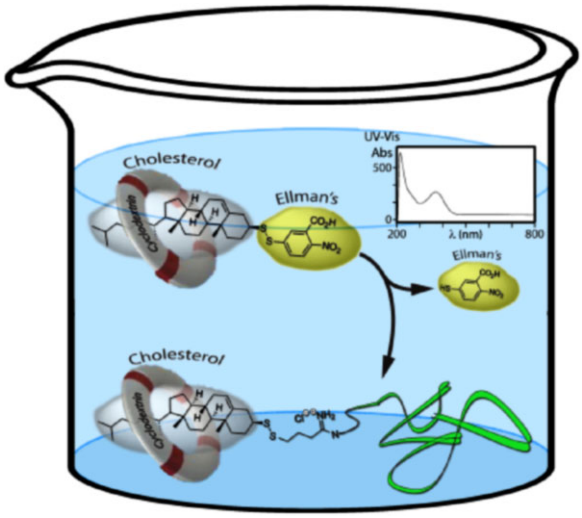
Cholesterol Modification of (Bio)Polymers Using UV-Vis Traceable Chemistry in Aqueous Solutions

Kasper F. Rasmussen, Anton A. A. Smith, Pau Ruiz-Sanchis, Katrine Edlund, Alexander N. Zelikin*

Cholesterol modification of synthetic and biological polymers is achieved using of thiocholesterol (TC) and thiol-disulfide exchange. TC is reacted with Ellman's reagent to produce a mixed disulfide (TC-ER) which is activated towards thiol-disulfide exchange. TC-ER is used to obtain an inclusion complex with methyl-β-cyclodextrin, which is then employed to achieve cholesterol functionalization of a model peptide, synthetic polymers, and physical hydrogels based on poly(vinyl alcohol). It is anticipated that the established techniques will significantly broaden the use of cholesterol in bio- and nanotechnology and specifically biomedicine.



1. Introduction

Cholesterol is an important building block in nature and a recognized tool of bio/nanotechnology.[1] Functionalization with cholesterol is pursued for diverse biomedical and biotechnological needs such as PEGylation of liposomes through cholesterol-aided anchoring of the polymer into the lipid bilayer,[2–4] RNAi techniques wherein conjugation of the nucleic acid with cholesterol enhances cellular uptake,[5] increases stability to degradation by nucleases,[6] and aids in targeting to the desired tissues,[7] specifically the liver. Modification of water-soluble polymers with hydrophobic groups such as cholesterol aims to induce formation of micelles for drug delivery.[8] Also, cholesterol-aided linkages between polymers and liposomes are used to create sub-compartmentalized assemblies as cell mimics.[9] These applications necessitate facile, reliable techniques in bioconjugation using cholesterol. However, while multiple approaches are indeed available, there are several fundamental challenges associated with cholesterol chemistry. These include i) solubility profile of cholesterol – which is not compatible with physiological conditions, ii) "invisible" nature or an ill-defined signature of this molecule in most characterization techniques (UV-Vis, NMR, FTIR, etc.), and iii) purification difficulties, that is, removal of non-specifically bound cholesterol from chemically conjugated product. The main objective of this work was to investigate opportunities in

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bioconjugation with cholesterol and (bio)polymers in aqueous conditions and using chemistries with facile tracing such as UV-Vis spectra.

With a broader interest in macromolecular design, bioconjugation, and hydrogel biomaterials,[10–12] as partners to cholesterol in developing conjugation techniques we used water-soluble synthetic polymers – poly(vinylpyrrolidone) (PVP), and poly(vinyl alcohol) (PVA). These polymers are among the most well-characterized materials in biomedicine and have regulatory approval for clinical applications in humans yet scant opportunities in bioconjugation. For PVP, opportunities in the synthesis of copolymers with ''activated esters'' for subsequent modification of the polymer chains via polymer analogous reaction[13] are limited due to incompatibility of the monomers. Inertness of the lactam ring to modification or ring opening further explains failure of this polymer to enter the mainstream of polymer therapeutics or polymer-protein conjugation techniques. Success of ''NVP-alike'' monomers in production of copolymers amenable for subsequent chemical modification and/or bioconjugation[14–16] was also moderate, largely due to laborious synthesis of monomers. For PVA, polymer synthesis proceeds via hydrolysis of the parent polymer, poly(vinyl acetate), which is typically performed at conditions which are incompatible with labile esters or activated disulfide functionalities[13,17–19] thus minimizing post-conjugation opportunities.[20] PVA offers numerous hydroxyl groups for bioconjugation and indeed, conjugation of PVA with cholesterol has been achieved,[8] however, this conjugation strategy relied on non-physiological conditions for bioconjugation. Also, hydroxyls are significantly disadvantaged compared to the classic sites of bioconjugation (amines and thiols) in terms of specificity of reactions. Finally, our specific interests lie in investigating physical hydrogels based on poly(vinyl alcohol) toward their use in creation of intelligent biointerfaces.[21–25] These matrices are labile and their chemical modification is possible only in aqueous conditions.

As an approach to bioconjugation with PVP and PVA, we use RAFT derived polymers and associated opportunities in the design of terminal groups.[26] For PVP, we have previously achieved polymer conjugation with oligopeptides and oligomeric nucleic acids, specifically using *O*-ethylphthalimidomethyl xanthate RAFT agent and removal of the terminal phthalimide (Phth) groups using hydrazine hydrate to afford amine-functionalized polymer chains.[27] We took advantage over adsorptive behavior of PVP toward silica surfaces and performed bioconjugation reactions as on-bead, solid support reactions and this afforded ease of purification and facilitated product analysis via flow cytometry. Subsequently, amine- and thiol-containing telechelic PVP was synthesized as a linker for immobilization of antibodies on the surface of polymer hydrogel capsules for targeting applications.[10] For PVA, we used the same RAFT agent as mentioned above and produced amine-terminated PVA chains for facile fluorescence labeling of chains,[11] site-specific thiolation,[22] and bioconjugation with oligopeptides.[28] In the latter case, bioconjugation was performed under physiological conditions using both, polymer chains in solution and physical hydrogels as surface-adhered biointerfaces thus establishing novel opportunities in functionalization of these matrices for biomedical applications. We note that for cholesterol functionalization in particular, RAFT-associated techniques have already documented success in growing polymer chains from cholesterol-derived RAFT agents[29,30] and using cholesterol (meth) acrylate for direct polymer synthesis.[1,24] However, due to the above mentioned limitations of synthesis, these techniques have not been implemented for cholesterol-functionalization of PVP and PVA. For RAFT derived polymers, a readily available site for conjugation is the thiol functionality produced via hydrolysis of the RAFT agent[31,32] yet this approach too is futile for PVP and PVA due to a well-documented rearrangement of the Z-group-derived terminal thiol.[33,34]

The outline of this report is as follows. First, we revisit the synthesis of amine-functionalized PVP and PVA with an emphasis on removal of the phthalimide group from the RAFT derived polymer chains. Subsequently, we investigate cholesterol-modification of polymers via carbamate linkages. Next, we outline several approaches to polymer conjugation with thiocholesterol via thiol-disulfide reshuffling with an emphasis on producing water-soluble form of cholesterol and performing reactions in aqueous conditions. Finally, we achieve cholesterol attachment onto matrices comprised of labile, physical hydrogels based on PVA. Taken together, this work contributes significantly to the development of methods to literally link materials science and a nature-derive building block of nanotechnology, cholesterol.

2. Experimental Section

2.1. Materials and Instrumentation

Unless stated otherwise, all chemical were obtained from Sigma-Aldrich and used without purification. ^{1}H NMR spectra were recorded on a Varian Mercury 400 MHz spectrometer. Spectra were referenced to the residual solvent peak: $CDCl_3$ ($\delta = 7.26$), D_2O ($\delta = 4.79$), and DMSO-d_6 ($\delta = 2.50$). IR spectra were obtained on an iD5 ATR integrated (Thermo Scientific, USA) in a Nicolet iS5 (Thermo Scientific, USA). HRMS was performed on a Micromass LC-TOF spectrometer with positive electrospray ionization.

Bz-Gly-Phe-OMe ($C_{19}H_{20}N_2O_4Na$, m/z 363.132) or Boc-Ser(OBn)-Ser-Leu-OMe ($C_{25}H_{39}N_3O_8Na$, m/z 532.2635) were used as internal standards. Analytical HPLC was performed on a Shimadzu LC-2010A HT equipped with a Shimadzu SPD-M-20A PDA detector and Peptide ES-C18 column with 2.7 μm particles, a length of 150 mm and an internal diameter of 3 mm from Sigma-Aldrich. The temperature was fixed at 40 °C; acetonitrile and water [with 0.045% trifluoroacetic acid (TFA)] were used as mobile phases. The gradient was changed from 30 to 100% MeCN within 15 min and then 100% MeCN was used for an additionally 30 min. Polymer characterizations were performed on a Shimadzu gel permeation chromatography (GPC) instrument consisting of: an auto sampler (SIL-10AF), a solvent pump (LC-20AD), a column oven (CTO-20A), a light scattering (LS) detector (DAWN HELEOS 8), a differential refractive index detector (RID-10A), and last a PDA detector (SPD-M-20A). The used column for DMF measurements was a Mz-Gel SDplus Linear column with 5 μm particles, a length of 300 mm and an internal diameter of 8 mm. The eluent was DMF (with 0.010 M LiBr) and the samples were run at 30 °C with a flow rate of 1 mL min^{-1}. The column used for measurements in water was a HEMA-Bio Linear column with 10 μm particles, a length of 300 mm and an internal diameter of 8 mm. The eluent was purified MQ water (with NaN_3 300 ppm).

2.2. Syntheses

2.2.1. *O*-Ethyl-*S*-Phthalimidylmethyl Xanthate (1)

A solution of *N*-(bromomethyl)phthalimide (233.8 mg, 0.98 mmol) in $CHCl_3$ (6 mL) was added dropwise to a solution of *O*-ethylxanthic acid potassium salt (296.1 mg, 1.85 mmol) in $CHCl_3$ (5 mL). The solution was stirred at room temperature for 14 h. The reaction was followed by thin-layer chromatography (TLC, $R_f = 0.6–0.7$ $CHCl_3$). The reaction was diluted with $CHCl_3$ (5 mL) and the solution was washed with water (2 × 10 mL) and brine (15 mL). The organic layer was dried over magnesium sulfate and then filtered, and the solvent was removed under vacuum affording the target compound, a pale yellow solid product (170.4 mg, 0.6 mmol, 72%,). ^{1}H NMR ($CDCl_3$): $\delta = 1.45$ (t, $J = 7.1$ Hz, 3H, $C\underline{H}_3CH_2-$); 4.67 (q, $J = 7.1$ and 7.1 Hz, 2H, $CH_3C\underline{H}_2-$); 5.33 (s, 2H, $S-C\underline{H}_2-N$); 7.74 (q, $J = 3.1$ and 5.4 Hz, 2H, $C\underline{H}_{ar}$), 7.87 (q, $J = 3.1$ and 5.4 Hz, 2H, $C\underline{H}_{ar}$). ^{13}C NMR (CDCl3): $\delta = 13.7$ (q, $\underline{C}H_3CH_2-$); 41.2 (t, $N-\underline{C}H_2-S$); 70.5 (t, $CH_3\underline{C}H_2-$); 123.6 (2d, $\underline{C}H_{ar}$); 131.81 (2s); 134.39 (2d, $\underline{C}H_{ar}$); 166.6 (2s, $\underline{C}=O$); 210.2 (s, $\underline{C}=S$).

2.2.2. α-Phthalymidyl-ω-(*O*-ethylcarbonodithiotyl)-PVP (2)

1 (132.5 mg, 471 μmol), azobisisobutyronitrile (AIBN) (7.3 mg, 44.4 μmol), and NVP (10 mL, 1.043 g, 9.9 mmol) were combined in toluene (20 mL). The reaction mixture was degassed by three freeze-pump-thaw cycles and then heated for 16 h at 60 °C. The reaction was quenched by rapidly cooling with liquid nitrogen. The product was isolated by precipitation into diethyl ether, filtration and removal of solvent on the vacuum line to give **2** in 4.5 g, as colorless solid. GPC analysis yielded a $\overline{M}_n$ of 13 kDa and a $Đ$ of 1.13. ^{1}H NMR ($CDCl_3$): $\delta = 0.50–2.50$ ($-C\underline{H}_2C\underline{H}_2C\underline{H}_2CON-$, $C\underline{H}_2CH-N$, $C\underline{H}_3C\underline{H}_2O-$); 3.22 ($-C\underline{H}_2CH_2CH_2CON-$); 3.74 ($CH_2C\underline{H}-N$); 7.71 (m, 2H, $C\underline{H}_{ar}$); 7.82 (m, 2H, $C\underline{H}_{ar}$).

2.2.3. α-Aminomethyl-PVP (3)

2 (2.010 g, 13 kDa) and $NH_2NH_2 \cdot H_2O$ (270 mg, 263 μL, 5.39 mmol) were combined in MeOH (8 mL). The mixture was heated for 1 h at 60 °C. The product was obtained by precipitation in Et_2O and filtration to give 1.742 g of **3**, a colorless solid. ^{1}H NMR ($CDCl_3$): $\delta = 0.50–2.50$ ($-CH_2C\underline{H}_2C\underline{H}_2CON-$, $C\underline{H}_2CH-N$, $C\underline{H}_3C\underline{H}_2O-$); 3.20 ($-C\underline{H}_2CH_2CH_2CON-$); 3.71 ($CH_2C\underline{H}-N$).

2.2.4. α-Cholesterylcarbonylaminomethyl-PVP (4)

A solution of cholesteryl chloroformate (22.2 mg, 24.7 μmol) in dry dichloromethane (DCM, 1 mL) was added dropwise to a solution of triethylamine (TEA, 2 μL, 1.45 mg, 14.3 μmol) and **3** (204.4 mg, 13.3 kDa) in dry DCM (1 mL) at 0 °C. The reaction was stirred for 12 h at room temperature. The solvent was removed and the crude was re-dissolved in $CHCl_3$; the polymer was precipitated in Et_2O and filtrated. The obtained compound **4** was a colorless solid (71.0 mg, cholesteryl ester loading = 91%). ^{1}H NMR ($CDCl_3$): $\delta = 0.67$ (s, 3H, $-C-C\underline{H}_3$); 0.85 (d, $J = 1.6$ Hz, 3H, $-CH(C\underline{H}_3)_2$); 0.86 (d, $J = 1.6$ Hz, 3H, $-CH(C\underline{H}_3)_2$); 0.92 (d, $J = 6.9$ Hz, 3H, $-CH-C\underline{H}_3$); 0.99 (s, 3H, $-CH-C\underline{H}_3$); 1.00–2.50 ($-C\underline{H}_2C\underline{H}_2C\underline{H}_2CON-$, $C\underline{H}_2CH-N-$, $C\underline{H}_3C\underline{H}_2O-$); 3.20 ($-C\underline{H}_2CH_2CH_2CON-$); 3.72 ($CH_2C\underline{H}-N$); 5.35 (m, 1H, $-C=C\underline{H}-$). IR (ATR, solid): $\nu = 1\,662$ cm^{-1} (vs, $>N-C=O$).

2.2.5. α-*N*-{4-[2-(3-carboxy-4-nitrophenyl)dithio]-1-iminobutyl]aminomethyl}-PVP (5)

3 (253.6 mg), Traut's reagent (20.1 mg, 146 μmol) and Ellman's reagent (80.9 mg, 204 μmol) were suspended in buffer 0.1 $NaHCO_3$ (10 mL, pH = 8.3). The reaction was stirred for 14 h. A NAP-25 column was used for purification and the collected fraction was freeze-dried to give a pale red solid **5** (163.2 mg). The Ellman's reagent loading was estimated by UV-measurements (179%). ^{1}H NMR ($CDCl_3$): $\delta = 1.20–2.50$ ($-CH_2C\underline{H}_2C\underline{H}_2CON-$, $C\underline{H}_2CH-N$, $C\underline{H}_3C\underline{H}_2O-$, $-S-C\underline{H}_2-C\underline{H}_2-C\underline{H}_2-C-$); 3.13 ($-C\underline{H}_2CH_2CH_2CON-$); 3.66 ($CH_2C\underline{H}-N$); 7.74 (b s, 2H, ar); 8.06 (b s, 1H, $C\underline{H}_{ar}$).

2.2.6. α-{*N*-[4-(thiocholesteryl)thio]-1-iminobutyl] aminomethyl}-PVP (6)

5 (100 mg) was suspended in $CHCl_3$ (2 mL) and degassed for 30 min. A solution of thiocholesterol (47.2 mg, 11.7 μ mol) in $CHCl_3$ (5 mL), which has been previously degassed for 30 min, was added to the PVP solution. The reaction was stirred for 14 h. A red solid, **6** (72.4 mg) was obtained by precipitation in Et_2O. ^{1}H NMR ($CDCl_3$): $\delta = 0.65$ (s, 3H, $-C-C\underline{H}_3$); 0.84 (s, 3H, $-CH(C\underline{H}_3)_2$); 0.86 (s, 3H, $-CH(C\underline{H}_3)_2$); 0.89 (d, $J = 6.6$ Hz, 3H, $-CH-C\underline{H}_3$); 0.98 (s, 3H, $-CH-C\underline{H}_3$); 1.00–2.50 ($-CH_2C\underline{H}_2C\underline{H}_2CON-$, $C\underline{H}_2CH-N$); 3.19 ($-C\underline{H}_2CH_2CH_2CON-$); 3.72 ($CH_2C\underline{H}-N$); 5.31 (b s, 1H, $-C=C\underline{H}-$).

2.2.7. 5-[(Thiocholesteryl)thio]-2-nitrobenzoic acid (7)

A solution of Ellman's reagent (99.8 mg, 0.252 mmol) in degassed tetrahydrofuran (THF, 5 mL) and TEA (200 μL) were added to a degassed solution of thiocholesterol in THF (103.4 mg in 5 mL, 0.257 mmol). The color of the solution immediately changed from

colorless to strong orange. The solution was stirred for 14 h at room temperature. THF was removed under vacuum giving an orange oil. The product was isolated trough a column chromatography (DCM/MeOH, 9:1) affording **7**, a pale yellow solid (76.0 mg, 51%). ^{1}H NMR ($CDCl_3$): $\delta = 0.65$ (s, 3H, —C$\underline{H}_3$); 0.85 (s, 3H, —C$\underline{H}_3$); 0.87 (s, 3H, —C$\underline{H}_3$); 0.89 (s, 3H, —C$\underline{H}_3$); 0.91 (s, 3H, —C$\underline{H}_3$); 1.71–2.04 (m, C$\underline{H}_2$); 2.26 (b s, 2H, C$\underline{H}_2$); 2.67 (s, 1H, —C$\underline{H}$—S—); 5.21 (s, 1H, =C$\underline{H}$—); 7.32–7.65 (m, 3H, C$\underline{H}_{ar}$). HRMS (ESI+) calculated for $C_{34}H_{49}NO_4S_2Na$ m/z ($M + Na^+$) 622.2995, found 622.3001.

2.2.8. α-{*N*-[4-(thiocholesteryl)thio]-1-iminobutyl} aminomethyl-PVP (8)

Traut's reagent (9.8 mg, 71 μmol) and **3** (229.1 mg, 22.9 μmol) were dissolved in 0.1 M $NaHCO_3$ (4 mL, pH = 8.28). A solution of **7** (42 mg, 70 μmol) in THF (2 mL) and buffer (0.1 mL, $NaHCO_3$, 0.1 M, pH = 8.28) was added. The reaction was stirred overnight at room temperature. THF was removed under vacuum, the precipitate was centrifuged (14 000 rpm, 2 min) and the supernatant filtered (0.2 μm filter). The product was isolated by NAP-25 column and freeze-dried to give **8**, a light brown solid (57.4 mg). ^{1}H NMR ($CDCl_3$): $\delta = 0.66$ (d, $J = 6.1$ Hz, 3H, —C—C$\underline{H}_3$); 0.84–0.86 (m, 6H, —CH—(C$\underline{H}_3$)$_2$); 0.88–0.94 (m, 3H, —CH—C$\underline{H}_3$); 0.99 (s, 3H, —CH—C$\underline{H}_3$); 1.00–2.70 (—CH_2C$\underline{H}_2$C$\underline{H}_2$CON—, C$\underline{H}_2$CH—N); 3.20 (—C$\underline{H}_2$$CH_2$$CH_2$CON—); 3.72 ($CH_2C\underline{H}$—N); 5.31 (m, 1H, —C=C$\underline{H}$—).

2.2.9. Inclusion Complex of Thiocholesterol in Methyl-β-cyclodextrin (9)

Thiocholesterol (1.4 mg) was dissolved in chloroform/methanol 2:1 (1.4 mL). Methyl-β-cyclodextrin (200 mg) was dissolved in deionized water (4 mL) to give a 5 wt% solution. Thiocholesterol solution was added in small (0.2 mL) aliquots to the solution at 80 °C, and reacted for 2 min per addition. The solution was filtered through a 0.2 μm filter and then the water was removed by freeze-drying to give **9**, a white solid (178.3 mg, thiocholesterol inclusion = 3 mg per 1 g solid).

2.2.10. Inclusion Complex of 5-[(Thiocholesteryl)thio]-2-nitrobenzoic acid in Methyl-β-cyclodextrin (10)

Methyl-β-cyclodextrin (1.0 mg, 0.76 mmol) was dissolved in H_2O (20 mL) affording a 5 wt% solution. A solution of **7** (35.0 mg, 58.3 μmol) in a mixture of $CHCl_3$/MeOH (2:1, 3.5 mL) was added in small aliquots (0.25 mL) at 80 °C, and reacted for 2 min per addition. The solution was filtered (0.2 μm filter) and freeze-dried to give **10**, a white solid (978 mg, 30 mg of TC-TNB per 1 g solid).

2.2.11. Aqueous Thiocholesterol Modification of Amine-Containing PVP (Thiol Disulfide Exchange Between 10 and 3)

Solutions of amine-containing PVP (100 g L^{-1}, 100 μL), Traut's reagent (1 g L^{-1}, 860 μl) and **10** (50 g L^{-1}, 3 ml) 0.1 M carbonate buffer, pH = 8.3 were combined in a reaction vial and incubated on a rotary shaker for 4 h. The solution was purified by dialyzing with, first, carbonate buffer (38 h), then, MilliQ water (36 h). The crude was freeze-dried giving a colorless solid, re-dissolved, filtered (0.4 μm filter), and freeze-dried again affording a colorless solid (14.77 mg). ^{1}H NMR ($CDCl_3$): $\delta = 0.67$ (s, 3H, —C—C$\underline{H}_3$); 0.84–0.86 (m, 6H, —CH—(C$\underline{H}_3$)$_2$); 0.89–0.91 (m, 6H, —CH—C$\underline{H}_3$); 0.99–2.50 (—CH_2C$\underline{H}_2$C$\underline{H}_2$CON—, C$\underline{H}_2$CH—N); 3.19 (—C$\underline{H}_2$$CH_2$$CH_2$CON—); 3.40–4.10 (CD); 4.99 (CD).

2.2.12. Aqueous Conjugation of Thiocholesterol with GSH

For kinetic measurements, in an Eppendorf tube, stock solutions of **10** and GSH in 0.1 M carbonate buffer, pH = 8.3 were combined to afford 10 g L^{-1} concentration of the inclusion complex and 5×10^{-3} M GSH. Reaction mixture was incubated on a rotary shaker taking UV-Vis measurements on a NanoDrop instrument at indicated time points. To determine an optical density corresponding to a full conversion of thiol-disulfide exchange, dithiothreitol (DTT, 1 μL, 100 g L^{-1}) was added instead of GSH.

For estimation of GSH thiol-to-disulfide conversion, aqueous solutions were prepared in 0.1 M $NaHCO_3$ buffer (pH = 8.3) to a final concentration of the inclusion complex of 10 g L^{-1} and a varied concentration of GSH. Mixtures were incubated on a rotary shaker for 14 h upon which UV-measurements were performed to estimate the reaction conversions. Optical density corresponding to a full reaction conversion was estimated using DTT as described above.

2.2.13. Hydrogel Functionalization

Surface adhered, micro-structured PVA hydrogels were prepared via micro-transfer molding using a sample of reversible addition/fragmentation chain transfer (RAFT)-derived, amine-containing PVA (31 kDa) as described in detail previously.[28] In brief, a drop of 12 wt% polymer solution was placed between a glass cover slip and a poly(dimethylsiloxane) stamp with cylindrical cavities (diameter 5 μm, center-to-center spacing 10 μm, depth 2 μm) and clamped at finger tight pressure overnight. Disassembly of the clamps afforded surfaced adhered materials which underwent a treatment using 0.5 M sodium sulfate for 1 h and rehydration in phosphate-buffered saline (PBS, pH = 7.4) to afford a final preparation, surface adhered hydrogels.

Terminal amine groups on polymer chains comprising hydrogel samples were converted into thiol groups via immersion of the specimen into a 0.010 M solution of iminothiolane in carbonate buffer pH = 8.3 supplemented with sodium sulfate to 0.25 M (here and below denoted as carbonate-sulfate buffer) and incubation for 30 min. Following this, cover slips were washed with copious amounts of carbonate-sulfate buffer. Reaction with Ellman's reagent was performed via adding 10 μL of 10^{-3} M solution of Ellman's reagent to 200 μL of carbonate-sulfate buffer covering the hydrogel sample and reaction times 15 or 60 min. Reaction with inclusion complex **7** was performed through adding 10 μL of a 63 g L^{-1} solution of **7** (corresponding to $\approx 10^{-3}$ M TC-ER) to 200 μL carbonate-sulfate buffer covering the hydrogel samples and incubation time 15 or 60 min. Progression of reactions was monitored via UV-Vis spectroscopy, specifically quantifying optical density at 412 nm.

3. Results and Discussion

3.1. Polymer Synthesis

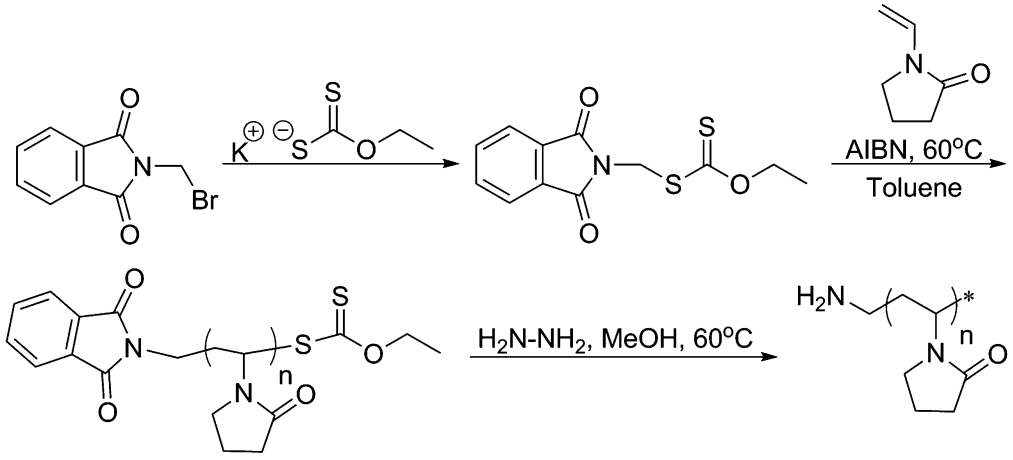
The synthesis of PVP with controlled molecular weight and appropriate end-group for further synthetic modifications

was accomplished using *O*-ethyl-*S*-(phthalimidylmethyl) xanthate as a RAFT agent. The latter was synthesized starting from *N*-(bromomethyl)phthalimide and potassium ethyl xanthate, Scheme 1.[35] Polymerizations were conducted combining RAFT agent, 1-vinyl-2-pyrrolidone, and AIBN as initiator in toluene at 60 °C. Molecular weights of the resulting polymers were independently estimated on the basis of ^{1}H NMR, comparing integrals corresponding to phthalimide and pyrrolidone protons, and via a gel permeation chromatography performed in DMF containing lithium bromide, as described in the Experimental Section.

For removal of the terminal Phth group, polymer samples were incubated in methanolic hydrazine. Resulting polymer was recovered via precipitation into diethyl ether, however, this purification technique alone was insufficient and resulting polymer samples contained significant amount of impurities. Polymer was dissolved in water and additionally purified on a size exclusion column (NAP-25) and recovered via freeze-drying. Polymer analysis was performed using GPC and a combination of multi-angle light scattering and full spectrum UV-Vis detectors. We have previously shown that this set-up is essential for a reliable analysis of polymers and specifically their end group conversion.[22,28] In particular, this combination set-up allows distinguishing between spectrally identical solutions of a true bioconjugate and a mixture of components. Herein, we use this combination of detectors to illustrate a successful removal of the terminal Phth group. Indeed, the parent polymer exhibits a pronounced absorbance at 285 nm which corresponds to the Phth functionality (Figure 1A and B). In contrast, UV-Vis spectrum of the elution volume corresponding to the hydrazine-treated polymer bears no characteristic signature of Phth and the solution has <10% absorbance of that registered for Phth-PVP (Figure 1B). Removal of the terminal group was also verified via ^{1}H NMR spectroscopy. This method revealed otherwise identical spectra for polymer before and after hydrazinolysis and confirmed disappearance of the signals corresponding to Phth group (Figure 1C) thus, corroborating above discussed GPC analysis.

3.2. Conjugation with Cholesteryl Chloroformate

For proof-of-concept functionalization of polymers with cholesterol via terminal amine groups, we chose a well-established synthetic approach based on cholesterol chloroformate, a derivative which is reactive toward classic sites of bioconjugation, amines. This reagent has previously been used in the design of lipopolymers,[36,37] pH-responsive polymeric micelles,[38] and cholesterol-containing polyamine lipids.[39] Reaction with amine-containing PVP was conducted in dichloromethane in the presence of triethylamine (Scheme 2), resulting polymer was recovered via precipitation into diethyl ether. NMR characterization (Figure 2A) revealed a 90% loading of polymer chains with cholesterol. The recovered solid did not contain cholesteryl chloroformate, which was verified via FTIR spectroscopy (Figure 2B). We note that reaction yield obtained herein (in terms of number of ligands per polymer chain) is comparable to that achieved in conjugation using terminal thiol groups obtained via hydrolysis of RAFT agent thioester.[40] This notion illustrates a practical utility of the conjugation approach developed in this work. However, this reaction also highlights the limitations and challenges of conjugation reactions with cholesterol. Thus, reaction analysis relies on rather ill-defined NMR signature of cholesterol and a semi-quantitative FTIR spectroscopy. Furthermore, progression of reaction does not produce or



Scheme 1. Synthesis of PVP using *O*-ethyl-*S*-(phthalimidylmethyl) xanthate as a RAFT agent and subsequent removal of phthalimide functionality to afford amine-functionalized polymer chains.

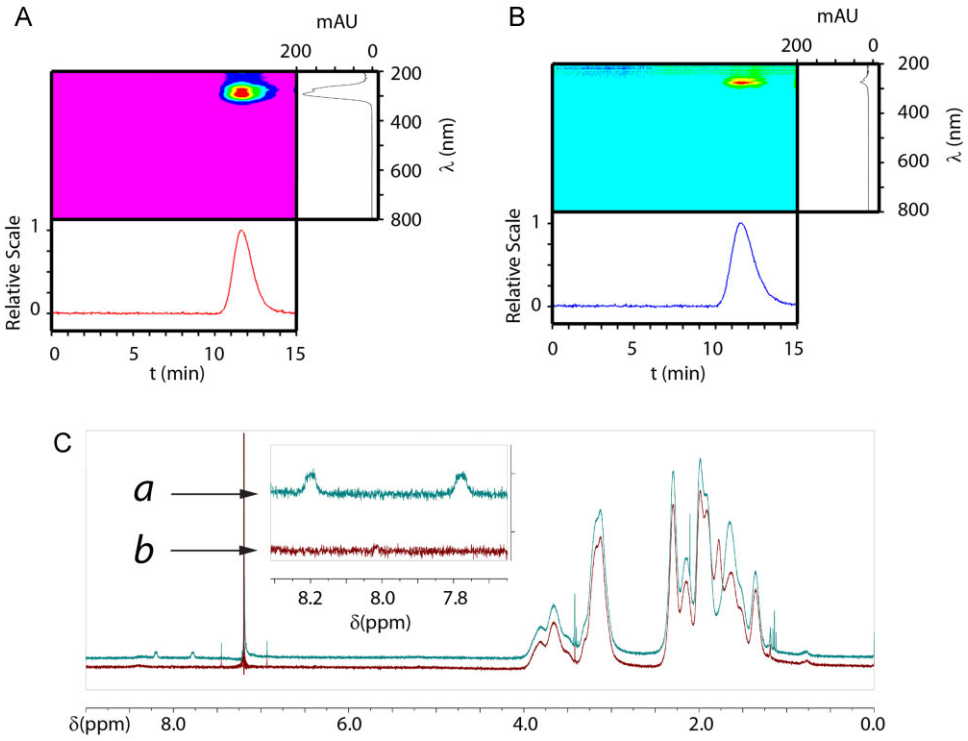


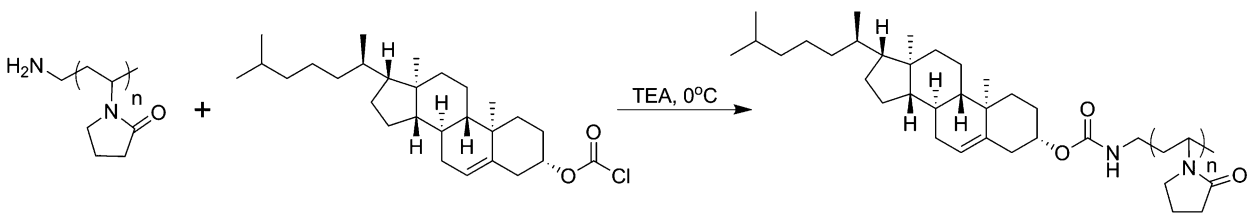
Figure 1. A,B) GPC characterization of RAFT-derived PVP before (A) and after (B) hydrazinoysis using a set-up with MALS and full-spectrum UV-Vis detectors. C) Corresponding ^{1}H NMR characterization of the polymers [before (a) and after (b) hydrozynolysis].

consume any species with defined UV, fluorescent, or other spectroscopic properties to allow facile monitoring of the process. Also, analysis of the product does not discriminate between true conjugated species and impurities adsorbed onto the polymer sample. Finally, this reaction was conducted in organic solvent and not at physiological conditions.

Our prior experience in bioconjugation suggested that an approach to circumvent the above discussed limitations can be found in thiol-disulfide chemistry, specifically interconversion facilitated by the use of activated thiols, for example, mixed disulfides with Ellman's reagent.[41,42] This chemistry can be accomplished in a range of solvents including physiological buffers thus, being attractive for bioconjugation. Release of 5-thio-2-nitrobenzoic acid (TNB) chromophore provides a facile means of monitoring progression of reaction and in doing so significantly increases the confidence in successful bioconjugation, i.e., synthesis of cholesterol derivatives. Finally, contributing to the overall feasibility of this approach, thiol containing analogue of cholesterol, thiocholesterol, is inexpensive and readily available from commercial sources. Below, we present our data on the use of thiocholesterol and thiol-disulfide interconversion in cholesterol modification of synthetic polymers.

3.3. Cholesterol Modification Via Thiol-Disulfide Exchange

Thiol-disulfide interconversion presents several opportunities in terms of starting reagents to produce the final disulfide linkage. First, we aimed to use commercial, non-



Scheme 2. Cholesterol conjugation to amine-functionalized PVP through carbamate linkage using cholesterol chloroformate.

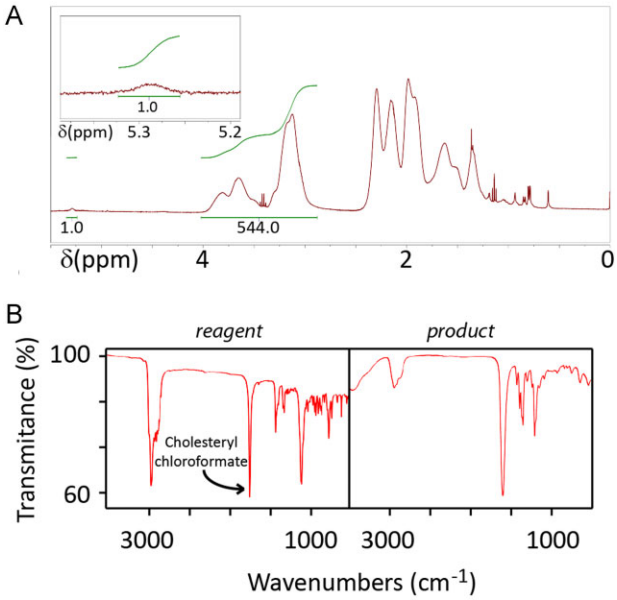
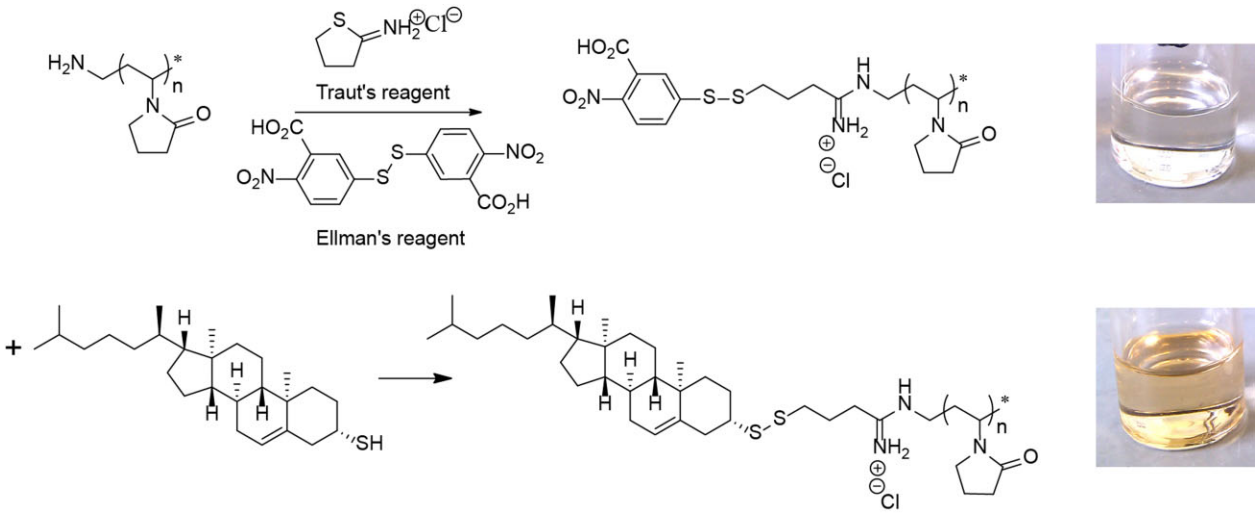


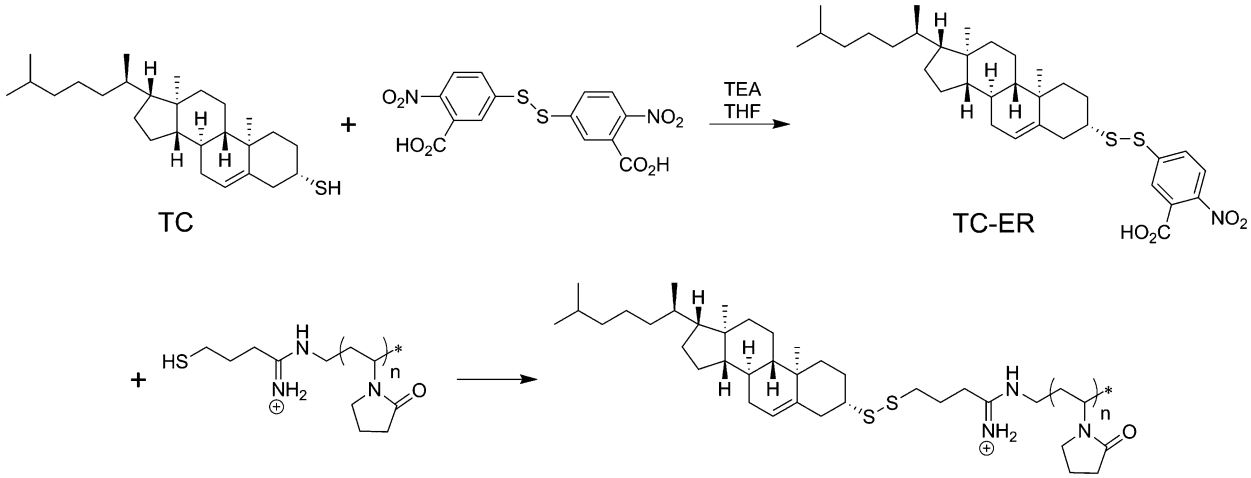
Figure 2. A) ^{1}H NMR and B) FT-IR characterization of cholesterol conjugation to amine-functionalized PVP through carbamate linkages.

modified thiocholesterol. To this end, PVP underwent a one-pot conversion using Traut's reagent (iminothiolane) and Ellman's reagent (Scheme 3). The first step of this reaction affords thiol-containing polymer chains which then immediately react with Ellman's reagent. This yields polymers which contain terminal mixed disulfide activated toward further reactions with thiol-containing molecules (Scheme 3). We have previously accomplished this on RAFT derived, amine-containing PVA and showed that the resulting polymer delivers versatile options in bioconjugation using this polymer.[21,28] Optimized reaction conditions for this reaction were carbonate buffer (pH = 8.0–8.3) as reaction medium and 1:11:8 ratio of the polymer to iminothiolane to Ellman's reagent. Resulting polymer was purified using a size-exclusion column (NAP-25) and recovered via freeze-drying. Content of the terminal mixed disulfide was estimated through a reaction with DTT, a quantitative reaction which liberates TNB, a chromophore with characteristic absorbance at 412 nm. Isolated polymers revealed an apparent degree of thiolation of over 100%. To explain this, one has to consider that terminal thioester group inherited with the RAFT agent affords a thiol group upon hydrazynolysis[43] and is also detected in the above employed assay. However, the thioester-derived thiol undergoes spontaneous rearrangement in aqueous solution of RAFT-derived PVP and polymer chains gradually lose these thiol groups thus, leading to a varied amount of R-group derived thiols[33] and thus being an un-reliable site for conjugation.

For cholesterol modification, above obtained PVP was reacted with thiocholesterol. In this reaction, we exploit excellent solubility of PVP in organic solvents which allowed performing this conversion in chloroform. Reaction was significantly accelerated in the presence of triethylamine. Polymer purification was accomplished via repetitive precipitation into diethyl ether. Polymer characterization revealed cholesterol modification at 75–95%, as estimated by ^{1}H NMR spectroscopy. This reaction provides a proof-of-concept conjugation of polymers with



Scheme 3. Synthesis of disulfide-based conjugate of cholesterol with PVP. Reaction was conducted using PVP with terminal thiol groups, activation of the latter towards thiol-disulfide exchange using Ellman's reagent, and thiolcholesterol. Target conjugate was obtained via thiol-disulfide reshuffling. Corresponding images demonstrate a colour change accompanying the progression of reaction.



Scheme 4. Syntheses of disulfide-based conjugate of cholesterol with PVP using thiocholesterol, Ellman's reagent to activate thiocholesterol toward disulfide reshuffling, and polymer chains equipped with a single terminal thiol group.

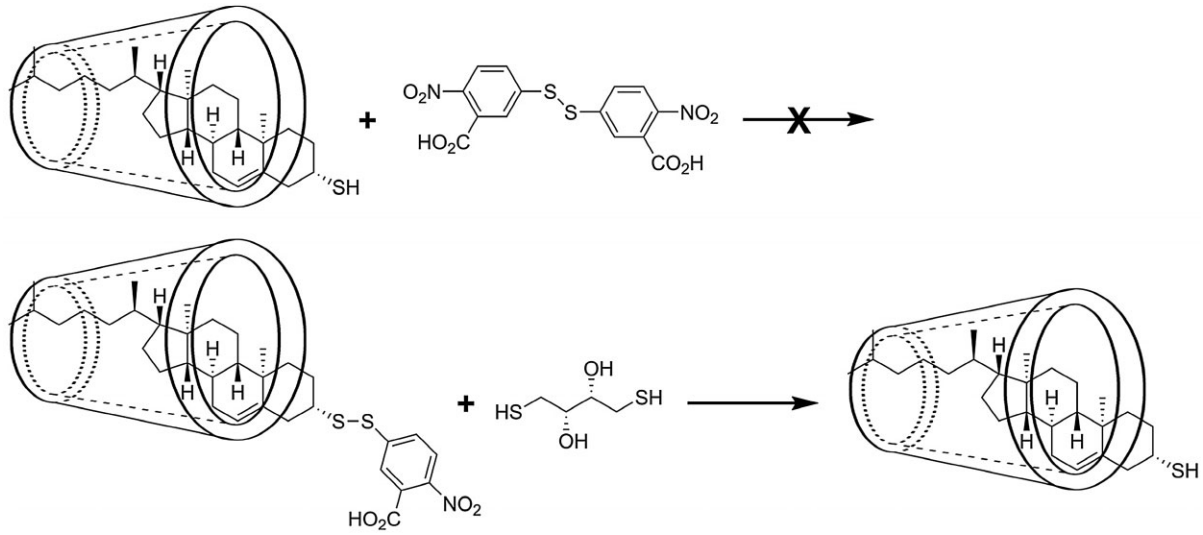
cholesterol via disulfide linkages but as described, fails to facilitate monitoring the progress and quantification of the product of this conversion. Also, above reaction can only be performed in organic solvents thus, significantly limiting its scope for biomedicine.

In an attempt to prepare water-soluble derivative of thiocholesterol, this compound was reacted with Ellman's reagent in THF in the presence of TEA to produce thiocholesterol-5-thio-2-nitrobenzoic acid, TC-ER (Scheme 4). Despite expectations, target cholesterol derivative did not exhibit solubility in water or physiological buffers to accommodate aqueous phase bioconjugation. Nevertheless, amine-containing PVP was treated with Traut's reagent to produce thiolated polymer chains. Conjugation with TC-ER was conducted in mixed solvents, aqueous tetrahydrofurane, and afforded yield of cholesterol modification not exceeding 60%.

3.4. Water-Soluble Thiocholesterol and Aqueous Thiol-Disulfide Exchange

To further increase solubility of thiocholesterol in aqueous media, we turned to well-described inclusion complexes of steroidal compounds with cyclodextrins (CD). Synthesis of inclusion complexes using TC was accomplished following Williams's protocol[23] with slight modifications and using methyl-β-cyclodextrin (M-β-CD) or hydroxyl-propyl-β-cyclodextrin (HP-β-CD). Surprisingly, the CD-TC adduct revealed only a minor content of thiol groups, as verified using Ellman's reagent. Apparent TC loading was established at ≈3 mg per gram of inclusion complex, which is only ≈10% of cholesterol content in commercial preparations of CD-based "water-soluble cholesterol." While it is plausible that TC loading into the structure of CD is indeed significantly lower that for pristine cholesterol, we hypothesize that it is more likely that Ellman's test underestimates the presence of TC. Specifically, it is plausible that thiol groups may undergo oxidation and coupling into disulfide linkages during preparation of the inclusion complex thus escaping Ellman's test. However, our efforts to obtain TC-CD and quantify thiol groups under inert conditions did not afford significantly higher values of thiol content.

A more plausible explanation lies in that within TC-CD inclusion complex, the thiol group (more specifically, thiolate anion, the active species in a thiol-disulfide exchange reaction) is positioned well within the cavity of CD thus being sterically hindered and un-available for Ellman's test. Supporting this hypothesis, CD-based inclusion complex with TC-ER successfully underwent a thiol-disulfide conversion with DTT thus, revealing accessibility of the disulfide linkage to further chemical modification (Scheme 5). The adduct of cyclodextrin with TC-ER was obtained via a protocol similar to that described above for pristine TC and afforded a thiocholesterol loading of 30 mg of TC-TNB per gram of compound, similar to commercial preparations of cholesterol-CD adducts. ^{1}H NMR characterization of this compound in deuterated water revealed a well pronounced peak at $\delta = 5.5$ characteristic of cholesterol, and two groups of peaks, from $\delta = 0.92$ to 1.69 and from $\delta = 7.62$ to 8.14, corresponding to the methyl groups and aromatic protons of TC-ER, respectively (Figure 3A). CD-based inclusion complex with TC-ER was fully water soluble and presented a UV signature in its aqueous solution matching that of Ellman's reagent. To verify that the adduct was indeed that between CD and TC-ER, not un-reacted ER or impurities and derivatives thereof, we further characterized the inclusion complex using HPLC and a water-acetonitrile gradient as a mobile phase (Figure 3B). This analysis revealed a UV-Vis signature



Scheme 5. Schematic illustration of thiol-disulfide exchange between cyclodextrin – based inclusion complexes of thiocholesterol (top) and Ellman's reagent – activated thiocholesterol (bottom) with Ellmans' reagent and DTT, respectively.

for [CD/TC-ER] matching that of Ellman's reagent and an elution time matching that of TC-ER (Scheme 3B). This result implies that in the presence of acetonitrile, TC-ER is removed from the inclusion complex and also demonstrates that inclusion complex indeed contains TC-ER thus, substantiating successful incorporation of the thiocholesterol derivative into the structure of cyclodextrin.

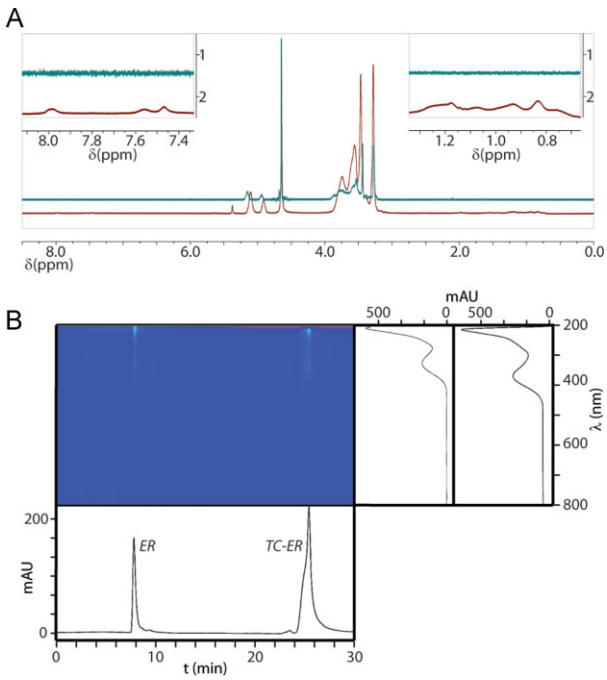


Figure 3. A) ^{1}H NMR characterization of an inclusion complex between M-β-CD and TC-ER in deuterated water: i) CD, ii) [CD/TC-ER]; B) overlaid HPLC elution traces for ER and [CD/TC-ER].

To illustrate the utility of developed water soluble thiocholesterol derivative for modification of biomolecules at physiological conditions, we used a readily available, natural thiol-containing tripeptide, GSH. This peptide is found in mammalian cells at concentration of 5×10^{-3} M and is typically used to probe degradation of disulfide-stabilized drug carrier vehicles and also to probe novel bioconjugation techniques relying on thiol chemistry.[27,41,44] The progress of reaction between GSH and CD-complexed TC-ER was monitored via UV-Vis spectroscopy monitoring absorbance of TNB chromophore, and using DTT to set the 100% scale mark. This reaction proved to be highly efficient and fast, achieving a near-complete conversion in excess GSH within minutes, Figure 4A. For modification of biomolecules, complete conversion of target thiols is the prime aim, and with GSH, near-full conversion of GSH into cholesterol containing disulfide was achieved with as little as twofold excess of [CD/TC-ER] over GSH. Taken together, results in Figure 4 present the newly synthesized water-soluble thiocholesterol as an effective tool for bioconjugation.

For PVP and PVA, aqueous solutions of amine-containing polymers were charged with [CD/TC-ER] and iminothiolane for an in situ conversion of amine terminal groups into thiols and subsequent conjugation reaction (Scheme 6). In optimizing reaction conditions we observed that solutions buffered to pH = 8 (e.g., carbonate buffer) favor spontaneous ring opening of the Traut's reagent and reaction of the products of this reaction with [CD/TC-ER]. This was overcome by using phosphate buffers with pH = 7.3. Lower concentration of reagents also facilitated formation of the desired polymer-containing product. Reaction mixtures were incubated for 14 h, upon which solutions were first

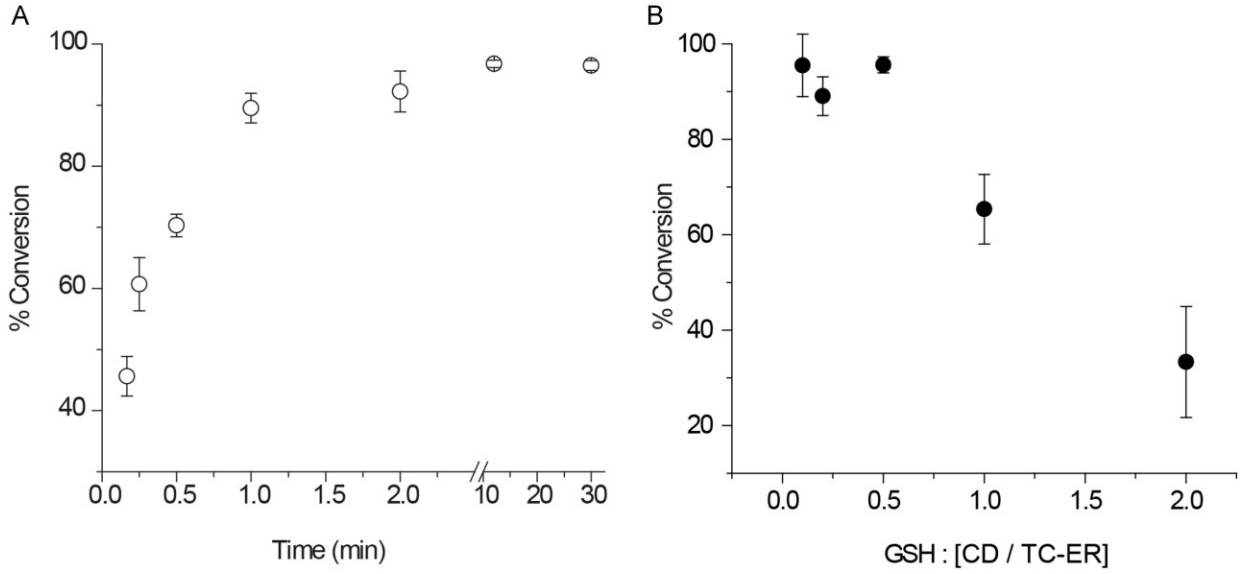
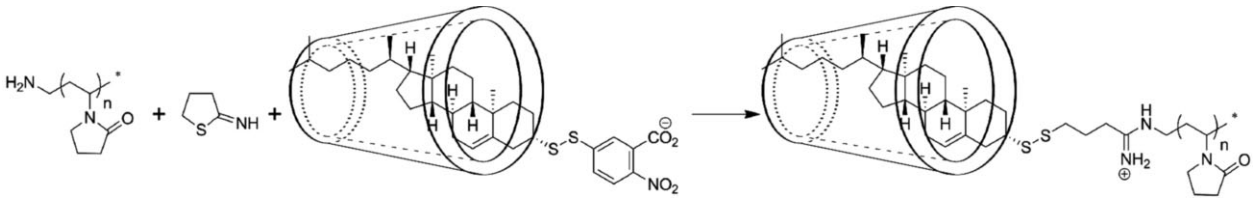


Figure 4. A) Kinetic profile of bioconjugation in aqueous solutions of [CD/TC-ER] (inclusion complex between methyl-β-cyclodextrin and Ellman's reagent activated thiocholesterol) and glutathione (5 g L^{-1} GSH, carbonate buffer pH = 8.3). B) Conversion of thiol groups within GSH into disulfide linkages with thiocholesterol in aqueous solutions of GSH and [CD/TC-ER] as a function of the ratio of reagents.

purified on a desalting column followed by additional dialysis for 48 h. Finally, the polymers were recovered via freeze-drying and analyzed for cholesterol content using NMR spectroscopy. Polymer loading with cholesterol reached almost 100% for PVP and ≈80% for PVA thus, illustrating successful modification of the polymers. We note that overlapping peaks make direct NMR quantification of remaining CD challenging, however, low affinity of CD to solutes forming inclusion complexes suggests that dialysis step removes an overall majority of CD from the polymer termini.

Finally, to illustrate possibilities in cholesterol modification of supramolecular materials, we tested [CD/TC-ER] for modification of physical hydrogels based on PVA. Despite decades of development, characterization, and biomedical applications, these hydrogels present scant opportunities in quantification of polymer within the hydrogel matrix and only limited opportunities for biochemical characterization. Over the past few years, we developed novel tools and methods for micro-fabrication using PVA hydrogels and their biomedical characterization and functionalization.[22,25,28] Specifically, we presented a novel method for conjugation of PVA with oligopeptide cargo wherein conjugation relies on thiol-disulfide chemistry and is conducted at physiological conditions.[28] Herein, we capitalize on these findings and use surface adhered, micro-structured physical PVA hydrogels for their functionalization with cholesterol. We hypothesized that running bioconjugation on pre-formed hydrogels offers opportunities in facile isolation of the product, specifically through simple withdrawing the hydrogel from the reaction medium. To illustrate this possibility, amine-containing PVA was used to assemble the surface-adhered hydrogel samples. Subsequently, hydrogels were immersed in a buffered solution of iminothiolane for 30 min to achieve an in situ thiolation of hydrogels. Specimen were removed from the reaction milieu and washed with copious amounts of fresh buffer.



Scheme 6. Schematic illustration of conjugation of thiocholesterol to PVP through a UV-Vis traceable chemistry and conducted in aqueous, physiological conditions. Reaction was performed using methyl-β-cyclodextrin based inclusion complex with Ellman's reagent activated thiocholesterol and a RAFT-derived PVP with a single terminal amine group which was converted in situ into a thiol using iminothiolane. For experimental details, see Experimental Section.

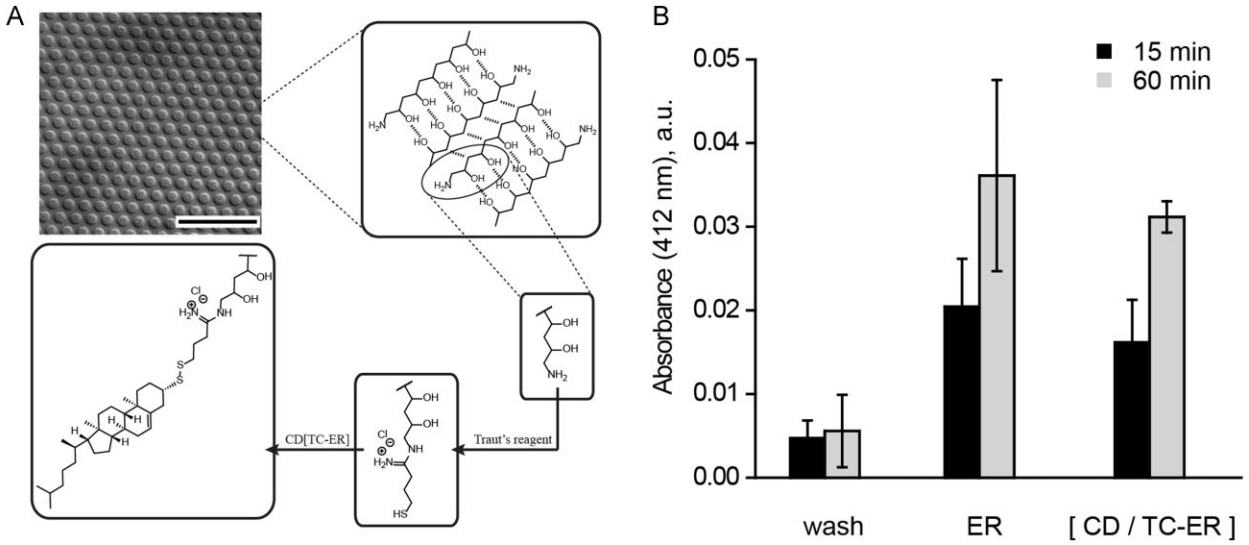


Figure 5. A) Differential interference contrast image of micro-structured, surface adhered physical hydrogel based on PVA (scale bar: 50 μm) and schematic illustration of chemical modification of PVA chains within the structure of hydrogels using water-based chemical transformations: thiolation using Traut's reagent and subsequent bioconjugation with thiocholesterol using a water-soluble preparation, [CD/TC-ER] (inclusion complex between methyl-β-cyclodextrin and Ellman's reagent activated thiocholesterol). B) UV-Vis data on the reaction between the terminal thiol groups on PVA chains comprising physical hydrogels with Ellman's reagent or [CD/TC-ER].

Subsequently, hydrogel samples were immersed in a solution of Ellman's reagent upon which supernatants attained a characteristic yellow color. This indicated a successful reaction of Ellman's reagent with the thiol groups immobilized within the hydrogel matrix, that is, terminal groups of PVA chains, thus, demonstrating successful thiolation of the hydrogel (Figure 5). Supernatants drawn from above the hydrogel samples immediately before this analysis revealed a significantly lower absorbance which verifies that Ellman's test indeed registered thiols groups as part of hydrogel biomaterials. When [CD/TC-ER] was introduced onto thiolated hydrogels instead of Ellman's reagent, resulting absorbance was similar to that registered with ER only indicating a highly efficient conversion of the thiol groups within hydrogel matrix into cholesterol-containing disulfides. We believe that a high degree of hydration of the hydrogels greatly facilitates exchange of solutes between the hydrogel and the solution bulk thus, contributing to the feasibility of the reaction. We strongly believe that this is the first described example of an in situ thiolation of PVA physical hydrogels and also the first report on cholesterol-functionalization of these biomaterials, both goals achieved under physiological conditions. We note that while no attempt was made in this work to quantify the removal of cyclodextrin and "unmasking" cholesterol, typical binding constants for these inclusion complexes are in the 10^{-1} to 10^{-3} L mol^{-1} range[45] and CD removal is likely to proceed spontaneously upon washing and/or storage of the hydrogel samples. We are now investigating utility of cholesterol-containing hydrogels for immobilization of liposomes as drug deposits for surface-mediated drug delivery[46] and for biosensing applications[47] as well as tools to achieve controllable adhesion of mammalian cells towards the use of PVA hydrogels as matrices in tissue engineering.

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