

A Journal of the Gesellschaft Deutscher Chemiker A Deutscher Chemiker GDCh International Edition www.angewandte.org

Accepted Article

- Title: Hetero-Diels–Alder Cycloaddition with RAFT Polymers as Bioconjugation Platform
- Authors: Ana Beloqui, Shivshankar R Mane, Marcel Langer, Mathias Glassner, Dennis M Bauer, Ljiljana Fruk, Christopher Barner-Kowollik, and Guillaume Delaittre

This manuscript has been accepted after peer review and appears as an Accepted Article online prior to editing, proofing, and formal publication of the final Version of Record (VoR). This work is currently citable by using the Digital Object Identifier (DOI) given below. The VoR will be published online in Early View as soon as possible and may be different to this Accepted Article as a result of editing. Readers should obtain the VoR from the journal website shown below when it is published to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

To be cited as: Angew. Chem. Int. Ed. 10.1002/anie.202005747

Link to VoR: https://doi.org/10.1002/anie.202005747

WILEY-VCH

COMMUNICATION

WILEY-VCH

Hetero-Diels–Alder Cycloaddition with RAFT Polymers as Bioconjugation Platform

Ana Beloqui,^[a,b,c,d] Shivshankar R. Mane,^[a,b] Marcel Langer,^[b] Mathias Glassner,^[b] Dennis M. Bauer,^[e] Ljiljana Fruk,^[e,f] Christopher Barner-Kowollik,^{*[b,g,h]} and Guillaume Delaittre^{*[a,b,i]}

[a]	Dr. A. Beloqui, Dr. S. R. Mane, Prof. Dr. G. Delaittre
	Institute of Biological and Chemical Systems (IBCS)
	Karlsruhe Institute of Technology (KIT)
	Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany
[b]	Dr. A. Beloqui, Dr. S. R. Mane, Dr. M. Langer, Dr. M. Glassner, Prof. Dr. C. Barner-Kowollik, Prof. Dr. G. Delaittre
	Macromolecular Architectures
	Institute for Chemical Technology and Polymer Chemistry (ITCP)
	Karlsruhe Institute of Technology (KIT)
	Engesserstr. 18, 76131 Karlsruhe, Germany
[c]	Dr. A. Beloqui
	Department of Applied Chemistry (UPV/EHU)
	Avda. Manuel de Lardizabal 3, E-20018 Donostia - San Sebastian, Spain
[d]	Dr. A. Beloqui
	IKERBASQUE, Basque Foundation for Science
	Maria Diaz de Haro 3, E-48013 Bilbao, Spain
[e]	Dr. D. M: Bauer, Dr. L. Fruk
	Center for Functional Nanostructures (CFN)
	Karlsruhe Institute of Technology (KIT)
	Wolfgang-Gaede-Straße 1a, 76131 Karlsruhe, Germany
[f]	Dr. L. Fruk
	Department of Chemical Engineering and Biotechnology
[1	West Cambridge Site, Philippa Fawcett Drive, Cambridge CB3 UAS, United Kingdom
[9]	Prot. Dr. C. Barner-Nowollik
	Centre of Materials Science, Physics and Mechanical Engineering
	Queensiand Oniversity of rechnology (QUT)
[0]	Disballe, QLD 4000, Australia
[9]	School of Chemistry, Dhysics and Mechanical Engineering
	Ouensland University of Technology (OLIT)
	Brichane OI D 4000 Australia
	E-mail: Christopher barnerkowollik@gut edu au
ſil	Prof. Dr. G. Delaittre
1.1	Organic Functional Molecules. Organic Chemistry
	University of Wuppertal
	Gaußstrasse 20, 42119 Wuppertal, Germany
	E-mail: delaittre@uni-wuppertal.de

Supporting information for this article is given via a link at the end of the document.

Abstract: We introduce the bioconjugation of polymers synthesized by RAFT polymerization, and bearing no specific functional end group, by means of hetero-Diels–Alder cycloaddition through their inherent terminal thiocarbonylthio moiety with a diene-modified model protein. Quantitative conjugation occurs over the course of a few hours, at ambient temperature and neutral pH, and in the absence of any catalyst. Our technology platform affords thermoresponsive bioconjugates, whose aggregation is solely controlled by the polymer chains.

Proteins are essential compounds in modern medicine and biotechnology. However, their physicochemical characteristics brings about critical limitations, particularly in terms of solubility and stability.¹ One of the most important ways of addressing these issues relies on the attachment of synthetic polymer chains, in order to produce so-called protein–polymer conjugates (PPCs).^{2–6} The pioneering and so

far most employed polymer for PPCs is polyethylene glycol (PEG). Yet, utilizing other polymers than PEG gives access to a wider range of properties and may elude some shortcomings of PEG,7-12 notably its immunogenicity.13,14 In this context, reversible addition-fragmentation transfer (RAFT) polymerization is one the most powerful synthetic techniques to access macromolecules with defined chain length and (endgroup) functionality.^{15–17} One of the methods to achieve RAFT-based PPCs involves reacting one end of the synthetic polymer with one or several residues on the protein surface. The reactive end of the RAFT polymer is typically introduced through the reinitiating fragment - the so-called R group - of a specifically designed chain transfer agent (CTA).¹² The cycloaddition (RAFT-HDA) RAFT-hetero-Diels-Alder emerged about a decade ago as a complementary and highly efficient method for chain-end conjugation of RAFT polymers without the need for introducing functional R groups.^{18–22}.

WILEY-VCH

COMMUNICATION



Scheme 1. Synthesis of bovin serum albumin polymer conjugates by RAFT-HDA, as described in the current contribution.

RAFT-HDA relies on RAFT agents possessing a C=S double bond with a specifically adjusted electron deficiency. The latter should be sufficiently high to enable HDA with a range of dienes, yet not too high in order for a well-controlled RAFT polymerization to take place. While RAFT-HDA in organic solvents requires heat, catalysts, or highly active diene partners (e.g., cyclopentadiene or 0quinodimethanes),18-22 we have previously demonstrated that a fast RAFT-HDA is achieved in aqueous solutions simply by mixing the components at ambient temperature and in the absence of a catalyst, even with less reactive dienes.²³ Such mild conditions seem ideal for the functionalization of proteins, which are generally sensitive to heat or additives. Importantly, most if not all cycloadditions are biorthogonal, thus offer an ideal platform to specifically conjugate polymers to biomolecules.²⁴ In the present contribution, we report the first protein-polymer conjugates obtained through the RAFT-HDA pathway (Scheme 1).

A range of water-soluble acrylic polymers based on ethylene glycol side chains were first synthesized: (i) homopolymers of triethylene glycol methyl ether acrylate (PmTEGA) and (ii) copolymers of diethylene glycol ethyl ether acrylate and oligoethylene glycol methyl ether acrylate (P(eDEGA-*co*-mOEGA)). PmTEGA is water-soluble in the useful temperature range of PPCs (< 55–70 °C)^{25–28} and may impart a stealth character and improved solubility to proteins. P(eDEGA-*co*-mOEGA)s exhibit a lower critical solution temperature (LCST), which depends on the exact comonomer ratio,^{29,30} and will lead to thermoresponsive PPCs at possibly useful temperatures. Responsive PPCs^{31,32} are particularly interesting for control of biomolecular activity,33 triggered reversible self-assembly into biohybrid nanostructures³⁴⁻³⁸ and fast removal/recovery of proteins from solution.^{39,40} Interestingly, similar polymers with short oligoethylene glycol side chains were shown not to exhibit the non-desired antigenicity of PEG.41 2-cyanoprop-2-yl diethoxyphosphoryldithioformate (CPDPDT) was employed as CTA because it simultaneously enables the controlled polymerization of acrylates and the synthesis of polymers with a terminal C=S bond sufficiently electron-deficient for a rapid HDA cycloaddition to occur.23 As seen in Figure 1, PmTEGAs with number-average molar masses M_n of 2000 and 6000 g mol^{-1} and narrow dispersities (D = 1.1-1.2) were obtained (noted as PmTEGA2000 and PmTEGA6000, respectively). Similarly, P(eDEGA-co-mOEGA)s CoP15000 and CoP18000, with $M_{\rm p}$ of 15000 and 18000 g mol⁻¹ (D = 1.3), respectively, were produced by **CPDPDT**-mediated RAFT copolymerization. All polymers displayed the classic maximum of absorption at 327 nm (Figure S8), characteristic of the π π* transition in the diethoxyphosphoryldithioformate end group.

For conjugation reactions not based on natural amino acids, reactive proteins can be obtained either by genetic engineering⁴² or simple post-translational chemical



Figure 1. (Top) Synthetic route for oligoethylene glycol-based polyacrylates by RAFT polymerization in the presence of 2-cyanoprop-2-yl diethoxyphosphoryldithioformate (CPDPDT). (*i*) mTEGA, AIBN, ethanol, 60 °C. (*ii*) eDEGA:mOEGA 80/20 mol/mol, AIBN, ethanol, 60 °C. (Bottom) Corresponding SEC traces.

scepted

WILEY-VCH

COMMUNICATION



Figure 2. Coomassie-stained SDS-PAGE gels of BSA conjugates obtained by RAFT-HDA with **PmTEGA6000** (up) and corresponding electrophoretograms (bottom). (A) Variation of the [**PmTEGA6000**]:[**dBSA**] ratio for a fixed reaction time of 12 h: 25 (c), 50 (d), 80 (e), and 100 (f). (B) Variation of the reaction time for a fixed [**PmTEGA6000**]:[**dBSA**] ratio of 100: 1 h (g), 2 h (h), 4 h (i), 6 h (j), and 8 h (k). Molecular weight protein ladder (a) and control sample (b, **dBSA**) are added as references.

modification.⁴³ Here, we have chosen the latter for its ease of implementation. To introduce diene moieties, the difunctional linker 2,5-dioxopyrrolidin-1-yl (hexa-2,4-dien-1-yl)succinate **DSS** (see Supporting Information), consisting of a sorbyl group and a succinimidyl ester on either side, was readily synthesized in two steps and reacted with the lysine residues of the model protein bovin serum albumin (BSA). The diene-functionalized BSA (**dBSA**) remained fully soluble in aqueous medium and did not show any significant change in circular dichroism (CD) (Figure S14). Mass spectrometry analysis



Figure 3. Number-based hydrodynamic diameter distributions for BSA conjugates obtained by RAFT-HDA with P(eDEGA-*co*-mOEGA) copolymers CoP15000 and CoP18000. dBSA is shown as reference.

showed the incorporation of an average of 6-7 diene tags per protein molecule, that is, x = 6-7 in Scheme 1 (Figure S10).

Protein-polymer conjugation was performed by simple incubation of dBSA with the RAFT polymers in aqueous buffers, in the absence of catalyst and at room temperature. During initial experiments, it was observed that the characteristic purple color of the RAFT polymer stock solutions in aqueous media faded with time. It is known that RAFT end groups are sensitive to a range of reagents, mostly primary amines and basic conditions⁴⁴ and that discoloration of RAFT polymers implies end-group loss. For this reason, we monitored this phenomenon by UV-Vis spectroscopic measurements of PmTEGA6000 solutions in various aqueous buffers. As expected, basic conditions led to the fastest decrease in absorption at 327 nm and consequently the fastest deactivation (Figure S11). Particularly, incubation in bicarbonate buffer at pH 9.15 or Tris buffer at pH 8.1 led to instantaneous full degradation. Phosphate buffer at the same pH was less damaging. Decreasing pH further led to slower degradation. Eventually, significantly sodium phosphate buffer (50 mM at pH 6.0) was chosen for bioconjugation as it offered the best compromise between the slow degradation and close-to-neutral conditions. Before protein-polymer conjugation was addressed, model HDA conjugation reactions with DSS were performed. It was observed that absorption at 327 nm decreased significantly faster in reaction mixtures of PmTEGA6000 and DSS compared to the polymer alone (Figure S12). Higher amounts of DSS (2 and 3 equivalents) led to faster disappearance of the characteristic RAFT moiety absorption, a clear sign of the HDA reaction occurring at the C=S double bond.²³

A range of conditions were assessed in order to determine the optimum conditions for polymer bioconjugation. Mixtures with various PmTEGA6000:dBSA molar ratios were prepared in sodium phosphate buffer at pH 6.0 for overnight reactions. As observed in gels obtained by SDS-PAGE and the corresponding intensity plots (Figure 2A), higher polymer:protein ratios generated species with higher molar masses. With 80 eq. and above, conjugates with an average of 6 to 7 grafted polymer chains were obtained, as deduced from the \approx 40 kDa shift. It can thus be assumed that in these conditions all accessible diene groups within the protein have been coupled via HDA reaction. Moreover, the kinetics of the reaction was monitored using a 100-fold molar excess of polymer (Figure 2B). The reaction was arrested at various incubation times by removing the non-reacted PmTEGA6000 by size-exclusion centrifugation. We observed that the conjugation of the first two polymer chains to dBSA occurred within just 1-2 h, with the fully conjugated protein obtained after 6 h. The first easily reachable diene moities might react fast, while the attachment of further polymer chains is certainly slowed down for more buried dienes and due the increasing steric constraints imposed by already grafted polymer chains. Moreover. the successful preparation of dBSA-PmTEGA2000 and dBSA-PmTEGA6000 conjugates was also confirmed by a shift in the hydrodynamic diameter distribution compared to that of the free protein, as measured by dynamic light scattering (DLS) (Figure S13). Again, the protein secondary structure was not affected by the modification, as demonstrated by CD (Figure S14). Albeit not an enzyme, BSA possesses an esterase activity that can be

WILEY-VCH

COMMUNICATION



Figure 4. (A) Evolution of the turbidity measured at 670 nm with temperature for aqueous solutions of **CoP15000** and **CoP18000** (15 μ M in PBS). (B) Evolution of number-average hydrodynamic diameters of **dBSA-CoP15000** and **dBSA-CoP18000** conjugates with temperature as measured by DLS. *In both cases, measurements were carried out with 1 °C increments and a stabilitization time of 5 min before acquisition.*

exploited to further assess modifications. In a colorimetric glyceryl acetate-based esterase assay (Figure S16), no significant difference between BSA and **dBSA-PmTEGA6000** were observed, which confirms the conservation of the protein structure and stability.

The conjugation of functional polymers to proteins leads to PPCs with specific properties. Here, the RAFT-HDA conjugation was carried out with the thermoresponsive P(eDEGA-co-mOEGA) copolymers **CoP15000** and **CoP18000** (see Figure 1). DLS measurements revealed a significant increase of the average hydrodynamic diameter from 6.5 ± 0.8 nm for **dBSA** to 7.9 ± 1.2 and 9.1 ± 1.5 nm after the conjugation reaction with **CoP15000** and **CoP18000**, respectively (Figure 3), confirming successful conjugation. As for **PmTEGA6000**, CD measurements revealed no alteration in the secondary structure of BSA through the grafting of **CoP15000** and **CoP18000** (Figure S15).

The thermoresponsive behavior of the newly generated BSA conjugates was subsequently evaluated. P(eDEGA-*co*-mOEGA) copolymers typically exhibit a tunable thermoresponsive behavior over the 25–75 °C range, depending on their comonomer composition and molar mass.^{29,30} The thermal transition is readily detectable via an increase in the turbidity of the sample, which can be measured within a UV-Vis spectrophotometer (Figure 4A). We applied a

heating ramp from 40 to 55 °C to aqueous solutions of **CoP15000** and **CoP18000** polymers and monitored the absorbance at 670 nm, a wavelength at which no interference with possible chromophores may occur. We observed that while **CoP18000** showed turbidity at temperatures higher than 45 °C, the turbidity of **CoP15000** sample was detected only above 48 °C. We measured cloud points (temperature at 50% of the maximal absorbance) of 49.5 and 46.5 °C for **CoP15000** and **CoP18000** polymers, respectively.

Finally, we assessed how the thermoresponsive properties of P(eDEGA-co-mOEGA) copolymers transferred to their corresponding BSA conjugates. Note that BSA was previously shown to be stable in the considered temperature range (*i.e.*, below 60 °C)^[45,46] and that one could in any case modulate the transition temperatures by varying the compositions of the copolymers. As the turbidity of the media implied the temperature-induced aggregation of the polymeric components, the overall size of the PPCs is expected to increase at temperatures higher than LCST, with the protein component stabilizing the aggregates.³⁴ Purified PPCs were thus subjected to a heating ramp and the hydrodynamic diameter of the particles was simultaneously measured. As shown in Figure 4B, the average hydrodynamic diameter increased from 7.9 \pm 1.2 and 9.1 \pm 1.5 nm at 40 °C to 69 \pm 5 and 77 ± 4 nm at 55 °C for dBSA-CoP15000 and dBSA-**CoP18000** conjugates, respectively. Interestingly, the onsets of aggregation of the PPCs match well those measured for the polymers alone, and the cloud points only slightly decrease: 48.6 and 45.8 °C for dBSA-CoP15000 and dBSA-CoP18000. respectively, versus 49.5 and 46.5 °C for their corresponding free polymers. The polymers are physically bound to the protein counterpart and the protein does not seem to significantly interfere in the temperature-induced physical aggregation of the polymers.

In conclusion, we introduce the application of the RAFT-HDA chemistry for catalyst-free protein-polymer conjugation under mild conditions (ambient temperature, neutral pH). Using this method, we have decorated the surface of BSA with up to 7 hydrophilic polymer chains, as well as with thermoresponsive polymers. The reaction of the dienefunctionalized protein with the **RAFT-derived** diethoxyphosphoryldithioester polymer is relatively fast, pHdependent, and can be monitored by UV-Vis spectroscopy and SDS-PAGE. Finally, we demonstrate that the thermoresponsive properties of the polymers are transferred to the protein-polymer conjugates, showing similar phaseseparation temperatures. The current procedure entails preconditioning of the protein through covalent anchoring of reactive diene tags. Further control of the bioconjugation degree and location is certainly achievable by the introduction of genetically encoded unnatural aminoacid containing the diene group.42,45

Acknowledgements

A. B. thanks the Spanish Research Agency (AEI) for the funds within Ramón y Cajal program (RYC2018-025923-I) and Retos call (PID2019-110239RB-I00). C.B.-K. acknowledges funding by the Australian Research Council (ARC) in the context of a Laureate Fellowship and continued key support

10.1002/anie.202005747

COMMUNICATION

from the Queensland University of Technology (QUT). G. D. thanks the German Federal Ministry of Education and Research (BMBF, Grant No. 031A095C) for funding (Molecular Interaction Engineering program, Biotechnologie 2020+). The Levkin lab (IBCS, KIT) is thanked for access to DLS. Frank Kirschhöfer, Boris Kühl, and Dr Gerald Brenner-Weiss (IFG, KIT) are acknowledged for providing access to and support on MALDI-ToF spectrometry.

Conflict of interest

The authors declare no conflict of interest.

Keywords: Diels–Alder cycloaddition • polymer • protein • end group • bioconjugation

- [1] M. C. Manning, D. K. Chou, B. M. Murphy, R. W. Payne, D. S. Katayama, *Pharm. Res.* 2010, 27, 544–575.
- [2] B. Le Droumaguet, J. Nicolas, Polym. Chem. 2010, 1, 563-598.
- [3] M. A. Gauthier, H.-A. Klok, *Polym. Chem.* **2010**, *1*, 1352–1373.
- [4] J. H. Ko, H. D. Maynard, Chem. Soc. Rev. 2018, 47, 8998-9014.
- [5] A. J. Russell, S. L. Baker, C. M. Colina, C. A. Figg, J. L. Kaar, K. Matyjaszewski, A. Simakova, B. S. Sumerlin, *AIChE J.* **2018**, *64*, 3230– 3245.
- [6] T. A. Wright, R. C. Page, D. Konkolewicz, Polym. Chem. 2019, 10, 434– 454.
- [7] K. Knop, R. Hoogenboom, D. Fischer, U. S. Schubert, Angew. Chemie Int. Ed. 2010, 49, 6288–6308.
- [8] M. Barz, R. Luxenhofer, R. Zentel, M. J. Vicent, Polym. Chem. 2011, 2, 1900–1918.
- [9] E. M. Pelegri-ODay, E.-W. Lin, H. D. Maynard, J. Am. Chem. Soc. 2014, 136, 14323–14332.
- [10] Y. Qi, A. Chilkoti, Curr. Opin. Chem. Biol. 2015, 28, 181-193.
- [11] T. T. Hoang Thi, E. H. Pilkington, D. H. Nguyen, J. S. Lee, K. D. Park, N. P. Truong, *Polymers (Basel)*. **2020**, *12*, 298.
- [12] J. Morgenstern, G. Gil Alvaradejo, N. Bluthardt, A. Beloqui, G. Delaittre, J. Hubbuch, *Biomacromolecules* 2018, 19, 4250–4262.
- [13] R. P. Garay, R. El-Gewely, J. K. Armstrong, G. Garratty, P. Richette, *Expert Opin. Drug Deliv.* 2012, 9, 1319–1323.
- [14] H. Schellekens, W. E. Hennink, V. Brinks, Pharm. Res. 2013, 30, 1729– 1734.
- [15] C. Barner-Kowollik, Handbook of RAFT Polymerization, Wiley-VCH, 2008.
- [16] S. Perrier, *Macromolecules* **2017**, *50*, 7433–7447.
- [17] G. Moad, E. Rizzardo, S. H. Thang, Chem. An Asian J. 2013, 8, 1634– 1644.
- [18] S. Sinnwell, A. J. Inglis, T. P. Davis, M. H. Stenzel, C. Barner-Kowollik, Chem. Commun. 2008, 2052–2054.
- [19] A. J. Inglis, S. Sinnwell, M. H. Stenzel, C. Barner-Kowollik, Angew. Chemie - Int. Ed. 2009, 48, 2411–2414.
- [20] J. P. Blinco, V. Trouillet, M. Bruns, P. Gerstel, H. Gliemann, C. Barner-Kowollik, Adv. Mater. 2011, 23, 4435–4439.
- [21] M. Glassner, J. P. Blinco, C. Barner-Kowollik, Polym. Chem. 2011, 2, 83– 87.
- [22] K. K. Oehlenschlaeger, J. O. Mueller, N. B. Heine, M. Glassner, N. K. Guimard, G. Delaittre, F. G. Schmidt, C. Barner-Kowollik, *Angew. Chemie* - *Int. Ed.* 2013, 52, 762–766.
- [23] M. Glassner, G. Delaittre, M. Kaupp, J. P. Blinco, C. Barner-Kowollik, J. Am. Chem. Soc. 2012, 134, 7274–7277.
- [24] G. Delaittre, N. K. Guimard, C. Barner-Kowollik, Acc. Chem. Res. 2015, 48, 1296–1307.
- [25] F. Hua, X. Jiang, D. Li, B. Zhao, J. Polym. Sci. Part A Polym. Chem. 2006, 44, 2454–2467.
- [26] O. Grimm, S. C. Maßmann, F. H. Schacher, *Polym. Chem.* 2019, 10, 2674–2685.

- [27] G. Vancoillie, D. Frank, R. Hoogenboom, Prog. Polym. Sci. 2014, 39, 1074–1095.
- [28] N. Badi, Prog. Polym. Sci. 2017, 66, 54-79.
- [29] K. Skrabania, J. Kristen, A. Laschewsky, Ö. Akdemir, A. Hoth, J. F. Lutz, Langmuir 2007, 23, 84–93.
- [30] C. Boyer, M. R. Whittaker, M. Luzon, T. P. Davis, *Macromolecules* 2009, 42, 6917–6926.
- [31] I. Cobo, M. Li, B. S. Sumerlin, S. Perrier, Nat. Mater. 2015, 14, 143–159.
- [32] B. Trzebicka, R. Szweda, D. Kosowski, D. Szweda, Ł. Otulakowski, E. Haladjova, A. Dworak, *Prog. Polym. Sci.* 2017, 68, 35–76.
- [33] T. Shimoboji, E. Larenas, T. Fowler, A. S. Hoffman, P. S. Stayton, *Bioconjug. Chem.* 2003, 14, 517–525.
- [34] C. Lavigueur, J. G. García, L. Hendriks, R. Hoogenboom, J. J. L. M. Cornelissen, R. J. M. Nolte, *Polym. Chem.* 2011, *2*, 333–340.
- [35] H. Li, M. Li, X. Yu, A. P. Bapat, B. S. Sumerlin, *Polym. Chem.* 2011, 2, 1531.
- [36] C. K. Wong, A. J. Laos, A. H. Soeriyadi, J. Wiedenmann, P. M. G. Curmi, J. J. Gooding, C. P. Marquis, M. H. Stenzel, P. Thordarson, *Angew. Chemie* **2015**, 127, 5407–5412.
- [37] D. Moatsou, J. Li, A. Ranji, A. Pitto-Barry, I. Ntai, M. C. Jewett, R. K. O'Reilly, *Bioconjug. Chem.* **2015**, *26*, 1890–1899.
- [38] L. Jiang, J. S. Bonde, L. Ye, *Macromol. Chem. Phys.* 2018, 219, 1700597.
- [39] I. Mukherjee, S. K. Sinha, S. Datta, P. De, *Biomacromolecules* 2018, 19, 2286–2293.
- [40] Y. Wu, Z. Cai, S. Wu, W. Xiong, S. Ma, Biopolymers 2018, 109, e23222.
- [41] Y. Qi, A. Simakova, N. J. Ganson, X. Li, K. M. Luginbuhl, I. Ozer, W. Liu, M. S. Hershfield, K. Matyjaszewski, A. Chilkoti, *Nat. Biomed. Eng.* 2016, 1, 2.
- [42] J. W. Chin, Nature 2017, 550, 53-60.
- [43] E. Baslé, N. Joubert, M. Pucheault, Chem. Biol. 2010, 17, 213–227.
- [44] A. B. Lowe, C. L. McCormick, in *Handb. RAFT Polym.*, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, **2008**, pp. 235–284.
- [45] A. Michnik, J. Therm. Anal. Calorim. 2003, 71, 509-519.
- [46] V. A. Borzova, K. A. Markossian, N. A. Chebotareva, S. Y. Kleymenov, N. B. Poliansky, K. O. Muranov, V. A. Stein-Margolina, V. V Shubin, D. I. Markov, B. I. Kurganov, *PLoS One* **2016**, *11*, e0153495.
- [47] A. H. St. Amant, F. Huang, J. Lin, K. Rickert, V. Oganesyan, D. Lemen, S. Mao, J. Harper, M. Marelli, H. Wu, et al., *Angew. Chem. Int. Ed.* **2019**, *58*, 8489–8493.