Octreotide End-Functionalized Diblock Copolymers Facilitated by RAFT Polymerization

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Abstract

The incorporation of biologically active targeting ligands into polymeric materials is a key challenge in drug delivery and bioimaging. Reported here is the synthesis of diblock copolymers end-functionalized with the Somatostatin analog Octreotide. This methodology employs a novel Octreotide functional reversible addition-fragmentation chain transfer (RAFT) polymerization chain transfer agent, which is used to mediate the polymerization of *N*-isopropylacrylamide and subsequent chain-extension with *N*,*N*-dimethylacrylamide.

Keywords

Polymer-peptide conjugate, RAFT polymerization, grafting-from, end-functionalized

Introduction

Modern controlled radical polymerization (CRP) techniques[1-3] have given polymer scientists the ability to prepare highly sophisticated polymeric architectures.[4] These techniques can be utilized to prepare functional nanoparticles with high levels of control, with the possibility to prepare multi-block copolymers with specific functional groups at desired locations along the polymer backbone and chain terminus. This has directly resulted in a great drive to develop novel polymeric nanoparticles for application in the fields of drug delivery[5] and bioimaging.[6] Perhaps the most significant advantage is the ability to prepare polymeric nano-medicines that are able to target a specific location.[7, 8] To achieve this goal biologically active targeting ligands are directly incorporated into polymeric structures.[9] The integration of the targeting ligand can be performed post-polymerization, a process known as "grafting-to",[10] or polymer chains can be grown from the targeting ligand, "grafting-from".[11]

The synthetic route employed is largely governed by the selected targeting ligand. "Grafting-to" is beneficial when the targeting ligand is unstable and would not survive the polymerization process, however, this method can often require additional purification steps, characterization can be challenging and there is an inherent steric barrier to overcome when linking together large macromolecules. "Grafting-from" overcomes the steric barrier as monomer units are polymerized directly from the targeting ligand. This method can also reduce the number of purification steps required. The main challenge with "grafting-from" lies in the initial synthetic objective of linking the targeting ligand to a CRP initiator.

Several small-molecule biologically relevant compounds have been incorporated into polymeric materials. Armes and Licciardi have reported the synthesis of folic acid end-functionalized diblock copolymers for targeted drug delivery utilizing atom transfer radical polymerization (ATRP) and subsequent coupling via amide-bond formation.[12] D'Agosto and Charreyre described the synthesis of a biotin functional chain transfer agent, which can be used to prepare end-functionalized polymers facilitated via reversible addition-fragmentation chain transfer (RAFT) polymerization.[13] Small-molecule targeting ligands such as folic acid and biotin are very appealing as their synthetic incorporation is relatively straight-forward, however, they often undergo non-selective interactions which can limit their targeting efficiency.[14]

Poly(peptides) and proteins can undergo very selective ligand-receptor interactions and the incorporation of more complex biological targeting ligands is now a field of great interest.[15] Polymer-protein conjugates represent a great synthetic challenge[16] and recently the Sumerlin group has reported the utilization of RAFT polymerization and the "grafting-to" approach to prepare polymer-protein conjugates through thiol-ene click chemistry[17] and amide bond formation.[18] The Sumerlin group has also investigated the synthesis of polymer-protein conjugates via RAFT medicated "grafting-from", they report the polymerization of *N*-isopropylacrylamide (NIPAm) from bovine serum albumin (BSA)[19] and acrylamide-based diblock copolymers from lysozyme.[20]

While proteins can permit site specific ligand-receptor interactions, their complex structures can result in synthetic and characterization challenges. Therefore, relatively low molecular weight poly/oligo(peptides) could provide an agreeable intermediate between larger proteins and small molecule targeting ligands. The tri-peptide Arg-Gly-Asp (RGD) and its derivatives[21] have received a lot of attention due to their targeting abilities. Cyclic RGD has been linked to amphiphilic block copolymers for the targeted delivery of platinum-based anti cancer drugs.[22] The linear targeting peptide GRGDS has been successfully incorporated into polymeric assemblies for targeted bioimaging.[23] Börner has shown that RAFT polymerization can be used to prepare oligopeptide-polymer conjugates.[24]

The Somatostatin analog Octreotide[25] is a very appealing cyclic peptide as it is known to actively target breast cancer cells,[26] small-cell lung cancer[27] and pancreatic cancer[28] through receptor-medicated targeting. Octreotide is commonly linked to macrocycles and chelated with ^{64}Cu for targeted positron emission tomography (PET) imaging.[29-31] Despite its very attractive targeting properties the incorporation of Octreotide into polymeric systems is rather limited. The Morelli group has developed a surfactant-type system that incorporates Octreotide and Gd^{3+} for magnetic resonance imaging (MRI).[32] Octreotide has also been "grafted-to" poly(ethylene glycol)-b-(lactic acid)via amide bond formation[33] and poly(ethylene glycol) via imine bond formation.[34] We have previously presented the synthesis of Octreotide functional covalently cross-linked branched polymeric nanoparticles for targeted magnetic resonance imaging. In this work Octreotide was coupled to pre-formed polymeric nanoparticles via amide bond formation.[35] While successful this synthetic approach was very challenging and required laborious purification steps.

Reported here is the direct preparation of Octreotide end-functionalized diblock copolymers via RAFT polymerization, this is the first example of an Octreotide functional CRP chain transfer agent/ initiator which can be used to facilitate the growth of Octreotide end-functionalized polymers. This synthetically straightforward route to Octreotide functional diblock copolymers could greatly increase this cyclic peptides potential in targeted drug delivery and bioimaging.

Experimental

Materials and Methods

All chemicals were purchased from Sigma Aldrich. *N*-Isopropyl acrylamide was purified by recrystallization in hexane prior to use. Azobisisobutyronitrile (AIBN) was recrystallized from methanol prior to use. ^{1}H and ^{13}C NMR spectra were recorded on Bruker 400 Ultra Shield spectrometer. High resolution mass spectrometry was performed on a Thermo Finnigan MAT 95XP HRSM spectrometer. DLS analysis was carried out on a Malvern Zetasizer Nano-ZS machine in pure H_2O at a polymer conc. of 1 mg/mL. LCSTs were determined by DLS heating from 20 °C to 40 °C, collecting data points every 0.2 °C for 2 min. Heating runs were repeated in triplicate. GPC was conducted on a Viscotek TDAmax consisting of a GPCmax integrated solvent and sample delivery module, a TDA 302 Triple Detector Array, and OmniSEC software. 2 x PLgel 5 µm Mixed-C (200-2,000,000) columns were applied in sequence for separation. DMF was used as the eluent at 0.8 mL/min with column and detector temperature at 60 °C, molecular weight values were determined against poly(styrene) standards.

Pentafluorophenyl Activated RAFT Chain Transfer Agent (DDMAT-PFP)

S-Dodecyl-*S'*-(α,α'-dimethyl-*α''*-acetic acid)trithiocarbonate (**DDMAT**, 2.55 g, 7 mmol, 1 eq) and diisopropylethylamine (1.81 g, 14 mmol, 2 eq) were dissolved in DMF (25 mL) and the solution cooled to 0 °C. Pentafluorophenyl trifluoroacetate (2.35 g, 8.4 mmol, 1.2 eq) was then added and the reaction stirred at 0 °C for 2 h. After this time diethyl ether (100 mL) was added and the solution washed with 1M HCl (100 mL), sat. $NaCl_{(aq)}$ (100 mL) and H_2O (100 mL) The organic layer was dried with Na_2SO_4, filtered and evaporated to dryness to afford a crude yellow oil, which was purified by column chromatography [SiO_2, Hexane–EtOAc (9:1)] to afford the desired product **DDMAT-PFP** as a yellow oil (2.25 g, 61 %). ^{1}H NMR ($CDCl_3$): δ 3.31 (t, J = 7.5 Hz, 2H), 1.86 (s, 6H), 1.69 (q, J = 7.5 Hz, 2H), 1.39 (m, 2H), 1.35-1.20 (m, 16H), 1.88 (t, J = 7.0 Hz). ^{13}C NMR ($CDCl_3$): δ 220.0, 169.7, 142.7, 141.0, 140.0, 139.2, 138.4, 136.7, 125.2, 55.5, 37.3, 32.1, 29.8, 29.7, 29.6, 29.5, 29.2, 29.1, 28.0, 25.6, 22.8, 14.3. ^{19}F NMR ($CDCl_3$): δ -151.71 (m, 2H), -157.93 (m, 1H), -162.53 (m, 2H).

Lysine Boc-Protected Octreotide (Lys(Boc)Octreotide)

Di-*tert*-butyl dicarbonate (0.65 g, 3.0 mmol, 1 eq) was dissolved in DMF (20 mL) and added dropwise to Octreotide acetate (3.06 g, 3.0 mmol, 1 eq) in DMF (50 mL). After stirring at room temperature for 2 h the reaction was evaporated to dryness. Diethyl ether (200 mL) was then added, precipitating the crude product from solution, which was isolated by filtration and purified by column chromatography [SiO_2, CH_2Cl_2–MeOH (9:1)] to afford the desired product **Lys(Boc)Octreotide** as a white solid (2.45 g, 73 %). ^{1}H NMR (DMSO-d_6): δ 10.74 (s, 1H), 8.75 (d, 1H), 8.50 (m, 3H), 8.39 (d, 1H), 7.75 (d, 1H), 7.60 (d, 1H), 7.43 (d, 1H), 7.33 (d, 1H), 7.28 (m, 4H), 7.16 (m, 4H), 7.07 (m, 3H), 6.99 (t, 1H), 6.93 (m, 1H), 6.71 (t, 1H), 5.15

(m, 2H), 4.92 (d, 1H), 4.68 (m, 1H), 4.63 (q, 1H), 4.50 (m, 1H), 4.19 (q, 1H), 3.99 (m, 2H), 3.90 (m, 1H), 3.67 (m, 1H), 3.54 (m, 2H), 3.43 (m, 2H), 3.06 (m, 1H), 3.00-2.65 (br m, 8H), 2.57 (m, 1H), 1.71 (m, 2H), 1.37 (s, 9H), 1.20 (m, 2H), 1.06 (m, 6H), 0.83 (m, 2H). HRMS (ESI) *m*/*z*: $[M + H]^+$ calculated for $C_{54}H_{75}N_{10}O_{12}S_2$, 1119.5002: found 1119.5005 (ppm 4.79).

Octreotide Functional RAFT Chain Transfer Agent (DDMAT-Octreotide)

S-Dodecyl-*S'*-(α,α'-dimethylpentafluorophenyl acetate)trithiocarbonate (**DDMAT-PFP**, 0.21 g, 0.4 mmol, 1 eq) and **Lys(Boc)Octreotide** (0.53 g, 0.48 mmol, 1.2 eq) were dissolved in DMF (20 mL) and the solution cooled to 0 °C. Diisopropylethylamine (0.52 g, 4 mmol, 10 eq) was then added and the reaction stirred at 0 °C for 8 h. The reaction was allowed to warm slowly to room temperature and was stirred for a further 16 h. After this time evaporation to dryness afforded a crude yellow oil which was purified by column chromatography [SiO_2, CH_2Cl_2–MeOH (9:1)] to afford the desired product **DDMAT-Octreotide** as a yellow solid (0.40 g, 68 %). ^{1}H NMR (DMSO-d_6): δ 10.75 (s, 1H), 8.72 (d, 1H), 8.66 (br s, 2H), 8.48 (m, 2H), 8.38 (d, 1H), 7.64 (m, 2H), 7.44 (d, 1H), 7.33 (d, 1H), 7.29 (d, 4H), 7.22 (m, 1H), 7.16 (m, 3H), 7.06 (m, 3H), 6.98 (t, 1H), 6.94 (m, 1H), 6.71 (t, 1H), 5.10 (br s, 1H), 5.05 (s, 1H), 5.01 (br s, 1H), 4.62 (q, 1H), 4.48 (m, 1H), 4.21 (q, 1H), 3.97 (br m, 2H), 3.72 (m, 1H), 3.65 (m, 1H), 3.51 (m, 1H), 3.39 (m, 1H), 3.27 (t, 2H), 3.10 (dd, 1H), 2.92 (m, 2H), 2.85-2.60 (br m, 9H), 1.64 (s, 6H), 1.59 (t, 2H), 1.37 (s, 9H), 1.23 (br s, 22 H), 1.05 (m, 6H), 0.85 (m, 5 H). HRMS (ESI) *m*/*z*: $[M + Na]^+$ calculated for $C_{71}H_{104}N_{10}NaO_{13}S_5$, 1487.6280: found 1487.6259 (ppm 1.42).

Octreotide End-Functionalized Poly(NIPAm) (Poly(NIPAm)-Octreotide)

Octreotide functional RAFT chain transfer agent (**DDMAT-Octreotide**, 110 mg, 75 µmol, 1 eq), *N*-isopropylacrylamide (0.85 g, 7.5 mmol, 100 eq) and AIBN (1.23 mg, 7.5 µmol, 0.1 eq) were added to a small Schlenk tube followed by DMF (3 mL). The reaction mixture was degassed via the freeze-pump-thaw technique and the vessel was backfilled with N_2. The reaction mixture was then placed in an oil bath at 70 °C, and the polymerization was quenched by rapid cooling after 4 h. The reaction mixture was dissolved in a minimal amount of THF and added dropwise to a large excess of ice cold diethyl ether to afford the desired product **Poly(NIPAm)-Octreotide** as pale yellow solid (0.60 g). ^{1}H NMR ($CDCl_3$): δ 7.16 (br, Octreotide aromatic protons), 6.50 (br, N*H*), 3.98 (br, C*H*$(CH_3)_2$), 2.91 (br, Octreotide aliphatic protons), 2.13 (br, C*H*CH_2, polymer backbone), 1.73 (br, CHCH_2, polymer backbone), 1.40 (br, Octreotide *boc* and RAFT aliphatic protons), 1.13 (br, CH(CH_3)$_2$).

Octreotide End-Functionalized Block Copolymer (Poly(DMA)-*b*-(NIPAm)-Octreotide)

Octreotide end-functionalized poly(NIPAm) (**Poly(NIPAm)-Octreotide**, 0.5 g, 44 µmol, 1 eq), *N,N'*-dimethylacrylamide (0.61 g, 6.2 mmol, 140 eq) and AIBN (0.73 mg, 4.4 µmol, 0.1 eq) were added to a small Schlenk tube followed by DMF (4 mL). The reaction mixture was degassed via the freeze-pump-thaw technique and the vessel was backfilled with N_2. The reaction mixture was then placed in an oil bath at 70 °C, and the polymerization was quenched by rapid cooling after 4 h. The reaction mixture was dissolved in a minimal amount of THF and added dropwise to a large excess of ice cold diethyl ether to afford the desired product **Poly(DMA)-*b*-(NIPAm)-Octreotide** as pale yellow solid (0.90 g). ^{1}H NMR ($CDCl_3$): δ 7.16 (br, Octreotide aromatic protons), 6.65 (br, N*H*), 3.97 (br, C*H*$(CH_3)_2$), 2.88 (br, N(CH_3)$_2$), 2.60-2.00 (br, C*H*CH_2, polymer backbone), 1.61 (br, CHCH_2, polymer backbone), 1.12 (br, CH(CH_3)$_2$).

Results and Discussion

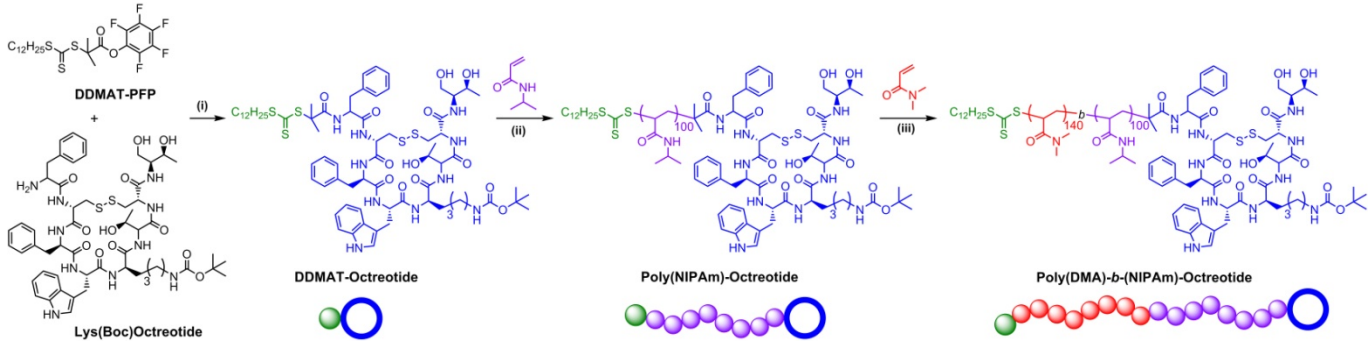

Fig 1. (i) Synthesis of Octreotide RAFT chain transfer agent (**DDMAT-Octreotide**) with DIPEA in DMF at 0 °C, (ii) polymerization of *N*-isopropylacrylamide (**Poly(NIPAm)-Octreotide**) with AIBN in DMF at 70 °C and (iii) subsequent chain-extension with *N,N*-dimethylacrylamide (**Poly(DMA)-*b*-(NIPAm)-Octreotide**) with AIBN in DMF at 70 °C.

The RAFT chain transfer agent *S*-dodecyl-*S'*-(*α,α'*-dimethyl-*α''*-acetic acid)trithiocarbonate (**DDMAT**) was selected for its ability to control the polymerization of acrylamide-based monomers[36, 37] and its capability to be modified to incorporate other functionalities.[38] **DDMAT** (1 eq) was treated with pentafluorophenyl trifluoroacetate (1.2 eq) in the presence of diisopropylethylamine (Fig 1) to afford the activated ester RAFT chain transfer agent **DDMAT-PFP** (61 % yield).[39] It is very important that the coupling of Octreotide to the RAFT chain transfer agent occurs at the amine site within the terminal phenylalanine residue, as the amine-functional lysine residue plays an active role in the targeting abilities of Octreotide.[27-32] Therefore, the lysine amino residue of Octreotide was *boc*-protected by treating Octreotide acetate (1 eq) with di-*tert*-butyl dicarbonate (1 eq) in DMF to furnish **Lys(Boc)Octreotide** (73 % yield).[40] **DDMAT-PFP** (1 eq) was then reacted with a slight excess of **Lys(Boc)Octreotide** (1.2 eq) in the presence of diisopropylethylamine (Fig 1) to afford the Octreotide functional RAFT chain transfer agent **DDMAT-Octreotide** via amide bond formation. The reaction was performed at 0 °C and then allowed to warm slowly to room temperature to avoid any undesirable ester formation. **DDMAT-Octreotide** was purified by column chromatograph (CH_2Cl_2: MeOH [9:1]) before characterization by ^{1}H NMR spectroscopy (Fig 2(a)). Integration of peaks 34 (2H) and 43 (6H) relative to the Octreotide proton peaks confirmed that each Octreotide peptide was coupled to one DDMAT molecule. Mass spectrometry (*m/z*: $[M + H]^+$ calculated for $C_{54}H_{75}N_{10}O_{12}S_2$, 1119.5002: found 1119.5005, ppm 4.79) also confirmed a successful synthesis.

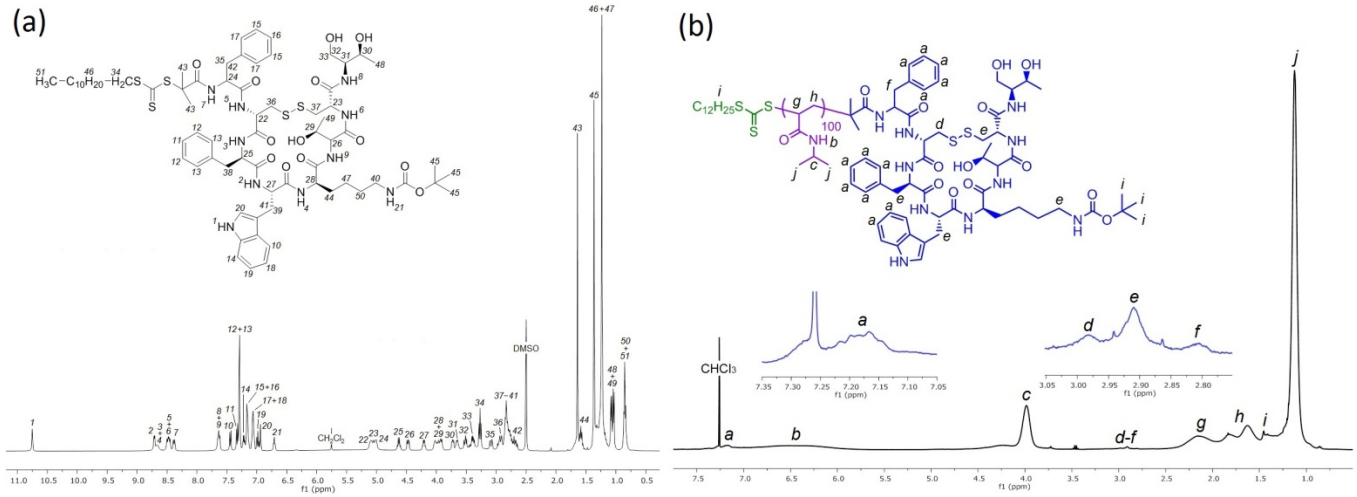

Fig 2. (a) ^{1}H NMR (DMSO-d_6) of **DDMAT-Octreotide** and (b) ^{1}H NMR ($CDCl_3$) of **Poly(NIPAm)-Octreotide**.

DDMAT-Octreotide (1 eq) was then used to mediate the RAFT polymerization of *N*-isopropylacrylamide (100 eq) with AIBN (0.1 eq) at 70 °C in DMF. Small samples were extracted at regular intervals and ^{1}H NMR spectroscopy confirmed a constant concentration of radicals throughout the polymerization (Fig 3(a)) and a final monomer conversion of 98 % (M_n^{theo} = 12,200 Da). The resulting polymer **Poly(NIPAm)-Octreotide** was characterized by gel permeation chromatography (Fig 3(b)) with DMF as the eluent which displayed a successful extension (purple trace) of the RAFT chain transfer agent **DDMAT-Octreotide** (blue trace) and a number average molecular weight (M_n = 13,400 Da, M_w = 15,150 Da, PDI = 1.13) similar to the theoretical value. These results confirm that the Octreotide functional RAFT chain transfer agent was able to successfully control the polymerization of *N*-isopropylacrylamide, affording a polymer-peptide conjugate with a narrow polydispersity indicative of RAFT control. ^{1}H NMR spectroscopy in $CDCl_3$ (Fig 2(b)) confirmed the presence of the Octreotide aromatic peaks at δ 7.35 – 7.10 and aliphatic peaks at δ 3.05 – 2.78.

It is well understood that poly(NIPAm) undergoes a temperature induced hydrophilic to hydrophobic phase transition, the temperature at which this switch occurs is known as the lower critical solution temperature (LCST). This physical characteristic can be measured by dynamic light scattering (DLS)[41] at the LCST the solvated linear polymer chains become hydrophobic and precipitate from solution, resulting in an increased intensity of scattered light. It is also commonly observed that the LCST of thermoresponsive polymers is affected by the relative hydrophobicity of the polymer end-groups.[42, 43] To further confirm the direct coupling of Octreotide within **Poly(NIPAm)-Octreotide** a control polymer (**Poly(NIPAm)**) was synthesized, under identical conditions, using **DDMAT** (1 eq) as the RAFT chain transfer agent and *N*-isopropylacrylamide (100 eq) with AIBN (0.1 eq) at 70 °C in DMF. It was hypothesized that **Poly(NIPAm)-Octreotide** would possess a higher LCST than that of **Poly(NIPAm)** due to the hydrophilic nature of the Octreotide cyclic peptide at its chain-terminus. Dynamic light scattering analysis (Fig 2(c)) of Poly(NIPAm) (green data points) displayed an LCST of 29 °C. This is slightly lower than previously reported for poly(*N*-isopropylacrylamide)[44], presumably due to the hydrophobic

nature of the -$C_{12}H_{25}$ end-group. The LCST (Fig 2(c)) of **Poly(NIPAm)-Octreotide** (purple data points) was found to be 32 °C, a significant 3 degrees higher than the analogous poly(*N*-isopropylacrylamide) without the presence of Octreotide. This relative increase in LCST further confirms the presence of Octreotide within the polymer-peptide conjugate.

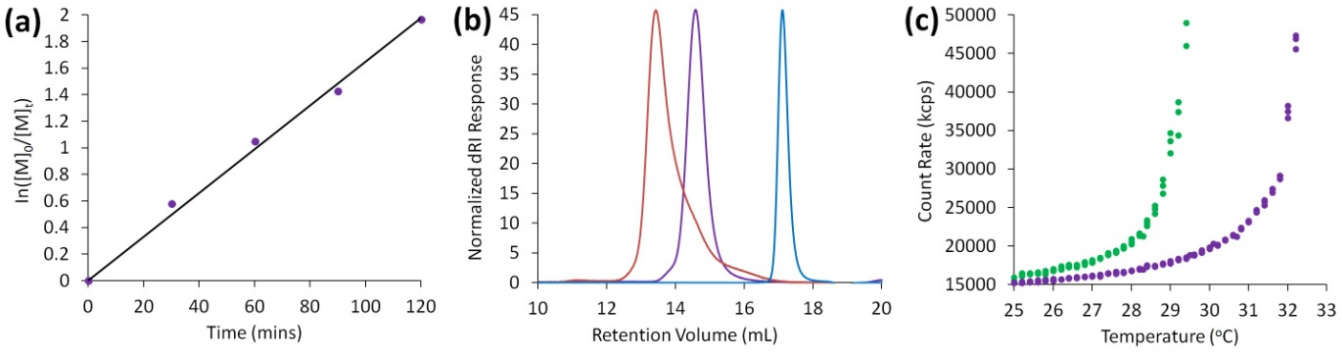

Fig 3. (a) Kinetics of N-isopropylacrylamide RAFT polymerization facilitated by **DDMAT-Octreotide**, (b) gel permeation chromatography (GPC) analysis of **DDMAT-Octreotide** (blue trace), **Poly(NIPAm)-Octreotide** (purple trace) and **Poly(DMA)-*b*-(NIPAm)-Octreotide** (red trace), (c) Lower critical solution temperature (LCST) determined by dynamic light scattering analysis of **Poly(NIPAm)** (green data) and **Poly(NIPAm)-Octreotide** (purple data).

The ability to prepare diblock copolymers is crucial for application in drug delivery or bioimaging, as multi-block copolymers are able to self-assemble into more complex architectures.[45, 46] To demonstrate the ability of this Octreotide-functional RAFT chain transfer agent to prepare diblock copolymers the **Poly(NIPAm)-Octreotide** homopolymer was chain extended with *N,N*-dimethylacrylamide. This polymerization was performed in DMF at 70 °C with **Poly(NIPAm)-Octreotide** (1 eq), *N,N*-dimethylacrylamide (140 eq) and AIBN (0.1 eq). ^{1}H NMR spectroscopy confirmed a similar conversion of 95 % (M_n^{theo} = 25,400 Da). The resulting polymer **Poly(DMA)-*b*-(NIPAm)-Octreotide** was characterized by gel permeation chromatography (Figure 2(b)) which displayed a successful chain extension (red trace) of the macro-RAFT chain transfer agent **Poly(NIPAm)-Octreotide** (purple trace) and a number average molecular weight (M_n = 22,200 Da, M_w = 36,800 Da, PDI = 1.66) similar to the theoretical value. The macro-RAFT chain transfer agent was fully consumed, which confirms the retention of the trithiocarbonate functionality and the living nature of **Poly(NIPAm)-Octreotide**. However, the obtained PDI of 1.66 could suggest a slight loss in RAFT control.

Conclusions

In summary, a novel RAFT chain transfer agent incorporating the very appealing biologically active targeting ligand Octreotide has been synthesized and used to facilitate the controlled radical polymerization of *N*-isopropylacrylamide. This polymer-peptide conjugate was subsequently chain-extended with *N,N*-dimethylacrylamide, demonstrating the ability of this system to prepare useful polymer-peptide building blocks which are useful in the preparation of self-assembled aggregates. Typically, the incorporation of Octreotide is performed post-polymerization which can result in steric barriers to overcome and requires additional purification steps. This approach is very robust and experimentally straightforward, and is currently being used to prepare functional polymeric nanoparticles for application in targeted drug delivery and bioimaging.

Acknowledgments

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