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In Vitro Imaging and *in Vivo* Liver Targeting with Carbohydrate Capped Quantum Dots

Raghavendra Kikkeri, Bernd Lepenies, Alexander Adibekian, Paola Laurino, and Peter H. Seeberger*

Laboratory for Organic Chemistry, Swiss Federal Institute of Technology (ETH) Zurich, Wolfgang-Pauli-Str. 10, 8093 Zurich, Switzerland

Received September 30, 2008; E-mail: seeberger@org.chem.ethz.ch

Quantum dots (QDs) are versatile inorganic probes with unique photophysical properties, including narrow and size-dependent luminescence with broad absorption spectra.[1] Surface functionalized QDs containing biomolecules such as DNA, proteins, and small molecules promise applications in cellular imaging, drug delivery, and as nanosensors.[2] Carbohydrate capped QDs can be used as building blocks for biocompatible materials. The immobilization of carbohydrates on the QD surface relies on electrostatic interactions, a template system, or a short thio-spacer between carbohydrates and the QD surface.[3] The cytotoxicity of the colloidal core of QDs necessitates an appropriate coating for *in vitro* or *in vivo* applications. PEGylated QDs provide an efficient alternative to previous approaches in preventing cytotoxicity. The hydrophilic nature of PEG increases the water solubility of QDs and does not interfere with biological studies.[4]

Here, we report a simple and convenient method to prepare different sugar-capped PEGylated QDs **1**, **2**, and **2a** that can be used for *in vitro* and *in vivo* applications (Figure 1). Furthermore, we show that QDs capped with D-galactose are preferentially taken up via asialoglycoprotein receptor (ASGP-R)-mediated endocytosis *in vitro*. The uptake of Gal-capped QDs can be partially inhibited by knockdown of ASGP-R1. Moreover, we demonstrate in the mouse model that QDs capped with D-mannose and D-galactosamine sequester specifically in the liver. D-Galactosamine is a known hepatotoxin leading to hepatocyte necrosis.[5] Upon i.v. injection of GalN-capped QDs we measured a significant increase in serum transaminases indicating that liver injury was selectively mediated by GalN-QDs. While the use of carbohydrate-capped QDs for *in vitro* applications has been described before,[4e,6] we report here that carbohydrate-capped QDs can be exploited for *in vivo* targeting as well.

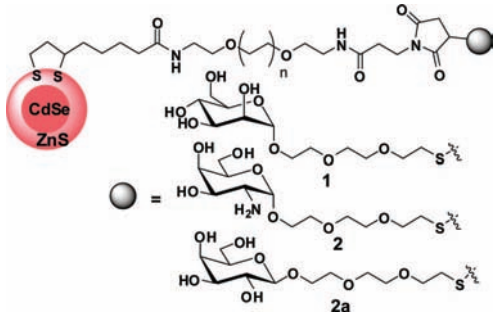
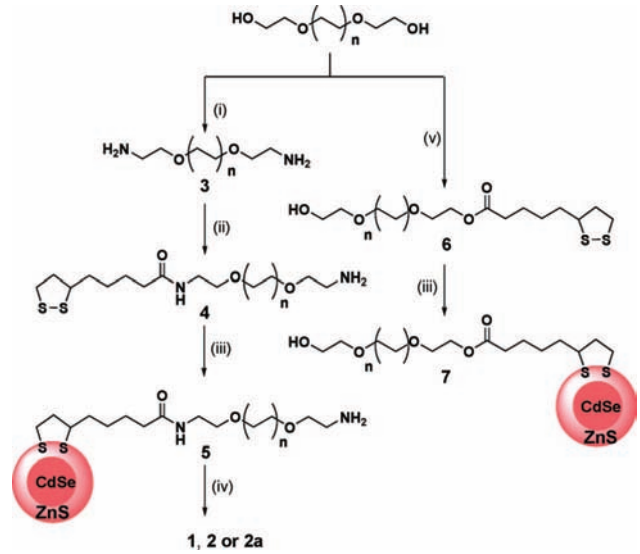


Figure 1. Quantum dots and sugars used in this study ($n = 45-50$).

Thiols have served primarily to anchor substrates onto QDs.[2] Recently, dithiols such as dihydrolipoic acid (DHLA) have been used to prepare PEG-QDs that are stable in aqueous solution and suitable for live cell imaging.[7] CdSe/ZnS core glyconanospheres were prepared starting from PEG_{2000} that was treated with methylsulfonyl chloride, sodium azide, and triphenyl phosphine to yield diamino-PEG_{2000} **3** (Scheme 1). Monoacylation of **3** by treatment with stoichiometric amounts of thioctic acid *N*-hydroxysuccinimide (NHS) ester gave TA-PEG_{2000}-NH_2 **4**. Reductive ring opening of **4**, followed by ligand exchange with tri-*n*-octyl phosphine/tri-*n*-octyl phosphine oxide (TOP/TOPO)-capped QDs afforded QD-PEG_{2000}-NH_2 **5**. The terminal amine was further reacted with 4-maleimidopropanoic acid NHS ester, before treating with mannose and galactosamine linked thiols to yield carbohydrate-capped QDs **1** and **2**, respectively. PEG_{2000}-capped QDs **7** were prepared from PEG_{2000} as control compounds by monoesterification with thioctic acid, reduction of **6**, and ligand exchange with TOP/TOPO-capped QDs. The glyco-QDs were physicochemically characterized by UV−vis and fluorescence spectroscopy, as well as TEM imaging (see Supporting Information, SI).

Scheme 1[a]



[a] Reagents and conditions: (i) MsCl, TEA, NaN_3, 12 h; Ph_3P, H_2O, 12 h; (ii) Thioctic acid, DIC, NHS, **3**, 12 h; (iii) $NaBH_4$, MeOH/H_2O; CdSe/ZnS, EtOH; (iv) 4-maleimidopropanoic acid NHS ester, pH 8.5; **9**, **11**, or **16** (see SI), pH 7.5; (v) DL-thioctic acid, DCC, DMAP, 12 h.

The asialoglycoprotein receptor (ASGP-R) is a glycoprotein that binds specifically to desialylated (galactosyl-terminal) glycoproteins and is expressed exclusively in hepatic parenchymal cells.[8] To analyze the specific uptake of QDs capped with galactose, we employed the hepatocellular carcinoma cell line HepG2 that expresses ASGP-R, but not the mannose receptor.[9] Flow cytometry after 2 h of incubation of the cells with QDs revealed that Gal-capped QDs were taken up by the HepG2 cells preferentially over PEG_{2000}-capped QDs **7** (Figure 2). The uptake of Gal-capped QDs

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could be inhibited partially by preincubation of HepG2 cells with poly-L-lysine-galactose polymer **18** (SI). This finding indicates that Gal-capped QDs are internalized by the HepG2 cells by receptor-mediated endocytosis.

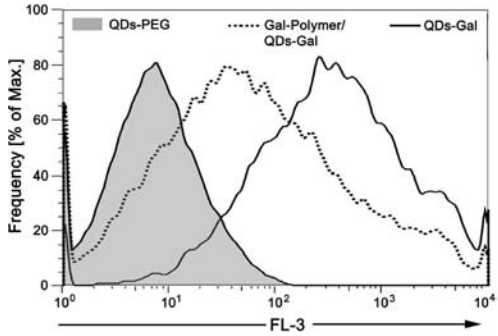

Figure 2. Specific uptake of D-Gal capped QDs by HepG2 cells. HepG2 cells were incubated for 2 h with either 20 nmol of PEG_{2000} QDs or 20 nmol of Gal-PEG_{2000} QDs. For inhibition of Gal-PEG_{2000} QDs binding and receptor-mediated uptake HepG2 cells were preincubated with a Gal-polymer before adding the Gal-PEG_{2000} QDs. Compared to the PEG_{2000} QDs (gray), Gal-PEG_{2000} QDs were preferentially taken up by the HepG2 cells (solid line). Uptake was partially inhibited by preincubating cells with 1 mg/mL of Gal-polymer (dotted line).

To analyze whether the Gal-QDs were internalized by ASGP-R-mediated endocytosis, ASGP-R1 knockdown in HepG2 cells was performed by siRNA. Western Blot analysis after 48 h indicated that ASGP-R1 expression was at least partially reduced (Figure 3A). After ASGP-R1 knockdown HepG2 cells were incubated with Gal-capped QDs for 2 h (Figure 3B). Flow cytometry revealed that compared to the uptake of Gal-QDs by wild-type HepG2 cells QD endocytosis was reduced upon knockdown of ASGP-R1. This finding indicates that ASGP-R contributes to the uptake of the Gal-QDs by receptor-mediated endocytosis.

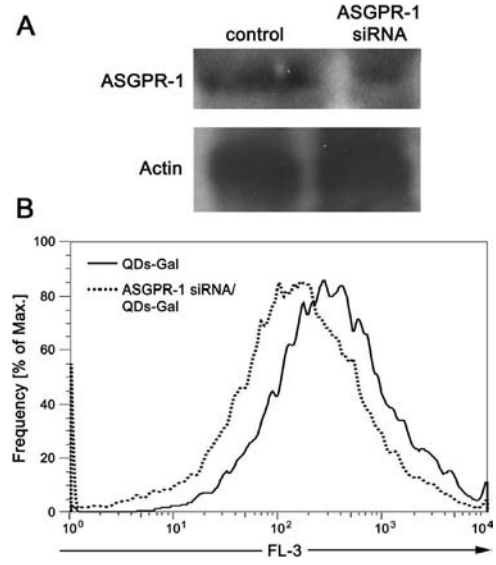

Figure 3. Inhibition of Gal-QDs uptake by ASGP-R1 knockdown. HepG2 cells were incubated with ASGP-R1 specific siRNA for 48 h. (A) Partial knockdown of ASGP-R1 was verified by Western Blot. The housekeeping gene Actin was detected as control. (B) Wild-type HepG2 cells (solid line) and HepG2 cells upon ASGPR-1 knockdown (dotted line) were incubated with Gal-QDs for 2 h. Gal-QDs uptake was partially inhibited after ASGP-R1 knockdown.

D-Galactosamine has been reported to accumulate selectively in the liver leading to necrosis of hepatocytes presumably induced by the depletion of intracellular uridine moieties.[5] Thus, we employed GalN-capped QDs to target hepatocytes *in vitro* and *in vivo*. To analyze the specific uptake of GalN-capped QDs **3** into hepatocytes, we incubated HepG2 cells with the GalN-capped QDs. Flow cytometry after overnight incubation of the cells with QDs revealed that few PEG_{2000}-capped QDs **7** were taken up (Figure 4). Man-PEG_{2000} QDs **2** uptake was only slightly increased compared to PEG_{2000}-capped QDs since HepG2 cells only express ASGP-R, but not the mannose receptor (data not shown). In contrast, QDs equipped with D-galactosamine were taken up preferentially by HepG2 cells (Figure 4). Similar results were obtained when cells were incubated with the QDs for 2 h (data not shown).

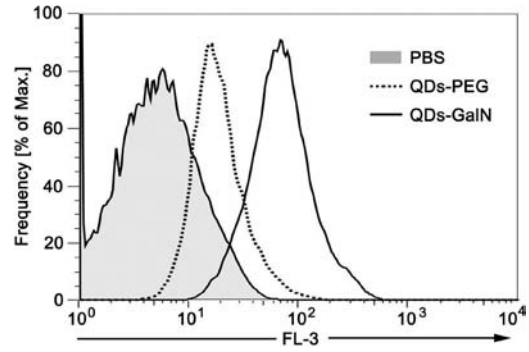

Figure 4. Specific uptake of D-GalN capped QDs by HepG2 cells. HepG2 cells were incubated overnight with 20 nmol of PEG_{2000} QDs or 20 nmol of GalN-PEG_{2000} QDs. As negative control, PBS was added to the cells. After washing, cells were collected and uptake of QDs was measured by flow cytometry. Compared to the negative control (gray), PEG_{2000} QDs were taken up only to a minor extent (dashed line). In contrast, GalN-capped QDs were taken up to a higher extent by HepG2 cells (solid line).

After demonstrating specific uptake of D-GalN-capped QDs *in vitro* we analyzed specific targeting of the liver *in vivo*. For this purpose, mice received either PEG_{2000} QDs or QDs capped with D-mannose or D-galactosamine by i.v. injection (PBS buffer was injected as a negative control). A low level of unspecific sequestration was observed in the liver 2 h after injection of PEG_{2000}-capped QDs (Figure 5A). In contrast, injection of Man-PEG_{2000} and also GalN-PEG_{2000} capped QDs resulted in a high number of QDs sequestering in liver. This finding suggests binding and/or endocytosis of the QDs mediated by mannose receptor and ASGP-R. ASGP-R is expressed predominantly on hepatocytes, while the mannose receptor is strongly expressed on Kupffer cells and sinusoidal endothelial cells in the liver.[10] Fluorescence microscopy revealed that sequestration of Man-PEG_{2000} and GalN-PEG_{2000} capped QDs into the liver was three times higher compared to PEG_{2000} QDs (Figure 5B). This finding indicates that carbohydrate−protein interactions exhibit specificity and may be exploited for targeted drug delivery *in vivo*. To the best of our knowledge, this is the first study highlighting the potential of exploiting carbohydrate−protein interactions for specific targeting of tissues or organs *in vivo* by carbohydrate-capped QDs. Approaches to analyze the potential of QD-conjugates with more complex oligosaccharide structures for cell-specific targeting and their detection by *in vivo* imaging techniques are currently ongoing.

Finally, to analyze whether the GalN-capped QDs exhibit liver toxicity, we employed a combined model of LPS/GalN-induced liver injury and measured serum transaminases ALT and AST (Figure 6 and data not shown). LPS alone induced only a marginal increase in ALT level that was slightly increased by the injection of Gal-capped QDs. This finding indicates that the QDs themselves

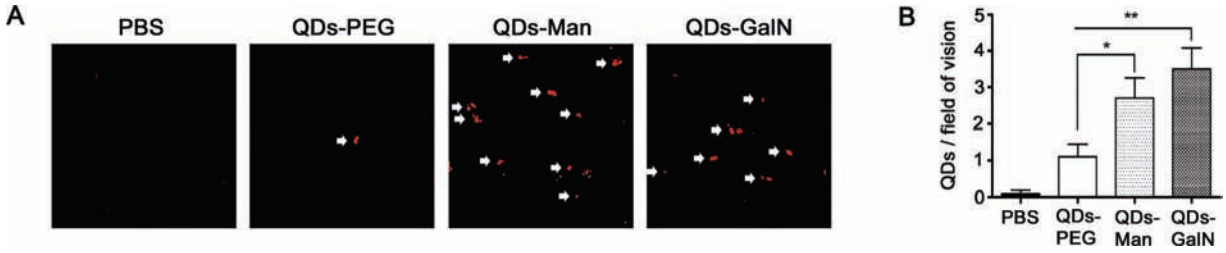

Figure 5. Specific liver sequestration of D-Man and D-GalN capped QDs in liver. Mice were injected with PBS or 2.5 nmol of either PEG_{2000} QDs or QDs capped with D-mannose or D-galactosamine ($n = 2$). 2 h after injection mice were sacrificed and livers were perfused. (A) Paraffin sections of the livers were prepared, and QD sequestration in the liver was visualized by fluorescence microscopy. Arrows indicate QDs sequestered to liver tissue. (B) Statistical analysis of QD sequestration in the liver was performed by counting 10 microscopic fields of vision for each mouse. Data are presented as mean ± SEM for each group ($*P < 0.05$, $**P < 0.01$).

are somewhat hepatotoxic possibly due to the release of the CdSe core or metabolism of the QDs. However, when the GalN-capped QDs were injected i.v., the ALT serum level was significantly increased (Figure 6). This finding indicates that GalN-capped QDs not only sequester specifically in the liver but also exhibit a specific biological function in this organ.

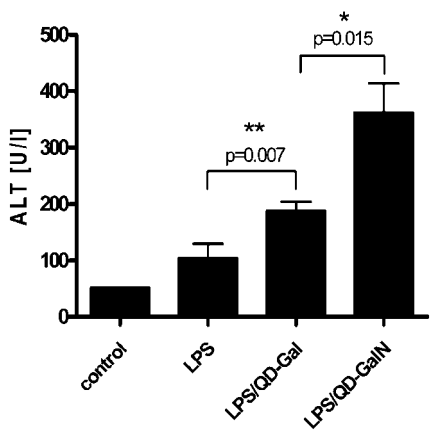

Figure 6. Hepatotoxicity by GalN-capped QDs in a model of liver injury. Mice were injected with PBS, 2 mg/kg LPS, or LPS together with 2.5 nmol of Gal-PEG_{2000} QDs or GalN-PEG_{2000} QDs. 8 h after injection ALT levels were analyzed in sera. Data are presented as mean ± SEM for each group of mice ($n = 4$).

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Supporting Information Available: Preparation and physical properties of PEGylated QDs and detailed methods of *in vitro* and *in vivo* experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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