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Synthesis of a Pyridyl Disulfide End-Functionalized Glycopolymer for Conjugation to Biomolecules and Patterning on Gold Surfaces

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Abstract

A pyridyl disulfide end-functionalized polymer with *N*-acetyl-D-glucosamine pendant side-chains was synthesized by atom transfer radical polymerization (ATRP). The glycopolymer was prepared from a pyridyl disulfide initiator catalyzed by a Cu (I)/Cu (II)/2,2'-bipyridine system in a mixture of methanol and water. The final polymer had a number-average molecular weight (M_n) of 13.0 kDa determined by ^{1}H NMR spectroscopy and a narrow polydispersity index (1.12) determined by gel permeation chromatography (GPC). The pyridyl disulfide end-group was then utilized to conjugate the glycopolymer to a double-stranded short interfering RNA (siRNA). Characterization of the glycopolymer-siRNA by polyacrylamide gel electrophoresis (PAGE) showed 97% conjugation. The activated disulfide polymer was also patterned on gold via microcontact printing. The pyridyl disulfide allowed for ready immobilization of the glycopolymer into 200 μm sized features on the surface.

Introduction

Controlled radical polymerization (CRP) techniques such as atom transfer radical polymerization (ATRP),[1,2] reversible addition-fragmentation chain transfer (RAFT) polymerization,[3–5] and nitroxide-mediated polymerization (NMP)[6] provide tools for the facile preparation of well-defined polymers. These techniques are tolerant to a wide range of monomers, temperatures, functional groups and solvents, including water. Polymers with targeted molecular weights, narrow molecular weight distributions, end and/or pendant group functionality and various architectures are easily prepared. End-group functionality can be readily achieved without the need for post-polymerization modifications by incorporating functional groups into the initiators or chain transfer agents (CTAs).[7–9] We and other researchers have exploited this to conjugate polymers to biomolecules such as proteins, peptides and double-stranded short interfering RNA (siRNA).[8,9] Furthermore, this strategy can be used to attach polymers to surfaces. Initiators or CTAs containing functional groups such as maleimide, pyridyl disulfide, aminooxy, azide, and biotin are of particular interest because they react with thiols, ketones or aldehydes, alkynes and streptavidin, respectively. Among these, the pyridyl disulfide group was chosen for our study not only because of its selectivity towards thiol groups and reversibility of the formed bond, but because it can also be used to pattern polymers on gold surfaces. In particular, we were interested in studying pyridyl disulfide end-functionalized polymers with pendant saccharides. So called glycopolymers have been shown to mimic natural saccharides and polysaccharides, which are involved in numerous biological processes.[10,11] Thus, we envisioned that methods to synthesize pyridyl disulfide glycopolymers would be useful to prepare materials for diverse applications. For example, the resulting polymers could be employed to make bioconjugates

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where the glycopolymer moiety could help stabilize or target the biomolecule. In addition, the resulting polymers might be useful to modify surfaces with sugars to direct cell adhesion or for microarray technology.

Well-defined glycopolymers have been synthesized by ATRP,[12–28] RAFT polymerization,[29–44] cyanoxyl-mediated free radical polymerization[45–52] and ring opening metathesis polymerization[53–61] (ROMP). These techniques have also been used to prepare end functionalized glycopolymers. Haddleton and coworkers have synthesized glycopolymers by ATRP with NHS or maleimide end-functional groups.[27,62] Kiessling synthesized a glycopolymer with a fluorescent end-group by ROMP to study its interaction with Concanavalin A.[59] Armes prepared a glycopolymer by ATRP with an aldehyde end-group which was incorporated through a post-polymerization modification.[15] An azide-functionalized glycopolymer synthesized by ATRP and its conjugation to a virus was discussed by Sen Gupta et al.[21] Several groups prepared biotinylated glycopolymers[44] which were conjugated to streptavidin in solution,[24,52] on surfaces[49] or on gold nanoparticles.[32,38,43] However, to our knowledge glycopolymers with pyridyl disulfide end-groups synthesized by CRP techniques have not been reported.

Herein, we report the synthesis of a pyridyl disulfide end-functionalized polymer with *N*-acetyl-D-glucosamine (GlcNAc) side chains. GlcNAc is a known posttranslational modification of proteins and is added to serine or threonine amino acid residues by *O*-GlcNAc transferase.[63] This glycosylation has been detected on transcription factors, protein kinases and cytoskeletal proteins and is important in a range of diseases including diabetes and cancer.[63–65] Our group previously polymerized a methacrylate with GlcNAc side chains by ATRP from a biotin and an amino acid functionalized initiator.[22,23]

The sugar side chain in the glycopolymer can be used as a multivalent targeting moiety and/or the glycopolymer itself can act a solubilizing agent or protective moiety when attached to biomolecules leading to advantageous effects. This is particularly important for DNA and RNA where inefficient cellular uptake and low *in vivo* stability are considered as the major hurdles for effective therapeutic use. For example, enhanced plasmid DNA delivery to mammalian cells has been observed from cationic glycopolymer/DNA complexes.[66,67] In another example, it was shown that a galactose-cationic liposome enhanced the stability and facilitated cellular uptake of siRNA *in vivo*.[68] In this study, we demonstrate the feasibility of utilizing the pyridyl disulfide end-functionalized GlcNAc polymer to form high yields of reversible siRNA conjugates.

We also used the same pyridyl disulfide glycopolymer to modify gold surfaces. While a large volume of work has been published on glycopolymer preparation, surprisingly few papers have been reported on patterning these on surfaces. Yet, such surfaces are interesting to assemble different cell types on the same substrate and for microarray technology. Miura and coworkers were able to pattern glycopolymers using a photolithography method in multiple studies.[68,69] In their first study, the patterning was achieved through the use of a photomask and ultraviolet light to produce a patterned hydrophobic layer on a silicon surface.[69] Glycopolymers were then bound to the hydrophobic areas via self-assembly. In a later study, the same hydrophobic pattern was backfilled with a cationic layer providing a cationic/ hydrophobic pattern.[68] This was then used to bind a heparin polymer to the cationic areas of the pattern and a lactose-substituted styrene homopolymer to the hydrophobic region. This array preferentially bound hepatocytes to the glycopolymer region and fibroblasts to the heparin region. Chaikof and coworkers were able to eliminate the use of photolithography and pattern glycopolymers by microcontact printing;[49] a biotin-terminated glycopolymer was synthesized and subsequently bound to a surface patterned with streptavidin via microcontact printing. Herein, we describe a simple method of achieving glycopolymer patterning through the use of direct microcontact

printing of a pyridyl disulfide-terminated glycopolymer on gold. Microcontact printing was selected because of the ease of the technique and because it does not require clean room techniques. The pyridyl disulfide group can form a stable sulfur-gold bond between the polymer and surface, presumably releasing the 2-pyridinethione in the process.[70] Below, the details of the polymerization of a GlcNAc modified methacrylate, its conjugation to siRNA and the direct patterning of surfaces are discussed.

Materials and Methods

Materials

All chemicals were purchased from Aldrich or Acros and used as received, unless otherwise specified. Copper bromide (CuBr) was purified by stirring in glacial acetic acid for 12 h and rinsing with ethanol and diethyl ether. The pyridyl disulfide ATRP initiator **1**[71] and the glycomonomer **2**[22] were synthesized according to published procedures. Complementary 5'-thiol modified sense siRNA (5'ThioMC6-D-GCU GAC CCU GAA GUU CAU CUU-3') and antisense siRNA (5'-GAU GAA CUU CAG GGU CAG CUU-3') were purchased from Integrated DNA Technologies and annealed following the manufacturer's protocol. The goat-anti mouse Alexafluor-568 labeled antibody was purchased from Invitrogen while the mouse monoclonal [HGAC85] to *O*-linked *N*-acetylglucosamine was purchased from Abcam.

Analytical techniques

^{1}H NMR spectra were recorded on a Bruker Avance500 spectrometer. Gel permeation chromatography (GPC) was conducted on a Shimadzu HPLC system equipped with a refractive index detector RID-10A and two Polymer Laboratories PLgel 5 μm mixed D columns (with guard column). Lithium bromide (0.1 M) in *N*,*N*-dimethylformamide (DMF) at 40 °C was used as a solvent (flow rate: 0.8 mL/min). Near-monodisperse poly(methyl methacrylate) standards (Polymer Laboratories) were employed for calibration. Chromatograms were processed with the EZStart 7.2 chromatography software to obtain the number-average molecular weight (M_n) and polydispersity index (PDI).

Polymerization of glycomonomer 2 from pyridyl disulfide initiator 1

The polymerization was conducted in a 25 mL air free reaction tube, where **2** (0.15 g, 0.45 mmol), CuBr (3.2 mg, 0.023 mmol), $CuBr_2$ (5.0 mg, 0.023 mmol) and 2,2'-bipyridine (bipy, 0.014 g, 0.090 mmol) were evacuated-refilled with argon gas three times to create an oxygen-free atmosphere and then dissolved in a 3:1 (500 μL) mixture of degassed methanol:water. (Note: The 3:1 methanol:water mixture was prepared from anhydrous methanol and deionized water.) The pre-polymer mixture was placed in a 30 °C oil bath, and **1** (0.011 μL, 0.045 mmol, degassed with argon) was added to initiate the polymerization. Samples were taken from the mixture at defined time points. The methanol and water were removed, and the samples were diluted with DMSO-d_6 for ^{1}H NMR analysis or DMF for GPC analysis. The polymerization was terminated after 90 minutes by exposure to oxygen. Glycopolymer **3** was isolated after dialysis for 3 days against water (10 × 4 L). The polymer was collected as a white solid after lyophilization. ^{1}H NMR (D_2O, peaks are broad): δ 8.57 (end group, NC**H**), 7.99 (end group, SCC**H**C**H**), 7.45 (end group, NCHC**H**), 4.64 (**H**1), 4.29-3.50 (**H**2-6), 3.08 (end group, SSC$\mathbf{H}_2$) 2.19-1.98 (NCOC$\mathbf{H}_3$, C$\mathbf{H}_2$CCH_3), 1.12-0.92 (CH_2CC$\mathbf{H}_3$). M_n (^{1}H NMR): 13.0 kDa; M_n (GPC): 10.2 kDa; PDI (GPC): 1.12.

Conjugation of siRNA to pyridyl-disulfide end-functional glycopolymer

Conjugation of **3** to siRNA was performed following our reported procedure.[72] Double stranded (ds)-siRNA solution (0.0383 mM, 30 μL) was mixed with a freshly-prepared dithiothreitol solution (DTT, 5 μL, 200 mM) and kept for 2 h at 21 °C. ds-siRNA was purified

by addition of 3.5 μL of 5 M NaCl (3.5 μL, 5M) followed by the addition of 96.3 μL of ethanol (96.3 μL, 70 %). The solution was stored at −20 °C for 2 h, the siRNA pellet was then recovered by centrifuging the solution at 13200 rpm for 20 min at 4 °C. The siRNA pellet was resuspended in 30 °L of a glycopolymer solution (3.77 mg in 500 °L of 100 mM sodium bicarbonate buffer at pH 8.5) and left at 21 °C for 24 h before assessing the conjugation by polyacrylamide gel electrophoresis (PAGE). ds-siRNA was stained by incubating the gel in 1 X SYBR-Safe nucleic acid dye/TBE buffer. The polyacrylamide gel was visualized and quantified using a Versadoc Imaging System Model 500. The gel was irradiated with UV light and fluorescence was measured at 520 nm. The image intensity of polymer-conjugated (Ic) or unmodified siRNA (Is) was analyzed using the Quanti-One program (BIO-RAD). The conjugation efficiency of glycopolymer-siRNA was defined as: $\text{Conjuation efficiency} = \text{Ic} / (\text{Ic}+\text{Is})$ and was 97%.

Patterning

A polydimethylsiloxane (PDMS) stamp with 200 micron in diameter circular features was used for microcontact printing. The stamp was made as described in the literature.[73] The stamp was inked with a 1:1 water:methanol solution of glycopolymer (10 mM in end group) by incubating the solution on the stamp for 10 min, followed by drying under a stream of argon. The stamp was pressed into contact with a freshly piranha-cleaned [4:1 H_2SO_4: 30% H_2O_2, CAUTION] gold surface for 30 min at 60 °C followed by backfilling with triethylene glycol mono-11-mercaptoundecyl ether for 2 h at 60°C. The gold was then washed with copious amounts of absolute ethanol and dried with argon prior to incubating with a mouse monoclonal [HGAC85] to *O*-linked *N*-acetylglucosamine primary antibody for 30 min followed by a goat-anti mouse Alexafluor-568 labeled secondary antibody for 30 min. The surface was then imaged by fluorescent microscopy (signal to noise ratio of ~ 5:1). Fluorescent images were visualized using a Zeiss Axiovert 200 fluorescent microscope equipped with an AxioCam MRm monochrome camera, and pictures were acquired and processed using AxioVision LE 4.1. A control experiment was performed where the patterning and antibody staining process was repeated, but without incubation of the primary anti-GlcNAc antibody.

Results and Discussion

Synthesis of glycopolymer 3

The glycomonomer **2** was prepared in 3 steps starting from 2-acetamido-2-deoxy-β-D-glucopyranose 1,3,4,6-tetraacetate following a literature procedure.[22] The ATRP pyridyl disulfide initiator **1** was synthesized in two steps as described by Bontempo et al.[71] Initial polymerization attempts were performed in dimethyl sulfoxide (DMSO), but kinetic studies revealed high conversions (90% and higher) in less than 5 minutes. Polymerizations performed in methanol also proved unsuccessful. The rate of polymerization did decrease, but the glycopolymer solutions became cloudy at approximately 60% conversion (30 minutes into the polymerization). The reason for this was that, although low molecular weight chains were soluble in methanol, higher molecular weight chains were not; as the polymerization proceeded to form larger chains, the polymer precipitated from solution. To resolve this, the polymerization was conducted in a methanol-water solvent mixture. We first optimized the polymerization conditions for **2** in this solvent mixture utilizing ethyl 2-bromo-2-methyl propanoate as the initiator (data not shown). The best results were obtained when a 1:1 ratio of CuBr:$CuBr_2$ was used in a 3:1 methanol:water solvent system.

The monomer was then polymerized from the pyridyl disulfide initiator utilizing CuBr:$CuBr_2$:bipy as the catalyst in a methanol:water (3:1) mixture at 30 °C (Scheme 1). The initial [**2**]:[**1**]:[CuBr]:[$CuBr_2$]:[bipy] ratio was 10:1:0.5:0.5:2. Kinetic studies revealed a linear first order kinetic plot (Figure 1a) up to approximately 80 % conversion. The molecular weight

of the polymer also increased linearly up to 80% conversion, and the polydispersity index remained below 1.2 throughout the polymerization (Figure 1b). These results demonstrated that well-defined polymers are accessible with this technique. Once optimal polymerization conditions were found for **2**, the molecular weight by ^{1}H NMR and pyridyl disulfide end-group retention were investigated.

The polymer was dialyzed extensively against water for three days to remove any unreacted monomer and initiator and isolated. The purified glycopolymer **3** had a M_n of 10.2 kDa by GPC and 13.0 kDa determined by ^{1}H NMR. The GPC trace (Figure 2) demonstrated that the polymer was monomodal and that the molecular weight distribution was narrow (PDI of 1.12). The molecular weight was estimated by comparing the end group peaks to the main chain peaks in order to obtain the degree of polymerization. Specifically, the integration of the protons of the pyridyl ring (8.5 to 7.5 ppm) was compared to the integration of the backbone peak (1.12 to 0.92) and the proton at the anomeric position (4.64 ppm). The M_n was significantly higher than the expected M_n (3.2 kDa). We have seen similar differences with this particular monomer and a biotinylated initiator.[22] However, we have also obtained targeted molecular weights when **3** was polymerized from an amino acid ATRP initiator.[23] Thus, the likely reason for the observed molecular weight discrepancy was that the pyridyl disulfide initiator was inefficient. We have observed this for the polymerization of HEMA with initiator **1**, which resulted in higher molecular weights and narrow polydispersities.[71] The reason for the initiator inefficiency is not yet known.

End-group retention was determined by ^{1}H NMR. By comparing the integration of the protons in the pyridyl ring (8.5 to 7.5 ppm) versus the protons of the CH_2 group next to the disulfide bond (3.1 ppm) it was estimated that 87% of the pyridyl disulfide end-group was retained (Figure 3). This result indicated that some chain transfer to the disulfide occurred during the polymerization. This has also been observed by Bontempo et al. [71] and Tsarevsky et al.[74] with disulfide containing initiators and could possibly be eliminated by using CuCl instead of CuBr in the reaction to reduce the concentration of radicals.[71] The pyridyl disulfide end-group was then exploited to prepare conjugates and micropatterned surfaces.

Conjugation of siRNA to pyridyl-disulfide end-functional glycopolymer

The bioconjugation capability of the glycopolymer via disulfide bond exchange was then demonstrated by using siRNA (Scheme 2). siRNA is a double stranded RNA molecule, which can be chemically synthesized with designated end functionality. In this study, siRNA with a protected thiol group was used.[72] The thiol was deprotected by incubating with a reducing agent (100 mM DTT) for 2 h. Following deprotection it was critical to remove the DTT from the siRNA for efficient conjugation to the glycopolymer. Ethanol precipitation was utilized as an effective method to isolate pure siRNA with free thiol groups. A glycopolymer with an M_n (^{1} H NMR) of 13.5 kDa was synthesized and then incubated with free thiol-siRNA at 21 °C for 24 h to obtain polymer-siRNA conjugate **4**. The conjugation efficiency was assessed using polyacrylamide gel electrophoresis (PAGE) followed by staining with a nucleic acid-specific fluorescence dye. The PAGE displayed bands of higher molecular weight compared to free siRNA (Figure 4, lanes 2 and 3 compared to lane 1); only residual amounts of free siRNA were observed. Quantification of the image intensity of free siRNA and comparison to the intensity of the glycopolymer-siRNA on the PAGE, gave a conjugation of 97%. The reversibility of the polymer-siRNA conjugation was demonstrated by subjecting the conjugate to a DTT solution. After incubation, the siRNA-polymer was fully converted back to free siRNA (Figure 4, lanes 4 and 5). This demonstrated that the macromolecule was fully released and suggested the possibility of utilizing this polymer for delivery and then release of siRNA.

Glycopolymer surface patterning

The pyridyl disulfide-terminated glycopolymer was next patterned on a gold surface via microcontact printing as it has been shown that polymers terminated with pyridyl disulfide groups are able to form monolayers on gold.[70,73] The polymer was dissolved in a 1:1 water:methanol solution (10 mM) and applied to a polydimethylsiloxane stamp (with 200 uM features). The solvent was allowed to evaporate off, leaving a layer of glycopolymer on the stamp. The stamp was then pressed against a freshly piranha cleaned gold surface for 30 min at 60 °C to allow SAM formation. The surface was passivated by "backfilling" with a triethylene glycol-functionalized alkanethiol to minimize non-specific binding of the antibodies to the background. The glycopolymer was stained using a mouse monoclonal [HGAC85] to *O*-linked *N*-acetylglucosamine as the primary antibody followed by a goat-anti-mouse Alexa-Fluor 568-tagged secondary antibody and imaged by fluorescence microscopy. An array of red-fluorescent spots with 200 micron diameter was observed indicating that the glycopolymer had been patterned onto the surface (Figure 5a). A control experiment was performed to further confirm the presence of the glycopolymer. The patterning and antibody staining process was repeated, but incubation with the primary anti-GlcNAc antibody was omitted. When no anti-GlcNAc staining was performed, no red fluorescence was observed (Figure 5b). This indicated that the presence of the primary antibody was required for the binding of the fluorescent secondary antibody and that the red fluorescence observed in Figure 5a was derived from interaction with the GlcNAc moieties. This data demonstrates the feasibility of utilizing this approach to prepare patterns of sugars on the surface. Depending on the glycopolymer, the surfaces could then be used to study biological interactions, for selectively cell adhesive materials, or as saccharide microarrays.

Conclusions

A well-defined pyridyl disulfide end-functionalized polymer with *N*-acetyl-D-glucosamine pendant side-chains was synthesized by ATRP. Characterization of the polymer and the end group was achieved using ^{1}H NMR spectroscopy and GPC. This end-group was employed to prepare a glycopolymer-siRNA conjugate and was also used for surface patterning. Conjugation of the polymer to thiol-modified siRNA proved successful with 97% conjugation efficiency. The glycopolymer may lend stability to the siRNA conjugate during delivery. This highly efficient synthesis and one-step conjugation system also offers great potential for further study of sugar-targeted siRNA gene therapy. Depending on the sugar moiety, glycopolymer-siRNA conjugates may display preferable cellular uptake and enhanced *in vivo* stability compared to unmodified siRNA. Surface micropatterning of this glycopolymer on gold was also achieved through microcontact printing. This could lead to surfaces patterned with multiple glycopolymers on the micron scale. Such glycosurfaces could be applied in microarray technology or to mimic the extracellular matrix in order to control and manipulate cell behavior.

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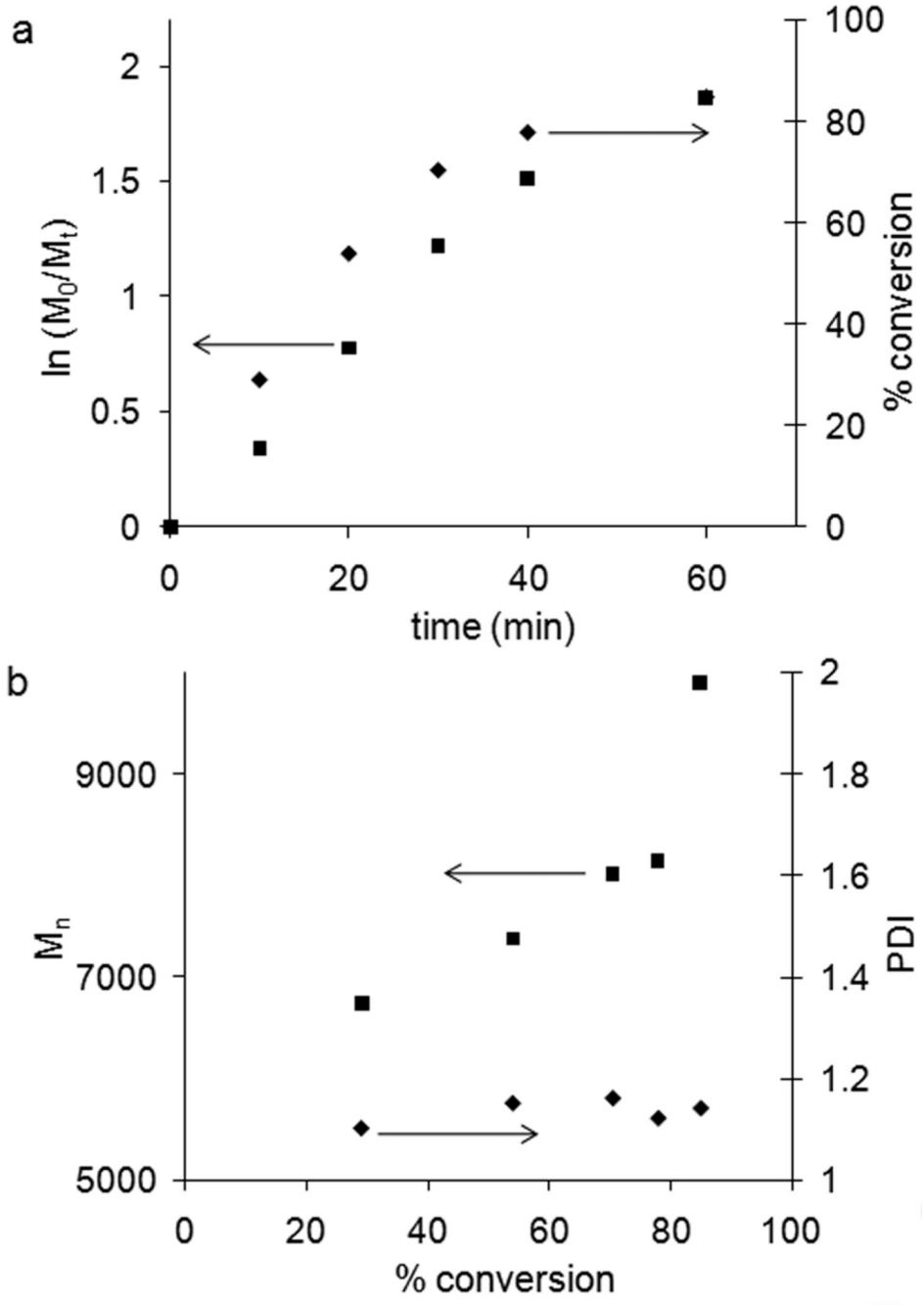


Figure 1.
Polymerization of glycomonomer **2** in methanol:water from initiator **1** at 30 °C [2]:[1]:$[CuBr]_0$:$[CuBr_2]_0$:$[bipy]_0$ = 10:1:0.5:0.5:2. a) Kinetic plot; b) experimental M_n (from GPC) and PDI vs. conversion.

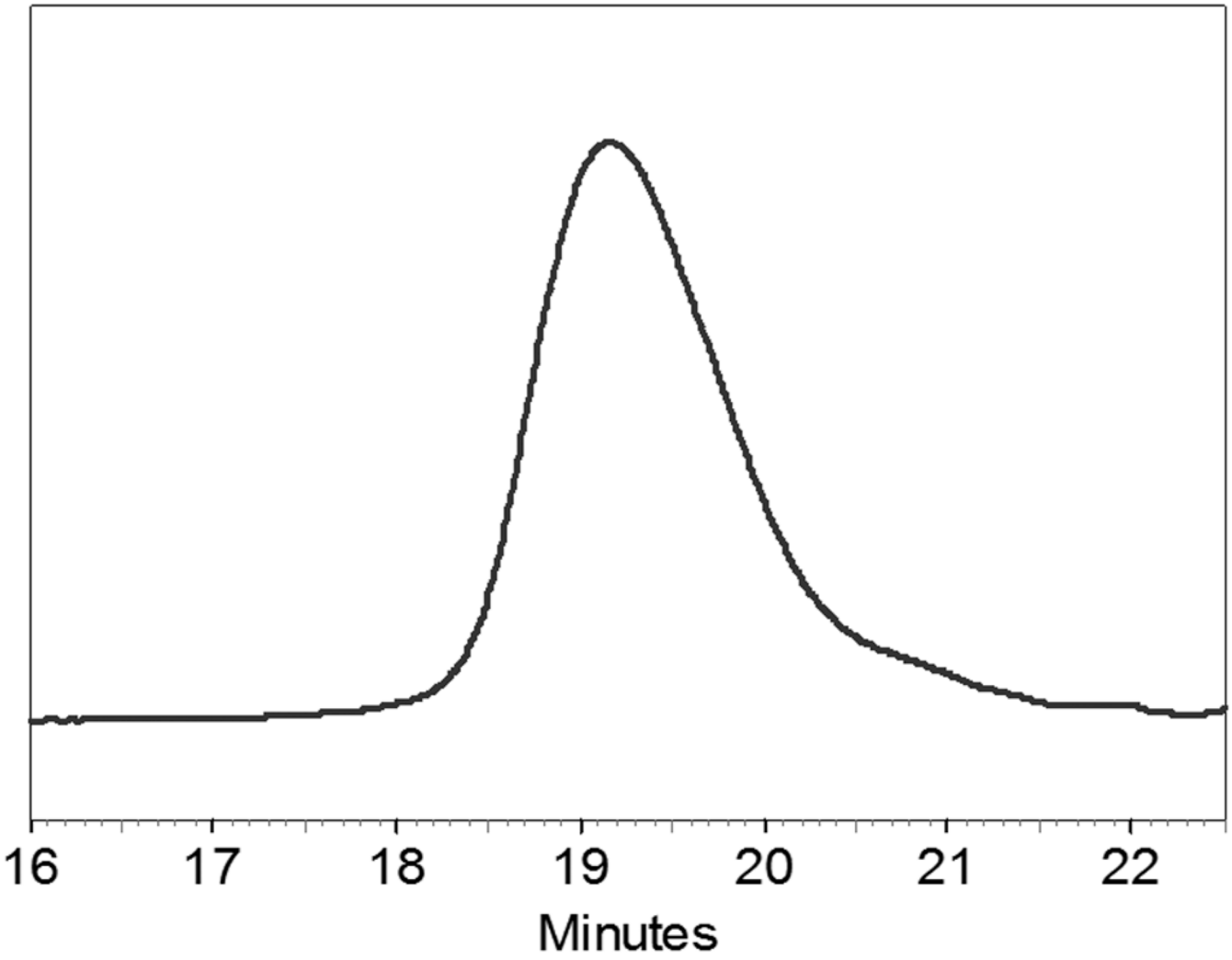


Figure 2.
GPC chromatogram of polymer **3**. Mobile phase: 0.1M LiBr DMF at 40 °C.

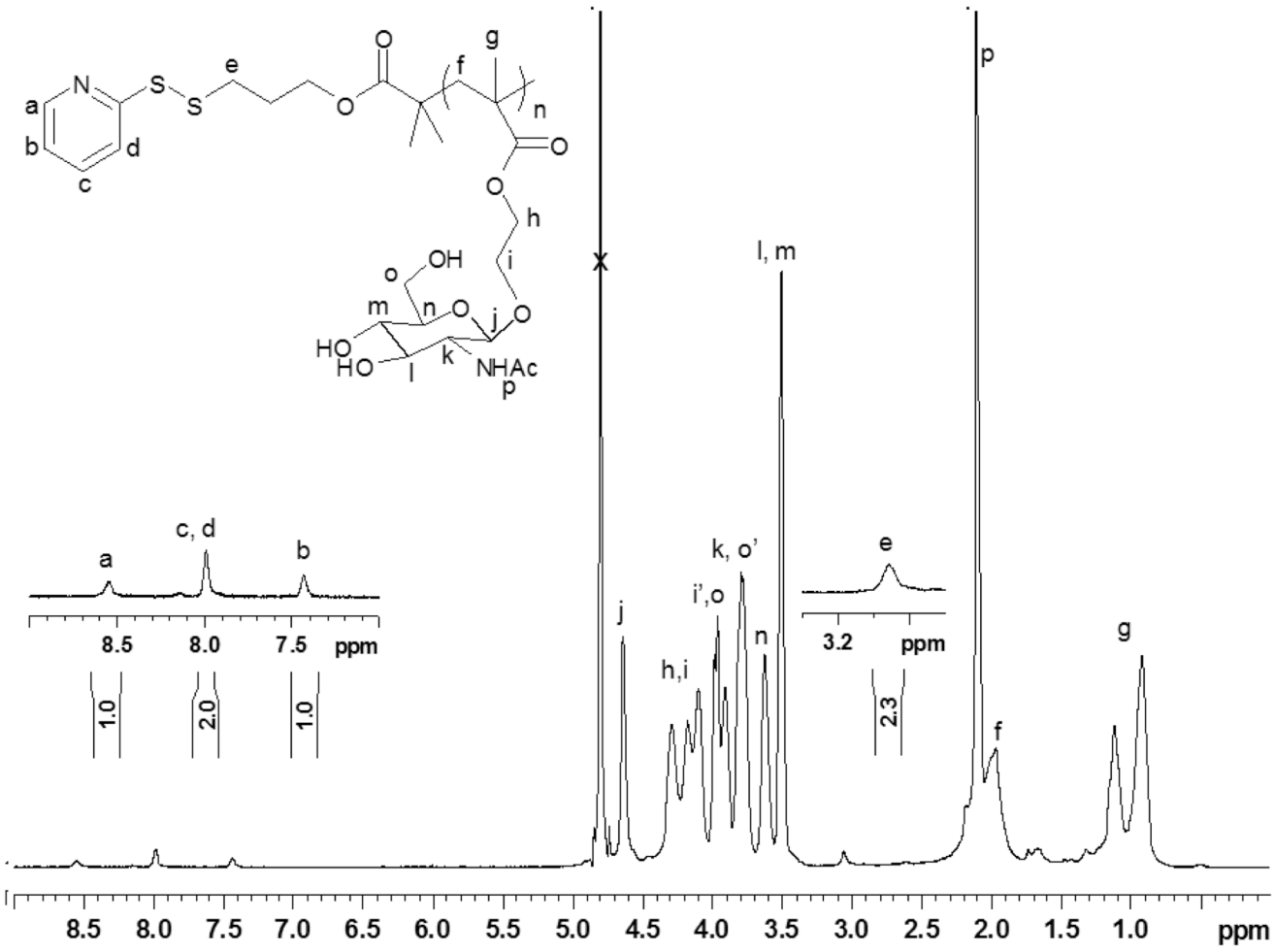


Figure 3.
^{1}H NMR spectrum (500 MHz) in D_2O of glycopolymer **3** ([**2**]:[**1**]:$[CuBr]_0$:$[CuBr_2]_0$:$[bipy]_0$ = 10:1:0.5:0.5:2).

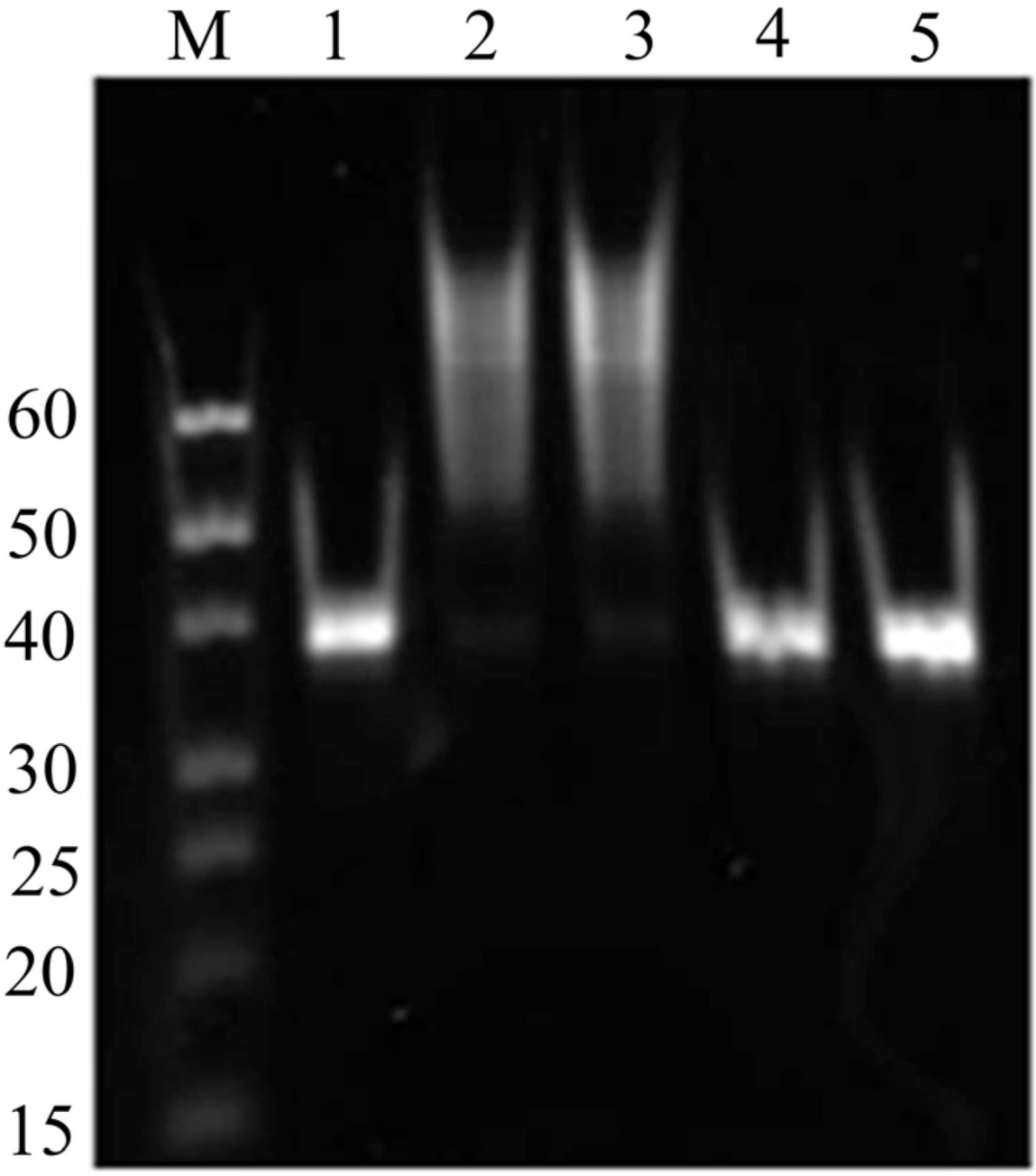


Figure 4. Polyacrylamide gel electrophoresis of the siRNA–pPEGAconjugates

Lane 1: unmodified double stranded siRNA. Lanes 2 and 3 (duplicate): siRNA-glycopolymer, lanes 4 and 5 (duplicate): siRNA-glycopolymer with DTT (100 mM) treatment. M: 10/60 oligo ladder (Integrated DNA Technologies, Inc., IA).

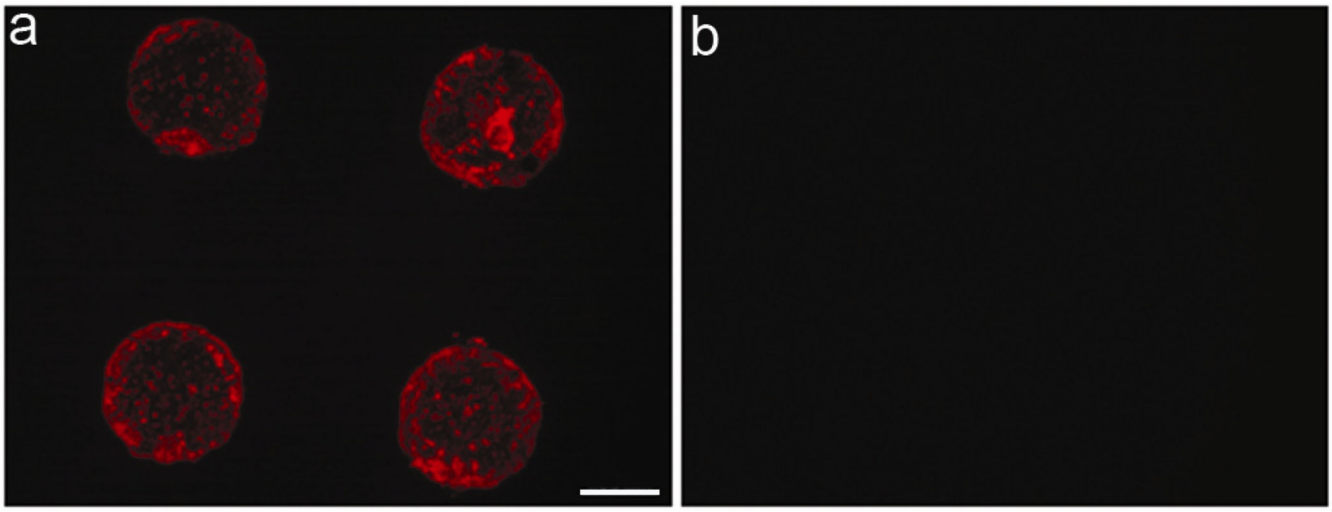
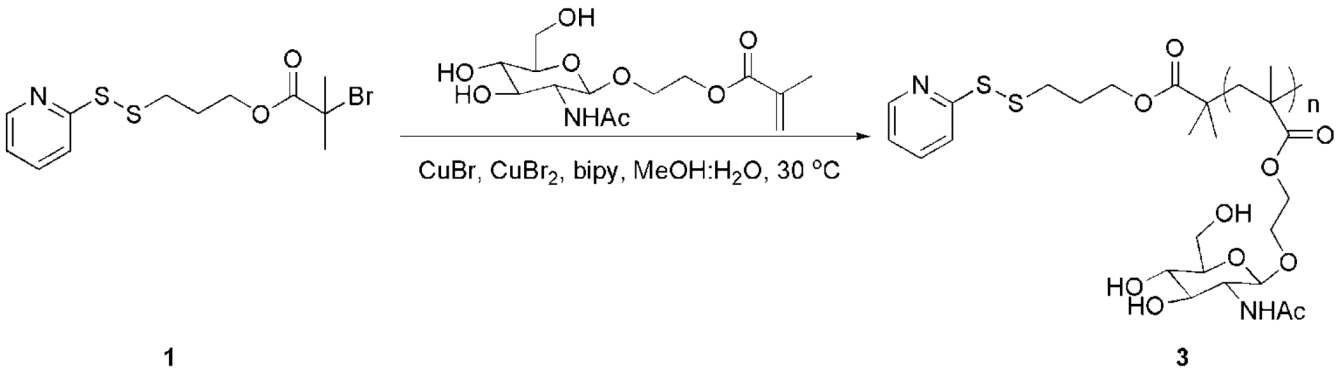
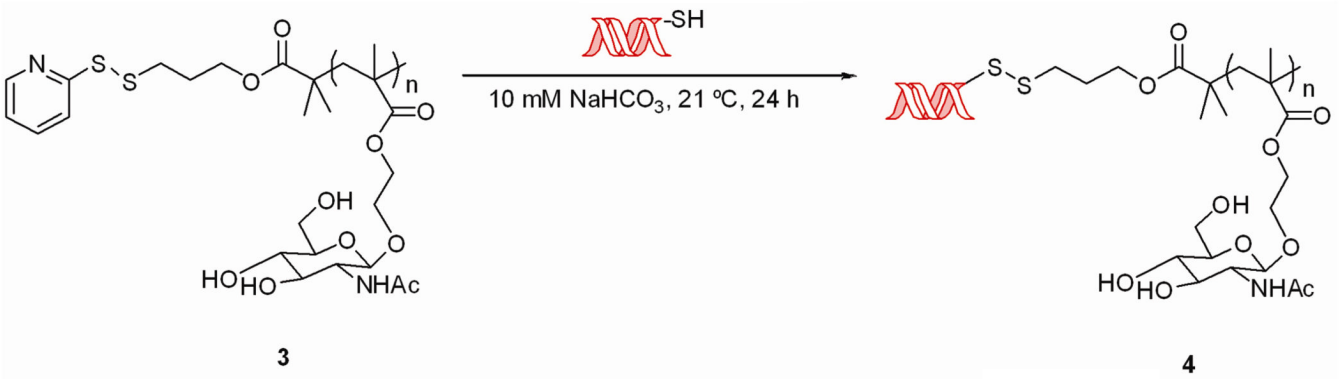


Figure 5.
Patterning of glycopolymer **3**. Patterns were made on a gold surface using a polydimethylsiloxane (PDMS) stamp. a) The glycopolymer was stained using an anti-GlcNAc antibody and an Alexa Fluor 568-tagged secondary antibody. b) Control, pattern was stained with an Alexa Fluor 568-tagged secondary antibody only. Scale bar = 100 μm.



Scheme 1.
Polymerization of glycomonomer **2** from ATRP initiator **1**.



Scheme 2.
Conjugation of siRNA to glycopolymer **2**.