the polysaccharides on the surface of the cell and as a fluorescence probe opens new opportunities for studying the carbohydrate–protein interactions.[8,14,15] Seeberger and co-workers[9] have shown that Gal-capped PEGylated (PEG2000) QDs are preferentially taken up via asialoglycoprotein receptor (ASGP-R)-mediated endocytosis in vitro. Moreover, they demonstrated in the mouse model that QDs capped with D-mannose and D-galactosamine are sequestered specifically by the liver. PEG2000 was introduced to prevent cytotoxicity and increase the water solubility. However, the preparation of oligosaccharides with a PEG-linker is complex and needs multi-steps synthesis. Moreover, the characterization of the glyco-QDs is also difficult.

We previously reported a convenient fabrication technique for glyco-QDs, in which 1-thiol sugar without a spacer was used as a functional assembly molecule to coat the QD surface.[10] Moreover, we have developed a strategy using Nuclear Magnetic Resonance (NMR) and Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) to analyze the coated polysaccharide molecules and the inner structure of the core and shell of the glyco-QDs. The biological assay showed that the lectin binding property of the polysaccharide on the glyco-QDs is maintained and this strategy can also dramatically enhance their binding activity through cluster effect.[11] This type of glyco-QDs is not toxic to human cancer cells (Hela cell) and normal cells (Human Umbilical Vein Endothelial Cells) even at high concentrations of 0.5 mg/ml and 0.05 mg/ml.

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Herein we also used ternary core/shell CdSeS/ZnS QDs to construct multivalent galactose-containing derivatives Lac-QDs (**1**) and Gal-QDs (**2**). Although GalNAc has stronger binding affinity to ASGP-R than galactose, most researches used lactose and galactose because of their availability. Using NMR and ICP-OES, we determined that about 157 galactose or 150 lactose were coated on the QDs. The selective uptake of glyco-QDs by HepG2 and its interaction with the ASGP-R were investigated using Flow cytometry (FCM) and fluorescence microscopy. The experiments showed that the galactose-containing glyco-QDs can selectively bind to HepG2, and the agglutination of the glyco-QDs after endocytosis could be clearly visualized under a microscope.

2. Experimental

2.1. Materials and methods

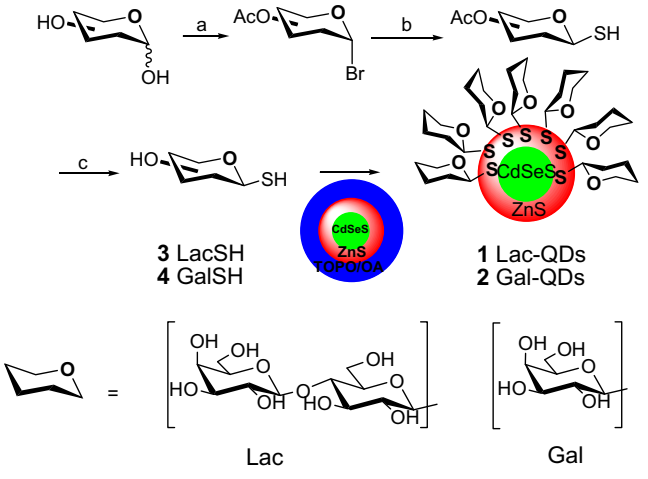
All starting materials, reagents, and solvents were obtained from commercial suppliers and used as supplied without further purification. NMR spectra were recorded on a Bruker AMX-400 (400 MHz) or JEOL-300 (300 MHz) spectrometer. Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) was recorded on a Thermo-iCAP 6000 spectrometer. Nikon E200 microscope was used to study the adhesion and receptor-mediated endocytosis. The uptake of glyco-QDs was measured by Flow Cytometry (Guava EasyCyte Mini System) and analyzed with the Guava ExpressPro software. Hepatocellular carcinoma cell line (HepG2) was obtained from Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, China).

2.2. Preparation of QDs

Ternary core/shell CdSeS/ZnS QDs capped with trioctylphosphine oxide (TOPO) and oleic acid (OA) were prepared according to our previous report with minor adjustment of the amount of Se.[10] Green (λ_{em} = 534 nm) QDs were synthesized and characterized by ICP-OES. Transmission Electron Microscopy (TEM) revealed that the QDs have a diameter of 4.5 ± 0.5 nm (see Supplementary data).

2.3. Preparation of GalSH and LacSH

1-Thiol sugar LacSH (**3**) and GalSH (**4**) were synthesized with three steps from lactose[10] and galactose[12] (Scheme 1). No purification was necessary for each step (Supplementary data).



Scheme 1. Synthesis of GalSH and LacSH. Reagents and conditions: (a) AcBr/MeOH, Ac_2O/AcOH, 95%; (b) (i) NH_2CSNH_2; (ii) $Na_2S_2O_5$, 50%; (c) NaOMe, MeOH, 98%.

2.4. Preparation of Gal-QDs and Lac-QDs

The exchange of TOPO/OA-capped QDs with **3** and **4** were carried out by phase transfer reaction between chloroform phase and aqueous phase of phosphate-buffered saline (PBS) (Scheme 1). QDs (10 mg) were dispersed in chloroform (10 ml), and 200 mg 1-thiol sugar was dissolved in PBS and then added in the reaction system. The mixture was stirred at room temperature until the luminescent QDs were fully transferred from the chloroform phase in the bottom to the upper PBS layer. The upper aqueous layer was purified with Sephadex G-70 and lyophilized to give a yellow powder as the final product.

2.5. Determination of the weight of Gal and Lac on QDs

^{1}H NMR of Lac-QDs **1** (400 MHz, 5 mg in D_2O with 1 μL acetonitrile as internal standard): δ 1.94 (s, 8.37H, CH_3CN); 3.12–4.08 (m, 12H); 4.33 (d, 1H, J = 8 Hz, H-1′); 4.48 (d, 1H, J = 10 Hz, H-1). LacS was calculated to be 48.7% of the total weight.

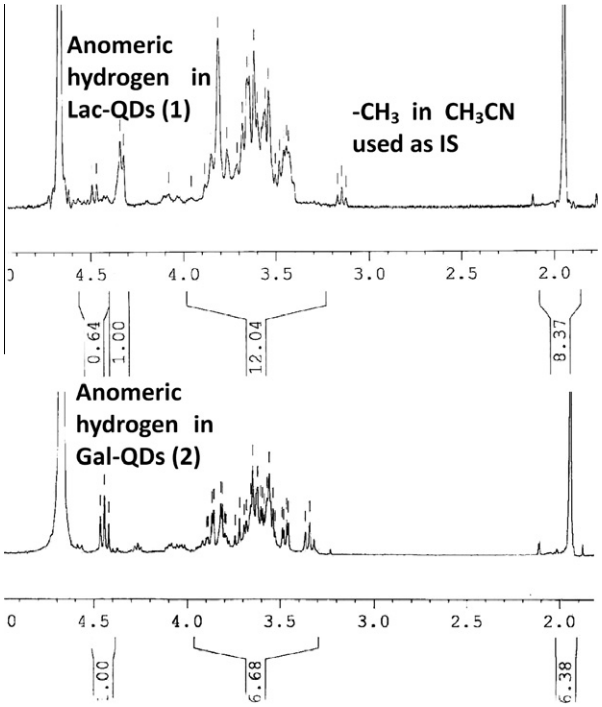


Figure 1. ^{1}H NMR of **1** and **2**.

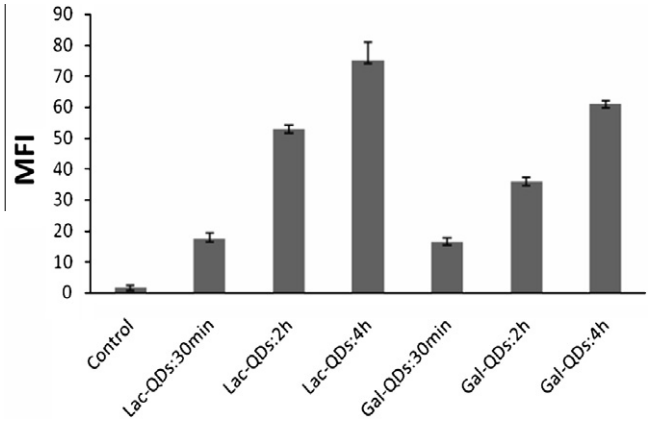


Figure 2. Uptake of LacSH- and GalSH-coated glyco-QDs by HepG2 cells. PBS was added to the HepG2 cells as negative control.

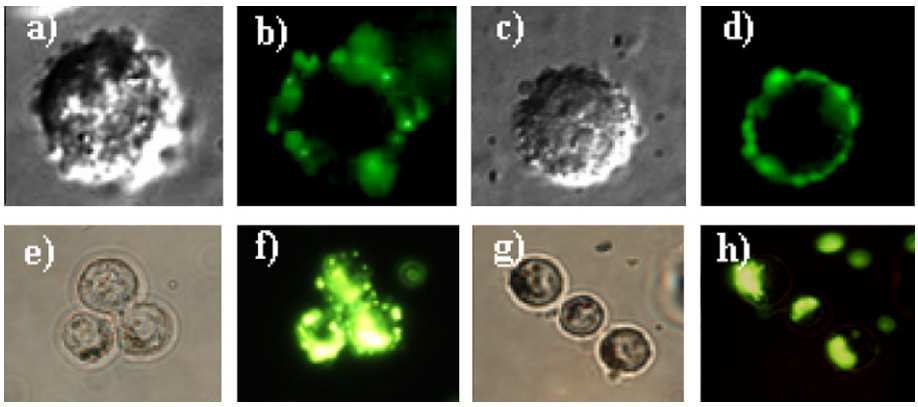


Figure 3. HepG2 cells were incubated for 0.5 h with 40 μM of Lac-QDs or Gal-QDs. (a), (b) Lac-QDs bind to HepG2; (c), (d) Gal-QDs bind to HepG2. HepG2 cells were incubated for 4 h with 40 μM of Lac-QDs or Gal-QDs. (e), (f) Lac-QDs taken up by HepG2; (g), (h) Gal-QDs taken up by HepG2.

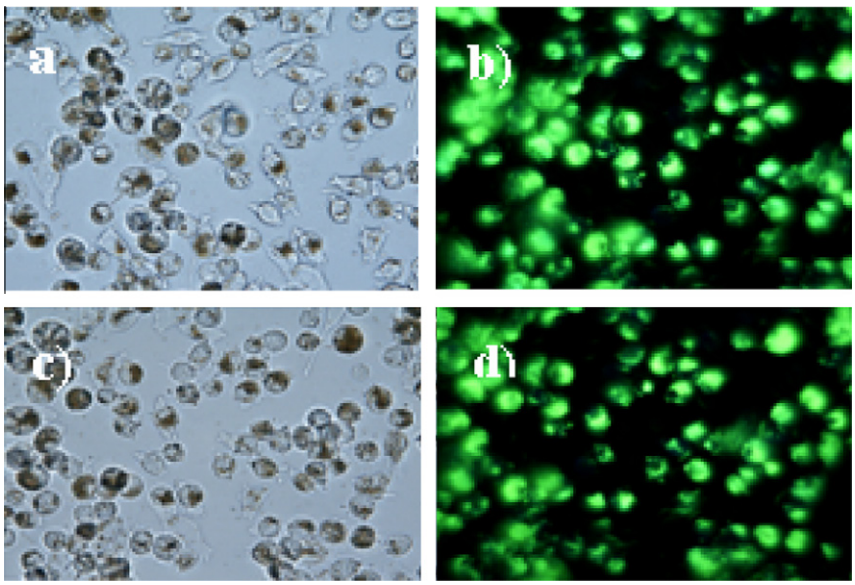


Figure 4. HepG2 cells were incubated for 12 h with 40 μM Lac-QDs or Gal-QDs. (a), (b) Endocytosis of Lac-QDs by HepG2; (c), (d) endocytosis of Gal-QDs by HepG2.

^{1}H NMR of Gal-QDs **2** (400 MHz, 5 mg in D_2O with 1 μL acetonitrile as internal standard): δ 1.95 (s, 6.38H, CH_3CN); 3.34–3.89 (m, 6H); 4.44 (t, 1H, J = 8.4 Hz, H-1). GalS was calculated to be 35.3% of the total weight.

2.6. Uptake of glyco-QDs by HepG2 cells in vitro

2.6.1. Flow cytometry

HepG2 cells were cultivated in DMEM culture medium. Cells were collected at the concentration of 10^5/ml. **1** and **2** (40 μM) were added to the cells. After incubation for 0.5 h, 2 h, and 4 h, the cells were washed three times with cold PBS. The uptake of glyco-QDs was measured by flow cytometry.

2.6.2. Fluorescence microscopy

After the washing step, the cells were transferred onto glass slides for bright field images and fluorescent imaging.

3. Results and discussion

3.1. Design of the glyco-QDs

It has been shown that nanoparticles that are comparable in size to proteins and coated with protein-binding ligands may be effective at disrupting protein/protein interactions.[13] Particle-based displays of multiple ligands can create a high local concentration of binding molecules (called statistical effect). Consequently, binding equilibrium between a surface-bound ligand and receptor favors the formation of ligand–receptor pairs (called receptor clustering effect).

Our previous work showed that the removal of the linker did not affect the binding property of the glyco-QDs. On the contrary, the directly coated glyco-QDs showed higher affinity to its receptor than that coated via a PEG (n = 3) linker. Based on these principles, we design our multivalent glyco-QDs **1** and **2** as a fluorescent probe to investigate their interactions with ASGP-R.

3.2. Determination of the number of sugar molecules on QDs

Traditionally the amount of the carbohydrate on nanoparticles is chemically analyzed by phenol–sulfuric acid method.[9,14] However, the procedure is complex and the result is strongly affected by the heating time and the duration of the experiment. To quantitatively determine the weight of sugar on QDs surface, we developed a facile strategy using NMR. Acetonitrile was used as the internal standards (IS). By peak integration we calculated that about 6.8 μmol of LacSH or 9 μmol GalSH was loaded onto the QDs surface. Together with the ICP-OES and the calculated size of the QDs and 1-thiol sugar,

we calculated that the formula of **1** was $[Cd_{171}Se_{12}S_{356}\text{-}(ZnS)_9]@(LacS)_{150}$ and **2** was $[Cd_{171}Se_{12}S_{356}\text{-}(ZnS)_9]@(GalS)_{157}$. The number of the sugar molecules on the QDs surface was in agreement with the previous reports for the carbohydrate-encapsulated 5 nm diameter QDs[15] and 6 nm gold nanoparticles.[16]

^{1}H NMR of **1** and **2** was basically similar to that of LacSH and GalSH. Sometimes, the anomeric hydrogen of the glyco-QDs became multiple peaks, because of the instability of nanocrystals coated with hydrophilic thiols (Fig. 1).[17] The luminescence emission wavelength of the glyco-QDs was around 534 nm (30 nm, full width at half height, Supplementary data).

3.3. Uptake of glyco-QDs by HepG2 cells in vitro

Two types of cells, Hela cells that do not express the ASGP-R[18] and HepG2 cells, were used to study the association (binding and uptake) with the glyco-QDs. At first the ASGP-R-mediated uptake of compounds **1** and **2** was quantitatively evaluated by flow cytometry. The cellular uptake of the glyco-QDs was evaluated by comparing the shift in median intensity of fluorescence (MFI) between untreated cells (background fluorescence) and treated cells. The MFI of cells incubated with compounds **1** and **2** at hours ranging from 0.5 to 4 h indicated the uptake of the glyco-QDs into HepG2 cells, but no uptake by Hela cells was observed (data not shown) (Fig. 2).

Fluorescence microscopy was used to assess the binding of **1** and **2** with the cell surface. Binding of **1** and **2** with HepG2 cells was observed after 0.5 h (Fig. 3). To determine if endocytosis of **1** and **2** was involved, cells after 4 h and 12 h treatment were also observed. It is clear that **1** and **2** were taken into the cells through endocytosis after 12 h (Fig. 4). It was observed under the bright field that the glyco-QDs were agglutinating in the cells after endocytosis.

A number of studies have indicated that ASGP-R-based recognition is highly dependent on spatial presentation. In other words, the Gal residues connected by flexible spacers with appropriate lengths are needed. It is important to note that these results are based on small molecules, in which the sugar density is limited by the conjugation points of the linker. In our study, we used nanoparticle as the fluorescent anchor, which allows the sugar molecules to be more densely coated on the surface of the glyco-QDs. Because the exact mechanism of the interactions of multivalent glyco-QDs with proteins is not yet clear, we further reasoned that the sugar molecules on the glyco-QDs might be assembled in different orientations, which can provide a variety of spatial presentations for the interactions with ASGP-R.

4. Conclusion

In this study we prepared fluorescent and multivalent Gal-containing glyco-QDs as ligands for the ASGP-R. Using flow cytometry, we have shown that compounds **1** and **2** exhibit selective uptake via the ASGP-R by HepG2 cells. The formation of distinct endocytic vesicles was clearly visualized under a fluorescence microscope. QDs represent a valuable carrier to assemble sugars to exhibit maximal multivalency effects. The convenient synthesis and characterization of the glyco-QDs allow the study of various carbohydrate–protein interactions.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.06.002.

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