Glucose Single-Chain Polymer Nanoparticles for Cellular Targeting

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Supporting Information

ABSTRACT: Naturally occurring glycoconjugates possess carbohydrate moieties that fulfill essential roles in many biological functions. Through conjugation of carbohydrates to therapeutics or imaging agents, naturally occurring glycoconjugates are mimicked and efficient targeting or increased cellular uptake of glycoconjugated macromolecules is achieved. In this work, linear and cyclic glucose moieties were functionalized with methacrylates via enzymatic synthesis and used as building blocks for intramolecular cross-linked single-chain glycopolymer nanoparticles (glyco-SCNPs). A set of water-soluble sub-10 nm-sized glyco-SCNPs was prepared by thiol-Michael addition cross-linking in water. Bioactivity of various glucose-conjugated glycopolymers and glyco-SCNPs was evaluated in binding studies with the glucose-specific lectin Concanavalin A and by comparing their cellular uptake efficiency in HeLa cells. Cytotoxicity studies did not reveal discernible cytotoxic effects, making these SCNPs promising candidates for ligand-based targeted imaging and drug delivery.

Biological functions of carbohydrates range widely from highly specific cellular recognition and communication, to the supply of energy. Carbohydrate transport through the cell membrane is facilitated by specific membrane-bound carbohydrate transporters, that is, GLUT 1–14, which have therefore become popular targets for cell targeting with nanomaterials. Furthermore, bacteria and viruses exhibit surfaces covered with carbohydrate-binding proteins, that is, lectins, with high binding affinity toward carbohydrates displayed on the targeted (human) cells. This mimicry hinders recognition by the immune system and clearance of bacteria and viruses. Hence, functionalization of (polymer) nanomaterials with carbohydrates is a popular strategy to avoid immune response and furthermore may introduce targeting capabilities and increase cellular uptake. For example, glucose-modified micelles were recently found to be taken up to an increased extent by cells with overexpressed GLUT 1 receptors and to cross the blood–brain barrier in fasting mice. For successful brain uptake, glycaemic control of the mice diet and conjugation on the C6 position instead of the C3 position proved crucial, which suggests a transporter-mediated uptake mechanism. For the interaction between glucose and the transporter, mainly the C1–C3 hydroxyl groups are assumed to play a role. Similarly, substitution of galactose either on C1 or C6 position was influential for the endocytic pathway of conjugated nanoparticles.
To obtain glycopolymers and nanoparticles, carbohydrate functionalities can either be attached postpolymerization or employed in the form of glycomonomers. Controlled polymerization techniques, such as via reversible addition–fragmentation chain transfer (RAFT), offer superior structural control in both strategies. In case of postpolymerization functionalization, full substitution is impeded by steric hindrance and multistep protective group chemistry is typically required. Alternatively, glycomonomers are obtained via protective group chemistry or via enzymatic pathways providing chemo-, regio-, and stereoselectivity.

The carbohydrate recognition efficacy of transporters depends on the architecture of the glycomacromolecule and varies with the carbohydrate hydroxyl group substitutions and carbohydrate linker density and spacing. Not only in nature, but also synthetically, a variety of glycomacromolecule topologies is known, ranging from dendrimers to nanogels. Multivalency in such structures is a striking factor to achieve selective binding toward the target site. One way to evaluate the selective recognition of glycomacromolecules is to test their binding affinity to lectins. Concanavalin A (ConA) from Canavalia ensiformis (Jack bean) is one of the most studied lectins for modeling receptor interactions of glycomacromolecules, as it is structurally similar to a large number of bacterial and animal lectins in cell communication events. Dendritic structures and micelles have shown superior binding as compared to linear polymer chains, as evaluated through ConA binding assays. Whereas the complexity of the well-defined dendrimers increases with every generation, polymeric structures offer ease of synthesis, to certain extents at the cost of loss in structural control.

Single-chain polymer nanoparticles (SCNP) are prepared through exclusive intramolecular cross-linking of polymer chains and typically measure around 10 nm in diameter. SCNPs are comparable in size to dendrimers, while the combination of polymer preparation techniques greatly alleviates synthetic effort. Recently, SCNPs have also been prepared from glycopolymers, and current investigations are focused on controlling the structures of SCNPs and their biomedical applications.

Glucose-based molecules were conjugated with methacrylate moieties via enzymatic coupling reactions based on literature procedures. Bead immobilized lipase from Candida antarctica catalyzed the conjugation at the C6 position as established by Davis and co-workers, whereas β-glucosidase from almonds was used for C1 conjugation as described by the Loos group. Conjugation at the C1 position blocks the opening of glucose to its linear form and due to the enzymatic reaction, only the β-anomer is formed G1MA (M3), as confirmed by the absence of the H1-α signal in 1H NMR spectroscopy. C6 conjugation was performed on methyl glucoside resulting in the formation of the mG6MA monomer (M1), where the glucose is fixed in its α-ring pyranose form, as well as on glucose, resulting in the formation of the G6MA monomer (M2), where the molecule can still interconvert between its open-chain form and the α/β anomers after conjugation. The thiol-functional monomer, xanthate methacrylate (XMA), was prepared following established literature procedures and combined via RAFT co polymerization with the glucose monomers. Aliquots of the polymerizations were taken at regular intervals to confirm equal consumption of the monomers and hence to confirm a random copolymerization. Copolymers P1–P3, prepared from monomers M1–M3, were obtained with molecular weights of 40 kDa (P1α) and 100 kDa (P1β–P3) and with typical xanthate incorporation ratios of 15–20% (Table 1). Low dispersity indices (PDI) were achieved for the polymers (D ~ 1.2), except for P2 (D = 1.5), as analyzed by gel permeation chromatography (GPC), which may suggest interaction between M2 and the chain transfer agent (CTA).

SCNP formation from glucose-containing precursor polymers was based on a protocol that we developed previously for glycerol-xanthate copolymers. After deprotection of the xanthate moiety with hydrazine, as observed with 1H NMR spectroscopy (Figure S3), the obtained thiol polymers were evaluated in relation to the mode of glucose functionalization. Precursor copolymers were prepared from enzymatically synthesized C1- and C6-functionalized glucose monomers and the corresponding SCNPs were subsequently obtained via thiol-Michael cross-linking of the precursor glycopolymers (Scheme 1). Bioactivities of the resulting SCNPs were compared based on their lectin binding abilities, reductive properties, cell toxicity, and uptake by HeLa cells.

Scheme 1. Schematic Representation of Glyco-SCNPs by Thiol-Michael Addition

Glucose-based molecules were conjugated with methacrylate moieties via enzymatic coupling reactions based on literature procedures. Bead immobilized lipase from Candida antarctica catalyzed the conjugation at the C6 position as established by Davis and co-workers, whereas β-glucosidase from almonds was used for C1 conjugation as described by the Loos group. Conjugation at the C1 position blocks the opening of glucose to its linear form and due to the enzymatic reaction, only the β-anomer is formed G1MA (M3), as confirmed by the absence of the H1-α signal in 1H NMR spectroscopy. C6 conjugation was performed on methyl glucoside resulting in the formation of the mG6MA monomer (M1), where the glucose is fixed in its α-ring pyranose form, as well as on glucose, resulting in the formation of the G6MA monomer (M2), where the molecule can still interconvert between its open-chain form and the α/β anomers after conjugation. The thiol-functional monomer, xanthate methacrylate (XMA), was prepared following established literature procedures and combined via RAFT copolymerization with the glucose monomers. Aliquots of the polymerizations were taken at regular intervals to confirm equal consumption of the monomers and hence to confirm a random copolymerization. Copolymers P1–P3, prepared from monomers M1–M3, were obtained with molecular weights of 40 kDa (P1α) and 100 kDa (P1β–P3) and with typical xanthate incorporation ratios of 15–20% (Table 1). Low dispersity indices (PDI) were achieved for the polymers (D ~ 1.2), except for P2 (D = 1.5), as analyzed by gel permeation chromatography (GPC), which may suggest interaction between M2 and the chain transfer agent (CTA).
increased radius and hence to intramolecular cross-linking. Plotting cross-linking, which is related to a reduced hydrodynamic radius, displayed an increased molar mass upon cross-linking, therefore excluding multichain-aggregates and thus pointing to exclusive intramolecular cross-linking. Differential molar mass weight fractions of NP2 confirmed the coexistence of multichain aggregates of NP2 with higher \( M_n \) than the original polymer. The formation of multichain aggregates precludes a reduction of \([\eta]\), but the differential intrinsic viscosity weight fraction does reveal presence majority of fractions with reduced \([\eta]\). Despite the presence of intermolecular cross-links (<20%, Figure S9), intramolecular cross-linking is predominant.

Furthermore, intrinsic viscosities \([\eta]\) were reduced after cross-linking, which is related to a reduced hydrodynamic radius and hence to intramolecular cross-linking. Plotting differential weight fraction against molar mass does not indicate increases in molar mass upon cross-linking, therefore excluding multichain-aggregates and thus pointing to exclusive intramolecular cross-linking. However, NP2 displayed an increased \( M_n \) indicating formation of intermolecular cross-links. Differential molar mass weight fractions of NP2 confirmed the coexistence of multichain aggregates of NP2 with higher \( M_n \) than the original polymer. The formation of multichain-aggregates and thus pointing to exclusive intramolecular cross-linking. Differential weight fraction against molar mass does not indicate increases in molar mass upon cross-linking, therefore excluding multichain-aggregates and thus pointing to exclusive intramolecular cross-linking. However, NP2 displayed an increased \( M_n \) indicating formation of intermolecular cross-links. Differential molar mass weight fractions of NP2 confirmed the coexistence of multichain aggregates of NP2 with higher \( M_n \) than the original polymer. The formation of multichain aggregates precludes a reduction of \([\eta]\), but the differential intrinsic viscosity weight fraction does reveal presence majority of fractions with reduced \([\eta]\). Despite the presence of intermolecular cross-links (<20%, Figure S9), intramolecular cross-linking is predominant.

For polymers P1a, P2, and P3 (\( M_n \geq 90 \text{ kDa} \)), size reductions were observed in dynamic light scattering (DLS) upon SCNP formation (Table 1 and Figure S8). In the case of the shortest precursor polymer (P1b, 40 kDa), a reduction in size was not detectable with DLS. However, additional measurements with diffusion-ordered spectroscopy (DOSY) NMR on P1a and NP1a revealed a slight particle size reduction from 3.9 to 3.7 nm. Furthermore, transmission electron microscopy (TEM) on negatively stained NP1a revealed particles of around 5 nm in radius with a uniform particle shape and size (Figure S10).

The reducing characteristics of the glucose monomeric units in the prepared polymers and SCNPs were evaluated with Benedict’s reagent (Figure 2), a citrate solution that complexes copper(II), resulting in a blue color.\(^{50}\) Formation of red copper precipitates in Benedict’s solution with P2 and NP2 confirms preservation of reducing properties of the glucose monomeric units. Hence, ring-opening of glucose in M2 is not blocked by polymerization or cross-linking. For the other polymers and nanoparticles, no color change was expected as the glucosides are present in the pyranose, nonreducing form. Surprisingly, polymers P1 and P3 result in green colors when performing the reduction assay, while the NP1 and NP3 solutions stayed blue as the negative control. Diethylthiocarbamates, comparable to the CTA- and xanthate moieties on polymers P1 and P3, have been reported earlier to form copper complexes with CuCl2, resulting in color changes from green to brown.\(^ {51}\) As controls, the RAFT homopolymers pHEMA and a glycerol methacrylate-co-XMA copolymer were tested with Benedict’s reagent. Whereas pHEMA did not result in a color change, the glycerol methacrylate-co-XMA copolymer displayed a blue color, indicating the presence of reducing properties.

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### Table 1. Comparison of Molecular Weights of Glycopolymers and Their Corresponding Nanoparticles

| \( M_{\text{eliner}} \) (kg/mol) | \( M_{\text{GPC}} \) (kg/mol) | \( M_{\text{MALS}} \) (kg/mol) | \( \chi_{\text{SH}} \) | \( D \) | \( \Delta M_{\text{app}} \) (%) | \( r_{\text{FLIXY}} \) (nm) | \( r_{\text{FLIXY, c}} \) (nm) | \( [\eta]^{\#} \) (mL/g) |
|-------------------------------|-------------------------------|-------------------------------|----------------|-----|----------------|-----------------|-----------------|----------------|
| P1a                           | 40                            | 32                            | 14             | 1.13| 3.4            | 3.9             |                  |                |
| Np1a                          | 22                            |                               | 1.16           | 30   | 3.4            | 3.7             |                  |                |
| P1b                           | 108                           | 154                           | 102            | 1.31| 6.4            | 9.4             | 33.4            |                |
| Np1b                          | 101                           | 91                            | 1.13           | 34   | 2.8            | 8.0             | 26.8            |                |
| P2                            | 105                           | 89                            | 70             | 1.45| 5.9            | 7.8             | 23.6            |                |
| NP2                           | 63                            | 87                            | 1.46           | 30   | 3.2            | 12.4            | 28.1            |                |
| P3                            | 102                           | 105                           | 83             | 1.23| 5.7            | 8.8             | 27.2            |                |
| Np3                           | 80                            | 65                            | 1.10           | 24   | 1.9            | 6.2             | 19.4            |                |

\(^{a}\)Determined by \(^1\)H NMR. \(^{b}\)Determined in DMF by GPC, relative to PEG standards. \(^{c}\)Determined in DMF by GPC, by MALS measurements. \(^{d}\)Determined in DMF by GPC, by a viscometer. \(^{e}\)Reduction in apparent number-average molecular weight, calculated as \(\Delta M_{\text{app}} = -(M_{n,\text{NP}} - M_{n,P})/M_{n,P} \times 100\). \(^{f}\)Measured in H2O/D2O.

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Figure 1. Overlay of GPC traces for the glycopolymers (P1–3) and corresponding nanoparticles (NP1–3) with different chain lengths and their corresponding nanoparticles (NP1–3).

Figure 2. Benedict's assay with glycopolymers (P1–3) and corresponding nanoparticles (NP1–3) (sucrose = negative control, glucose = positive control, pHEMA = polymer with same CTA, pXMA = copolymer containing XMA).
in a color change, the XMA copolymer revealed a similar color shift as polymers P1 and P3. Presumably, the color change is therefore due to copper interactions with the xanthate moieties and is not caused by the glucose functionalities. Neither monomer synthesis, nor polymerization or SCNPs formation, affected the nonreducing and reducing properties of the glucoside and glucose monomeric units, respectively.

ConA is a lectin with four binding sites at pH 7, which are specifically sensitive to glucose moieties. Various carbohydrate conjugated positional isomers have been thoroughly investigated, demonstrating specificity of ConA toward α-glucose over β-glucose, while substitution of hydroxyl groups at the 3-, 4-, and 6-carbon positions has shown to disrupt carbohydrate recognition. Therefore, the binding ability of C1- and C6-functionalized glucose repeating units in polymers P1–P3 and nanoparticles NP1–NP3 was tested in a quantitative ConA precipitation assay. In agreement with literature reports, polymers with glycomonomers functionalyzed at the C6 position (P1 and P2), and the corresponding SCNPs (NP1 and NP2) showed a lower binding affinity toward ConA. Interestingly, ConA did not even precipitate when exposed to P2 or NP2 and precipitation was inhibited, comparable to monomeric glucose. In contrast, P3 and NP3 with C1-functionalized glucose in its β-configuration were indeed recognized by ConA, where NP3 displayed a significantly decreased binding affinity toward ConA as compared to its linear precursor P3 (Figure S11). P3/NP3 not only contain a C1-conjugated glucose, but also a longer linker (one methylene unit) between glucose moiety and acrylate group than in the case of P1/NP1 and P2/NP2, which might enhance ConA recognition. Since the SCNPs is no longer linear, but rather collapsed and cross-linked, the glucose moieties are likely less available for formation of clusters with lectins. The decreased lectin binding upon SCNPs formation is in contrast with the findings of Becer and co-workers with C-shaped mannoside SCNPs, where the mannose units are incorporated in the middle segment of the polymer, and only the end blocks are cross-linked. The difference between the studies highlights the importance of the secondary structure of glycomacromolecules on molecular recognition. Here, the ConA binding studies confirm the positional dependency of glucose functionalization on molecular recognition and point toward reduced flexibility of the polymer chain after crosslinking. However, interaction of glycomacromolecules with a single lectin is not representative for the wide range of interactions between cell receptors, proteins, and transporters. In order to further evaluate the effects of the positional isomers of glycoconjugates, in vitro studies were performed with each conjugate.

HeLa cells express predominantly GLUT1 and GLUT3 as glucose transporters and were therefore selected for our investigations. Cytotoxicity of the different SCNPs was evaluated at concentrations up to 200 μg/mL. None of the polymers or SCNPs displayed significant toxicity, even at the highest concentrations, after 24 or 48 h and likewise no concentration dependency could be discerned (Figure S12). Additional evaluation of P1 and NP1 on human endothelial brain cells (hCMEC/D3) also did not reveal notable effects on cell viability.

In order to facilitate cell uptake studies, particles were fluorescently labeled with 5-(4,6-dichlorotriazinyl)-aminofluorescein (DTAF), which was confirmed by GPC equipped with fluorescence detector. As free label was still detectable after extensive dialysis, the samples were purified by FPLC prior to cell experiments. NP1–3 were incubated with HeLa cells and FACS experiments were conducted at different incubation time points to quantify nanoparticle uptake (Figure 3). For all nanoparticles, cellular uptake was confirmed and increased with incubation time. Highest uptake was observed for NP2, comprising glucose with a free C1 position. Lowest uptake was clearly observed for NP3, with glucose moieties conjugated at the C1 position. NP1, which is composed of C6-conjugated glucose with a modified C1 position, is taken up to an intermediate amount.

To further investigate the uptake mechanism, the nanoparticle uptake to HeLa cells was studied in combination with confocal microscopy. In agreement with the FACS results, all three nanoparticles NP1–3 were observed inside of the cells, while the fluorescence intensity is substantially reduced in the case of NP3. As the confocal images show clear vesiculation of the nanoparticles, early endosomes, late endosomes, and lysosomes were stained in addition to the nucleus and membrane (Figures 4 and S14–17). After 4 h of incubation, fluorescence of the SCNPs only partially overlapped with early endosomes, but strong colocalization with the late endosome and lysosome staining was observed for all nanoparticles. Furthermore, the intensity of the endosome and lysosome staining is strongly increased in the case of NP1 and NP2 incubation in contrast to the control cells.

In combination with the differing cellular uptake for the three glyco-SCNPs, these results strongly suggest receptor-mediated uptake to the HeLa cells. For cellular recognition, the C1 position of the glucose hydroxyl groups is more relevant than the C6 position. However, methylation of the C1 hydroxyl does not impede uptake drastically, which supports that not one single hydroxyl group is solely responsible for recognition. Further, the similar cellular uptake behavior of NP1 and NP2 also implies that the linear structure of glucose is not essential for recognition and that the pyranose form participates in receptor binding.

In this work, we describe the synthesis and incorporation of C1- and C6-glucose-derived methacrylates into RAFT copolymers and into SCNPs. Furthermore, the resulting nanoparticles were compared with regards to molecular recognition and targeting behavior. The reductive properties of the C6-glucose moiety were preserved upon RAFT copolymerization, as well as cross-linking via thiol-Michael addition and are, hence, expected to be also accessible in cellular environments. Evaluation of the glyco-SCNPs in ConA binding assays revealed strongest binding when the C6 position of glucose remains accessible. While all three glyco-

![Figure 3. FACS analysis of uptake of glyco-SCNP (NP1–3) to HeLa cells: (a) Histogram after 8 h of incubation; (b) Uptake index (UI) over time (UI = mean fluorescence intensity (MFI) of treated cells/ MFI of control cells).](Image)
SCNPs were taken up via the endocytic pathway, the efficiency of their uptake to HeLa cells differed. Comparison of the cellular uptake efficiency of SCNPs with glucose moieties conjugated through the C1 and C6 position, in their blocked pyranose and in their partly linear form, demonstrates that the effect of a reducing glucose is minor in comparison to the position of conjugation. Accordingly, equipping SCNPs with glycoligands provides control over cell targeting, which may be exploited in tumor targeting.

**ASSOCIATED CONTENT**

**Supporting Information**
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmacrolett.8b00812.

Experimental details, polymerization plots, ^1^H NMR spectra, DLS/GPC data, TEM image, cytotoxicity/lectin binding studies, and confocal images (PDF).

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All authors have given approval to the final version of the manuscript.

**Notes**
The authors declare no competing financial interest.

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Figure 4. Overlaid confocal images of HeLa cells incubated 4 h with the glyco-SCNPs NP1−3 (in green, nuclei and membrane = blue, cell compartment = red).
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