Cellular Uptake Behavior of Glycoconjugated and Micellar-Encapsulated Quantum Dots on HeLa Cervical Cancer Cells

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ABSTRACT

Glyconanomaterials are of great interest for bio-imaging to understand biological processes, for instance metabolism, cell-cell or virus-cell interactions, gluconeogenesis, and cancer. Herein, we present a strategy for the biofunctionalization of fluorescent quantum dots (QDs) from continuous-flow reactor with carbohydrates. This flow reactor enables the reproducible synthesis of a large amount of QDs, with controlled surface functionalization. These QDs act as fluorescent biomarkers and as structural scaffolds for the presentation of glycoclusters to lectins, receptors and cells. Before the phase transfer into water takes place, the carbohydrates are covalently attached to an amphiphilic poly(isoprene)-*b*-poly(ethylene glycol) diblock copolymer (PI-*b*-PEG) using click-chemistry as previously described by us. These functionalized polymers are subsequently used for the encapsulation of the QDs under preservation of their unique optical properties in a continuous flow system. Binding of glycoconjugated QDs to the human cervical cancer cell line HeLa was characterized using confocal microscopy. Depending on the terminal group of the polymer (namely: D-maltose, D-glucose, carboxyl, and amine), the uptake of the functionalized QDs can be controlled and directed. Functionalization with maltose yields very high uptake in low incubation times and low concentrations. Although serum is known to inhibit the cellular response of artificial nanostructures, we observe reduced but significant cellular uptake of the maltose functionalized nanocontainers in serum containing media. This encapsulated materials have already been tested to be suitable for *in vivo* tumor targeting, due to their lack of toxicity as well as extraordinary stability. Though this method relies on highly reproducible continuous flow systems, which yield high amounts of well defined, functional, non-toxic and highly stable nanoparticles this method has extraordinary industrial, biological and medical relevance.

Keywords: cellular uptake, carbohydrates, diblock copolymer, continuous flow reactor, glycopolymer

INTRODUCTION

In the human organism carbohydrates play an essential role in many biological processes, for instance as structural device in the DNA and RNA, in the energy metabolism, as posttranslational modifications of proteins influencing tumor genesis, bacterial/ viral infection, inflammation, immune response, and cell-cell communication.[1-2] Many processes are mediated *via* interactions between sugar-binding proteins, so called glycoreceptors, and carbohydrates.[3] These interactions are difficult to analyze and to monitor with a single monosaccharide moiety due to the weak affinity.[4] This binding strength can be increased about several orders of magnitude by the combination of nanomaterials with carbohydrates. These gyconanomaterials present many sugars to the glycoreceptor. The strong binding of glycoclusters is a result of polyvalent interactions.[5] By combining the carbohydrate with a fluorescent quantum dot (QD), the localisation of the composite in the tissue can be monitored and the influence of carbohydrates on such composites may be studied. QDs are used in biomedicine for imaging, because of their excellent optical properties.[6] QDs benefit from high photostability and of their adjustable emission wavelength correlating with the particle size in contrast to organic dyes.[7-8]

Nonetheless, only a few articles in scientific literature can be found on glycoconjugated QDs.[9-11] Recently, we published a micellular encapsulation technique which is applicable for different NPs (QDs, iron oxides and gold NPs) using an amphiphilic poly(isoprene)-*b*-poly(ethylene glycol) diblock copolymer (PI-*b*-PEG).[12] The polymers can be cross-linked which leads to a high stability, preserving the physical properties of the particle.[13-14], Due to the synthesis of the diblock copolymer by living anionic polymerization (LAP) the chain end can be tailored by different strategies.[12] More recently, we presented the attachment of azido-modified carbohydrates to PI-*b*-PEG with an alkyne end group using copper catalyzed Huisgen's 1,3-dipolar cycloaddition (*click*-chemistry).[15] The

glycosylated polymers were successfully used for the encapsulation of QDs and used for lectin-binding assays.
Here, we performed uptake studies of the glycoconjugated QDs on HeLa cells *in vitro*. Their binding to the cervical cancer cell line was characterized using confocal microscopy. Depending on the terminal group of the polymer, (D-maltose, D-glucose, carboxy, and amine) the uptake of the functionalized QDs can be controlled. This may have a high potential for specific targeting of cervical cancer cells and further tumor entities *in vivo*.

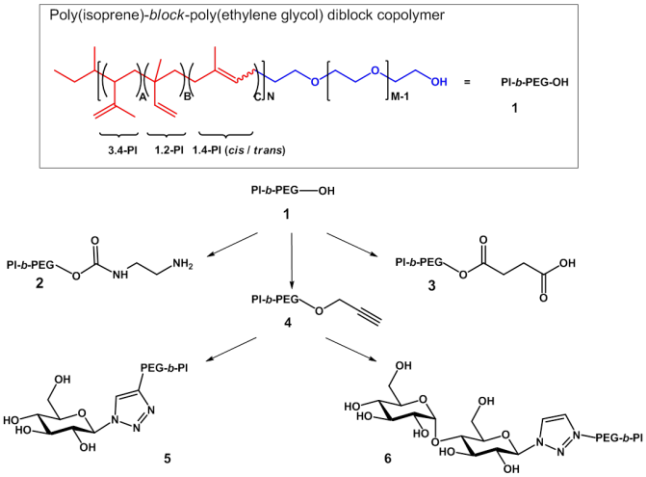
EXPERIMENTAL SECTION

Synthesis. CdSe/CdS/ZnS core/shell/shell nanoparticles were synthesized in a flow reactor as described previously.[16-17] The block copolymers PI and PI-*b*-PEG **(1)** were synthesized by living anionic polymerization (LAP) as previously described.[12] PI-DETA was synthesized by CDI-activation and coupling to diethylenetriamine (DETA).[18]
Functionalization of PI-*b*-PEG. PI-*b*-PEG-NH_2 (**2**), PI-*b*-PEG-COOH (**3**), and PI-*b*-PEG-C≡CH (**4**) the glycosylation *via* click-chemistry were realized according to Schmidtke *et al*.[12, 19],
Phase Transfer into Water. The QDs were encapsulated with PI-*b*-PEG diblock copolymer as reported previously.[12]
Confocal Microscope Imaging. HeLa cells were cultured to 30% confluency in Lab-Tek thin bottom chambers in Dulbecco/Vogt modified Eagle's minimal essential medium (DMEM, c(glucose) = 4.5 g/L) medium containing 10% FCS at a temperature of 37 °C in a humidified atmosphere containing 5% CO_2. Medium was replaced with serum-containing or serum free DMEM. Subsequently, the cells were exposed to 0.1 µM of QDs for one hour and to the endosomal marker Cy5-transferrin. The cells were fixed using repeated rinsing with 4% paraformaldehyde and mountened with *Prolong Antifade Reagent with DAPI*.
Instrumentation: Confocal microscopy images were collected on an inverted Nikon TE2000-E2 Eclipse C1si (Nikon Instruments Inc., U.S.) equipped with a Nikon CFI Plan Apochromat TIRF 60x oil immersion objective (NA 1.49, 0.12 mm WD) and imaged with a Cool SNAP HQ2 monochrome camera (Photometric). DAPI, Cy5 and a QD filters (excitation 400-440 nm, emission 540-580 nm) were used.

RESULTS AND DISCUSSION

Phase Transfer

Since most high-quality NPs with a high crystallinity and monodispersity are produced in organic solvents at high temperatures *via* bottom-up procedure, a phase transfer into aqueous media is necessary.[20] In our case, we synthesized CdSe/CdS/ZnS core/shell/shell QDs in a flow-reactor.[16] These QDs can be transferred into water using an amphiphilic, cross-linkable poly(isoprene)-*b*-poly(ethylene glycol) diblock copolymer (PI-*b*-PEG) as previously reported.[18] We have already shown that the functionalization of the polymer can be done prior to the phase transfer (pre-assembly). This strategy is useful to incorporate functional groups (amine (**1**), alkyne (**2**), or carboxyl (**3**)) or small affinity molecules (e.g. biotin or carbohydrates).[12] We adopted this method for the glycosylation of the diblock copolymer with D-glucose (**5**) and D-maltose (**6**) using Huisgen's 1,3-dipolar cycloaddition[21] under copper catalyzed conditions[22],[23] between a glyco-azide (2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl azide and 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-*α*-D-glucopyranosyl-1→4-*β*-D-glucopyranosyl azide) and a polymeric alkyne (PI-*b*-PEG-C≡CH (**4**)).[15] The reaction is not affected by other biological relevant end groups and the resulting triazole ring is very stable against hydrolysis/enzymatic degradation. Sharpless *et al.* defined this kind of reactions as click-chemistry which are simple, selective, avoiding byproducts and running under mild conditions in high yields.[24] Taking the highly selective structure/effect relationships between glycoreceptors and sugars into account, we synthesized functional polymers with the same connectivity to the anomeric carbon atom of the carbohydrate.



Scheme 1: Schematic representation of the functionalization / glycoconjugation strategy and chemical structures of the resulting compounds.

These functionalized polymers were successfully used for the micellar encapsulation of QDs in water with excellent fluorescence quantum yields.[13] No spectral shifts were obtained by UV-vis spectroscopy, neither in the absorption nor in the emission maximum. Furthermore, the good fluorescence properties of the glyco-QDs showing that the toxic copper catalyst is well separated during the purification of the polymers, because even smallest quantities of copper ions quench the QDs immediately.[14, 25-27]

Cellular Uptake of encapsulated QDs on human cervical carcinoma cell line HeLa

Cervical cancer rates fourth in cancer related death in women. Therefore, the human cervical carcinoma cell line

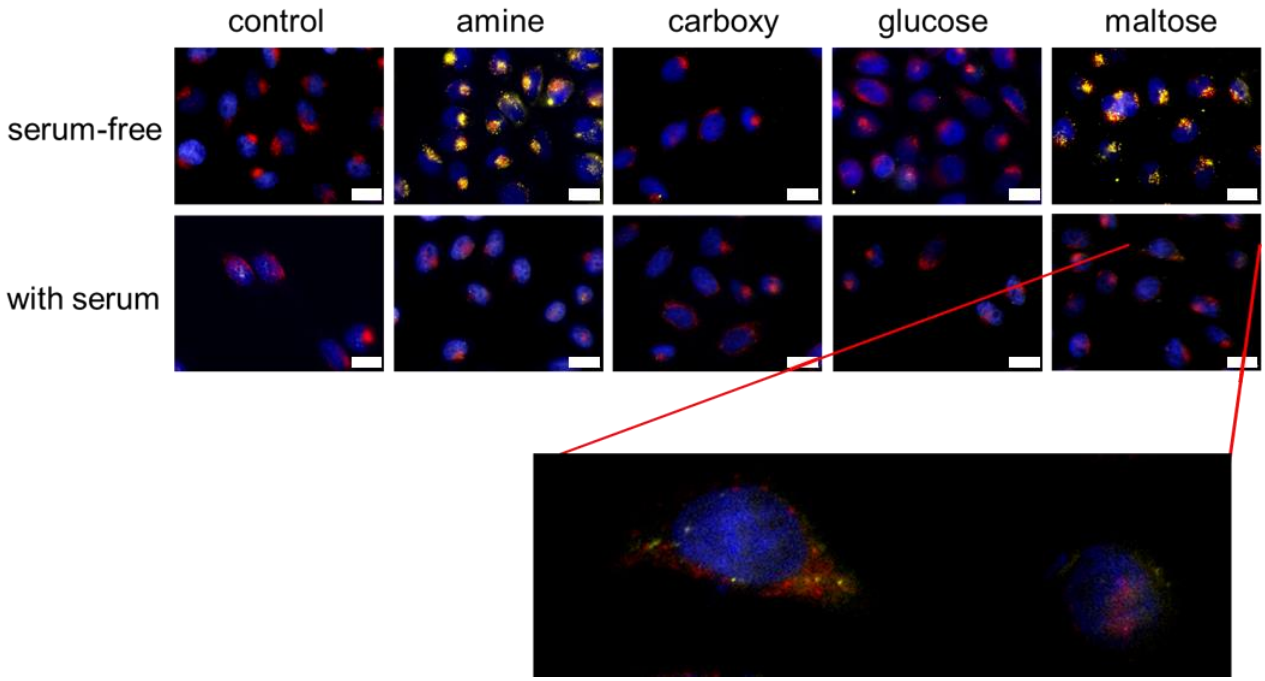


Figure 1: Confocal microscopy images of HeLa cells after exposure to different functionalized QDs. Top row: serum-free media, middle row: serum containing media, bottom: enlargement of HeLa cells incubated with maltose functionalized QD in serum-containing media. The color code represents blue: DAPI, red: Cy5, yellow: QD, the scale bar represents 20 µm.

HeLa is highly attractive for *in vitro* binding and uptake studies to achive cancer specific targeting. Many important biological processes like inflammation, cancer and cancer metastasis, are frequently mediated by glycoreceptor−glycan interactions.

The influence of different functional groups and carbohydrates on the surface of the QDs, namely: amine, carboxyl, D-glucose, and D-maltose were studied.

We performed the uptake-studies on HeLa cells, where the functional QDs were incubated on the cells for one hour in serum-containing and serum-free medium. The latter prevents a possible protein corona formation on the NP or an impaired uptake mechanism.[28] Using confocal microscopy, the cell-nanoparticle interactions were investigated and the fluorescent quantum dots were located intracellularly (see Figure 1). QDs were identified by both spectral position and by wavelength scans, if necessary. Surprisingly, we observed cellular uptake of maltose-functionalized QDs in serum-free as well as serum-containg media, even though cellular uptake in serum-containing media was significantly reduced. Furthermore, we observed a strong uptake in serum-free media for the QDs with amine, whereas the carboxylated and glucose functionalized NPs showed none. These results are in accordance to our previous published results.[29] Colocalization with the endosomal marker Cy5-transferrin indicates endosomal uptake in both cases. We interpret the cellular uptake of maltose-QDs medium as specific, due to comparable low incubation times and concentrations compared to our recent reports.[29] The reduced, but significant uptake of maltose-QDs in serum containing media supports this assumption. This is also supported by a recent report of Song *et al.*, where enhanced cellular uptake of maltose-modified liposomes in HeLa cells in serum-free medium is reported.[30] In a more recent report, the influence of the number of glucose units in a maltooligosaccharide-QD construct on the rate of nuclear import was studied. There microinjection was used to transfer QDs into the cells and an enhanced rate of nuclear import of maltooligo-QDs with more than three glucose units was found in comparison to (mono)-glucose functionalized QDs.[31] These reports underline the importance of polysaccharides in the cellular response of glyco-QD constructs. Interestingly, eukaryotic cells do not express endogenous maltose receptors since maltose is broken down to glucose by maltase (alpha-glucosidase) in the small intestine.[32] It can be hypothesed that maltose is transported via its terminal glucose moiety by the faciliative glucose receptors (GLUTs) that are frequently overexpressed in cancer cells to overcome the elevated need for energy and that the glucose functionalized QDs are not identified due to the triazole.[33] Further investigations will elucidate the effects of carbohydrate presentation on the NP's surface and their receptor interactions in detail.

CONCLUSION

In conclusion, we could show a simple method to couple different carbohydrates (D-maltose and D-glucose) to the PI-*b*-PEG diblock copolymer *via* click-chemistry. The glycosylated polymers were successfully used for the phase transfer of fluorescent QDs preserving their optical properties. Both QD synthesis as well as phase transfer

were carried out in automated flow systems, allowing highly reproducible synthesis of a big amount of nanomaterials. The resulting glyco-QDs have proven to be non-toxic assessed by several toxicity assays (WST8 and LDH) at concentrations as high as 1 µM. Uptake studies presented in this article, show that these glyconanomaterials can be used to target HeLa cells. These results are promising and underline the potential of glyconanomaterials for further *in vivo* studies.

ACKNOWLEDGEMENT

This work was supported by the EU within the FP 7 program (Vibrant, EU 228933). J.-P.M. acknowledges the support of the Chemical Industry Fund, VCI: German Chemical Industry Association, the German-American Fulbright Program and furthermore travel fellowships of the Freunde- und Förderverein Chemie der Universität Hamburg e.V. and of the University of Hamburg. We would like to thank Matthias Wulf from the Institute of Organic Chemistry (University of Hamburg) for providing us the maltose-derivate.

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