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Polymer-protein conjugation *via* a 'grafting to' approach – a comparative study of the performance of protein-reactive RAFT chain transfer agents†

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Efficient polymer-protein conjugation is a crucial step in the design of many therapeutic protein formulations including nanoscopic vaccine formulations, antibody-drug conjugates and to enhance the *in vivo* behaviour of proteins. Here we aimed at preparing well-defined polymers for conjugation to proteins by reversible addition–fragmentation chain transfer (RAFT) polymerization of both acrylates and methacrylamides with protein-reactive chain transfer agents (CTAs). These RAFT agents contain either a *N*-hydroxysuccinimide (NHS) or pentafluorophenyl (PFP) ester moiety that can be conjugated to lysine residues, and alternatively a maleimide (MAL) or pyridyl disulfide (PDS) moiety that can be conjugated to cysteine residues. Efficiency of the bioconjugation of these polymers to bovine and avian serum albumin was investigated as a function of stoichiometry, polymer molecular weight and the presence of reducing agents. A large molar excess of polymer was required to obtain an acceptable degree of protein conjugation. However, protein modification with *N*-succinimidyl-*S*-acetylthiopropionate (SATP) to introduce sulfhydryl groups onto primary amines, significantly increased conjugation efficiency with MAL- and PDS-containing polymers.

Introduction

Polymer-protein conjugation strategies have received increasing interest owing to the ability to engineer proteins with a wide variety of properties, by simply coupling protein-reactive polymers to certain amino acid residues.[1–5] For example the conjugation of linear poly(ethylene glycol) to proteins, commonly known as PEGylation, results in prolonged body-residence time and a reduction of protein immunogenicity by blocking the adhesion of opsonins present in blood serum. In this way the proteins are masked from phagocytic cells and opsonization is strongly reduced.[6–8] Besides using hydrophilic polymers for protein conjugation, also conjugating stimuli-responsive polymers is of interest.[9] For example, temperature-responsive polymers conjugated to proteins can self-assembly into nanoparticles above the lower critical solution temperature (LCST), due to their amphiphilic character.[10–12] This controlled and reversible aggregation of proteins could *e.g.* be exploited for vaccine delivery. Formulating protein antigens as nanoparticulate carriers has proven to be a promising strategy to modulate and increase the adaptive antigen-specific CD8+ T-cell responses.[13–15] In this regard, Stayton *et al.* described the assembly of micellar nanoparticles from amphiphilic diblock copolymers, composed of a pH-responsive ampholytic core-forming block and a corona block with thiol-reactive pyridyl disulfide groups to attach antigens or immunostimulatory adjuvants.[16] *Via* the interstitial flow and lymphatic capillaries, ultra-small nanoparticles and albumin-binding amphiphiles, are efficiently transported to the draining lymph nodes to target lymph node–resident dendritic cells.[17,18]

Controlled radical polymerization offers an excellent tool to synthesize polymers with well-defined composition, chain length, narrow dispersity and functional end-groups that can be used for protein conjugation.[19,20] This significantly increases versatility and reproducibility over classical free radical

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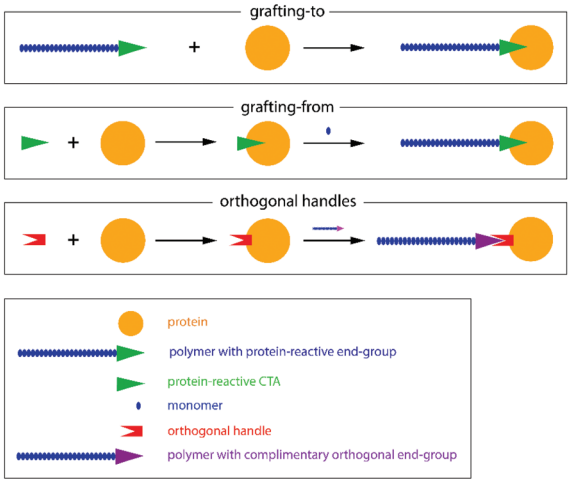
polymerization, which is important with respect to both the design of multifunctional polymeric architectures as well towards regulatory affairs. Reversible addition–fragmentation chain transfer (RAFT) polymerization in particular, has shown to be tolerant to many chemical groups and offers a straightforward route to synthesize polymers with a protein-reactive end-group *via* the use of a functional chain transfer agent (CTA).[21,22] Sumerlin *et al.* reported on the use of a *N*-hydroxysuccinimide containing RAFT agent to synthesize poly(*N*-isopropylacrylamide) (polyNIPAm) with a protein-reactive end-group that allowed conjugation to lysine residues.[23] Depending on the polymer to protein ratio, multisite attachment (*i.e.* multiple polymer chains grafted onto one protein chain) can be obtained. However, for some applications, single site attachment, *i.e.* one polymer chain per protein molecule, might be more advantageous. In this regard, Velonia and co-workers used a protected maleimide functionalized RAFT CTA that can react with less abundantly present free cysteine residues.[24] Alternatively, the Maynard group used a pyridyl disulfide group to introduce reversibility into the conjugate bonds.[25] In addition to these conventional protein-reactive groups, increasing synthetic effort is dedicated to develop newly emerging conjugation chemistries based on reactive moieties such as thiazolidine-2-thione,[26] pentafluorophenyl[27,28] and dibromomaleimide.[29] To our knowledge, no direct comparison of these different conjugation strategies has been reported to date.

Different approaches have been proposed to design polymer-protein conjugates (Scheme 1) including the 'grafting-to' method, where a pre-formed reactive polymer is conjugated to a protein; and the 'grafting-from' method, where the polymer chain is grown from a protein macroinitiator (or macroCTA).[21] Both strategies can be followed using protein-reactive RAFT agents.[30–32] The advantage of grafting-to is the use of pre-synthesized polymer, which allows thorough characterization of the polymer and avoids exposure of the protein to potentially denaturing polymerization conditions. An additional limitation of the grafting-from method is the possible sterical hindrance during polymerization, leading to a substantial amount of unreacted protein-bound CTA. The major advantage of the 'grafting-from' approach is that the prepared conjugates only need to be purified from low molecular weight compounds (*i.e.* unreacted monomer, initiator, *etc*...). This can easily be done by dialysis, unlike the removal of unreacted polymer, involved in 'grafting-to', which is often tedious and requires preparative gel filtration chromatography. An alternative approach to conventional conjugation chemistries is the use of so-called orthogonal handles, *e.g.* based on the copper(I) cyclo-addition between an azide and an alkyne[21] (*i.e.* the Huisgen-Sharpless 'click' reaction[33]). This involves a two-step modification strategy, where first the protein is modified with an orthogonal (*i.e.* a group that does not interfere with any of the amino acid residues) functional group that is then in a second step used to conjugate to the functional end-group of a polymer. The latter end-group is also of a kind which does not interfere with any natural amino acid residue. Different groups described the synthesis of an azide-functionalized RAFT agent that can couple to alkyne-modified biomolecules.[34,35] Alternatively, Maynard and co-workers modified a protein backbone with ketone moieties that allowed subsequent conjugation to an aminooxy end-functionalized polymer *via* oxime bond formation.[36] However, these methods require the introduction of reactive handles on the protein before polymer conjugation can be performed.

In this paper, we report a head-to-head comparison of the performance of various protein-reactive RAFT CTAs, to afford polymer-protein conjugation *via* a grafting-to approach. These protein-reactive RAFT CTAs contain either a *N*-hydroxysuccinimide (NHS) or pentafluorophenyl (PFP) ester moiety that can conjugate to lysine residues, and alternatively a (furan-protected) maleimide (FpMAL) or pyridyl disulfide (PDS) moiety for conjugation to cysteine residues. First we investigated the polymerization kinetics of these RAFT agents for the polymerization of a model hydrophilic acrylate monomer 2-hydroxyethylacrylate (HEA). The obtained polymers were then evaluated for their ability to conjugate to two types of serum albumin (*i.e.* avian and bovine). Next we evaluated the most promising conjugation chemistries for the synthesis of CTA's that allow RAFT polymerization of methacrylamides with biomedical relevance, namely 2-hydroxypropylmethacrylamide (HPMA) and its thermo-responsive dilactate derivate (HPMA-lac$_2$). These monomers are currently intensively studied for biomedical applications, in particular for anti-cancer drug formulations.[37–41]



Scheme 1 Schematic overview of the different RAFT-based strategies that are available for designing polymer-protein conjugates.

Materials and methods

Materials

Organic solvents dichloromethane (DCM, anhydrous and HPLC grade), toluene, methanol, chloroform, hexane, ethylacetate (EtOAc), dimethylacetamide (DMA, anhydrous), dimethylformamide (DMF, anhydrous) and chemicals *N*-hydroxy-

succinimide, pentafluorophenol, mercaptoethanol, furan, 2-(2-amino-ethoxy)-ethanol), bovine serum albumin, $MgSO_4$, $NaSO_4$, $NaHCO_3$, NaSCN, hydroxylamine-HCl, ethylenediaminetetraacetic acid (EDTA), ninhydrin reagent (2% solution) were obtained from Sigma Aldrich and used without purification. Azobisisobutyronitrile (AIBN, 98%, Aldrich) was recrystallized from MeOH (twice) and stored in a freezer. 2-Hydroxyethyl acrylate (HEA, 96%, Aldrich) was destabilized by passing the monomer over a column with inhibitor remover (Aldrich) prior to polymerization. The RAFT agent 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid (C*D*TPA) was purchased from Sigma Aldrich whilst 2-propanoic acid butyl trithiocarbonate (PABTC) and 4-cyano-4-[(ethylsulfanylthiocarbonyl)sulfanyl]pentanoic acid (C*E*TPA) were prepared according to established procedures.[42,43] Chemicals 2,2′-dipyridyl disulfide, 4-dimethylaminopyridine (DMAP), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and *N*,*N*′-diisopropylcarbodiimide (DIC) were purchased from TCI Europe. Maleic anhydride from Merck Germany was used as received. 2-Hydroxypropylmethacrylamide (HPMA) was obtained from Polysciences, whilst HPMA-dilactate was synthesized according to established procedures.[44] Ovalbumin (OVA) and *N*-succinimidyl-*S*-acetylthiopropionate (SATP) were purchased from Worthington and Thermo scientific respectively.

Instrumentation

Kinetic studies of the RAFT polymerizations were performed using a Chemspeed ASW2000 automated synthesizer equipped with 16 parallel reactors of 13 mL, a Huber Petite Fleur thermostat for heating/cooling, a Huber Ministat 125 for reflux and a Vacuubrand PC 3000 vacuum pump following a recently reported protocol.[45,46] Stock solutions of all components were prepared and bubbled with argon for at least 30 minutes before being introduced into the robot system and then kept under argon atmosphere. The reactors were degassed through ten vacuum-argon cycles and subsequently flushed with argon to ensure an inert atmosphere. The hood of the automated synthesizer was continuously flushed with nitrogen. Stock solutions were transferred to the reactors using the syringe of the automated synthesizer while the reactors were cooled to 10 °C, after which the reactors were heated to 70 °C to start the polymerizations. During the reactions samples were taken at preset time intervals for GC, SEC and NMR analysis. The polymerizations were stopped by cooling the reactors to 10 °C.

Gas chromatography was performed on a 7890A from Agilent Technologies with an Agilent J&W Advanced Capillary GC column (30 m, 0.320 mm, and 0.25 μm). Injections were performed with an Agilent Technologies 7693 auto sampler. Detection was done with a FID detector. The injector and detector temperatures were kept constant at 250 and 280 °C, respectively. The column was initially set at 50 °C, followed by two heating stages: from 50 °C to 100 °C with a rate of 20 °C min^{-1} and from 100 °C to 300 °C with a rate of 50 °C min^{-1}, and then held at this temperature for 0.5 minutes. Conversion of HEA was determined based on the integration of the monomer peak using DMA as internal standard.

Size exclusion chromatography was carried out on an Agilent 1260 system, equipped with an 1260 ISO-pump, an 1260 diode array detector (DAD) and an 1260 refractive index detector (RID). Measurement were done in DMA containing 50 mM LiCl at 50 °C and with a flow rate of 0.593 mL min^{-1}. The two PL gel 5 μm mixed-D columns were calibrated with polymethylmethacrylate (PMMA) standards (Polymer standards service) in a molecular weight (Mn) range of 1980 Da to 372 000 Da.

^{1}H-NMR an ^{19}F-NMR spectra were recorded on a Bruker 300 MHz FT-NMR spectrometer using $CDCl_3$ as solvent.

Column chromatography was performed on a Grace Reveleris X2 flash chromatography system using silica Reveleris flash cartridges.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 4–20% polyacrylamide gradient gel, using the Mini-PROTEAN Tetra Cell from Bio-Rad.

Methods

Synthesis of *N*-hydroxysuccinimide containing CTA. *N*-Hydroxysuccinimide (NHS) PABTC was synthesized as reported by Sumerlin *et al.*[23] PABTC (1.430 g, 6 mmol) and NHS (690.54 mg, 6 mmol) were introduced in a round-bottom flask and dissolved in anhydrous dichloromethane (DCM, 50 mL). The reaction mixture was cooled to 0 °C in an ice bath and a solution of DIC (757 mg, 6 mmol) in anhydrous DCM (10 mL) was added drop-wise while vigorously stirring. The reaction mixture was stirred in an ice bath for 2 h and subsequently at room temperature overnight. The resulting solution was filtered (whatman grade 2), and the solvent was evaporated under vacuum. The crude product was purified by column chromatography on silica gel with a mobile phase of EtOAC–hexane 1/1 (v/v). The first fraction was collected and the solvent was removed under reduced pressure to obtain the product as a yellow oil (986 mg, yield 49%).

^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 5.13 (q, J = 7.5, 1 H, –C***H***CH_3), 3.36 (t, J = 7.4 Hz, 2 H, –SC***H*****$_2$**–), 2.82 (s, 4 H, –COC***H*****$_2$**C***H*****$_2$**CO–), 1.73 (d, J = 7.5 Hz, 3 H, –CHC***H*****$_3$**), 1.68 (tt, J = 7.4 Hz, 2 H, –SCH_2C***H*****$_2$**–), 1.42 (app. sext, J = 7.4 Hz, –C***H*****$_2$**CH_3), 0.92 (t, J = 7.4 Hz, 3H, –CH_2C***H*****$_3$**).

Synthesis of pentafluorophenol containing CTA. Pentafluorophenol (PFP) PABTC was synthesized as described by Stenzel *et al.*[27] PABTC (1.192 g, 5 mmol), PFP (1.012 g, 5.5 mmol) and DMAP (61 mg, 0.5 mmol) were introduced into a round-bottom flask and dissolved in anhydrous dichloromethane (DCM, 50 mL). The reaction mixture was cooled to 0 °C in an ice bath and a solution of DIC (694 mg, 5.5 mmol) in DCM (10 mL) was added drop-wise while vigorously stirring. The reaction mixture was stirred in an ice bath for 2 h and subsequently at room temperature overnight. The resulting solution was filtered (whatman grade 2), and the solvent was evaporated under vacuum. The crude product was purified by column chromatography on silica gel using chloroform as eluent. The first fraction was collected and the solvent was removed under reduced pressure to obtain the product as an orange/red oil (1.490 g, yield 67%).

^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 5.10 (q, J = 7.4, 1H, –C***H***CH_3), 3.38 (t, J = 7.4 Hz, 2 H, –SC***H***$_2$–), 1.76 (d, J = 7.4 Hz, 3 H, –CHC***H***$_3$), 1.69 (tt, J = 7.4 Hz, 2 H, –SCH_2C***H***$_2$–), 1.44 (app. sext, J = 7.4 Hz, –C***H***$_2CH_3$), 0.93 (t, J = 7.4 Hz, 3H, –CH_2C***H***$_3$). ^{19}F-NMR (300 MHz, $CDCl_3$) δ (ppm): −152.27 (d, 2 F), −157.40 (t, 1 F), −162.07 (t, 2F).

The same procedure was used to functionalize CDTPA (yield 67%) and CETPA (yield 64%) with PFP. CDTPA ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 3.33 (t, J = 7.4 Hz, –SC***H***$_2$–), 3.03–2.96 (band, 2 H, –CC***H***$_2$–), 2.73–2.45 (band, 2 H, –COC***H***$_2$–), 1.92 (s, 3 H, –CC***H***$_3$), 1.75–1.64 (band, 2 H, –SCH_2C***H***$_2$–), 1.44–1.21 (band 18 H, –C_9***H***$_{18}CH_3$) 0.87 (t, J = 6.4 Hz, 3 H, –CH_2C***H***$_3$). CETPA ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 3.36 (q, J = 7.4 Hz, 2 H, –C***H***$_2CH_3$), 3.03–2.97 (band, 2 H, –CC***H***$_2$–), 3.75–2.45 (band, 2 H, –COC***H***$_2$–), 1.92 (s, 3 H, –CC***H***$_3$), 1.36 (t, J = 7.4 Hz, 3 H, –CH_2C***H***$_3$). CDTPA ^{19}F-NMR (300 MHz, $CDCl_3$) δ (ppm): −152.49 (d, 2 F), −157.29 (t, 1 F), −161.86 (t, 2F). CETPA ^{19}F-NMR was identical.

Synthesis of the pyridyl disulfide containing CTA

Synthesis of hydroxyethyl pyridyl disulfide. Hydroxyethyl pyridyl disulfide (HEPDS) was synthesized as described by Thayumanavan *et al.*[47] 2,2′-dipyridyl disulfide (25 g, 113 mmol) was dissolved in 120 mL of methanol containing 1.7 mL of glacial acetic acid. A solution of mercaptoethanol (4.434 g, 57 mmol) in methanol (25 mL) was added drop-wise at room temperature while vigorously stirring. The reaction was continued at room temperature for additional 3 hours. The resulting solution was filtered (whatman grade 2), and the solvent was evaporated under vacuum. The crude product was purified by column chromatography on silica gel with a gradient of EtOAC–hexane 30/70–50/50 (v/v). The second fraction was collected and the solvent was removed under reduced pressure to obtain the product as a colorless oil (7.5 g, yield 70%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 8.45 (m, 1H, ***H***$_{ortho\text{-}N}$), 7.55 (m, 1H, ***H***$_{meta\text{-}N}$), 7.37 (m, 1H, ***H***$_{para\text{-}N}$), 7.10 (ddd, J = 1.1, 5.2 and 7.4 Hz, 1H, ***H***$_{ortho\text{-}disulfide}$), 5.98–4.65 (m, 1H, –O***H***), 3.76 (t, J = 5.2 Hz, 2H, –C***H***$_2$OH), 2.91 (t, J = 5.2, 2H, –C***H***$_2CH_2$OH).

Synthesis of pyridyl disulfide containing CTA. HEPDS was used to synthesize the pyridyl disulfide (PDS) RAFT agent as reported by Maynard *et al.*[25] PABTC (2.86 g, 12 mmol) and HEPDS (1.87 g, 10 mmol) were introduced into a round-bottom flask and dissolved in anhydrous dichloromethane (DCM, 50 mL). The reaction mixture was cooled to 0 °C in an ice bath while vigorously stirring. EDC (2.12 mL, 12 mmol) and DMAP (122 mg, 1 mmol) were then added in one portion. The reaction was stirred in an ice bath for 2 h and subsequently at room temperature overnight. The resulting solution was filtered (whatman grade 2), and the solvent was evaporated under vacuum. The crude product was purified by column chromatography on silica gel with a mobile phase of EtOAC–hexane 1/2 (v/v). The second fraction was collected and the solvent was removed under reduced pressure to obtain the product as a yellow oil (2.287 g, yield 56%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 8.46 (m, 1 H, ***H***$_{ortho\text{-}N}$), 7.70–7.60 (band, 2 H, ***H***$_{meta\text{-}N}$ and ***H***$_{para\text{-}N}$), 7.09 (ddd, J = 1.7, 4.9 and 6.6 Hz, 1 H, ***H***$_{ortho\text{-}disulfide}$), 4.80 (q, J = 7.4 Hz, 1 H, –C***H***CH_3), 4.38 (t, J = 6.4 Hz, 2 H, –OC***H***$_2$–), 3.34 (t, J = 7.3 Hz, 2 H, –$SCH_2C_3H_7$), 3.03 (t, J = 6.4 Hz, 2 H, –C***H***$_2CH_2$O–), 1.66 (app. quint, J = 7.3 Hz, 2 H, –SCH_2C***H***$_2$–), 1.57 (d, J = 7.4 Hz, 3 H, –CHC***H***$_3$), 1.41 (app. sext, J = 7.3 Hz, –C***H***$_2CH_3$–), 0.91 (t, J = 7.3 Hz, 3H, –CH_2C***H***$_3$).

The same procedure was used to functionalize ECT with PDS (yield 46%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 8.50–8.45 (m, 1 H, ***H***$_{ortho\text{-}N}$), 7.68–7.56 (band, 2 H, ***H***$_{meta\text{-}N}$ and ***H***$_{para\text{-}N}$), 7.14–7.07 (m, 1 H, ***H***$_{ortho\text{-}disulfide}$), 4.37 (t, J = 6.4 Hz, 2 H, –OC***H***$_2$–), 3.34 (q, J = 7.4 Hz, 2 H, –C***H***$_2CH_3$), 3.04 (t, J = 6.4 Hz, 2 H, –OCH_2C***H***$_2$–), 2.65–2.45 (band, 2 H, –$COCH_2$C***H***$_2$–), 2.41–2.21 (band, 2 H, –COC***H***$_2$–), 1.86 (s, 3 H, –CC***H***$_3$), 1.35 (t, J = 7.4 Hz, 3 H, –CH_2C***H***$_3$).

Synthesis of the (furan protected) maleimide containing CTA

*Synthesis of 4,10-dioxatricyclo[5.2.1.0(2,6)]dec-8-ene-3,5-dione [**1**].* 4,10-Dioxatricyclo[5.2.1.0(2,6)]dec-8-ene-3,5-dione was synthesized as reported by Velonia *et al.*[24] Maleic anhydride (30.0 g, 306 mmol) was suspended in 150 mL of toluene and the mixture was warmed to 80 °C in an oil bath. Furan (33.4 mL, 459 mmol) was added *via* syringe while vigorously stirring and the resulting turbid solution was stirred for 6 h. The mixture was then cooled to ambient temperature and the stirring was stopped. After 1 h, the formed white crystals were collected by filtration and washed with 30 mL of hexane to obtain the product as small white crystals (44 g, yield 87%, melting point 114–115 °C). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 6.57 (t, J = 0.9 Hz, 2H, –C***H***$_{vinyl}$–), 5.45 (t, J = 0.9 Hz, 2H, –C***H***O–), 3.17 (s, 2H, –C***H***–).

*Synthesis of 4-[2-(2-hydroxy-ethoxy)-ethyl]-10-oxa-4-aza-tricyclo-[5.2.1.0(2,6)]dec-8-ene-3,5-dione [**2**].* The anhydride [**1**] (16.00 g, 96.3 mmol) was suspended in methanol (250 mL) and the mixture was cooled in an ice bath. A solution of 2-(2-amino-ethoxy)-ethanol (9.64 mL, 96.3 mmol) in 20 mL of methanol was subsequently added dropwise (15 min) while vigorously stirring. Next, the reaction was stirred at ambient temperature for 30 min and finally refluxed for 4 h. After cooling the mixture to ambient temperature, the solvent was removed under reduced pressure, and the residue was dissolved in 2 × 150 mL of DCM (in two portions) and washed with 2 × 100 mL of water separately. The organic layers were combined, dried over $MgSO_4$ and filtered. Removal of the solvent under reduced pressure yielded the product as a highly viscous yellow residue (6.88 g, yield 28%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 6.49 (t, J = 0.9 Hz, 2H, –C***H***$_{vinyl}$–), 5.27 (t, J = 0.9 Hz, 2H, –C***H***O–), 3.71–3.67 (band, 2 H, –C***H***$_2$OH), 3.65–3.60 (band, 4 H, –NC***H***$_2$C***H***$_2$–), 3.53–3.50 (band, 2 H, –C***H***$_2CH_2$OH), 2.85 (s, 2H, –C***H***–), 2.57–2.22 (m, 1 H, –O***H***).

*Synthesis of 2-butylsulfanylthiocarbonylsulfanyl-propionic acid 2-[2-(3,5-dioxo-10-oxa-4-aza-tricyclo[5.2.1.0(2,6)]dec-8-en-4-yl)-ethoxy]-ethyl ester [**3**].* A solution of compound [**2**] (2.5 g, 9.87 mmol) and PABTC (2.82 g, 11.8 mmol) in dichloromethane (20 mL) was cooled in an ice bath while vigorously stirring. Solutions of EDC (1.84 g, 11.8 mmol) and DMAP (0.15 g, 1.18 mmol) in DCM were then added drop-wise *via* syringe over 10 minutes. The reaction was stirred in an ice

bath for 2 h and subsequently at room temperature overnight. The product was diluted with dichloromethane (100 mL), washed with distilled water (3 × 100 mL), dried with sodium sulfate (Na_2SO_4), filtered and finally dried under reduced pressure to get product [**3**]. Finally the product was purified by column chromatography on silica gel using a mobile phase of EtOAC–hexane 1/1 (v/v). The third fraction was collected and the solvent was removed under reduced pressure to obtain the product as a yellow oil (2.24 g, yield 48%).

^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 6.50 (t, J = 0.8 Hz, 2 H, –C$\boldsymbol{H}_{vinyl}$–), 5.25 (t, J = 0.8 Hz, 2H, –C$\boldsymbol{H}$O–), 4.82 (q, J = 7.3, 1H, –C$\boldsymbol{H}$$CH_3$), 4.23 (m, 2 H, –COOC$\boldsymbol{H}_2$–), 3.70–3.59 (band, 6 H, –NC$\boldsymbol{H}_2$C$\boldsymbol{H}_2$OC$\boldsymbol{H}_2$–), 3.35 (m, 2 H, –SC$\boldsymbol{H}_2$–), 2.86 (s, 2H, –C$\boldsymbol{H}$CON–), 1.67 (app. quint, J = 7.4, 2 H, –SCH_2C$\boldsymbol{H}_2$–), 1.59 (d, J = 7.3 Hz, 3 H, –CHC$\boldsymbol{H}_3$), 1.42 (app. sext, J = 7.4 Hz, –C$\boldsymbol{H}_2$$CH_3$–), 0.92 (t, J = 7.3 Hz, 3H, –CH_2C$\boldsymbol{H}_3$).

RAFT polymerization of HEA with the protein-reactive CTA's

Kinetic study in Chemspeed robot. The functionalized PABTC CTAs were used for the RAFT polymerization of HEA in DMF (2.5 M) at 70 °C, using AIBN as initiator at a HEA : CTA : AIBN ratio of 100 : 1 : 0.2 or 100 : 1 : 0.1, respectively. Samples were taken at various time points (T0 = 0 min, T1 = 10 min, T2 = 20 min, T3 = 30 min, T4 = 40 min, T5 = 50 min, T6 = 60 min, T7 = 90 min, T8 = 120 min, T9 = 150 min, T10 = 180 min) and were evaluated *via* GC to follow HEA conversion and SEC to follow the number average molecular weight (M_n) and dispersity ($Đ$) of the polymers in time.

Synthesis of functional poly(2-hydroxyethylacrylate) for protein conjugation. The HEA : CTA : AIBN ratio was kept at 100 : 1 : 0.1 and polymerizations were stopped after 30 min (FpMAL CTA), 90 min (PFP CTA) and 120 min (NHS and PDS CTA). An example RAFT polymerization was as follows. HEA (10 mmol, 1.149 mL), CTA (0.1 mmol) and AIBN (0.01 mmol, 1.642 mg) were transferred into a schlenktube and dissolved in anhydrous DMF (2.9 mL, 2.5 M). After bubbling with nitrogen for 30 min., the solution was heated at 70 °C in an oil bath for the predetermined time before being quenched by cooling it in an ice water bath and exposing the polymerization solution to air. The reaction solutions were diluted with DCM in a 1 : 1 ratio, and the polymeric product was precipitated into hexane and dried under vacuum to yield protein-reactive polymers.

Deprotection of the furan-protected maleimide group. Deprotection was accomplished by a retro Diels–Alder reaction.[24] After precipitation, the polymer was dissolved in dioxane (1 g/25 mL) and the solution was heated to 110 °C in an oil bath. Next, dioxane was removed under reduced pressure to give the final polymer. To investigate the effect of cleavage of the trithiocarbonate end group, the latter was cleaved into a thiol by aminolysis. After 30 minutes of polymerization, the reaction mixture was cooled to room temperature and an excess of propylamine in molar ratio of 1 : 30 CTA : Propylamine was added to the schlenktube. The solution was stirred for 2 hours at room temperature and the formed product was precipitated 3 times in hexane. Next, the same deprotection procedure was performed as described above.

RAFT polymerization of HPMA with the protein-reactive CTA's

Kinetic study in Chemspeed robot. The functionalized PFP CDTPA and PFP CETPA CTA's were used for the RAFT polymerization of HPMA in DMAc (2 M) at 70 °C, using AIBN as initiator at a HPMA : CTA : AIBN ratio of 100 : 1 : 0.2 or 200 : 1 : 0.2, respectively. Stock solution of AIBN, CTA and monomer were bubbled with nitrogen for 30 min. and the polymerization reactions were performed in the reactors of the Chemspeed robot under argon atmosphere. Samples were taken at various time points (T0 = 0 min, T1 = 30 min, T2 = 60 min, T3 = 90 min, T4 = 120 min, T5 = 180 min, T6 = 240 min, T7 = 300 min, T8 = 360 min, T9 = 480 min, T10 = 600 min) and were evaluated *via* ^{1}H-NMR spectroscopy to follow monomer conversion and SEC to follow the number average molecular weight (M_n) and dispersity ($Đ$) of the polymers in time. The conversion of HPMA was determined according to literature by comparing the ^{1}H-NMR integration areas of resonances from the vinyl protons of HPMA at 5.30 ppm and the methine protons of HPMA at 3.65 ppm of the crude reaction mixture.[48]

Synthesis of functional poly(N-hydroxypropylmethacrylamide) for protein conjugation. An example RAFT polymerization with PDS CETPA CTA was as follows. HPMA (6.98 mmol, 1 g), CTA (0.07 mmol for DP100; 0.035 mmol for DP200) and AIBN (0.014 mmol for DP100; 0.007 mmol for DP200) were transferred into a schlenktube and dissolved in anhydrous DMAc (3.5 mL, 2 M). After bubbling with nitrogen for 30 min., the solution was heated at 70 °C in an oil bath for 10 h. The polymers were isolated by precipitation in diethyl ether and dried under vacuum. The same conditions were used to polymerize HPMA-lac_2.

Conjugation of protein-reactive polymers to OVA and BSA

Protein conjugation to OVA and BSA. An example conjugation procedure is as follows. Stock solutions of protein (1.16×10^{-4} M, 5 mg OVA or 7.7 mg BSA mL^{-1}) and polymer (10 mg mL^{-1}) were prepared in a bicarbonate buffer (0.1 M, pH 8.2). The stock solutions were combined to obtain a molar ratio of protein : polymer 1 : 10 or 1 : 20. Subsequently, the reaction mixture was diluted with buffer solution to obtain a protein concentration of 9.30×10^{-6} M. For conjugation reactions with SATP-modified protein, a deacetylation solution (0.5 M hydroxylamine-HCl/25 mM EDTA in PBS) was added to the reaction in a 300-fold molar excess of hydroxylamine to protein. For conjugation reactions with polyHPMA-lac_2, a 1 M NaSCN solution was used to enhance polymer solubility by "salting in". Next, the mixture was incubated overnight at room temperature (or on ice for HPMA-lac2) with continuous stirring. Conjugation efficiency was evaluated by native SDS-PAGE. β-Mercaptoethanol was used to check reversibility of the PDS- and MAL-conjugates. Quantification of protein conjugation was done by automated integration of optical density by ImageJ software and calculating the ratio of bound protein to total protein content per lane (Fig. S1†).

SATP-modification of OVA and BSA. Prior to conjugation, part of the lysines of OVA and BSA was modified with *N*-Succi-

nimidyl *S*-Acetylthiopropionate (SATP) (Scheme S1†). SATP (20 mg mL^{-1} in anhydrous DMSO) was added to a stock solution of OVA and BSA (120 μM in PBS) in a molar ratio of protein : SATP 1 : 20. The mixture was incubated at room temperature for 45 min. and unreacted SATP was removed using a disposable PD10 desalting column (Sigma). After freeze-drying, the degree of modification was calculated by determination of the moles of free amines per mol of protein before and after SATP modification using the ninhydrin assay.[49]

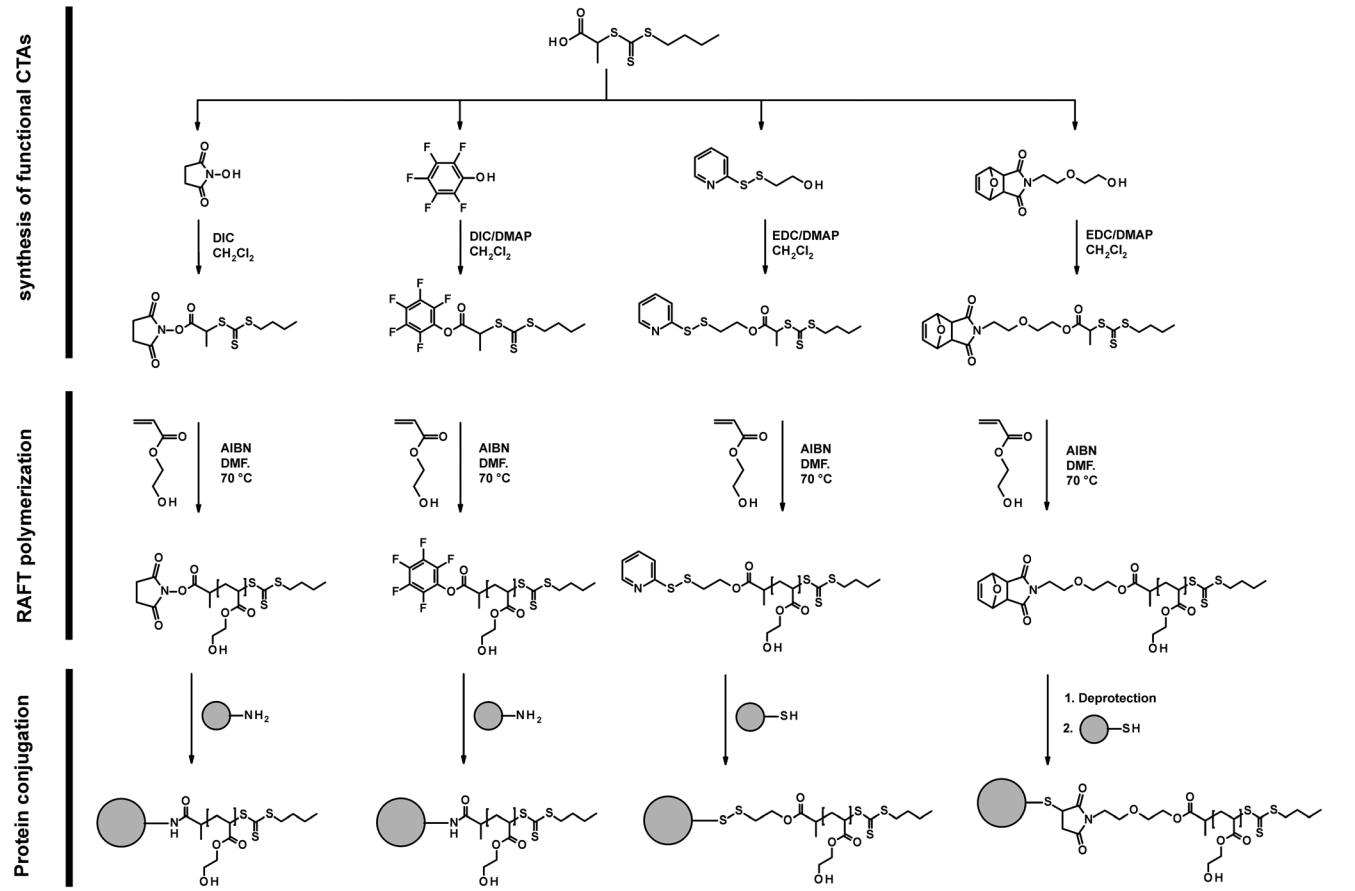
Results and discussion

Synthesis of protein-reactive CTAs for acrylate RAFT polymers

Four different trithiocarbonate-based CTAs for acrylate/acrylamide RAFT polymerization bearing a protein-reactive functional group at the R-position were synthesized. This was done by carbodiimide-mediated esterification of the carboxylic acid group of 2-(*n*-butyltrithiocarbonylthio)propionic acid (PABTC) with either *N*-hydroxysuccinimide (NHS), pentafluorophenol (PFP), hydroxethylpyridyldisulfide (PDS) or 4-[2-(2-Hydroxy-ethoxy)-ethyl]-10-oxa-4-aza-tricyclo[5.2.1.0(2,6)]dec-8-ene-3,5-dione. The latter contains a furan-protected maleimide (FpMAL) that requires deprotection, revealing the maleimide, prior to protein-conjugation. Note that the furan protection group is required to avoid side-reactions by radical addition to the maleimide during RAFT polymerization. Scheme 2 summarizes the synthesis of these different CTAs, and illustrates how they conjugate to either lysine or cysteine residues to afford protein conjugation. For the sake of clarity the respective CTAs will be further denoted as NHS-PABTC, PFP-PABTC, PDS-PABTC and FpMAL-PABTC (Fp: furan-protected). Further on, the polymer with a deprotected maleimide group will be referred to as MAL.

RAFT polymerization of HEA with protein-reactive CTAs

The respective CTAs with a protein-reactive functional group were subsequently used for RAFT polymerization of 2-hydroxyethylacrylate (HEA) (Scheme 2) with a targeted degree of polymerization (DP) of 100. Note that this DP was chosen arbitrary, and in future work we are aiming at investigating the influence of polymer chain length on the conjugation efficiency of the respective functional RAFT polymers. Polymerization kinetics were studied using an automated synthesis robot that allows to run multiple polymerization reactions,



Scheme 2 Synthesis of the different functional PABTC CTAs used in this work for RAFT polymerization of 2-hydroxyethylacrylate and subsequent protein conjugation. From left to right: *N*-hydroxysuccinimide (NHS) and pentafluorophenyl (PFP) esters that can conjugate to lysine residues, and alternatively a pyridyl disulfide (PDS) and maleimide (MAL) for conjugation to cysteine residues.

including sampling, in parallel. This set-up minimizes the experimental error and batch-to-batch variation. Polymerizations were run in duplicate at 70 °C and with two different AIBN to CTA (*i.e.* 0.1 and 0.2) ratios. Although it is well-documented that polymerization of acrylate monomers at 70 °C can cause side reactions (*e.g.* backbiting),[50] we have chosen work conditions that have been reported in literature to provide good control over the polymerization.[51,52] This will also be confirmed by our kinetic data, *vide infra*. Samples were analyzed by gas chromatography (GC) for monomer conversion and by size exclusion chromatography (SEC) for their molecular weight and dispersity. The kinetic data in Fig. 1 depict that NHS-, PFP- and PDS-PABTC provide, for both AIBN/CTA ratios, good control over the polymerization as indicated by the linear pseudo-first order kinetic plots and the linear growth of molecular weight with monomer conversions. A double molar mass shoulder in the SEC elugrams was only observed above 90% conversion, indicative of termination by chain coupling as commonly observed for radical polymerization at high monomer conversion. As expected, the RAFT polymerization kinetics are, within experimental error, independent of the CTA as the initiating fragments are very similar. Furthermore, using more AIBN results in faster polymerization due to the higher concentration of radicals. However, it should be noted that in case of FpMAL-PABTC, side-reactions were observed above 50% conversion, as witnessed by the emergence of a multimodal distribution in the SEC elugrams and an increase in dispersity. This is probably due to *in situ* deprotection of the maleimide at the polymerization temperature of 70 °C and its subsequent incorporation into the polymer chains by radical addition. This issue could not be fully solved by using V70 initiator (10 hour half-life at 30 °C) and performing the polymerization reaction at 50 °C (data not shown).

Next, a larger amount of polyHEA was synthesized with the different RAFT CTAs and, based on the kinetic data, conversion was stopped at 90% when using NHS-PABTC, PFP-PABTC and PDS-PABTC as chain transfer agent and 50% when using FpMAL-PABTC to obtain well-defined polymers for further polymer-protein conjugation studies. The obtained polymers were purified by threefold precipitation and drying under high vacuum, followed by storage at −20 °C under nitrogen prior to further use. The properties of the polymers are listed in Table 1. All polymers had narrow molecular weight distributions (PDI < 1.2), indicative of a well-controlled RAFT polymerization process. Note that the molecular weights (M_n) determined by SEC are largely overestimated, due to differ-

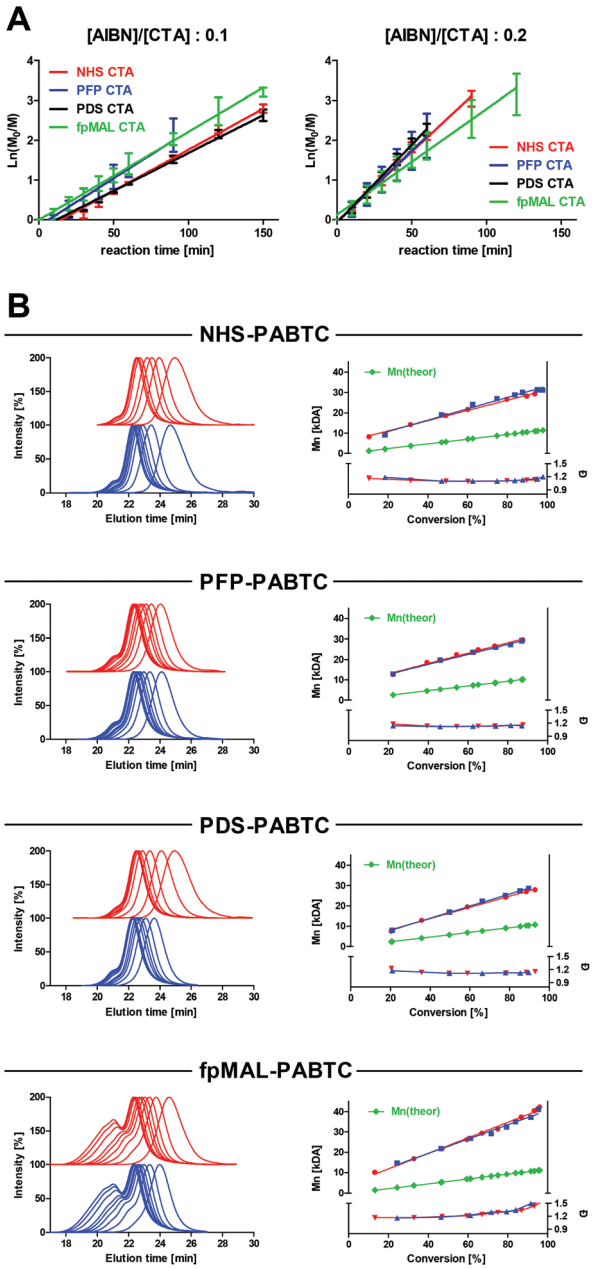


Fig. 1 RAFT polymerization of HEA, using 4 different protein-reactive CTAs in DMF at 70 °C with a HEA : CTA : AIBN molar ratio of 100 : 1 : 0.1 (left graph in **A**, red curves in **B**) or 100 : 1 : 0.2 (right graph in **A**, blue curves in **B**) (**A**) Kinetic curve *versus* polymerization time; (**B**) SEC traces at different timepoints (left), and molecular weight (M_n) and PDI ($Đ$) of the polymers *versus* monomer conversion (right). The green curve corresponds to the theoretical molecular weight (M_n(theor)) based on monomer conversion.

Table 1 Protein-reactive polyHEA polymers, synthesized in DMF at 70 °C with AIBN as an initiator and functionalized PABTC as a RAFT CTA

CTA	[HEA]/[CTA]	$t_{reaction}$	DP^{GC}	M_n^{GC}	M_n^{SEC}	$Đ$
NHS-PABTC	100	120 min	94	10.9 kDa	32 kDa	1.18
PFP-PABTC	100	90 min	94	10.9 Da	29 kDa	1.16
PDS-PABTC	100	120 min	93	10.8 kDa	28 kDa	1.15
FpMAL-PABTC	100	30 min	56	6.5 kDa	21 kDa	1.20
MAL-PABTC	—	—	—	—	25 kDa	1.20

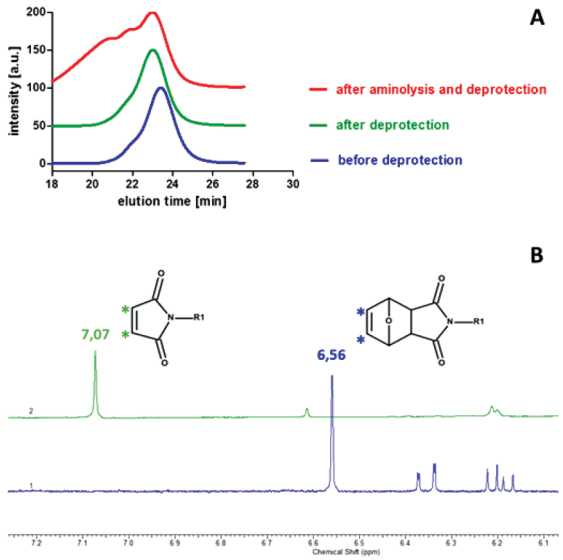


Fig. 2 Deprotection of fpMAL-polyHEA. **(A)** SEC elugram of polyHEA synthesized using fpMAL-PABTC as chain transfer agent before (blue curve) and after deprotection (green curve) of the maleimide end-group. As control, the trithiocarbonate end-group was cleaved into a thiol prior to the deprotection reaction, leading to a multimodal distribution (red curve). **(B)** ^{1}H-NMR (500 MHz, DMSO) of fpMAL-polyHEA (blue curve) and after deprotection (green curve) where the peak of the furan vinyl protons (6.56 ppm) diminishes and the maleimide vinyl protons appear (7.07 ppm).

ences in hydrodynamic volume with respect to the PMMA standards.

In case of polyHEA synthesized using Fp-MAL-PABTC as chain transfer agent, the maleimide group needed to be deprotected prior to protein-conjugation. This was done by refluxing the purified polymer in dioxane at 110 °C. Note that in literature this reaction is mostly reported in toluene,[24] however this was not possible due to the insolubility of polyHEA in this solvent. SEC analysis (Fig. 2A) shows that the polymer could be recovered with only slight alteration in the molecular weight (possibly due to a change in end-group polarity) and dispersity, while ^{1}H-NMR analysis revealed the disappearance of the signal from the furan moiety (Fig. 2B). To investigate the effect of cleavage of the trithiocarbonate end-group, we have converted this group into a thiol by aminolysis (*i.e.* addition of propylamine), followed by threefold precipitation and high vacuum to remove the propylamine. When subsequently the deprotection of the maleimide was performed, a multimodal elugram was observed by SEC analysis (Fig. 2A). This suggests that under the conditions used for deprotection of the maleimide end-group, the trithiocarbonate moiety at the other polymer chain end most likely remains intact, which is essential to obtained defined MAL-polyHEA.

Protein conjugation using functional polyHEA

In this part of work we aimed at evaluating the performance of the respective functional polymeric end-groups with respect to protein conjugation. For this purpose ovalbumin (OVA) and bovine serum albumin (BSA) were chosen as model proteins. The reason for choosing OVA is due to the availability of a large number of *in vitro* and *in vivo* immuno-biological assays for OVA-based formulations.[13,53,54] This makes OVA a highly relevant model vaccine antigen for the future research in elucidating the adjuvant effect of (stimuli-responsive) polymer conjugation to OVA. BSA was used as reference protein, as it has been extensively reported in literature for the evaluation of polymer-protein conjugation.[30,32,55] All conjugation reactions were performed in a 0.1 M sodium bicarbonate buffer of pH 8.2. This buffer has been reported by various groups as being an optimal conjugation buffer for the respective functional groups.[23,25,56] Here we incubated OVA and BSA in aqueous medium with the different functional polyHEA's in a protein-to-polymer molar ratio of 1 : 10 or 1 : 20, respectively. After overnight reaction, the conjugates were analyzed by SDS-PAGE and after staining, the gels were imaged and processed by ImageJ software. In the Materials and Methods section, the quantitative analysis of the SDS-PAGE data by ImageJ is explained into detail. Note that to preserve the disulfide bonds that are formed between the PDS-polyHEA and the proteins, SDS-PAGE was run under non-reductive conditions (in absence of β-mercaptoethanol).

For OVA, conjugation efficacy was relatively low, especially when using activated ester functionalized polymers. Of these two activated ester containing polymers (*i.e.* bearing NHS, respectively PFP as end-group), the PFP-functional polymers appeared to perform the best. This can likely be ascribed to the higher hydrolytic stability of PFP-esters *versus* NHS-esters.[57,58] This would provide more opportunity for the PFP-esters to react with lysine moieties, whereas the NHS-esters will be more prone to rapid hydrolysis in aqueous medium into carboxylic acid moieties. The best performing functional group for OVA appeared to be the PDS which afforded over 50% of the protein to become conjugated (Fig. 3). For BSA, the overall conjugation efficacies were higher and PFP was the best performing functional group, allowing over 80% of the protein

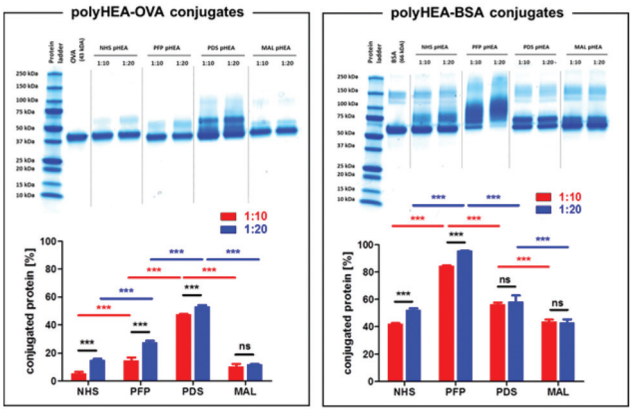


Fig. 3 SDS-PAGE results from the conjugation of OVA (43 kDa) and BSA (66 kDa) with protein-reactive polyHEA polymers (NHS, PFP, PDS and MAL) in a molar ratio of protein : polymer 1 : 10 and 1 : 20. ***P < 0.0001; one-way ANOVA.

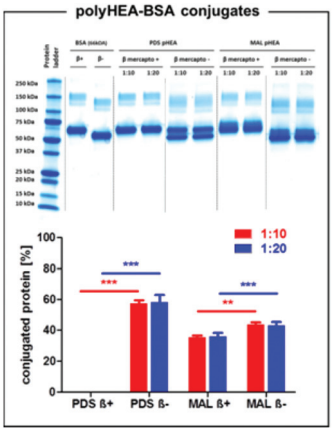


Fig. 4 SDS-PAGE results from the conjugation of BSA with PDS- and MAL-containing polyHEA with and without β-mercaptoethanol, in a molar ratio of protein : polymer 1 : 10 and 1 : 20. β+ = reductive SDS-AGE, β− = native SDS-PAGE. ***$P < 0.0001$, **$P < 0.001$; one-way ANOVA.

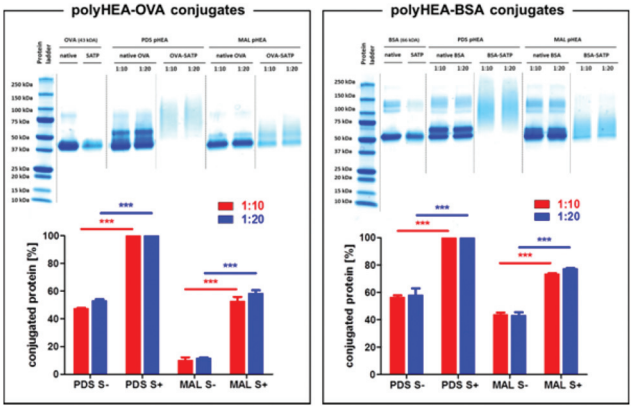


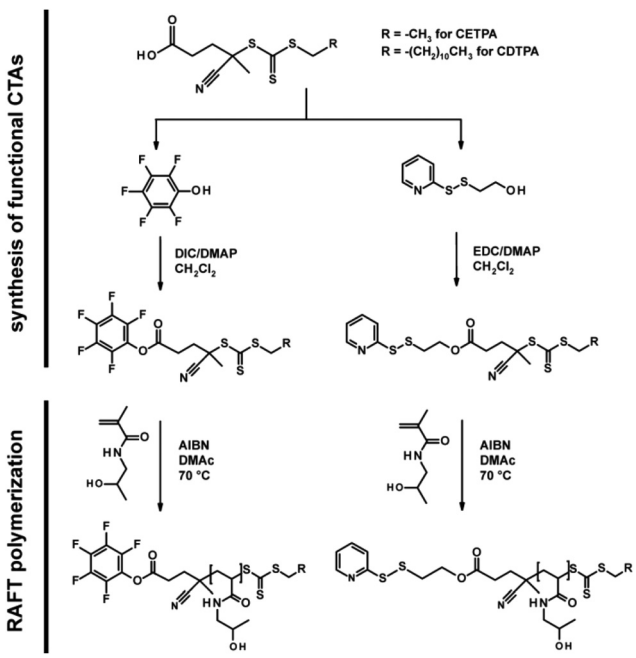
Fig. 5 SDS-PAGE results from the conjugation of SATP-modified OVA and BSA with PDS- and MAL-containing polyHEA in a molar ratio of protein : polymer 1 : 10 and 1 : 20. S− = native protein, S+ = SATP-modified protein. ***$P < 0.0001$; one-way ANOVA.

to become conjugated. The limited increase in protein-binding when doubling the polymer-to-protein ratio from 10 : 1 to 20 : 1 highlights the current limitations of the 'grafting-to' approach. This offers a window of opportunity for either elaborating onto alternative strategies (*e.g.* 'grafting from') or to develop more quantitatively strategies involving more reactive orthogonal groups, *e.g.* in combination with recombinantly engineered proteins that express complimentary reactive groups on a well-accessible site on the protein periphery. To evaluate the reversibility of the PDS- and MAL-conjugates, reductive SDS-PAGE in presence of β-mercaptoethanol was performed. As expected, the disulfide bonds formed between the proteins and the PDS-polyHEA were fully cleaved upon reduction (Fig. 4). For MAL, the BSA conjugates remained intact in the presence of β-mercaptoethanol. Although it is common knowledge that a thio–ether bond by Michael-type addition is irreversible, some recent findings have suggested limited stability of thiol-maleimide bonds under physiological conditions.[59] Similar results were observed for OVA (results not shown). It should be noted that the protein retention by SDS-PAGE was slightly increased with the use of β-mercaptoethanol.

To further enhance the conjugation efficiency for PDS- and MAL-moieties, OVA and BSA were modified with SATP, which adds sulfhydryl groups onto primary amines (*i.e.*, lysine residues and the N-terminus) of proteins in a protected form. Deprotection by deacylation to generate a free sulfhydryl is accomplished by treatment with an excess of hydroxylamine. Quantification of the free amines before and after SATP modification (molar ratio 1 : 20) by ninhydrin assay showed a conversion of 79% and 78% for BSA and OVA respectively. Due to the extensive modification of the lysine residues, staining of the gels became less efficient as coommassie dyes have a high complexation affinity for lysine moieties.[60] Nonetheless, SDS-PAGE analysis revealed that the introduction of sulfhydryl groups onto primary amines groups of proteins strongly increases conjugation efficiency with PDS and MAL containing polyHEA (Fig. 5). PDS polyHEA reaches 100% of conjugation for both OVA and BSA, even at a protein-to-polymer ratio of 1 : 10.

Synthesis of protein-reactive CTA's for methacrylamide RAFT polymers

Next we aimed at exploring the applicability of the two most promising conjugation strategies, *i.e.* based on PFP and PDS containing RAFT agents, to obtain protein-reactive poly(*N*-hydroxypropylmethacrylamide) (polyHPMA) (Scheme 3), which is a highly relevant polymer in view of biomedical appli-



Scheme 3 Synthesis of the different functional CTAs used in this work for RAFT polymerization of *N*-hydroxypropylmethacrylamide (HPMA).

cations.[37,38] In addition we also evaluated the polymerization of *N*-hydroxypropylmethacrylamide-dilactate (HMPA-lac$_2$), which yields temperature responsive polymers (phase transition temperature T_{CP} = 10 °C) that witness a gradual increase in T_{CP} upon degradation of the dilactate side chains and which are currently under investigation for a number of biomedical applications, including anti-cancer drug delivery and tissue engineering.[39–41,61]

Successful application of the RAFT process requires the appropriate selection of a RAFT agent for a particular monomer.[19] Indeed, it has been reported that RAFT polymerization of methacrylates and methacrylamides requires the presence of a cyano moiety adjacent to the thiocarbonate moiety.[62] This was confirmed by us as well, as PABTC was found incapable of yielding well-defined pHMPA. Several groups have reported on RAFT of pHMPA using trithio- and dithiocarbonate based chain transfer agents.[48,63] In our present work we opted for a cyanotrithiocarbonate CTA for its reported higher hydrolytic stability[64,65] and its lower susceptibility to retardation at the initial phase of the polymerization.[66] Hennink and co-workers used the commercially available 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid (CDTPA) CTA for the polymerization of HPMA.[48] However, to minimize in future studies the contribution of the aliphatic dodecyl chain to potential self-assembly behaviour in aqueous medium by hydrophobic interaction, we compared the performance of this CTA to 4-cyano-4-[(ethylsulfanylthiocarbonyl)-sulfanyl]pentanoic acid (CETPA) which might be preferred due to the shorter ethyl chain length of the Z-group. CETPA was successfully functionalized with respectively PFP and PDS by carbodiimide chemistry.

RAFT polymerization of HPMA with protein-reactive CTAs

Kinetic studies of HPMA polymerization carried out in an automated synthesis robot showed for both the PFP-CDTPA and PFP-CETPA chain transfer agents high control over the polymerization reaction with linear pseudo-first order kinetic plots and a linear growth of molecular weight with monomer conversions (Fig. 6). Overall, conversions were significantly lower than for HEA polymerization, which can be likely ascribed to the lower reactivity of methacrylamides *versus* acrylates. Therefore we also investigated higher DP values, *i.e.* DP = 200, to obtain higher molecular weight polymers at similar conversions. As shown in Fig. 6, this was found to be successful. These polymerization conditions were then used to synthesize well-defined PFP- and PDS-polyHPMA and PDS-polyHPMA-lac$_2$ using functionalized CETPA as RAFT agent. The specifications of the synthesized polymers are listed in Table 2. Note that the molecular weights (M_n) determined by SEC are largely overestimated, due to differences in hydrodynamic volume with respect to the PMMA standards.

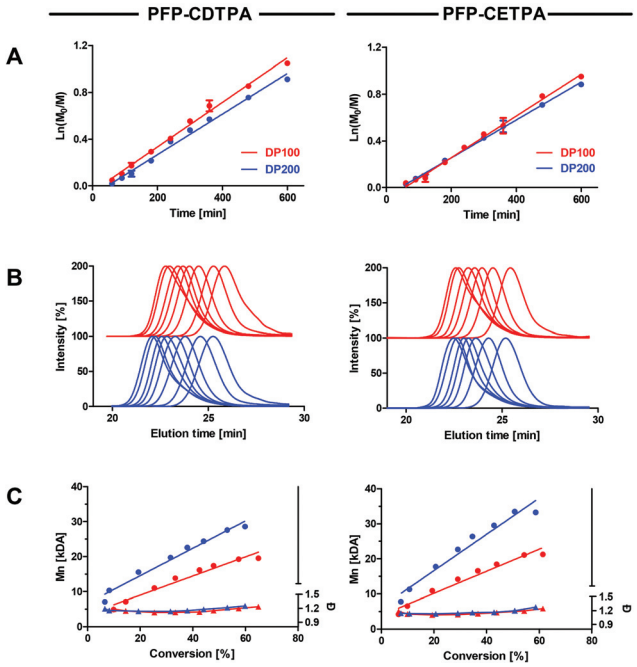


Fig. 6 RAFT polymerization of HPMA, using PFP-CDTPA (left) and PFP-CETPA (right) in DMAc at 70 °C with a HPMA : CTA : AIBN molar ratio of 100 : 1 : 0.2 (red curves) or 200 : 1 : 0.2 (blue curves). **(A)** Kinetic curve *versus* polymerization time; **(B)** SEC traces at different timepoints; **(C)** Molecular weight (M_n) and PDI of the polymers *versus* monomer conversion.

Protein conjugation using functional polyHPMA

In analogy with the protein-conjugation experiments with polyHEA, we now used PFP-polyHMPA and PDS-polyHMPA of different molecular weights to conjugate to OVA and BSA, in a 1 : 10 and 1 : 20 protein-to-polymer ratio. Protein conjugation was performed under identical conditions as described earlier for polyHEA. Interestingly, SDS-PAGE analysis (Fig. 7) of the reaction mixture yielded similar trends as observed for the conjugation experiments with polyHEA. Again, conjugation of PFP-functionalized polymer was much more efficient for BSA conjugation than for OVA conjugation, whereas PDS-polyHPMA was much more efficient in conjugating to OVA than to BSA.

Table 2 Protein-reactive polyHPMA and polyHPMA-lac2 polymers synthesized in DMAc at 70 °C with AIBN as an initiator and functionalized CETPA as a CTA

PR CTA	Monomer	[M]/[CTA]	$t_{reaction}$	DP^{NMR}	M_n^{NMR}	M_n^{SEC}	PDI
PFP	HPMA	100	10 h	59	8.4 kDa	21 kDa	1.24
PFP	HPMA	200	10 h	123	17.6 kDa	33 kDa	1.27
PDS	HPMA	100	10 h	30	4.3 kDa	13 kDa	1.20
PDS	HPMA	200	10 h	46	6.6 kDa	16 kDa	1.24
PDS	HPMA-lac2	100	10 h	33	4.7 kDa	13 kDa	1.19

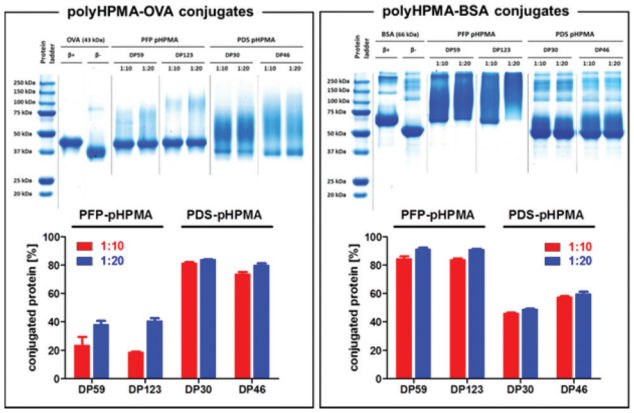


Fig. 7 SDS-PAGE results from the conjugation of OVA and BSA with PFP- and PDS-containing polyHPMA of different molecular weights, in a molar ratio of protein : polymer 1 : 10 and 1 : 20.

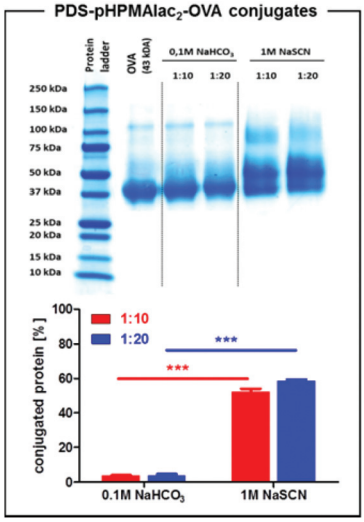


Fig. 8 SDS-PAGE results from the conjugation of OVA with PDS-containing polyHPMA-lac$_2$ in a molar ratio of protein : polymer 1 : 10 and 1 : 20 in the standard 0.1 M $NaHCO_3$ conjugation buffer and in a 1 M NaSCN "salting in" solution. ***$P < 0.0001$; one-way ANOVA.

This points out that differences in conjugation efficiency are most likely due to differences in protein structure, especially the availability of reactive groups rather than polymer chemistry, at least for the systems studied in this work. Moreover, the conjugation products with PFP-polyHPMA of DP123 clearly exhibited an increase in molecular weight as compared to those with PFP-polyHPMA of DP59. For the PDS-polyHPMA polymers this effect was less clear, due to the smaller difference in molecular weight.

For conjugation experiments with polyHPMA-lac$_2$, we found that performing conjugation below the T_{CP} of the polymer (*i.e.* at 0 °C) did not yield polymer-protein conjugation, while performing the conjugation reaction at room temperature (*i.e.* above the T_{CP}) with the polymer in collapsed state was unsuccessful too. To address this issue we added sodium thiocyanate (NaSCN) which is known from the Hofmeister series to exert a strong 'salting in effect' and is capable of increasing the phase transition temperature of polymers.[67,68] We observed that in presence of 1 M NaSCN polyHMPA-lac$_2$ became fully soluble in an ice bath and the consequent conjugation efficiency of PDS-polyHMPA-lac$_2$ to OVA was found to be drastically improved (Fig. 8).

Conclusions

A series of RAFT chain transfer agents for both acrylates and methacrylamides were functionalized with different reactive moieties that can be conjugated to either lysine or cysteine residues to afford polymer-protein conjugates. RAFT polymerization with these protein-reactive CTA's provided high control over the polymerization with linear pseudo-first order kinetic plots and a linear growth of molecular weight with monomer conversions, for both acrylates and methacrylamides. The chain transfer agent bearing a (protected) maleimide, however, was prone to side-reactions and therefore, polymerization had to be stopped at 50% conversion to be able to obtain well-defined polymers. This can probably be attributed to deprotection of the maleimide during polymerization inducing its participation in the polymerization process. Moreover, an additional deprotection step is necessary to activate the protein-reactive maleimide group. These setbacks make the maleimide group a less attractive conjugation strategy. Pentafluorophenyl- and pyridyl disulfide-functionalized polymers exhibited the highest conjugation efficiency with BSA and OVA respectively, as observed for both polyHEA and polyHPMA, and therefore are the preferred conjugation chemistries for BSA and OVA respectively. The improved conjugation efficiency of PFP-esters *versus* NHS-esters can likely be ascribed to their higher hydrolytic stability. The disulfide bond in the PDS-polymer-protein conjugates was fully cleaved upon reduction, whereas maleimide-based conjugates remained intact under reductive conditions. Introduction of additional thiol moieties by converting lysine residues with SATP, strongly increased protein-conjugation efficiencies for both pyridyl disulfide- and maleimide-functional polymers. This can be exploited for PDS-polymer-BSA conjugates, when a reversible disulfide bond is preferred to the stable amide bonds obtained with PFP-functionalized polymers. Finally, we were also able to conjugate the thermo-responsive dilactate derivate of HPMA (*i.e.* HPMA-lac$_2$) to OVA *via* a PDS CTA, when using a 'salting-in' approach.

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