CONTROLLED RADICAL POLYMERIZATION OF *N*-ISOPROPYLACRYLAMIDE AND OF ACTIVATED ESTERS FOR THE SYNTHESIS OF POLYMER-PROTEIN AND POLYMER-DRUG CONJUGATES

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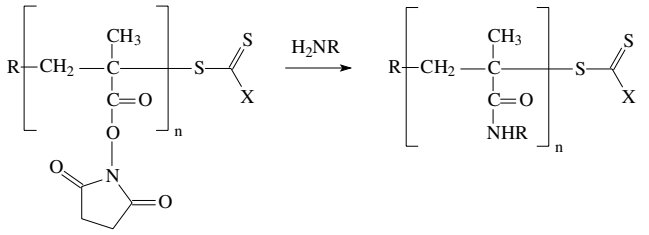
Introduction

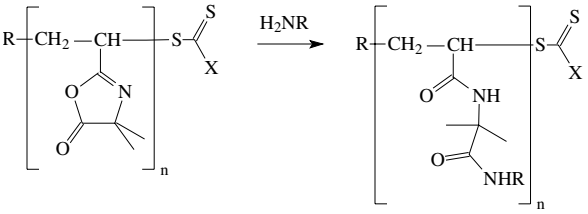
Recently, the synthesis of polymers via reversible addition-fragmentation chain transfer (RAFT) polymerization has gained importance due to its great versatility, its compatibility with a wide range of monomers, the control of the molecular weights, and the low polydispersities of the resultant polymers. Besides, it offers all advantages of conventional free radical polymerization. The polymerization is carried out in the presence of thiocarbonylthio compounds of general structure Z-C(=S)S-R and results in the formation of end-functionalized polymers.[1]

Poly(*N*-hydroxysuccinimide methacrylate) is widely used in the synthesis of watersoluble block copolymers for polymer-drug conjugation.[2] The drug is bound to the polymer via a hydrolizable oligopeptide linker, which in turn is bound to the polymer through the reaction of the *N*-hydroxysuccinimide moiety (**Scheme 1**). If LCST polymers are sequentially copolymerized with functional monomers, such as *N*-hydroxysuccinimide methacrylate, short blocks are obtained which provide binding sites for the amino functions of proteins.



Scheme 1. Conjugation of amino moiety of a drug or protein to *N*-hydroxysuccinimide methacrylate block.

Poly(2-vinyl-4,4-dimethyl-5-oxazolone) is used for the conjugation of amino moieties to the oxazolone unit (**Scheme 2**).[3] The unit is an active ester that adds to amines under ring opening.



Scheme 2. Conjugation of amino moiety to oxazolone block.

Poly(*N*-isopropylacrylamide) (polyNIPAAm) shows LCST (lower critical solution temperature) behaviour in aqueous solutions and a sharp phase transition is observed at 32 °C in water. With the conjugation of a peptide or protein to the polymer, thermoresponsive systems can be created.[4,5] Their application ranges from the controlled release of enzymes to the adjustment of the LCST to a certain temperature. Alternatively, poly(acrylic acid) can be used to enable response to pH and ionic strength. Proteins conjugated to block copolymers of NIPAAm and acrylic acid can be investigated in terms of their response to combined external stimuli. These block copolymers form micelles in selective solvents. The solubility of the acrylic acid block, for example, can be controlled via the pH value. It precipitates in water at pH < 4, whereas NIPAAm precipitates in water at temperatures above 32 °C. Micelles are suitable for the inclusion of drugs in the micellar core and the controlled release thereof. The polymers can also be used for the creation of both thermo- and pH-responsive systems by conjugating drugs or proteins to them. With the conjugation, the system can be tuned by external stimuli. The poly(acrylic acid) block can be used to enable response to ionic strength or pH.

Different methods of polymer-protein conjugation can be employed: (a) RAFT polymerization in order to obtain sulfur-containing endgroups which are hydrolyzed to the corresponding thiols and can be conjugated to thiol functions of proteins; (b) synthesis of block copolymers with short blocks of oligo(active ester)s for conjugation to the amino functions of proteins. Two methods for the conjugation of protein thiol groups are predominant in biochemistry and can be used to create protein-polymer links: (i) Bismaleimide spacer between polymer and protein, (ii) direct disulfide coupling via 2,2'-dithio(5-nitropyridine).[6,7]

Poly(2-vinyl-4,4-dimethyl-5-oxazolone), poly(*N*-hydroxysuccinimide methacrylate), and poly(*N*-isopropylacrylamide) as well as NIPAAm/AA and NIPAAm/oxazolone block copolymers have been prepared via RAFT polymerization with narrow MWD for the use in polymer-protein conjugation.

Experimental

Materials. *N*-isopropylacrylamide (Aldrich, 97 %) was recrystallized twice from benzene/hexane 3:2 (v:v) and dried under vacuum prior to use. Acrylic acid was purified according to the standard procedure. The monomers 2-vinyl-4,4-dimethyl-5-oxazolone (TCI Tokyo) and *N*-hydroxysuccinimide methacrylate were used as received. Azobisisobutyronitrile (AIBN, Fluka, purum) was recrystallized from methanol and dried under vacuum prior to use. The initiator 1,1'-azobis(cyclohexanecarbonitrile) (DuPont) was used as received.

Instrumentation. Gel permeation chromatography (GPC) on the THF soluble polymers was performed on a Waters Associates liquid chromatograph equipped with an RI detector and a UV detector (λ=254 nm). PSS SDVgel columns (30 x 8 mm, 5 µm particle size) with 10^2, 10^3, 10^4, and 10^5 Å pore sizes were used. THF was used as an eluent (flow rate 0.5 mL/min) at a temperature of 25 °C. The injection volume was 100 µL and a Spectra Physics P 100 pump was used. As an internal standard, *o*-dichlorobenzene was used. Polystyrene standards were used for calibration. GPC on the DMF soluble polymers was performed on a Shimadzu SCL-10A VP system equipped with a Dawe EOS light scattering detector with Optilab DSP refractometer (both set at 690 nm) and with a series of four StyraGel columns HT2, HT3, HT4 and HT5. The oven temperature was 80 °C. The solvent was DMF + 0.05 M LiBr with 1.0 mL/min as flow rate.

UV spectra were recorded on a Lambda15 UV-vis spectrophotometer (Perkin-Elmer) in the wavelength range from 190 to 550 nm.

General polymerization procedure. The reagents were mixed in a vial and aliquots were transferred to ampoules, which were degassed by three freeze-thaw-evacuate cycles and then flame sealed under vacuum. The ampoules were immersed completely in an oil bath at the specified temperature.

Synthesis of poly(*N*-hydroxysuccinimide methacrylate). The polymerization was conducted using the chain transfer agents (CTAs) 1-cyanoisopropyl dithiobenzoate, benzyl 1-pyrrolecarbodithioate, and cumyl 1-pyrrolecarbodithioate. For the polymerizations, 1.67 mg (0.010 mmol) AIBN and 0.50 g (2.730 mmol) *N*-hydroxysuccinimide methacrylate as well as 31.12 mg (0.141 mmol) 1-cyanoisopropyl dithiobenzoate, 32.90 mg (0.141 mmol) benzyl 1-pyrrolecarbodithioate, and 36.86 mg (0.141 mmol) cumyl 1-pyrrolecarbodithioate, respectively, was used. The polymerizations were conducted for 10 h or 16 h in 2.0 mL DMF at a temperature of 60 °C. The products were characterized by DMF GPC.

Synthesis of poly(*N*-isopropylacrylamide). The polymerization was performed with either benzyl 1-pyrrolecarbodithioate or cumyl 1-pyrrolecarboditihoate as chain transfer agent. Benzyl 1-pyrrolecarbodithioate (0.413 g, 1.77 mmol) and cumyl 1-pyrrolecarbodithioate (0.512 g, 1.96 mmol), respectively, was dissolved in 100 mL 1,4-dioxane along with AIBN (0.115 g, 0.70 mmol) and NIPAAm (20.37 g, 0.18 mol). The solution was degassed by three freeze-thaw evacuation cycles. The polymerization mixture was heated to 60 °C (temperature of oil bath) for the specified time. The polymerization was conducted under nitrogen atmosphere and samples were drawn at different time intervals. The samples were immediately immersed into liquid nitrogen and subsequently freeze-dried. The polymers were characterized by THF GPC with 0.25 wt.-% tetrabutylammonium bromide added to the eluent.

Synthesis of poly(acrylic acid)-*b*-poly(*N*-isopropylacrylamide). For the synthesis of the block copolymers, poly(acrylic acid) was used as a macromolecular chain transfer agent. The homopolymer was obtained by RAFT polymerization of 2 mL (2.102 g, 0.029 mol) acrylic acid, 0.804 mg ($3.3 \cdot 10^{-3}$ mmol) 1,1'-azobis(cyclohexanecarbonitrile), and 45.66 mg (0.21 mmol) 1-cyanoethyl 2-pyrrolidone-1-carbodithioate in a mixture of 2.44 mL MeOH and 0.56 mL H_2O at 90 °C for 3 h. The block copolymers were synthesized using 0.223 g (0.045 mmol, M_n = 7900 g/mol) of the poly(acrylic acid) homopolymer as chain transfer agent along with 3.44 mg (0.021 mmol) AIBN and 0.5071 g (4.481 mmol) *N*-isopropylacrylamide in 3.0 mL methanol at 60 °C for 10 h or 16 h. The polymer samples were characterized by DMF GPC.

Synthesis of poly(2-vinyl-4,4-dimethyl-5-oxazolone)-*b*-poly(*N*-isopropylacrylamide). The synthesis of the block copolymers was carried out using poly(2-vinyl-4,4-dimethyl-5-oxazolone) as a macromolecular chain transfer agent. The homopolymer was obtained by polymerizing 2.0 g (0.0144 mol) 2-vinyl-4,4-dimethyl-5-oxazolone, 9.80 mg (0.06 mmol) AIBN, and 143.58 mg (0.65 mmol) 1-cyanoisopropyl dithiobenzoate in 2.0 g (2.12 mL) benzene at 65 °C for 16 h. The block copolymers were synthesized by polymerizing 0.5062 g (4.473 mmol) *N*-isopropylacrylamide along with 0.0801 g (0.036 mmol, M_n = 2200 g/mol) of the oxazolone homopolymer as chain transfer agent and 0.637 mg ($3.88 \cdot 10^{-3}$ mmol) AIBN in 2 mL benzene at 65 °C. The polymerization time was 16 h or 22 h. The polymers were characterized by THF GPC.

Hydrolysis of the RAFT endgroups. The hydrolysis of the dithiocarbamate endgroups was carried out using MeOH / 28 % aq. NaOH (7:3) in the presence of a small amount of EDTA in order to prevent oxidation of the resultant polymeric thiol. The reaction was perfomed under nitrogen. After complete hydrolysis, the mixture was acidified with 88 % formic acid and subjected to ultrafiltration using PES-004H (nominal size exclusion 4 kDa, Celgard GmbH) membranes and methanol as an eluent. The hydrolytic cleavage of the dithiocarbamate endgroup was proven by UV spectroscopy.

Results and Discussion

Poly(*N*-hydroxysuccinimide methacrylate). The polydispersities of the produced polymers were unexpectedly high ($1.47 \leq M_w/M_n \leq 2.34$, **Table 1**). This indicates a rather uncontrolled polymerization and is ascribed to the high reactivity of the monomer. It could be shown that the chain transfer agents were not used up completely in the polymerization process. In agreement with this observation, the molecular weights obtained were much higher than the theoretical ones (24,500 and 24,200 g/mol instead of 3,100 and 2,700 g/mol for 1-cyanoisopropyl dithiobenzoate as chain transfer agent).

Sample	CTA	Polym. Time [h]	Conv. [%]	M_n [g/mol]	$M_{n,theor}$ [g/mol]	PDI
1a	1-cyanoisopropyl dithiobenzoate	16	81	24500	3100	1.52
1b		10	70	24200	2700	1.47
2a	benzyl 1-pyrrolecarbodithioate	16	89	43500	3100	2.11
2b		10	74	41300	2600	2.34
3a	cumyl 1-pyrrolecarbodithoate	16	83	24100	3100	1.71
3b		10	60	22400	2300	1.78

Table 1. Experimental data of the RAFT polymerization of *N*-hydroxysuccinimide methacrylate.

Poly(*N*-isopropylacrylamide). The polymer samples show low polydispersities and good agreement between the experimental and calculated molecular weights (**Table 2**). Best control of molecular weight and narrow molecular weight distributions are obtained with the benzyl CTA.

Poly(acrylic acid)-*b*-poly(*N*-isopropylacrylamide). The acrylic acid block contains 110 monomer units and the NIPAAm block consists of 82 and 64 monomer units, respectively. Block copolymers were obtained with quite low polydispersities of 1.09 and 1.11, respectively.

Poly(2-vinyl-4,4-dimethyl-5-oxazolone)-*b*-poly(*N*-isopropylacrylamide). The length of the oxazolone block is 16 monomer units and that of the NIPAAm block is 73 and 97 monomer units, respectively. The block copolymers have polydispersities of 1.22 and 1.14.

Sample	CTA	Polym. Time [min]	Conv. [%]	M_n [g/mol]	$M_{n,theor}$ [g/mol]	PDI
1a	benzyl 1-pyrrolecarbodithioate	319	60	5700	7000	1.17
1b		366	72	6500	8400	1.14
1c		478	84	8700	9800	1.14
1d		1128	97	11400	11200	1.13
2a	cumyl 1-pyrrolecarbodithioate	310	13	2700	1600	1.26
2b		362	39	6000	4200	1.21
2c		419	60	8800	6300	1.19
2d		462	71	10100	7300	1.15

Table 2. Experimental data of the RAFT polymerization of *N*-isopropylacrylamide.

Hydrolysis of the RAFT endgroups. The hydrolytic cleavage of the dithiocarbamate endgroup with aqueous NaOH is proven by UV spectroscopy (**Figure 1**): The dithiocarbamate moiety absorbs in the UV-vis region with λ_{max}=296 nm. After hydrolysis, only the thiol moiety is left, which does not absorb in the range measured.

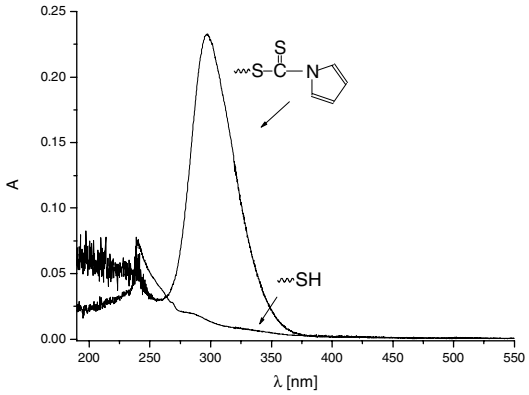


Figure 1. UV spectra of polyNIPAAm in methanol before and after hydrolysis of the dithiocarbamate endgroup.

Conclusions

RAFT polymerization is a powerful technique in the synthesis of low-polydispersity polymers with controlled structures. The presence of dithiocarbamate endgroups in the polymers allows for the conjugation of proteins or drugs after hydrolysis of the endgroups to the corresponding thiols. The conjugation can be achieved either via direct disulfide bonding with the aid of symmetric disulfides or via the use of spacer groups, such as bismaleimide. With the RAFT process, block copolymers containing active esters for conjugation to protein amino groups can be synthesized with controlled molecular weights and narrow polydispersities. Block copolymers consisting of poly(NIPAAm) and poly(acrylic acid) blocks are well suited for the creation of systems that respond to combined external stimuli, such as pH, ionic strength, and temperature.

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