Uptake of Well-Defined, Highly Glycosylated, Pentafluorostyrene-Based Polymers and Nanoparticles by Human Hepatocellular Carcinoma Cells[a]

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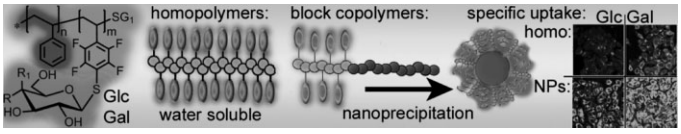
Chain length, size, composition, surface charge, and other properties of polymeric materials affect their recognition and uptake by cells and must be optimized to deliver polymers selectively to their target. However, it is often not possible to precisely modify selected properties without changing other parameters. To overcome these difficulties, well-defined poly(pentafluorostyrene)-based polymers are prepared that can be grafted via thiol/*para*-fluorine "click" reaction with 1-thio-β-D-glucose and 1-thio-β-D-galactose. Fluorescence microscopy and flow cytometry show that nanoparticles are taken up by HepG2 cells to a higher degree than the respective water-soluble polymers, and that internalization of both galactosylated homo- and nanoprecipitated block copolymers is enhanced.



1. Introduction

Synthetic polymers offer the possibility to introduce biologically active moieties and to design tailor-made macromolecules with well-defined architectures and properties.[1,2] Glycopolymers, consisting of a synthetic polymeric backbone and pendant sugar moieties, are currently applied for affinity separations, bioassays, and

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biocapture analysis, in clinical diagnostics, as well as in targeted drug delivery systems.[3] They represent a highly versatile tool allowing the precise modification of their material and biological traits for the synthesis of various, cell (organ)-targeting systems. Modern polymerization techniques enable the adjustment of the polymeric backbone composition and to control in this way the solubility behavior of the material as well as offer the possibility to introduce responsiveness to stimuli such as a change of temperature or pH values.[4] Since the carbohydrate units act as ligands for a broad spectrum of receptors, glycopolymers that actively target specific cells or organs can be obtained by selecting appropriate types of sugar moieties.[5] Recognition by cell type specific receptors is significantly enhanced by multivalent representation of the carbohydrate ligand along the polymeric backbone, exploiting the cluster glycoside effect.[6,7]

By introducing a hydrophobic block into an otherwise water soluble glycopolymer, materials that aggregate in water to nanoparticles (NPs) can be created. Their significance in biomedical fields for the delivery of drugs, genes as well as imaging agents has been thoroughly reviewed.[8,9] Adjustment of the formulation conditions during the preparation of NPs allows a tuning of the physicochemical properties (size, charge, and surface properties) over a wide range and in a high-throughput manner.[10] By fine-tuning of macromolecular composition and processing parameters, biodistribution and pharmacokinetics of particulate carriers can be modified to reach an enhanced accumulation within specific tissues.[11] NPs have also been used in the treatment of liver diseases.[12] In order to successfully target liver the particles should efficiently pass through the liver sinusoidal endothelium.[12] To be biocompatible they should not interact with serum proteins, avoid mechanical entrapment by the capillaries in the lung and the body, and evade uptake by macrophages or provoke immune responses. Glycopolymers have revealed some protein repellant properties, therefore, their interaction with serum can be minimized and formation of agglomerates that are captured in capillaries can be avoided.[13,14]

In order to study the interaction between polymers and cells well-defined and characterized materials are essential.[15] Chain length, composition, and topology are the factors that influence the spatial distribution of sugars on the backbone of the macromolecule, thus, affecting their recognition by cells.[16] The synthesis of glycopolymers can be performed via the polymerization of glycosylated monomers or the grafting of sugar moieties onto a preformed polymeric backbone.[17] In this study, the second approach was applied. Utilizing controlled polymerization techniques in combination with a highly efficient introduction of carbohydrate moieties, for example, by ''click chemistry,'' ensures a precise control over the structure of the glycosylated product.[18]

Poly(pentafluorostyrene)-based glycopolymers reveal high thermal stability (up to 220 °C) and do not decompose under acidic conditions.[19,20] In addition, the carbohydrate attachment via *S*-glycosidic bonds offers resistance toward enzymatic degradation.[21] Previously, the glucosylated, fluorescently labeled, water soluble, pentafluorostyrene (PFS)-based homopolymers were applied as coating for superparamagnetic iron oxide NPs.[20] It was shown that they act as an appropriate stabilizing agent without any cytotoxicity towards 3T3 fibroblasts. Furthermore, films prepared from the water insoluble polystyrene block copolymers were proven as synthetic biocompatible coatings on poly(propylene) (PP) substrates for culturing 3T3 fibroblasts and MC3T3-E1 preosteoblasts.[22] Both cell types showed stable adhesion and proliferation on the glycopolymer-coated surfaces.

In order to confirm that the cellular recognition of carbohydrates, attached via thiol/*para*-fluorine ''click'' reaction to poly(pentafluorostyrene) (PPFS), is maintained, we report in this contribution the interactions of a HepG2 human hepatocellular carcinoma cell (HCC) line with water soluble homopolymers as well as nanoprecipitated polystyrene block copolymers, carrying β-D-thioglucose or β-D-thiogalactose moieties. Internalization of fluorescently labeled water soluble compounds and NPs by a hepatocarcinoma cell line is studied by confocal laser scanning microscopy (CLSM) as well as flow cytometry (FC).

2. Experimental Section

2.1. Materials

Styrene (≥99%, Aldrich), PFS (99%, Aldrich) and BlocBuilder® (Arkema) were used as received. 2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose (>99%) was purchased from Glycon Biochem. GmbH, triethylamine (TEA) from Merck (for synthesis, ≥99%), *N,N*-dimethylformamide (≥99.5%) and *N,N*-dimethylacetamide (DMA) from Fluka and methanol (anhydrous 99.8%) from Aldrich. 2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-galactopyranose was synthesized as previously reported.[23] Fluorescein 5(6)-isothiocyanate (FITC, Sigma) and dry *N,N*-dimethylformamide (DMF) from Fluka were used for labeling. For nanoprecipitation of the polymers, distilled water and THF from Aldrich was utilized. Wheat germ agglutinin (WGA) coupled to Alexa Fluor 633 nm, which was applied to stain the cell membrane, was purchased from Molecular Probes/Invitrogen. For embedding fixed cells Moviol 4–88 solution containing 625 μg 1,4-diazabicyclo-(2,2,2)octane from Roth, was used.

2.2. General Methods and Instrumentation

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 300 MHz spectrometer, and ^{19}F NMR spectra on a Bruker Avance 200 MHz

spectrometer in deuterated DMF. The chemical shifts were calibrated with respect to residual DMF peaks. Size-exclusion chromatography (SEC) was measured on an Agilent Technologies 1200 Series SEC system equipped with a G131A isocratic pump, a G1329A autosampler, a G1362A refractive index detector, and both a PSS Gram 30 and a PSS Gram 1000 columns in series. 2.1% LiCl solution in DMA was used as eluent at 1 mL · min^{-1} flow rate at a column oven temperature of 40 °C. The reported number-average molar masses were determined by using polystyrene standards. Thermogravimetric analyses were performed on a Netzsch TG 209 F1 Iris with 10 °C min^{-1} heating rates from room temperature up to 900 °C under nitrogen flow. Dynamic light scattering measurements (DLS) were performed on a Zetasizer Nano ZS device from Malvern Instruments (Worcestershire, UK). In these measurements, a 633 nm He/Ne laser beam was used and scattered light was detected at an angle of 173°. Scanning electron microscopy (SEM) images were recorded on a LEO-1450 VP SEM (Leo, Oberkochen, Germany), operating at 10 kV. For platinum coating of the sample a BAL-TEC SCD005 sputtering device (Balzers, Lichtenstein) was used, applying a current of 60 mA for 80 s. Cryo-TEM images were recorded using a Technai G2 Sphera (FEI) transmission electron microscope (TEM) with an acceleration voltage of 200 kV. Fluorescence excitation and emission spectra of the polymers and NPs were obtained with a Cary Eclipse fluorescence spectrofluorometer (Varian, Darmstadt, Germany) using Hellma quartz cuvettes. The slit width of the emission and excitation monochromator was adjusted such that the resulting resolution was 5 nm. Excitation spectra were recorded at an emission wavelength of 515 nm. The emission spectra were measured by exciting the polymers at 488 nm. Fluorescence images were obtained with confocal laser-scanning microscopes (LSM 510 Meta and LSM 710, Zeiss, Jena, Germany), using a Plan-Apochromat 63× oil immersion objective (NA 1.4, Zeiss) and a C-Apochromat 40× water immersion objective (NA 1.2, Zeiss). FITC was excited with the 488 nm line of the argon laser. The emitted fluorescence was collected with a 505 nm longpass filter (LSM510) or with the built-in grating in the 505–550 nm wavelength range (LSM710). To excite the WGA Alexa Fluor 633 membrane stain, the He/Ne 633 nm laser was used. Fluorescence was recorded in the 640–700 nm range using the built-in grating (LSM710). To allow a comparison, all images of a series were captured under identical conditions and instrument settings (laser power, pinhole diameter and detector gain). Quantitative image analysis was performed on grayscale converted images using the ImageJ software. FC was measured on a Beckmann Coulter Cytomics FC-500 equipped with Uniphase Argon ion laser, 488 nm, 20 mW output and analyzed with the Cytomics CXP software.

2.3. Glycopolymer Synthesis

The glycopolymers were synthesized as previously reported.[22] Briefly, acetylated carbohydrate thiols were grafted onto a PPFS or a polystyrene-*block*-PPFS backbones, which were prepared by nitroxide-mediated, living, radical polymerization.[19] The reaction was carried out in DMF in presence of TEA yielding polymers with high degrees of functionalization (≥90% of substitution). The glycopolymers were obtained by subsequent deprotection of the acetyl groups, using sodium methoxide as base.

2.4. Glycopolymer Labeling

Labeling was achieved by an analogous method to the previously reported for glucosylated homopolymers.[20] In an oven-dried, round bottom flask, glycopolymer and FITC (0.5 mol equiv. per polymer chain) were weighed in and dried for 1 h under vacuum. DMF (10 mL) was added, and the sealed mixture was stirred for 24 h in the dark. The reaction was quenched by dropping the mixture into ethanol. The precipitated polymers were centrifuged and washed at least six times with ethanol until the fluorescence in the supernatant disappeared completely as shown by thin-layer chromatography (TLC) monitoring.

2.5. Nanoprecipitation Procedure

8 mg of the labeled polystyrene block glycopolymers were dissolved in a mixture of 1 mL THF and 1 mL of distilled water. After 10 min stirring, 7 mL water were added dropwise to cause nanoprecipitation of the materials. THF was evaporated at 60 °C under reduced pressure and the solution was sterile filtered using a 2 μm filter.

2.6. Characterization of Nanoparticles

2.6.1. DLS Characterization

Dynamic light scattering was used to determine the size and zeta potentials of the particles. For this purpose, 20 μL of the suspension were added to 1 mL of demineralized, filtered water (pH = 6.0) and this solution was transferred into a polycarbonate zeta cell. For size measurements, three runs were applied for 150 s, and for the zeta potential three runs for 10 s.

2.6.2. SEM Characterization

For the SEM measurements one droplet (15–20 μL) of the suspension was placed on a mica surface and lyophilized for 3 h. Finally, the sample was sputtered with platinum applying a current of 60 mA for 80 s.

2.6.3. Cryo-TEM Characterization

Samples for cryo-TEM were prepared using a FEI Vitrobot system. 3 μL of the sample solution were transferred onto a Quantifoil (R2/2) grid, and blotting was performed at 3 mm and 3.5 s of blotting time. Samples were rapidly transferred into liquid ethane and stored in liquid nitrogen until the measurements were performed using a Gatan cryo holder.

2.7. Measurements of Fluorescence Spectra

To measure the fluorescence by the spectrofluorometer (Varian, Darmstadt, Germany), the water soluble homopolymers were dissolved in phosphate-buffered saline (PBS), pH = 7.4, at the indicated concentrations. The aqueous suspensions of the NPs, obtained from the block copolymers, were diluted to the indicated concentrations with PBS.

2.8. Uptake Studies

2.8.1. Cell Culture

The human hepatocarcinoma cell line HepG2 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). For uptake studies, 8×10^4 cells were initially seeded into 6 well plates in 4 mL of RPMI medium supplemented with 10% fetal calf serum (FCS), 100 U · mL^{-1} penicillin, and 100 μg · mL^{-1} streptomycin (all components from Biochrom, Berlin, Germany). To assess the polymer uptake in adherent HepG2 cell monolayers by CLSM, cells were seeded on sterile glass coverslips. The cells were grown at 37 °C in a humidified atmosphere containing 5 vol% CO_2 for 48 h until 75% confluency was reached and then subjected to incubation with the polymers.

2.8.2. Incubation of Cells With Polymers

The cells were incubated separately with different concentrations (0.10, 1.00, and 10.00 μg · mL^{-1}, respectively) of the sugar containing polymers for 24 h at 37 °C under 5% CO_2 atmosphere. Control cells were incubated with fresh culture medium. After incubation, the solutions were aspirated from the wells and any unbound conjugates were removed by washing the cell layer three times with PBS. Subsequently, the cells were subjected to further treatment for CLSM and FC analysis.

2.8.3. Analysis of Glycopolymer and Nanoparticle Uptake by CLSM

After incubation with the sugar containing polymers, the cells were fixed directly onto the glass coverslips for 10 min at room temperature using 4% paraformaldehyde (PFA) dissolved in PBS and subsequently washed twice with PBS. The glass coverslips were mounted on glass slides using 25 μL Moviol 4-88 solution containing 625 μg 1,4-diazabicyclo-(2,2,2)octane. After 24 h CLSM images were recorded using a Carl Zeiss 510. Quantification of the cell-associated fluorescence signal was performed using 8 bit, grayscale-converted images and ImageJ software. The cell boundaries were identified by the user and the average fluorescence intensity per pixel was obtained. Values from at least 100 cells were averaged.

2.8.4. Proof of Glycopolymer and Nanoparticle Internalization by CLSM

To show that the fluorescence resulted from internalized polymers and not from materials bound to the cell membrane, an aliquot of enzymatically detached and fixed cells was stained for 10 min with a solution of 5 μg WGA coupled to Alexa Fluor 633 nm in 1 mL phosphate-buffered saline (PBS). The cells were then centrifuged for 5 min at 1500 rpm and resuspended in 400 μL PBS. The suspension was transferred into a custom built microscope chamber and allowed to sediment. CLSM images were recorded on a Carl Zeiss 710. The emitted fluorescence was collected in the 505–550 nm wavelength range.

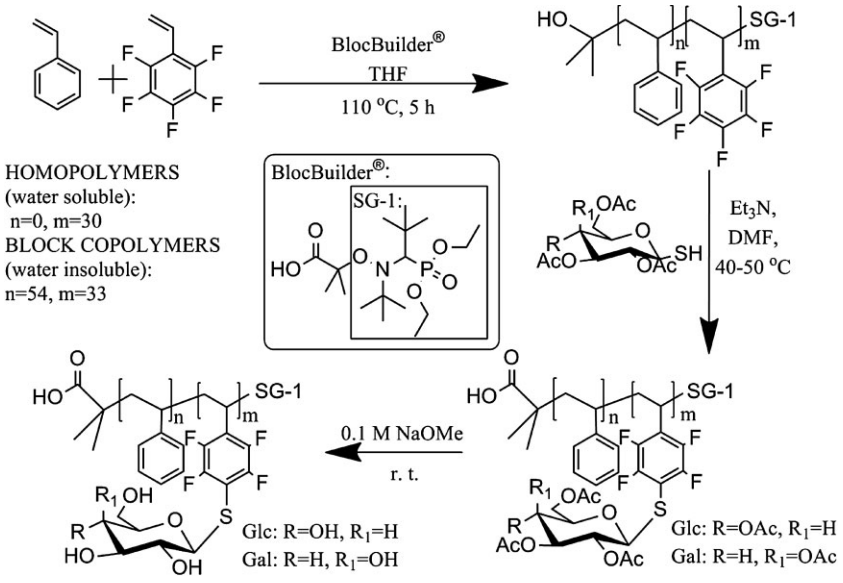
2.8.5. FC Analysis

After detaching the adherent cells by trypsin treatment, the cell suspension was washed twice with PBS supplemented with 10% FCS. For the investigation of cytotoxic effects, dead cells were labeled with a propidium iodide (PI) staining for 15 min at room temperature using 100 μg PI · mL^{-1} PBS. The cells were then incubated with 4% PFA dissolved in PBS for 10 min at room temperature and subsequently washed twice with PBS. A total of 2×10^4 cells were resuspended and subjected to FC using gates of forward and side scatters to exclude debris and cell aggregates.

3. Results and Discussion

3.1. Glycopolymer Synthesis and Labeling

The glycopolymers were synthesized as previously reported (Scheme 1).[22] In brief, glucosylated (hGlc) and galactosylated (hGal) homopolymers as well as the respective polystyrene block copolymers, bGlc and bGal, were obtained by post-polymerization modification using the thiol/*para*-fluorine "click" reaction to graft acetylated 1-thio-β-D-glucopyranose and 1-thio-β-D-galactopyranose onto a homopolymer of PFS as well as onto a block copolymer of styrene and PFS (PS-*b*-PFS). Subsequent deprotection of the carbohydrate moieties yielded well-defined, glucose- or galactose-modified polymers. The obtained glycopolymers were stable up to 220 °C, as confirmed by thermogravimetric analysis (see the Supporting Information). The post-polymerization modification approach, unlike the polymerization of glycosylated monomers, ensures that the materials investigated in this study are of the same degree of polymerization, since the grafting step was performed on the same batches of PFS and PS-*b*-PFS. In addition, the monomers used in the backbone synthesis are commercially available and thoroughly studied. Consequently, the synthetic problems connected with polymerization of unknown, sterically hindered carbohydrate containing units can be avoided.[24] The lengths of the obtained starting polymers were determined from ^{1}H NMR spectra, by integration of the signals derived from the initiator and the backbone (see Supporting Information) and SEC measurements. Furthermore, this method reduces the practical complications connected with the characterization of glycopolymers caused by the bulky carbohydrate moieties, which result in hydrodynamic diameter values that significantly deviate from the commonly applied SEC standard polymers (Table 1). In order to precisely determine the amount of attached carbohydrate moieties, after thiol/*para*-fluorine "click" reaction, ^{19}F NMR spectroscopy was used (Supporting Information).[19] The deprotected glycopolymers exhibited narrow molar mass distributions and carried equal amounts of carbohydrate units. Synthesis and characterization details for the obtained glycopolymers can be found in ref. [22] as well as in the Supporting Information (^{1}H, ^{13}C, and ^{19}F NMR spectroscopy).



Scheme 1. Schematic representation of the synthesis of the glucosylated and galactosylated homo and block copolymers.

Subsequently, the glycopolymers were labeled with FITC, as reported previously.[20] In order to ensure that the bioactivity of the carbohydrate moieties is maintained and the label is equally distributed, 0.5 equivalents of FITC per one polymeric chain were used for the labeling. SEC analyses of the fluorescent polymers (Figure 1) did not show any significant differences in the molar masses of the respective Glc and Gal derivatives. The low polydispersity index values were maintained for the labeled glycopolymers, indicating no coupling reactions of the polymeric backbone or its decomposition. Furthermore, the SEC traces show that the labeled homopolymers and block copolymers have the same molar masses and distributions as the respective non-labeled substrates. Successful introduction of the fluorescent label was further confirmed by the fluorescence spectra (Figure 2). For the same concentrations, the fluorescence of glucosylated polymer was 1.4 times higher than for the galactosylated one. This can be attributed to different amounts of FITC attached to the glycopolymers and has to be taken into account when interpreting the FC and CLSM measurements.

3.2. Preparation and Characterization of Nanoparticles

For the preparation of NPs the nanoprecipitation technique was chosen. The process of NP formation by solvent displacement method, i.e., mixing of the polymer solution with an anti-solvent that is miscible with the solvent, has already been applied for various synthetic polymers and biopolymers.[25–28] Important advantages are the fast and easy preparation and no necessity of surfactants. In particular, the absence of surface active agents like poly(vinyl alcohol) is essential for this study, as their

Table 1. Selected characterization data for the obtained glycopolymers.

Name	Structure	Carbohydrate amount[a)] [mol%]	$\overline{M}_{n\ theo}$ [g mol^{-1}]	$\overline{M}_{n,\ SEC}$[b)] [g mol^{-1}]	$\overline{M}_w/\overline{M}_n$[b)]
PFS	$PPFS_{35}$	0	7200	5700	1.06
hGlc	PTFSGlcOH	100	13 300	20 600	1.10
hGal	PTFSGalOH	99	13 200	19 300	1.06
PS-*b*-PFS	PS_{54}-*b*-$PPFS_{33}$	0	12 400	14 300	1.16
bGlc	PS-*b*-PTFSGlcOH	90	17 600	40 000	1.13
bGal	PS-*b*-PTFSGalOH	94	17 900	39 900	1.10

[a)]Calculated from ^{19}F NMR spectroscopy; [b)]Calculated according to polystyrene standards (PSS) using 2.1% LiCl solution in DMA as eluent.

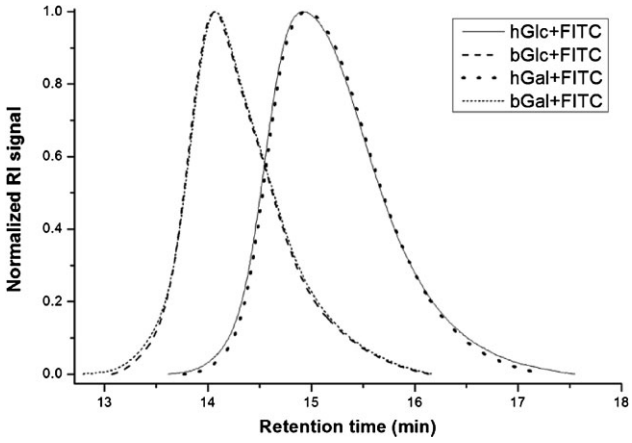


Figure 1. Normalized SEC traces of the FITC-labeled glycopolymers using 2.1% LiCl solution in DMA as eluent.

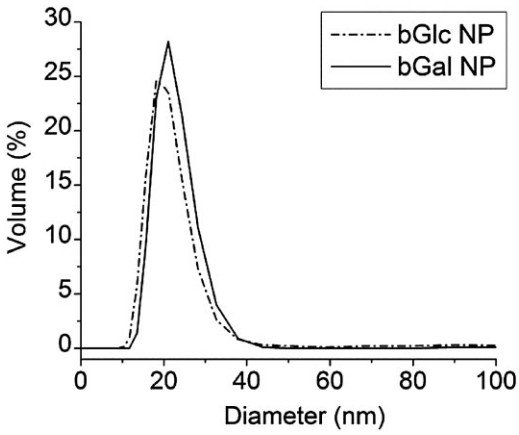


Figure 3. Volume size distributions of the fluorescent glycosylated nanoparticles, obtained by DLS.

presence may affect the cell uptake of the particles.[29] The particles for the cell study were obtained by dissolving the amphiphilic block glycopolymers in a water/THF mixture (1:1) and subsequent precipitation by successive dropwise addition of water to this solution (the homopolymers were water soluble and could, therefore, not be nanoprecipitated). THF provides good solubility for the PS block of the macromolecule and water is a good solvent for the glycosylated part, as shown for the homo-glycopolymers. Therefore, this solvent mixture gave visibly clear solutions of the amphiphilic block copolymers. The further addition of water caused the collapse of the polystyrene block, because of the water insolubility, and self-assembly of these hydrophobic parts into NPs. The resulting NP suspensions were characterized by DLS and the measurement of the zeta potential. The volume size distributions obtained by DLS are displayed in Figure 3. Both, glucosylated and galactosylated particles revealed the same hydrodynamic diameters of 20 nm. The zeta potentials of the particle suspensions (pH = 6) were determined to be −15 mV for bGlcNPs and −30 mV for bGalNPs. The negative zeta potential values can be attributed to the presence of electron rich hydroxyl groups of the sugar units as well as fluorine atoms, since the NPs prepared from unmodified, hydrophobic PS-*b*-PFS also revealed strongly negative (below −30 mV) values.[19] Apart from DLS investigations, the particles were characterized by electron microscopy techniques such as SEM and cryo-TEM (Figure 4). In the displayed images very small and spherical particles with diameters of 15–40 nm are visible, confirming the DLS results. The apparent larger sizes for glucosylated NPs result from the sample preparation technique and particle agglomeration during the drying process. Additional SEM images can be found in Supporting Information (Figure S7). In order to prove the long-term stability of the particles the suspensions were stored at 5 °C in the dark for twelve months. DLS measurements of these particles did not show any significant change in the size distribution. Furthermore, no aggregation or sedimentation of the particles was observed, confirming a very good stability of these glycosylated NPs in suspension. It is very important that the average diameters and size distributions are similar for both glycopolymers, in order to exclude any influence of the particle size on the cellular uptake.[30] Moreover, the shape of the particle as well as the morphology also affects its uptake.[31] The investigated NPs were all spherical with smooth surfaces; hence, the differences in their uptake can only result from the type of attached carbohydrate. The fluorescence of the particles at different concentrations in PBS was examined (Figure 5). The fluorescence intensities of the glucosylated NPs were, similarly to the water soluble glycopolymers, approximately 1.4-fold higher than of the galactose-functionalized NPs (also in this case the

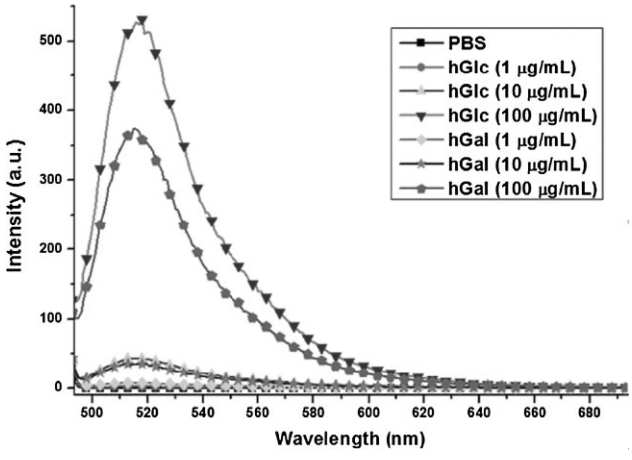


Figure 2. Fluorescence emission spectra of the FITC-labeled, water soluble glycopolymers.

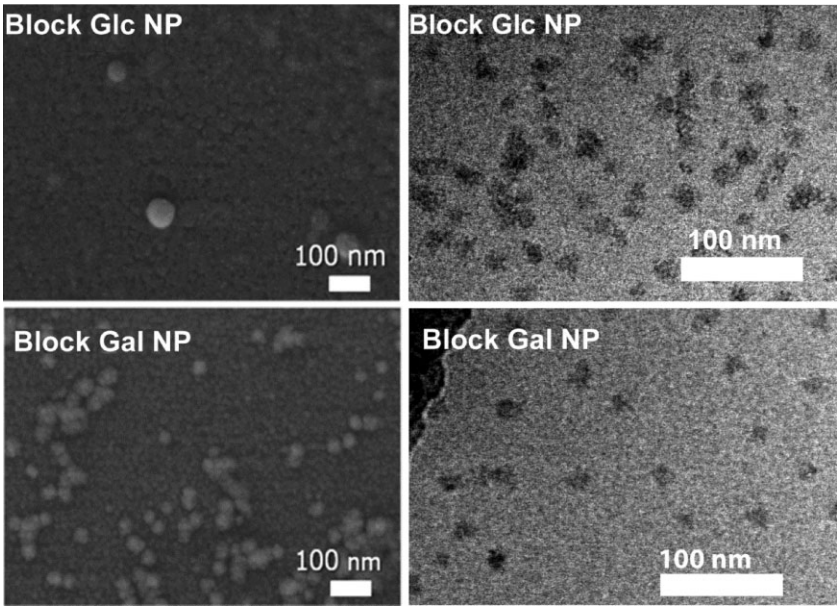


Figure 4. SEM (left) and cryo-TEM (right) images of the glycosylated nanoparticles, which reveal small and spherical particles with sizes between 15 and 40 nm.

difference has to be taken into account when evaluating the results of uptake studies performed by CLSM and FC).

3.3. Investigation of PFS-Based Glycopolymer Uptake by HepG2 Cells

3.3.1. CLSM Investigation of Uptake Dependence on Polymer Concentration and Type of Carbohydrate

Hepatocellular carcinoma HepG2 cells were seeded on sterile glass coverslips and incubated with the sugar containing polymers in order to investigate their interaction as described in the experimental section. Subsequently, the cells were fixed, using PFA, and the coverslips were mounted onto glass slides for microscopy observations. Representative CLSM micrographs of the cells are shown in Figure 6, left. The results reveal an increase of intracellular fluorescence after incubation with both, water soluble polymers and polymeric NPs in a concentration-dependent manner, confirming the uptake of the polymers by the HepG2 cells. To quantify the uptake, images were subjected to quantitative analysis using ImageJ software determining the mean fluorescence intensity per cell. At a concentration of $1\,\mu g \cdot mL^{-1}$ a clear increase of the fluorescence was observed for the internalized galactosylated, but not for the glucosylated materials (Figure 6, right). At higher concentrations the fluorescence resulting from the internalized NPs was much higher than from the water soluble homo-glycopolymers, but still galactosylated compounds were taken up to a higher degree than glucosylated ones.

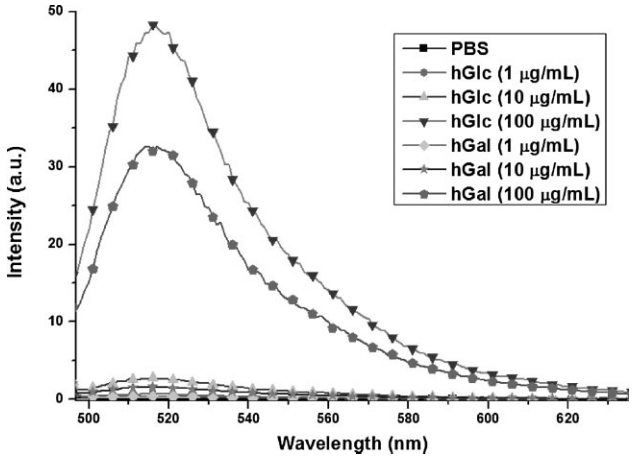


Figure 5. Fluorescence emission spectra of the FITC-labeled, glucosylated, and galactosylated nanoparticles.

3.3.2. Proof of Internalization by CLSM Investigations

To confirm that the particles as well as the soluble polymers did not adsorb to the outer leaflet of the plasma membrane but were truly internalized by the HepG2 cells, an aliquot of the cells was investigated by CLSM (Figure 7). To delineate the cell membrane, cells were stained with WGA conjugated to Alexa Fluor 633. WGA is a lectin (carbohydrate-binding protein), which selectively binds to the glycocalyx of cellular membranes. The overlay of the fluorescence originating from the labeled polymers (green) and the red stained membrane clearly shows that all particles are located inside the cells. This is further confirmed by z-stacks and a 3D reconstruction of the cells (see movie in Supporting Information). Both water-soluble polymers and NPs were accumulated in HepG2 cells.

3.3.3. Flow Cytometric Investigations of the Uptake

The uptake of the fluorescent labeled glycopolymeres was further quantified by FC measurements. For this purpose, cells were incubated with different concentrations (0.10, 1.00, and 10.00 $\mu g \cdot mL^{-1}$) of the materials for 24 h. Unbound polymers were removed by washing with PBS, and the cells were trypsinized, fixed with PFA and subjected to FC analysis. The mean fluorescence intensities per cells increased with increasing concentrations of the compounds. Since the intrinsic fluorescence intensities of the glucosylated compounds were 1.4 times higher than of the galactosylated materials (Figure 2 and 5), the results

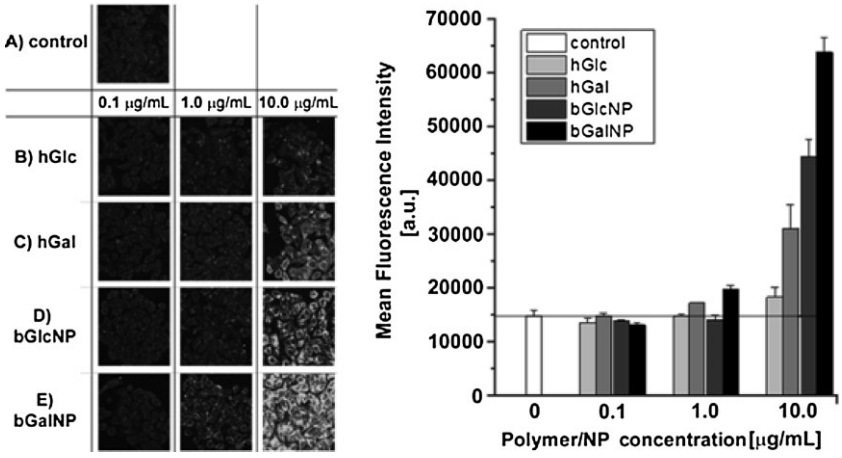


Figure 6. Confocal fluorescence microscopy images (left) of HepG2 cells after incubation with the water soluble polymers (hGlc and hGal) and polymeric nanoparticles (bGlcNP and bGalNP) for 24 h at 37 °C (left). Cells incubated with polymer free culture medium served as control. All images were obtained with identical instrument settings. Results from quantitative image analysis (right) of confocal fluorescence micrographs (left). Values for the mean fluorescence intensity per a HepG2 cell were assessed from grayscale-converted images using ImageJ software. A color version of this figure is available in the Supporting Information.

were normalized by this factor in order to enable an accurate comparison of the carbohydrate-specific uptake. The histogram plots (Figure 8) clearly show a concentration-dependent increase of the fluorescence intensity distributions (observable as a shift to the right) for all types of the polymers from a concentration of 1.0–10.0 $\mu g \cdot mL^{-1}$. The results, depicted in the bar chart, for the mean fluorescence intensities of the analyzed cell populations distinctly reveal the preferential uptake of the galactosylated compounds and confirm the results obtained from the image analysis of the CLSM micrographs (Figure 6). Furthermore, the fluorescence of the internalized glucose- and galactose-modified particles at concentration of 10 $\mu g \cdot mL^{-1}$ was much higher than of the respective homopolymers pointing to some additional uptake mechanism.

Additionally, FC investigation of cellular membrane integrity with PI exclusion assays showed that the analyzed materials did not affect the cell membrane integrity confirming the findings from CLSM experiments (see Supporting Information).[22]

The obtained results confirm the enhanced internalization of galactosylated PFS-based polymers and NPs. They are in good correlation with the previous studies, where both galactosylated and glucosylated materials show differences in their uptake via the asialoglycoprotein receptors (ASGPR).[32] Poly(*N*-*p*-vinylbenzyl-*O*-β-D-galactopyranosyl-[1 → 4]-D-gluconamide) (PVLA) was previously employed as a model ligand for ASGPR in order to examine the effect of the density of the carbohydrates attached to the polymeric backbone on binding and internalization of fluorescent polystyrene NPs by hepatocytes.[33] The uptake of particles (500 nm), coated with various concentrations of water soluble PVLA and non-galactosylated poly(vinylbenzyl-D-gluconamide), by rat hepatocytes, was found to be proportional to the galactose content.

These findings can be exploited to target liver cells selectively. So far, amphiphilic, galactose-functionalized polycarbonate block copolymers, prepared by ring opening polymerization, have been already employed to prepare doxorubicin-loaded micelles with diameters below 100 nm and narrow size distributions.[34] The micelles were taken up selectively by an ASGPR-expressing HepG2 HCC cell line and had a significantly higher cytotoxicity of the drug as compared to the ASGPR-negative HEK293 cell line. Herein, the fluorescence intensities of hGal and bGalNP, internalized by the HepG2 cells, are distinctly higher, at the concentration of 1.0 $\mu g \cdot mL^{-1}$, than the cellular autofluorescence (control) as well as the fluorescence of cells cultured with the glucosylated materials (Figure 6). Therefore, the galactosyl moieties attached to PFS backbones via thiol-*p*-fluorine "click" reaction retain their hepatocyte-uptake enhancing properties.

On the other hand, the uptake of glucosylated polymers and NPs was higher than expected, when comparing with similar glucose-modified glycopolymers having a hydro-

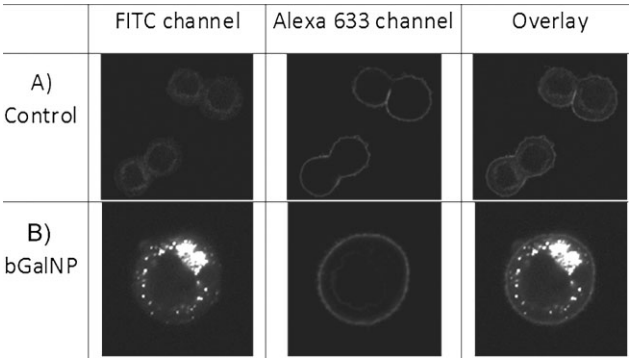


Figure 7. Representative CLSM micrographs of cells incubated with 10 $\mu g \cdot mL^{-1}$ of polymeric nanoparticles from bGal for 24 h at 37 °C. The cells were detached and stained with WGA conjugated to Alexa Fluor 633 to mark the plasma membrane. Fluorescence originating from the labeled polymers was collected in the FITC channel (1st column) whereas fluorescence originating from the plasma membrane was collected in the Alexa 633 channel (2nd column). The overlay of both channels (3rd column) clearly shows that the particles are located inside the cells. A color version of this figure is available in the Supporting Information.

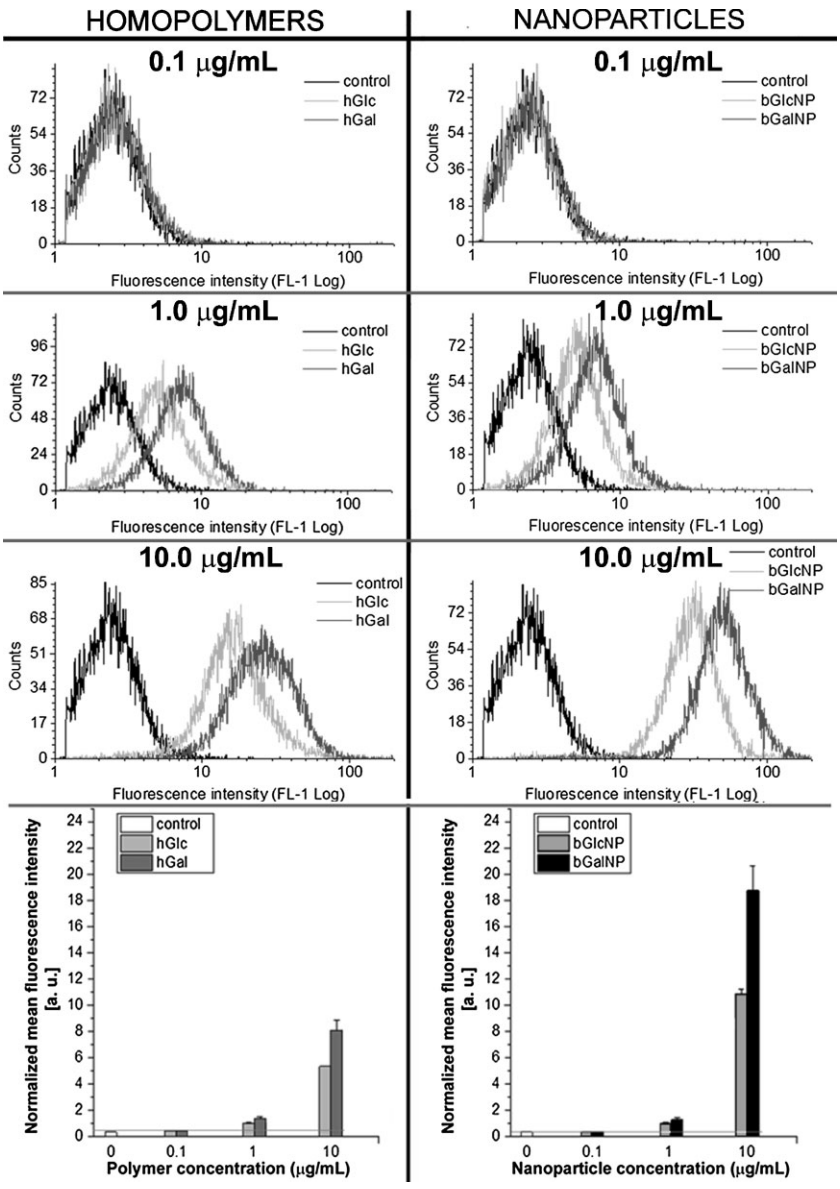


Figure 8. Histogram plots from flow cytometry on the uptake of water soluble polymers (hGlc and hGal) and polymeric nanoparticles (bGlcNP and bGalNP) by HepG2 cells after 24 h incubation at 37 °C. Cells incubated with polymer free culture medium served as control. The fluorescence intensity on the *x*-axis is plotted against the number of events on the *y*-axis. A shift of the histogram toward the right side demonstrates an increasing amount of FITC-labeled polymers/nanoparticles attached to or taken up by the cells. The bar charts depict the results for the mean fluorescence intensities, obtained from flow cytometry of the analyzed cell populations. Various polymer concentrations, physical forms (water soluble: hGlc and hGal; nanoparticles: bGlcNP and bGalNP), as well as carbohydrate moieties, are taken up to a different degree.

phobic polystyryl backbone, namely poly[*N*-*p*-vinylbenzyl-*O*-α-D-glucopyranosyl-[1 → 4]-D-gluconamide] (PVMA) and poly[3-*N*-*p*-vinyl-benzyl-D-glucose] (PVG).[35] These polymers carry glucose substituted at C-1 and C-3, respectively, and did not show any clear interaction with hepatocytes. However, hepatocytes took up the polymer with the carbohydrate attached via C6 (poly[*N*-*p*-vinylbenzyl-D-glucuronamide], PV6Gna) but to a lower degree than galactosylated PVLA.[36] It has to be taken into account that not only the type of sugar but also the rotational restriction, stiffness and hydrophobicity can influence the glycopolymer/hepatocyte interactions, as was shown for chitosan beads functionalized with lactonamide units.[37] Our observation concerning the higher uptake (at the concentration of 10 $\mu g \cdot mL^{-1}$) of nanoparticulate materials, as compared to the soluble homopolymers, is in line with studies on the interaction of poly(ethylene glycol)-*block*-poly(ε-caprolactone) block copolymeric micelles with HepG2. They have shown that even non-targeted particulated materials were taken up by hepatocellular carcinoma cells following 24 h of incubation.[38] This uptake was attributed to unspecific interactions and non-receptor-mediated endocytosis. The ratio between internalized non-glycosylated and galactosylated micelles, in ref.,[38] corresponds well to the ratio between bGlcNPs and bGalNPs taken up by the cells in this work.

4. Conclusion

Through nanoprecipitation of water-insoluble galactosylated or glucose-modified compounds NPs of the same sizes and similar morphologies were obtained. As a consequence, an influence of the polymeric architecture as well as of the particle diameter and shape on the interaction with cells could be excluded. Fluorescent labeling of the water soluble polymers and particles facilitated the use of CLSM and FC to confirm the carbohydrate specific uptake of the water soluble galactosylated polymer as well as of the NPs prepared from galactose-modified polystyrene block copolymer and show that internalization of galactosylated homo- and nanoprecipitated block copolymers is enhanced as compared to the respective glucose substituted compounds. NPs are taken up to a higher degree than respective water soluble polymers. All materials did not show any acute cytotoxicity.

Since these results revealed that the preferential internalization of β-D-galactose by HepG2 cells is maintained upon grafting onto PPFS-based polymers, these glycopolymers can find a multitude of potential applications in, for example, liver tumor-targeted chemotherapy, imaging, and as extracellular matrices for hepatocytes. Additionally, the PPFS-based glycopolymers can be modified with other thiolated glycosides for lectin-mediated drug targeting as reported for other classes of carbohydrate-containing polymers.

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