Adhesion of Preosteoblasts and Fibroblasts onto Poly(pentafluorostyrene)-Based Glycopolymeric Films and their Biocompatibility

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Introduction

Glycopolymeric materials, consisting of a synthetic polymeric backbone and pendant sugar moieties, have attracted researcher’s attention because of their unique properties and are currently applied, e.g., in clinical diagnostics, as targeted drug delivery systems, for affinity separations, bioassays, and biocapture analysis. The carbohydrate units act as ligands for a broad spectrum of protein receptors and, therefore, play a significant role in a variety of biological processes, like immunological recognition, interaction between bacteria or viruses with cells, or tissue growth and repair. Furthermore, the development of new methods for the synthesis of tailor-made polymers as well as sugar derivatives has given access to well-defined architectures and various techniques for their modification with carbohydrate moieties. Thus, synthetic glycopolymers represent a promising tool allowing the precise control of biological properties.
elucidation of various cell/surface interactions. Glycopolymeric coatings for cell attachment, proliferation, and differentiation are of significant interest since Schnaar et al. performed studies on the adhesion of chicken hepatocytes to poly(acrylamide) gels derivatized with N-acetylglucosamine.[12] The behavior of parenchymal liver cells on sugar-modified extracellular matrices has been thoroughly examined, and its dependence on the type of carbohydrate moieties as well as on the polymeric backbone and architecture was proven.[13–17] To further evaluate the potential of glycopolymer-coated substrates for cell cultivation, the attachment and proliferation of fibroblasts on hyperbranched glycoacrylate films and on polystyrene (PS) carrying N-acetylglucosamine residues was studied.[18,19] Both surfaces were proven to be non-cytotoxic, biocompatible, and to act as appropriate supports for fibroblast adhesion and proliferation. Different research activities concentrated on culturing erythrocytes on poly-[3-O-(4′-vinylbenzyl)-d-glucose].[20,22] The presence of reducing glucose moieties was crucial for a specific cell attachment. The adhesion of erythrocytes proceeded via type-1 glucose transporters and led to unique teardrop cell morphologies. Osteoblasts are responsible for bone formation and, therefore, of great interest for hard tissue engineering.[22] Their adhesion to various implant materials, such as calcium phosphate-based bioceramics, bioinert metals, metal alloys, or nondegradable synthetic polymers and their composites, has been extensively studied.[23–28] To the best of our knowledge, the adhesion of osteoblast to glycopolymeric substrates has not been examined yet.

In order to study the interaction between polymers and cells, well-defined materials are essential.[29] Different synthetic approaches to obtain glycopolymers, their advantages and disadvantages have been published and thoroughly reviewed recently.[4,30–34] The methods can be divided into two groups, namely the polymerization of glycosylated monomers or the grafting of sugar moieties onto a preformed polymeric backbone.[35] The polymerization of sugar-bearing moieties most often requires painstaking optimization of the reaction conditions and multi-step synthesis of the reactants.[36] In contrast, various living/controlled polymerization techniques have been applied to obtain well-defined, polymeric backbones with accurate molar masses and narrow polydispersity indices. Among them, the controlled radical polymerizations have been of significant interest to obtain tailor-made (block)copolymers, functional materials, and polymers for a wide range of biological applications.[37] In the reported research, nitroxide-mediated radical polymerization (NMP) was employed to synthesize pentafluorostyrene (PFS) homopolymers and PS block copolymers. NMP has been previously applied to obtain glycopolymers and copolymers from sugar-carrying, styrene-based monomers.[38–47] This method is suitable for the synthesis of biopolymers since it does not require any catalyst or metal salt to mediate the reaction, which represents a major disadvantage for most of the other techniques. However, the employed sugar substituted monomers required significant synthetic effort. Therefore, we have focused our research on post-polymerization functionalization of well-defined backbones obtained from commercially available monomers. The synthesis of functional polymers via this approach has been recently reviewed by Gauthier et al.[48] The most commonly employed coupling method for grafting of sugar moieties is the formation of amide bonds between polymers carrying activated carbonyl groups, e.g., N-hydroxysuccinimide esters or anhydrides and different aminosaccharides.[35] However, the N-glycosidic linkages produced by this approach may be susceptible to hydrolysis by different enzymes, such as amide hydrolyses and N-glycosyltransferrases.[49,50] Other post-polymerization functionalization methods belong to the group of “click” reactions, which proceed with high efficiencies and tolerate various other functionalities.[51–54] The most prominent click reactions are the copper-catalyzed azide/alkyne cycloaddition (CuAAC) and the thiol-ene coupling. Haddleton and coworkers have produced defined, alkyne-functionalized, polymeric architectures using controlled/living radical polymerization techniques. The polymers were subsequently reacted with various sugar azides in the presence of copper to yield glycopolymers.[55–59] A similar approach was reported recently by Lee et al. who clicked galactose residues onto a polycationic scaffold.[60] CuAAC was also used to modify azide-functionalized PS with galactose and lactose bearing triple bonds.[61] Since the carbohydrate-carrying polymers prepared via CuAAC require extensive purification from the remaining copper catalyst before application in biological studies, the thiol based click reactions provide an interesting alternative.[35] Thiol-ene coupling represents a versatile method for the functionalization of alkene-bearing polymeric scaffolds with a variety of mercaptans.[62] It has been employed to graft thiosugars onto a block copolymer of poly(ethylene glycol) methacrylate with alkene-grafted poly(2-hydroxyethyl methacrylate), a series of poly[2-(isopropyl/3-butenyl)-2-oxazolines] as well as 1,2-polybutadiene.[63–68] The method results in the formation of materials with S-glycosidic bound sugar residues, which are, unlike O- and N-glycosidic bonds, stable against enzymatic degradation.[69,70]

In this contribution, the thiol/para-fluoro click glycosylation was applied.[71] The reaction was carried out at slightly elevated temperatures (40 and 50 °C) to ensure high degrees of substitution of the para-fluorine moieties. The method comprises main advantages. It leads to uniform (co-)polymers with S-glycosidic bound sugar residues of different type, and the conversion can be quantified by 19F NMR spectroscopy. Also, the cytotoxicity of the prepared glycopolymers against 3T3 fibroblasts and MC3T3-E1...
preosteoblasts was investigated. Furthermore, thermal and film forming properties of these materials were discussed.

**Experimental Part**

**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-glucopyranoside (≥99%) was purchased from GLYCON Biochem GmbH, triethylamine (TEA) from Merck (for synthesis, ≥99%), N,N-dimethylformamide (DMF, ≥99.5%) and N,N-dimethylacetamide (DMA) from Fluka, methanol (analytical grade) from Aldrich. 2,3,4,6-Tetra-O-acetyl-1-thio-β-D-galactopyranoside was synthesized as previously reported.[72] Sodium methanolate was purchased from Fluka and stored under argon prior to use. All other chemicals were used as received, unless otherwise noted.

**Measurements**

$^1$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 DMF residual peaks. Size-exclusion chromatography (SEC) was measured on an Agilent Technologies 1200 Series gel permeation chromatography system equipped with a G1310A refractive index detector, a PSS Gram 1000 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 calculated according to PS standards. Thermogravimetric analyses (TGA) were performed on Netzsch TG 209 F1 Iris with 10 °C min$^{-1}$ heating rates from room temperature up to 900 °C under nitrogen flow. The water contact angle was measured with an OCA Dataphysics system. The film morphologies were observed with a WYKO NT9100 confocal white-light interferometer (Veeco Instruments, Tucson, Arizona, USA). Fluorescence of the surfaces was monitored using an Axiotech microscope (Zeiss AG, Jena, Germany) with filter sets 09 and 14. Photomicrographs were recorded using a CCD fluorescence imager MP 5000 (Intas, Göttingen, Germany). Imaging was supported by the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

**Synthesis of Poly(pentafluorostyrene) (PPFS)$_{55}$, 1** and PS$_{54}$

The synthesis of PS$_{54}$ was reported previously.[71] PPFS was synthesized analogously. PFS (7.11 mL, 51.5 mmol), BlocBuilder (393 mg, 1.03 mmol), and tetrahydrofuran (10 mL) were added in a 25 mL pressure-resistant round-bottom flask. The mixture was saturated with argon while stirring for at least 30 min. The flask was sealed and placed into a preheated oil bath (110 °C). The reaction was continued for 5 h. Subsequently, the reaction vessel was cooled down with tap water and the viscous reaction mixture was precipitated into cold methanol to remove the residual monomer. The isolated white powder was dried under vacuum for 24 h.

PPFS$_{55}$ (1): $^1$H NMR (DMF-d$_7$): $\delta$ = 1.42–0.80 (m, 10 × CH$_3$ initiator), 2.36–1.94 (broad signal, CH$_2$ backbone), 2.74–2.40 (broad signal, CH backbone), 3.57–3.45 (broad s, H$_2$O), 4.33–3.95 (CH$_2$ initiator).

$^{19}$F NMR (DMF-d$_7$): $\delta$ = −164.69 (broad signal, meta-F), −158.66 (broad signal, para-F), −140.96–145.32 (broad signal, ortho-F).

$^{13}$C NMR (DMF-d$_7$): $\delta$ = 27.6 (CH$_3$ initiator), 33.1–31.9 (CH backbone), 38.5–36.6 (CH$_2$ backbone), 146.4, 143.6, 138.8, 138.1, 135.4, 115.7 (broad signals, C$_{ac}$/F coupling).

Synthesis of Poly[4-(2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranosido)-2,3,5,6-tetrafluorostyrene] (PPFSGlAc, 2)

Prepared according to the previously described method.[71] In brief, 1 (500 mg, 2.58 mmol) and acetyl protected thiosugar (1.88 g, 5.15 mmol) were dissolved in 15 mL of dry DMF and TEA (1.08 mL, 7.73 mmol) was added to the solution. After stirring for 8 h at 40 °C the reaction mixture was concentrated on a rotary evaporator to ≈2.5 mL and precipitated into cold methanol. The precipitate was filtered, washed twice with methanol and dried in a vacuum oven to yield 1.00 g of a white powder, PFSGlAc (2, isolated yield = 93%).

$^1$H NMR (CDCl$_3$): $\delta$ = 1.38–0.71 (broad signals, initiator), 2.64–1.71 (backbone and CH$_3$ of acetyl groups), 5.88–3.54 (multiple broad signals of carbohydrate protons).

$^{19}$F NMR (CDCl$_3$): $\delta$ = −140.16–144.95 (broad signal, meta-F), −129.58–135.93 (broad signal ortho-F).

$^{13}$C NMR (CDCl$_3$): $\delta$ = 20.5 (CH$_3$-acetyl), 61.4, 68.0, 70.1, 73.7, 76.0, 85.3 (carbohydrate carbons), 170.6–169.1 (C acetyl).

Synthesis of Poly[4-(2,3,4,6-tetra-O-acetyl-1-thio-β-D-galactopyranosido)-2,3,5,6-tetrafluorostyrene] (PPFSGlAcGal, 3)

1 (250 mg, 1.288 mmol) and the protected sugar (1.00 g, 2.74 mmol) were dissolved in 9 mL of dry DMF. TEA (550 µL, 3.95 mmol) was added to the reagent solution. The reaction mixture was stirred for 10 h at 50 °C, concentrated to ≈2.5 mL, and precipitated into cold ethanol. The polymer was purified by reprecipitation from chloroform into ethanol (four times), filtered, and vacuum-dried to afford 610 mg of a white powder, PFSGlAcGal (3, isolated yield = 88%).

$^1$H NMR (DMF-d$_7$): $\delta$ = 1.42–0.76 (broad signals, initiator), 2.57–1.79 (backbone and CH$_3$ of acetyl groups), 3.52 (broad signal, H$_2$O), 6.09–3.78 (multiple broad signals of carbohydrate protons).

$^{19}$F NMR (DMF-d$_7$): $\delta$ = −163.69–166.37 (broad signal, meta-F, PPSs), −141.24–146.28 (broad signal, meta-F), −133.34–136.54 (broad signal, ortho-F).

$^{13}$C NMR (DMF-d$_7$): $\delta$ = 20.9 (CH$_3$-acetyl), 62.3, 68.3–69.4, 72.5, 75.7, 87.3 (carbohydrate carbons), 110.9, 123.3, 143.7, 147.3, 149.7 (broad signals, C$_{ac}$/F coupling), 171.4–170.5 (C acetyl).

Synthesis of Poly[4-(1-thio-β-D-glucopyranosido)-2,3,5,6-tetrafluorostyrene] (PPFSGlOH, 4)

The acetylated homopolymer (2) (1.00 g, 1.857 mmol) was dissolved in an oven-dried, round-bottom flask in 30 mL of dry DMF. Sodium methanolate (2.00 mL of 0.1 M solution in dry methanol) was added...
dropwise via a syringe to the solution. The reaction mixture was stirred at room temperature for 1.5 h, concentrated by evaporation to $\approx 3 \text{ mL}$, and precipitated into cold ethanol. The glycopolymer was purified by reprecipitation from water into cold ethanol and vacuum-dried to obtain 358 mg of white powder, \textit{PTFSGlcOH}, (4), isolated yield = 55\%.

$^1$H NMR (DMF-$d_7$): $\delta = 1.43 - 0.75$ (broad signals, initiator), 2.39 – 1.80 (backbone), 4.90 – 4.63, and 3.96 – 3.19 (multiple broad signals of carbohydrate protons), 6.05 – 5.57, 5.47 – 5.06, and 4.55 – 4.22 (broad signals of OH groups of the sugar).

$^{13}$F NMR (DMF-$d_7$): $\delta =$ (140.91 – 146.18) (broad signal, meta-$F$), (133.20 – 137.01) (broad signal, ortho-$F$), (163.62 – 166.25) (broad signal, meta-$F$), (133.53 – 137.46) (broad signal, ortho-$F$), (134.23) (broad signal, -galactopyranosido)-2,3,5,6-tetrafluorostyrene) (PS-$F$) 2011

Synthesis of Poly[1-thio-$\beta$-d-galactopyranosido]-2,3,5,6-tetrafluorostyrene (PTFSGaloH, 5)

Analogous to the synthesis of 4, 350 mg (0.650 mmol) of 3 were dissolved in 12 mL of dry DMF, and 700 $\mu$L of 0.1 $\mu$L sodium methanolate were added. 200 mg of white powder, \textit{PTFSGaloH}, (5), was obtained (isolated yield = 73\%). $^1$H NMR (DMF-$d_7$): $\delta = 1.42 - 0.75$ (broad signals, initiator), 2.38 – 1.80 (backbone), 4.89 – 3.32 (multiple broad signals of carbohydrate protons), 5.96 – 5.7, and 5.17 – 4.91 (broad signals of OH groups of the sugar units).

$^{13}$F NMR (DMF-$d_7$): $\delta =$ (163.62 – 166.25) (broad signal, meta-$F$, PPFS), (141.82 – 147.33) (broad signal, meta-$F$), (133.54 – 137.94) (broad signal, ortho-$F$).

$^{13}$C NMR (DMF-$d_7$): $\delta =$ 40.6 – 33.0 (backbone), 62.0 – 60.7, 70.1 – 69.3, 72.0, 76.35, 81.0 – 79.8, and 87.6 – 85.3 (carbohydrate carbons), 112.1, 121.7, 144.0, 146.9, 149.5 (broad signals, C$_{Ar}$/F coupling).

Synthesis of the Poly styrene-block-Poly-(pentfluorostyrene) (PS$_{b-a}$-PPFS)$_{33}$ Copolymer (6)

The synthesis of the block copolymer was published previously. \cite{[71]} Selected characterization data of the chosen polymer, PS$_{b-a}$-PPFS$_{33}$ (6), $^1$H NMR (DMF-$d_7$): $\delta =$ 1.12 – 0.75 (broad signal, CH$_3$ initiator), 1.80 – 1.35 (broad signal, CH$_2$ backbone), 2.46 – 1.81 (broad signal, CH backbone), 3.64 – 3.38 (broad signal, CH$_2$ backbone), 6.39 – 6.43 (broad signal, para-$H$, phenyl), 7.45 – 6.93 (broad signal, ortho- and meta-$H$, phenyl).

$^{13}$F NMR (DMF-$d_7$): $\delta =$ (165.01) (broad signal, meta-$F$), (158.87) (broad signal, ortho-$F$), (140.35 – 145.79) (broad signal, ortho-$H$).

$^{13}$C NMR (DMF-$d_7$): $\delta =$ 34.4 – 32.8 (CH backbone), 42.2 – 40.9 (CH$_2$ backbone), 126.9 (phenyl), 129.8 – 128.2 (phenyl), 117.1, 136.8, 140.2, 142.5, 144.7, 147.7 (broad signals, C$_{Ar}$/F coupling).

Synthesis of Poly styrene-block-Poly-[4-(2,3,4,6-tetra-O-acetyl-1-thio-$\beta$-d-glucopyranosido)-2,3,5,6-tetrafluorostyrene] (PS$_{b-b}$-PTFSGlcoAc, 7)

Prepared according to the previously described method. \cite{[71]} Briefly, 6 (1.00 g, 2.659 mmol PS units) and the protected thiogluco (1.40 g, 7.135 mmol) were dissolved in 30 mL of dry DMF. TEA (1.20 mL, 8.610 mmol) was added to the reaction solution. The mixture was stirred for 8 h at 40 $^\circ$C, concentrated afterwards on a rotary evaporator and precipitated into cold methanol. The polymer was purified by reprecipitation from chloroform into cold methanol (three times), filtered, washed with methanol, and vacuum-dried to give 1.20 g of a white powder, PS$_{b-b}$-PTFSGlcoAc (7), (isolated yield = 85\%). $^1$H NMR (CDCl$_3$): $\delta =$ 1.12 – 0.72 (broad signal, CH$_3$ initiator), 1.66 – 1.24 (broad signal, CH$_2$ backbone), 2.52 – 1.64 (backbone and CH$_2$ of acetyl groups, 5.86 – 5.97 (multiple broad signals of carbohydrate protons), 6.74 – 6.30 (broad signal, para-$H$, phenyl), 7.24 – 6.88 (broad signal, ortho- and meta-$H$, phenyl).

$^{13}$F NMR (CDCl$_3$): $\delta =$ (160.5 – 162.17) (broad signal, meta-$F$ of PPS), (153.51 – 154.54) (broad signal para-$F$ of PPS), (140.10 – 145.12) (broad signal, meta-$F$ of TFS-GlcAc and ortho-$F$ of PPS), (129.44 – 134.23) (broad signal, ortho-$F$ of TFS-GlcAc).

$^{13}$C NMR (CDCl$_3$): $\delta =$ 20.5 (CH$_3$-acetyl), 34.0 – 30.8 (broad signal, CH$_2$ backbone), 42.0 – 39.8 (CH$_2$-backbone), 61.6, 68.0, 69.4, 70.3, 76.0, 85.8 (carbohydrate carbons), 125.6 (phenyl), 129.0 – 126.8 (phenyl), 131.0, 122.2, 143.4, 145.3, 146.4 (broad signals, C$_{Ar}$/F coupling) 170.4 – 168.9 (C acetyl).

Synthesis of Poly styrene-block-Poly-[4-(1-thio-$\beta$-d-glucopyranosido)-2,3,5,6-tetrafluorostyrene] (PS$_{b-b}$-PTFSGlcoH, 9)

Similar to the preparation of 4, 700 mg (0.910 mmol of grafted PFS units) of copolymer 7 dissolved in 20 mL of dry DMF were used, and 910 $\mu$L of 0.1 $\mu$L sodium methanolate were added. The product was purified by reprecipitation from DMF into cold ethanol and vacuum-dried to result in 358 mg of a white powder, PS$_{b-b}$-PTFSGlcoH (9), isolated yield = 67\%.$^1$H NMR (DMF-$d_7$): $\delta =$ 1.23 – 0.91 (broad signal, CH$_3$ initiator), 2.34 – 1.27 (backbone), 4.86 – 4.64, 4.31 – 4.17, 4.07 – 3.88, and 3.83 – 3.41 (multiple broad signals of carbohydrate protons), 6.85 – 6.46 (broad signal, para-$H$, phenyl), 7.32 – 6.96 (broad signal, ortho- and meta-$H$, phenyl).

$^{13}$F NMR (CDCl$_3$): $\delta =$ (163.77 – 165.84) (broad signal, meta-$F$ of PPS), (157.20 – 159.04) (broad signal para-$F$ of PPS), (143.92 – 147.12) (broad signal, meta-$F$ of TFS-GlcOH and ortho-$F$ of PPS), (153.87 – 151.27) (broad signal, para-$F$ of TFS-GlcOH).
138.06) (broad signal, ortho-F of TFS-GlcOH). $^{13}$C NMR (CDCl$_3$): $\delta = 36.7$–32.9 (CH$_2$-backbone), 42.5–40.7 (CH-backbone), 60.9, 69.3, 72.8–71.4, 75.8, 80.2, 88.5–87.1 (carbohydrate carbons), 126.6 (phenyl), 129.3–128.0 (phenyl), 111.8, 121.5, 143.8, 147.5–145.7, 148.7 (broad signals, C$_{Ar}$/F coupling).

**Polystyrene-block-Poly[4-(1-thio-β-D-galactopyranosido)-2,3,5,6-tetrafluorostyrene] (P5-b-PTFSGalOH, 10)**

Analogous to the deprotection, 4,200 mg (0.264 mmol of grafted PFS units) of 8 were dissolved in 5 mL of dry DMF and 264 $\mu$L of 0.1 m sodium methanolate. The purification gave 126 mg of a white powder, P5-b-PTFSGalOH (10, isolated yield = 81%). $^1$H NMR (DMF-d$_2$): $\delta = 1.21$–0.75 (broad signal, CH$_3$ initiator), 2.34–1.27 (backbone), 4.86–4.64, 4.31–4.17, 4.07–3.88, and 3.83–3.41 (multiple broad signals of carbohydrate protons), 6.85–6.46 (broad signal, para-H, phenyl), 7.32–6.96 (broad signal, ortho- and meta-H, phenyl). $^{19}$F NMR (CDCl$_3$): $\delta = -(163.77$–$165.84)$ (broad signal, meta-F of PFS), $-(157.20$–$159.04)$ (broad signal para-F of PFS), $-(141.92$–$147.12)$ (broad signal, meta-F of TFS-GalOH and ortho-F of PFS), $-(133.87$–$138.06)$ (broad signal, ortho-F of TFS-GalOH).

$^{13}$C NMR (CDCl$_3$): $\delta = 36.7$–32.9 (CH$_2$-backbone), 42.5–40.7 (CH-backbone), 60.9, 69.3, 72.8–71.4, 75.8, 80.2, 88.5–87.1 (carbohydrate carbons), 126.6 (phenyl), 129.3–128.0 (phenyl), 111.8, 121.5, 143.8, 147.5–145.7, 148.7 (broad signals, C$_{Ar}$/F coupling).

**Film Formation in the 96-Well Poly(propylene) (PP) Microtiter Plates**

The non-glycosylated and the acetylated polymers (6 and 7) were drop-cast as 30 $\mu$L aliquots, a sufficient volume for covering the surface of the bottom of the well, of 10 mg $\cdot$ mL$^{-1}$ solutions in tetrahydrofuran (THF) into 96-well PP microtiter plates. The polymers carrying deprotected sugar moieties (9 and 10) were seeded as 30 $\mu$L aliquots of 10 mg $\cdot$ mL$^{-1}$ solutions in THF/water (4:1) into 96-well PP microtiter plates. The plates were dried in an oven at 37 °C for 1 h.

**Film Formation on Glass Slides**

Solutions (2 mg $\cdot$ mL$^{-1}$ in DMF) of the polymers carrying deprotected sugar moieties 9 and 10 were drop-cast onto 1 cm$^2$ glass slides in a sufficient volume to cover the whole surface and subsequently dried in an oven at 110 °C for 0.5 h.

**Cell Culture**

The 3T3 fibroblasts (from the DSMZ German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were cultured in Dulbecco’s modified eagle medium (DMEM) and MC3T3-E1 cells (from DSMZ) in alpha medium with 2 $\times$ 10$^{-5}$ M l-alanyl-l-glutamine (both media were supplemented with 10% fetal calf serum, 50 U $\cdot$ mL$^{-1}$ penicillin, and 50 $\mu$g $\cdot$ mL$^{-1}$ streptomycin, all components from Biochrom, Berlin, Germany) under 5% CO$_2$ atmosphere at 37 °C.

**3T3 Fibroblast and MC3T3-E1 Preosteoblast Viability and Proliferation**

The influence of the glycosylated polymers on viability and proliferation of 3T3-fibroblasts and MC3T3-E1 preosteoblasts was examined by using the fluorescein diacetate (FDA)/ethidium bromide (EtBr) viability assay.$^{[73,74]}$

For the water-soluble glycopolymer PTFSGlOH (4), the cell line was inoculated with 25 000 cells $\cdot$ cm$^{-2}$ into a 24-well cell-culture plate (Greiner Bio-One, Frickenhausen, Germany) and cultivated for 3 h at 37 °C. Then the culture medium was changed to different concentrations of 4 (10, 1, and 0.1 mg $\cdot$ mL$^{-1}$, respectively) in culture medium (four replicates). The tests were performed in duplicate after 1 and 4 d without changing the nutrient medium. The water-insoluble polymers, namely PFS (1) and P5-b-PTFSGlOH (9), were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 2 mg $\cdot$ mL$^{-1}$ and placed as 100 $\mu$L aliquots on six glass coverslips (15 mm diameter). After drying in an oven at 25 °C for 2 d, the coated surfaces were transferred into a 24-well microtiter plate, sterilized with 70% ethanol solution for 30 min, washed twice with PBS, and equilibrated in the culture medium. The cells were inoculated onto the coverslips analogously to the cell preparation for the testing of water-soluble polymer 4. The tests were performed in triplicate after 1 and 4 d with the renewal of the nutrient medium after 2 d. The previously prepared films of 9 and 10 were tested with MC3T3-E1 cells following the same procedure.

The viability staining followed the standard protocol for both water-soluble and -insoluble polymers. After 1 and 4 d the specimens were withdrawn and the cells were covered with PBS [amount assessed to avoid drying of the cells] and with an equal volume of twofold concentrated staining solution realizing the final concentrations of 15 $\mu$g $\cdot$ mL$^{-1}$ FDA and 4 $\mu$g $\cdot$ mL$^{-1}$ EtBr. The red and green fluorescence was observed after 1–10 min using fluorescence microscopy.

**Cell Adhesion Assays**

In order to analyze and compare the adhesion of fibroblast and preosteoblastic cells to the surfaces of the synthesized glycopolymer, the temporal behavior of the cell attachment was measured.$^{[75]}$ For this purpose, the number of cells that were firmly attached to the surface of the polymeric films and resisted the washing procedure was detected 30, 60, 90, and 120 min after seeding.

The 96-well microtiter plates with polymeric films were disinfected with 0.2 mL 70% ethanol. The ethanol was removed by two exchanges with PBS. Cells (3T3 and MC3T3-E1 cells in independent experiments) were harvested from precultures by trypsin/ethylenediaminetetraacetate (EDTA) treatment, and a suspension containing 80 000 cells $\cdot$ mL$^{-1}$ in their respective culture medium was prepared and held in a 50 mL poly(propylene) tube under occasional agitation at room temperature. From this suspension, every 30 min, four rows of eight cavities were inoculated with 0.2 mL of the cell suspension in each case. Starting the incubation of cells with a temporal shift allowed to subject the whole plate to the washing procedure at the end of the different adhesion times. Washing with PBS was performed automatically.
using the programmable ELISA washer Columbus (Tecan, Crailsheim, Germany) with defined wash steps (overflow mode: 0.3 mL, draw-down: 10 mm·s⁻¹). The procedure was finished with 0.05 mL PBS remaining in each well.

Relative numbers of adherent cells in the wells were determined thereafter by the CellTiter-Glo cell quantification kit (Promega, Mannheim, Germany) that lyzes cells and generates an ATP-dependent luminescence signal. The relative signal intensity reflects relative changes in cell numbers since the intracellular ATP content of unimpaired cells is in correlation to the cell number. The reaction was performed following instructions of the manufacturer. Briefly, 50 µL CellTiter-Glo reagent were added to each well, the plate was agitated for 5 min on a Mixmate (Eppendorf, Hamburg, Germany) at 1 000 rpm and the intensity of the luminescence signal was measured after 10 min with a microplate reader Genios Pro (Tecan, Crailsheim, Deutschland). In order to exclude the influence of the polymers on the enzymatic ATP detection, a separate experiment was performed, where the polymer-coated wells and empty wells respectively (four replicates) were incubated with 0.05 mL of ATP standard (ConCell, Nettetal, Germany), diluted to 8 ng·mL⁻¹. After 30 or 120 min 0.05 mL of CellTiter-Glo reagent were added to each well and the luminescence signals were measured as described above. The same level of ATP was detected for all samples, except for the wells containing the block copolymers carrying deprotected glucose (9) and galactose (10), where the luminescence was enhanced to a 1.34- and 1.39-fold, respectively, independent from the time of incubation. Hence, the measurement values from these polymers were corrected by dividing by those factors.

Results and Discussion

The synthetic approach to obtain well-defined glucosylated and galactosylated homo- and block copolymers is shown in Scheme 1 and 2. At first, the polymeric backbones were prepared via a nitroxide-mediated, controlled radical polymerization procedure. In the next step, the homo-
polymer of PFS (1) and the PS copolymer (6) were glycosylated either with the acetylated 1-thio-β-D-glucose or the acetylated 1-thio-β-D-galactose via thiol/para-fluorine substitution reaction in the presence of triethylamine using dry DMF as solvent.\(^\text{[71]}\) The glycosylation was followed by the deprotection of the carbohydrate moieties using catalytic amounts of sodium methanolate in dry DMF.

Preparation and Characterization of the PPFS Homopolymer (1) and the PS-b-PPFS (6) Copolymer

For the nitroxide-mediated, controlled radical polymerization, a β-phosphorylated alkoxyamine initiator (BlocBuilder from Arkema) was used. All polymerization reactions were carried out in a closed vessel, with styrene and PFS as the monomers, THF (saturated with argon) as solvent, BlocBuilder as initiator for the homopolymerizations and PS as macroinitiator for the block copolymer synthesis. The characterization data and SEC traces are presented in Table 1 and Figure 1, respectively. According to SEC the polymers exhibited narrow molar mass distributions, with polydispersity index (PDI) values < 1.2. The \(^1\)H and \(^19\)F NMR spectra of the obtained products confirmed their structures (Figure 2 and 3). The \(^13\)C NMR and HSQC-DEPT 2D NMR spectra of 1 and 6 can be found in the Supporting Information. The TGA of 6 revealed no thermal decomposition up to 380 °C (Figure 4).

Table 1. Polymerization, glycosylation and deacetylation conditions and SEC characterization of the synthesized polymers.

<table>
<thead>
<tr>
<th>No.</th>
<th>Structure</th>
<th>React. time</th>
<th>Temp.</th>
<th>Conv. (^a)</th>
<th>(\bar{M}_n) (^\text{theo})</th>
<th>(\bar{M}_n) (^\text{SEC})</th>
<th>(\bar{M}_w/\bar{M}_n) (^\text{b})</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>PPFS(_{35})</td>
<td>5</td>
<td>110</td>
<td>78</td>
<td>7 175</td>
<td>5 700</td>
<td>1.06</td>
</tr>
<tr>
<td>2</td>
<td>PTFSGlcAc</td>
<td>8</td>
<td>40</td>
<td>100</td>
<td>19 228</td>
<td>10 400</td>
<td>1.06</td>
</tr>
<tr>
<td>3</td>
<td>PTFSGalAc</td>
<td>10</td>
<td>50</td>
<td>99</td>
<td>19 039</td>
<td>10 400</td>
<td>1.06</td>
</tr>
<tr>
<td>4</td>
<td>PTFSGlcOH</td>
<td>1.5</td>
<td>r.t.</td>
<td>100</td>
<td>13 343</td>
<td>20 600</td>
<td>1.10</td>
</tr>
<tr>
<td>5</td>
<td>PTFSGalOH</td>
<td>1.5</td>
<td>r.t.</td>
<td>100</td>
<td>13 213</td>
<td>19 300</td>
<td>1.06</td>
</tr>
<tr>
<td>6</td>
<td>PS(<em>{54})-b-PPFS(</em>{33})</td>
<td>3.5</td>
<td>110</td>
<td>52</td>
<td>12 411</td>
<td>14 300</td>
<td>1.16</td>
</tr>
<tr>
<td>7</td>
<td>PS-b-PTFSGlcAc</td>
<td>8</td>
<td>40</td>
<td>90</td>
<td>22 638</td>
<td>23 500</td>
<td>1.15</td>
</tr>
<tr>
<td>8</td>
<td>PS-b-PTFSGalAc</td>
<td>8</td>
<td>50</td>
<td>94</td>
<td>23 093</td>
<td>23 900</td>
<td>1.17</td>
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<tr>
<td>9</td>
<td>PS-b-PTFSGlcOH</td>
<td>1.5</td>
<td>r.t.</td>
<td>100</td>
<td>17 645</td>
<td>40 000</td>
<td>1.13</td>
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<tr>
<td>10</td>
<td>PS-b-PTFSGalOH</td>
<td>1.5</td>
<td>r.t.</td>
<td>100</td>
<td>17 877</td>
<td>39 900</td>
<td>1.10</td>
</tr>
</tbody>
</table>

\(^a\)Calculated from \(^19\)F NMR spectroscopy for grafting and \(^1\)H NMR spectroscopy for deprotection; \(^b\)Calculated according to PS standards using 2.1% LiCl solution in DMA as eluent.

Figure 1. SEC traces of the homopolymers (left): PPFS\(_{35}\) (1), PTFSGalAc (3), PTFSGalOH (5), and the block copolymers (right): PS\(_{54}\)-b-PPFS\(_{33}\) (6), PS-b-PTFSGalAc (8), PS-b-PTFSGalOH (10).
Grafting of Thiolsugars onto PPFS

Thiols, as soft nucleophiles, have shown high reactivity in the nucleophilic substitution reaction of the para-fluorine. Previously, we have thoroughly studied the grafting of acetylated-1-thio-β-D-glucose onto PPFS. Online $^{19}$F NMR measurements of the reaction evidenced a conversion of more than 90% of fluorine moieties after 60 min at 40°C. In order to obtain the maximum degree of substitution, i.e., 100% grafting, the quantity of 1-thio-β-D-glucopyranose tetraacetate was increased to 2 equivalents with respect to PFS units, and the reaction was carried out for longer time (8 h). The quantitative substitution of para-fluorine moieties by thioglucose acetate is confirmed by the complete disappearance of the peaks belonging to the para-fluorine and by a shift of the signal of the meta-fluorines in the $^{19}$F NMR spectra (Figure 3). Furthermore, the appearance of proton signals from the carbohydrate...
moieties in the $^1$H NMR (Figure 2) spectrum as well as the increase in molar mass in the SEC (Figure 1) confirmed the successful grafting.

The aforementioned method was further used to graft 2,3,4,6-tetra-O-acetyl-1-thio-$\beta$-D-galactopyranose. Applying harsher conditions ($50^\circ C$) and longer reaction times (10 h) resulted in a conversion of 99% which was calculated from the ratio between the integral of the $^{19}$F signal of the two meta-fluorines of 1 and the integral of the two ortho-fluorines of 3 present in the $^{19}$F NMR spectrum (Figure 3). The $^1$H NMR (Figure 2) and $^{13}$C NMR (Supporting Information) spectra confirmed the presence of acetylated carbohydrate groups on the polymer. SEC analysis (Figure 1, Table 1) showed a clear increase from 5.7 to 10.4 kDa for the molar mass of the polymers, calculated according PS calibration. The low PDI value of 1.06 was maintained for the converted polymer.

Both thiosugars were successfully grafted onto the PS block copolymer applying the same conditions as for the homopolymers. High degrees of functionalization were obtained, 90% for glucose and 94% for galactose, as calculated from the $^{19}$F NMR spectra (Figure 3). The $^1$H NMR spectra (Figure 2) showed the presence of sugar protons as well as the PS backbone. SEC analysis results (Figure 1, Table 1) revealed a significant increase in the hydrodynamic volume of the grafted copolymers and narrow polydispersity indices. The TGA results provided further confirmation of the successful post-polymerization modification (Figure 4). The temperature of decomposition is lowered from 380 to 270 $^\circ C$ and the curve shows an additional inflection point at 380 $^\circ C$ corresponding to the degradation of the polymeric backbone.

Deprotection of the Carbohydrate Moieties

The final glycopolymers were obtained by applying the standard Zemplén procedure for sugar deprotection. For this purpose the polymers were dissolved in dry DMF, and catalytic amounts of sodium methanolate as 0.1 M solution in methanol were added dropwise to the mixture. The reaction was continued for 1.5 h at room temperature. Subsequently, the solution was concentrated and the polymers were precipitated into cold ethanol. The glycopolymers were purified by reprecipitation from either

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**Figure 5.** Confocal microscopy images of films formed by 10 drop-cast from THF/water (4:1) solution inside a microtiter well. Whole well (left), zoom into the scratched surface in the middle of the well (right).
water, for the homopolymers, or DMF, for the block copolymers, into cold ethanol. Complete deacylation was confirmed by the disappearance of the peaks of the protecting groups in the $^1$H (Figure 2) and $^{13}$C NMR spectra. The deprotected polymers and copolymers showed an increased hydrophilic character with respect to the non-grafted and protected ones. This dramatic change of solubility behavior causes the increase in the hydrodynamic volume of the glycopolymers in DMA, as clearly depicted in the SEC measurements by the shift toward higher molar masses (Figure 1). The polydispersity indices of the final polymers did not change significantly (Table 1). TGA analysis (Figure 4) showed a further decrease of the decomposition temperature (220 °C) and an inflection point corresponding to a degradation of the backbone.

Film Formation in the 96-Well PP Microtiter Plates

The films were obtained by means of a simple drop-casting method, as described in the Experimental Part. The topography of the dried surfaces was analyzed using a confocal interference microscope. Representative images for the resulting morphologies are shown in Figure 5. The usage of concentrated solutions (10 mg mL$^{-1}$) resulted in a complete coverage of the bottom surface of the wells. The obtained films showed the formation of “coffee ring” surface structures, with most of the polymer accumulated along the perimeter of the dried structure and a thinner layer of material present in the center. While the edges of the film exhibited a rougher morphology, the center of the films were more smooth and consisted of an $\approx$100 nm thick continuous layer, which was measured by means of scratching the film with the edge of a sheet of paper and measuring the height difference.

Film Formation on Glass Slides

The coatings were prepared by simple drop-casting as described in the Experimental Part. The film morphologies were observed with a confocal microscope, selected examples are depicted in Figure 6. All of the obtained films were relatively smooth and could be obtained in a reproducible manner. The coating of the entire 1 cm$^2$ surface was confirmed by a scratch test (Figure 7). The water contact angle measurement for slides coated with 9 indicated a significant increase in the hydrophobicity of the surface, as indicated by the change from 20° for the uncoated to 80° for coated glass slide. To further examine the chemical properties of the prepared films, the measurements were carried out after incubation of the glycopolymer-coated slide in water for 2 h and drying of the surface under a stream of argon. The water contact angle decreased by 20° (see Supporting Information Figure 8) signifying possible rearrangement of the functional groups.
on the surface. The film morphology was also analyzed by atomic force microscopy (AFM). No clear nanophase separation for the films of the block copolymer was found. Furthermore, no significant morphological changes were observed for the film after immersion in water (see Supporting Information, Figure S9).

3T3 Fibroblast and MC3T3-E1 Osteoblast Viability and Proliferation

To ensure the general biocompatibility of the obtained polymers, the cytotoxicity toward 3T3 mouse fibroblasts was examined. The water soluble homopolymer 4 was dissolved and administered to cells as described in the Experimental Part. The surfaces coated with 1 and 9 were directly inoculated with cells. By live/dead staining of the cells after 1 and 4 d with the fluorescence developing dyes FDA (labeling live cells) and EtBr (labeling the nuclei of dead cells), no signs of cytotoxicity could be detected (Figure 8). 3T3 fibroblasts attached and proliferated in the presence of 1, 3, and 9 confirming the suitability of the polymers to be tested in adhesion assays, where the maintenance of the average intracellular ATP-levels is a prerequisite. Furthermore, the absence of
contaminations, e.g., cytotoxic residues of not converted reactants, catalysts, or solvents within the analyzed polymers was verified. Also, any nonspecific cytotoxic effects from the synthesis and preparation techniques can be excluded.

Since the results of the adhesion assays differed between the cell types, MC3T3-E1 cells were also subjected to the viability test on 9 and 10 copolymer-coated, microscopic slides. The FDA/EtBr-staining of MC3T3-E1 cells cultured for 1 and 4 d generally resulted in less than 5% dead cells (Figure 9). The cell density on the glycopolymeric surfaces of about 25 000 cells · cm⁻² at day 1 increased to about 90 000–100 000 cells · cm⁻² at day 4. This result is only slightly lower than on the uncoated control of tissue culture polystyrene (TCPS) with about 130 000 cells · cm⁻² at day 4. This nearly fourfold increase of the cell number indicates a proliferation rate of the MC3T3-E1 cells on the examined polymers comparable to TCPS. However, it has to be mentioned that during long term cell culture over 7 d and more, initiated to study the differentiation processes of MC3T3-E1 cells (not shown), cell layers started to detach from the polymer surface. At first, they formed constricted areas and strands of aggregated cells and later detached completely as spheroids.

Cell Adhesion Assays

Cell adhesion is a very complex process and there are plenty of factors that regulate cell/material interaction like surface chemical composition, hydrophobicity/hydrophilicity, charge, roughness, and topography. Cell adhesion is the binding of cells to a surface, an extracellular matrix, or other cells, and occurs through the use of cell adhesion molecules (CAMs). CAMs are transmembrane proteins consisting of an extracellular ligand-binding domain and an intracellular signaling/regulatory domain. Most of the CAMs are highly glycosylated. Therefore, an investigation of the cell attachment to the carbohydrate-modified surfaces is of significant interest. Furthermore, different cell lines express additional sugar-binding receptors; and specific attachment through them has been confirmed, e.g., hepatocyte attachment to galactose residues via asialoglycoprotein receptor (ASGPR).

In the present study the temporal behavior of 3T3 fibroblast and MC3T3-E1 preosteoblast attachment onto five different surfaces is examined, namely PP (as reference), a fluorinated block copolymer of PS (6), the same copolymer carrying acetylated glucose (7), and deprotected glucose (9) or galactose (10) moieties. The results of the cell adhesion assays with 3T3 and MC3T3-E1 cells during a time period of 30 to 120 min are shown in Figure 10. The number of firmly attached fibroblasts and preosteoblasts is increased within 30 to 90 min after seeding on all polymer surfaces. In the case of fibroblasts, a better cell adhesion is found over the whole time period for the prepared polymers compared to the PP control surface. Furthermore, after 120 min, the number of attached cells is higher for the three glycosylated polymer surfaces (7, 9, 10) than for the non-glycosylated ones (PP, 6). Most efficient 3T3 cell adhesion is observed on both the deprotected galactosylated polymer (10) and the acetyl-protected glucosylated one (7). Compared to those polymers the deprotected glucosylated one (9) is less efficient to promote adhesion of fibroblasts.

In the case of preosteoblast cells, the differences in the adhesion behavior are less pronounced among the invest-
tigated polymers, and preosteoblasts are less influenced by different patterns of glycosylation as well as changes in the hydrophobicity of the polymer surface. Nevertheless, after 120 min both the deprotected galactosylated (10) and the acetyl-protected glucosylated polymer (7) exhibited most efficient adhesion of MC3T3-E1 cells, whereas cell adhesion on deprotected glucosylated polymer 9 is diminished compared to other glycosylated polymers and even non-glycosylated polymer 6.

Conclusion

We have presented an efficient route for the synthesis of fluorinated glycopolymers via post-polymerization functionalization. Both poly(pentafluorostyrene) homo- as well as PS block copolymers can be obtained using NMP. Applying the thiol/para-fluorine click reaction with acetylated thiosugars, high degrees (>90%) of carbohydrate substitution onto the polymeric backbone can be achieved. Furthermore, this method provides the opportunity to precisely calculate the amount of grafted sugar by means of $^{19}$F NMR spectroscopy. The obtained glycopolymers exhibited narrow molar mass distributions (homopolymers with PDI ≤ 1.10, block copolymers with PDI ≤ 1.15), are stable up to 220 °C and non-cytotoxic. Moreover, the carbohydrate attachment via a $\text{S}$-glycosidic bond offers stability toward enzymatic degradation. The water-insoluble PS block copolymers were drop-cast onto glass slides and PP wells to form stable films. The coated substrates were used to study the attachment of 3T3 fibroblasts and MC3T3-E1 preosteoblasts. For fibroblasts, an enhanced proliferation on glycosylated substrates, in particular on the galactosylated polymer, was observed, whereas preosteoblasts showed less pronounced specificity. In the future we will concentrate our research on the design of bone implant coatings that modulate cell adhesion and proliferation within the multicellular surrounding of the human body. The promotion of osteoblast adhesion combined with a relative suppression of fibroblast adhesion would be a valuable property of bone implants, avoiding unwanted growth of fibrous tissue. On the other hand, the prevention of stable adhesion of preosteoblasts can be helpful, e.g., in cases when implants have to be removed.

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