JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

pubs.acs.org/JACS

A Fluorescently Labeled Dendronized Polymer–Enzyme Conjugate Carrying Multiple Copies of Two Different Types of Active Enzymes

Andrea Grotzky,[†] Thomas Nauser,[‡] Huriye Erdogan,[‡] A. Dieter Schlüter,[†] and Peter Walde*[,†]

[†]Laboratory of Polymer Chemistry, Department of Materials and [‡]Laboratory of Inorganic Chemistry, Department of Chemistry and Applied Biosciences, ETH Zürich, Wolfgang-Pauli-Strasse 10, 8093 Zürich, Switzerland

Supporting Information

ABSTRACT: A hybrid structure of a synthetic dendronized polymer, two different types of enzymes (superoxide dismutase and horseradish peroxidase), and a fluorescent dye (fluorescein) was synthesized. Thereby, a single polymer chain carried multiple copies of the two enzymes and the fluorescein. The entire attachment chemistry is based on UV/vis-quantifiable bis-aryl hydrazone bond formation that allows direct quantification of bound molecules: 60 superoxide dismutase, 120 horseradish peroxidase, and 20 fluorescein molecules on an average polymer chain of 2000 repeating units. To obtain other enzyme ratios the experimental conditions were altered accordingly. Moreover, it could be shown that both enzymes remained fully active and catalyzed a two-step cascade reaction.

Polymer–protein conjugates are hybrids of polymers and proteins.[1−4] If synthetic polymers are linked with proteins, a macromolecular structure is created which typically has properties that are characteristic of both, the synthetic polymer as well as the protein. Depending on the chemical structure of the polymer and on the available functional groups on the surface of the protein, there are a number of different ways such conjugates can be prepared: by linking a presynthesized polymer with a protein or by growing a polymer chain from a protein macroinitiator.[5−7] In any case, to maintain the three-dimensional structure and therefore the overall properties of the protein intact, harsh reaction conditions during conjugate formation must be avoided. Furthermore, if the protein is an *enzyme*, retention of enzymatic activity is of particular importance.[8] Currently, polymer–enzyme conjugates are prepared with various aims, e.g. (i) for stabilizing the enzyme by attaching one or several polymer chains to the enzyme,[9,10] (ii) for making the enzyme soluble in a medium in which the native enzyme is not soluble,[11] and (iii) for immobilizing enzymes within a polymeric network or for binding enzymes to solid surfaces.[12]

The aim of the work presented was to prepare a water-soluble polymer–enzyme conjugate that contains multiple copies of two different types of enzymes along the polymer chain. The polymer serves as a macromolecular linking unit for bringing the two types of enzymes in close proximity. After binding to the polymer the enzymes should remain catalytically active to catalyze two steps of a cascade reaction. Such conjugates may be useful for the localization of enzyme-catalyzed multistep reactions in *in vitro* systems,[13] e.g. in vesicles. Entrapment of different types of enzymes at a defined ratio within one and the same vesicle is for statistical reasons difficult to obtain.[14] With the conjugate, however, this is possible.

The polymer used is the polycationic, water-soluble, first-generation dendronized polymer *de*-PG1$_{2000}$ (number average of repeating units (r.u.) ≈ 2000) that has a vast number of terminal amino groups (two per r.u.); see Figure 1.[15] These amino groups are protonated below pH ≈ 9 and help solubilize the polymer in aqueous media. The two enzymes superoxide dismutase (SOD, EC 1.15.1.1) from bovine erythrocytes and horseradish peroxidase (HRP, EC 1.11.1.7) were attached to the polymer *via* bis-aryl hydrazone (BAH) linkages that were formed upon reaction of a polymer-bound aryl hydrazine with a benzaldehyde moiety on the enzyme.[16,17] Successful bond formation was monitored by recording time-dependent UV/vis absorption spectra during conjugate formation since the newly formed bond has a characteristic absorption band centered around λ = 354 nm ($\varepsilon_{354\,nm}$ = 29 000 M^{-1} cm^{-1}).[17] It allows the direct quantification of the number of bound enzymes.[16] For quantification of the polymer by fluorescence spectroscopy and for possible imaging of the conjugate by fluorescence microscopy, fluorescein was also attached to the polymer; again *via* the same type of BAH bonds.

The conjugate *de*-PG1$_{2000}$-BAH-(FL,HRP,SOD) was prepared as follows (for experimental details, see Supporting Information (SI)). First, some of the amino groups at the periphery of *de*-PG1$_{2000}$ were modified with succinimidyl 6-hydrazinonicotinate acetone hydrazone (S-HyNic) at pH 7.6 to yield *de*-PG1$_{2000}$-HyNic. The modified polymer had on average 22 HyNic moieties per 100 repeating units; i.e. 11% of all available amino groups of the polymer were modified. In separate reaction vessels, some of the accessible lysine residues of SOD and HRP were modified with succinimidyl 4-formylbenzoate (S-4FB) at pH 7.2, yielding SOD-4FB and HRP-4FB. To avoid cross-linking during hybrid formation the ratio of 4FB moieties to enzyme was kept below 1. The experimental conditions were chosen such that on average 90% of the SOD molecules and 85% of the HRP molecules contained one 4FB moiety. This means that after enzyme modification the solution contained a mixture of enzyme molecules carrying one bound 4FB unit as well as unmodified enzymes. The presence of enzyme molecules carrying two or even more 4FB units was possible, but their amount is expected to be negligibly low, as demonstrated in the case of HRP by nanoESI-MS.[18]

Received: May 18, 2012
Published: June 28, 2012

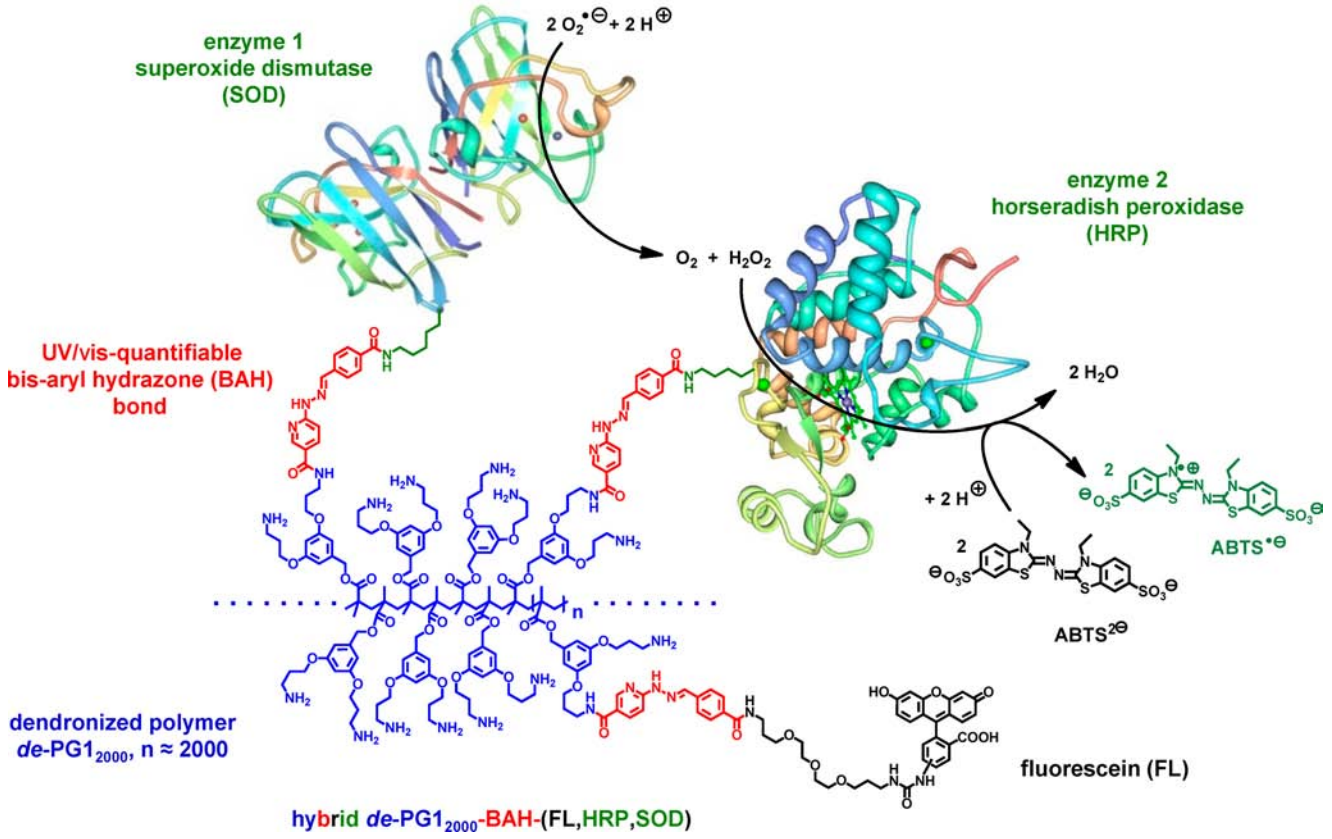

Figure 1. Schematic representation of the first generation dendronized polymer−enzyme hybrid (*de*-PG1$_{2000}$-BAH-(FL,HRP,SOD)) showing the chemical structure of the dendronized polymer (*de*-PG1$_{2000}$) with its polymethacrylate backbone and its dendritic side chains with terminal amino groups which are protonated below pH ≈ 9. Each polymer chain carries a number of fluorescent dyes (fluorescein, FL) and more importantly two different types of the enzymes superoxide dismutase (SOD, enzyme 1, PDB code: 1CB4) and horseradish peroxidase (HRP, enzyme 2, PDB code: 1W4Y) that may undergo a cascade reaction. All molecules are bound to the polymer *via* stable, UV/vis-quantifiable bis-aryl hydrazone (BAH) bonds. Also shown is the enzymatic cascade reaction whereby two superoxide radicals are disproportionated by SOD into hydrogen peroxide and dioxygen, followed by oxidation of HRP by hydrogen peroxide with a subsequent oxidation of two substrate molecules $ABTS^{2-}$ to give two $ABTS^{\bullet-}$ radicals which are detectable spectrophotometrically. Illustrated here is an idealized enzymatic cascade reaction that occurs with two enzymes in close proximity on the same polymer chain.

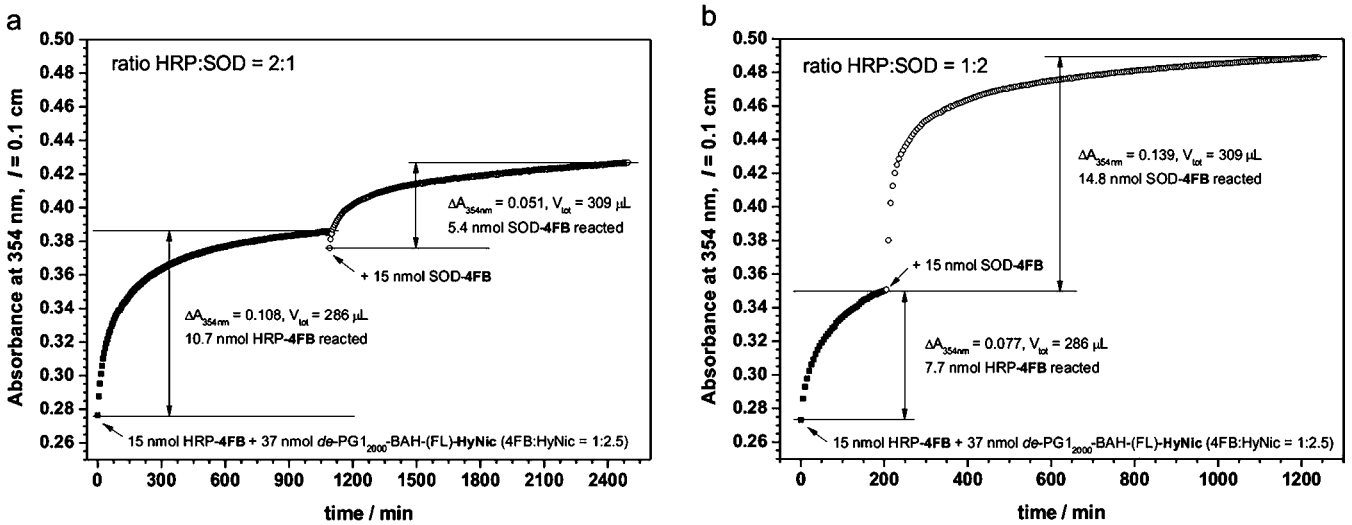

Figure 2. Following the bis-aryl hydrazone bond (BAH) formation with UV/vis-spectroscopy at λ = 354 nm during the conjugation reaction of *de*-PG1$_{2000}$-BAH-(FL)-HyNic with HRP-4FB and SOD-4FB: *de*-PG1$_{2000}$-BAH-(FL)-HyNic was first reacted with HRP-4FB, then SOD-4FB was added after (a) 18 h or (b) 3.5 h to give the desired conjugate *de*-PG1$_{2000}$-BAH-(FL,HRP,SOD) with a ratio of HRP/SOD of (a) 2:1 or (b) 1:2.

The actual conjugation reactions were then carried out at pH 4.7 consecutively. In the *first step*, *de*-PG1$_{2000}$-HyNic was reacted with a 4FB derivative of fluorescein, abbreviated as FL-4FB, to obtain *de*-PG1$_{2000}$-BAH-(FL)-HyNic with on average 1 fluo-

dx.doi.org/10.1021/ja304837f | J. Am. Chem. Soc. 2012, 134, 11392−11395

rescein molecule per 100 repeating units. The reaction was followed by recording the increase in absorbance at λ = 354 nm due to the formation of BAH bonds. The reaction was quantitative in the sense that, after a reaction time of 4 h, all initially added FL-4FB molecules had reacted (see SI).

In the *second step*, *de*-PG1$_{2000}$-BAH-(FL)-HyNic was incubated with HRP-4FB, again at pH 4.7, to yield *de*-PG1$_{2000}$-BAH-(FL,HRP)-HyNic, a fluorescently labeled polymer containing on average 6 HRP molecules per 100 repeating units. The progress of the reaction is shown in Figure 2a. After 18 h, the reaction between HRP-4FB and the HyNic linkers on the polymer slowed down dramatically; the increase in absorbance at λ = 354 nm leveled off although there were still available HRP-4FB molecules as well as reactive HyNic groups on the polymer (for control measurements, see SI). This observation, together with the control experiments and the fact that 100 r.u. carried on average 6 HRP molecules, showed that, after an 18 h reaction time, there was no free polymer *de*-PG1$_{2000}$-BAH-(FL)-HyNic, i.e. the polymer that did not carry HRP. In the *third step*, a solution of SOD-4FB was added to the reaction mixture which led to a renewed increase in absorbance at λ = 354 nm, indicating the formation of BAH bonds between SOD-4FB and the polymer carrying HRP and fluorescein. After another 24 h reaction time the desired conjugate *de*-PG1$_{2000}$-BAH-(FL,HRP,SOD) was obtained with 1 FL, 6 HRP, and 3 SOD molecules bound to 100 repeating units; the ratio of HRP/SOD was 2:1. An average polymer chain of 2000 r.u. (corresponding to a fully stretched polymer chain of ≈500 nm) had about 20 FL, 120 HRP, and 60 SOD molecules. Figure 2b shows the conjugation reaction where SOD-4FB was added after a reaction time of 3.5 h, instead of 18 h. In this case, the obtained hybrid contained approximately 1 FL, 4 HRP, and 8 SOD molecules per 100 repeating units, i.e. a ratio of HRP/SOD of 1:2. Thus, the ratio of the two polymer-bound enzymes could be controlled. Here, for detection reasons the ratio of HRP/SOD of 2:1 was best and used for all further experiments (see SI).

Removal of HRP, SOD, and unreacted HRP-4FB and SOD-4FB could be achieved easily by repetitive ultrafiltration. Figure 3 shows the size exclusion chromatograms of the unpurified and purified conjugate upon loading onto a Sepharose CL-6B column. In the latter case, the absence of free enzymes was clearly proven.

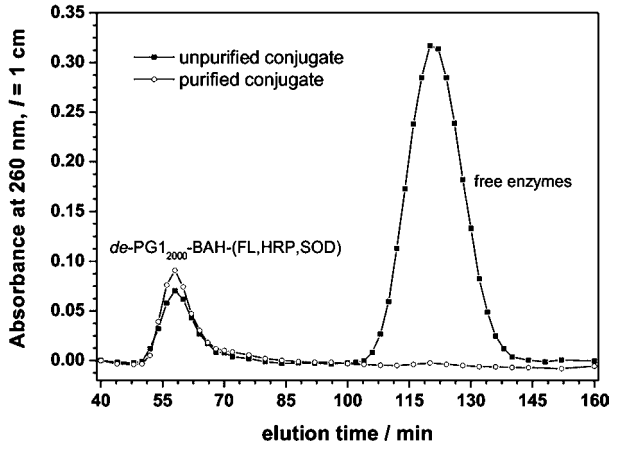

Figure 3. Size exclusion chromatography on Sepharose CL-6B: Elution diagram of the conjugate *de*-PG1$_{2000}$-BAH-(FL,HRP,SOD) before and after repetitive ultrafiltration (M_{cutoff} = 100 kDa).

The conjugate still contained ≈50% HyNic units; however, they could not react any further, neither with enzyme-4FB nor with 4-nitrobenzaldehyde. After ultrafiltration the actual concentrations of both enzymes in *de*-PG1$_{2000}$-BAH-(FL,HRP,SOD) were quantified by fluorescence spectroscopy with the help of polymer-bound fluorescein (see SI).

To test whether the polymer-bound enzymes were still active, enzymatic activity measurements were carried out. The enzymes' activities were investigated, both separately and together. The activity of bound HRP was determined with H_2O_2 as the oxidant and $ABTS^{2-}$ as the oxidizable substrate.[18] In a Michaelis–Menten type experiment the $ABTS^{2-}$ concentration was varied from 0.05 to 2 mM while the concentration of H_2O_2 was kept constant at 0.2 mM. The oxidation of $ABTS^{2-}$ to $ABTS^{\bullet-}$ was followed at λ = 414 nm for 1 min, and initial reaction rates were determined and plotted as a function of the starting concentration of $ABTS^{2-}$ (see SI). It could be shown that there was no difference between the free and polymer-bound enzyme; adding polymer to free HRP also had no effect on the activity. Thus, the catalytic activity of HRP in *de*-PG1$_{2000}$-BAH-(FL,HRP,SOD) could be *fully* preserved.

To demonstrate that the polymer-bound SOD was still active, an aqueous solution of the conjugate *de*-PG1$_{2000}$-BAH-(FL,HRP,SOD) was subjected to pulse radiolysis, i.e. a short pulse of high energy electrons that produced known amounts of superoxide radical anions ($O_2^{\bullet-}$) from water.[19] The formation and subsequent decay of $O_2^{\bullet-}$ was monitored at λ = 280 nm, the intensity being proportional to the amount produced which in turn is dependent on the dose of the applied pulse. SOD catalyzes the disproportionation (dismutation) of superoxide radicals into dioxygen and hydrogen peroxide. Taking into account the concentration of SOD, k_{cat} for SOD in the conjugate could be evaluated and compared with k_{cat} determined for the native enzyme. The values were $(1.5 \pm 0.2) \times 10^9$ M^{-1} s^{-1} for *de*-PG1$_{2000}$-BAH-(FL,HRP,SOD) and $(1.4 \pm 0.1) \times 10^9$ M^{-1} s^{-1} for native SOD. As a result, polymer-bound SOD remained *fully* active. Rate constants for native SOD previously reported vary in the range $(0.7–2.3) \times 10^9$ M^{-1} s^{-1} as the reaction is highly dependent on ionic strength.[19]

The enzymatic cascade reaction involving the conjugate *de*-PG1$_{2000}$-BAH-(FL,HRP,SOD) was measured using pulse radiolysis and by following the $ABTS^{\bullet-}$ formation spectrophotometrically. Thereby, SOD catalyzes the dismutation of 2 mol of $O_2^{\bullet-}$ into each 1 mol of O_2 and H_2O_2. The produced H_2O_2 oxidizes HRP which in turn is able to oxidize 2 mol of $ABTS^{2-}$ to $ABTS^{\bullet-}$; the stoichiometry of $O_2^{\bullet-}$ to $ABTS^{\bullet-}$ is therefore 1:1. Figure 4 shows the kinetic measurements of the cascade reaction expressed as the yield of $ABTS^{\bullet-}$. For the conjugated enzymes the yield was (92 ± 8)%; i.e., all initially formed superoxide radical anions result in the formation of $ABTS^{\bullet-}$ through a two-step cascade reaction. Furthermore, a comparison was made with the free enzymes (Figure 4). The $ABTS^{\bullet-}$ yield with free enzymes was found to be (92 ± 9)%. Although the cascade reaction seems to be somewhat faster with the free enzymes, no differences were detected regarding $ABTS^{\bullet-}$ yield after a 2.5 s reaction time. Hence, the conjugated enzymes are similarly efficient as the free enzymes.

In conclusion, we have shown that the preparation of a dendronized polymer–enzyme hybrid in which the two enzymes HRP and SOD are covalently bound to the same polymer chain was possible. Though there are few reports on the synthesis of polymer–protein conjugates containing per polymer chain (i) one copy of two different types of proteins[20] or (ii) multiple

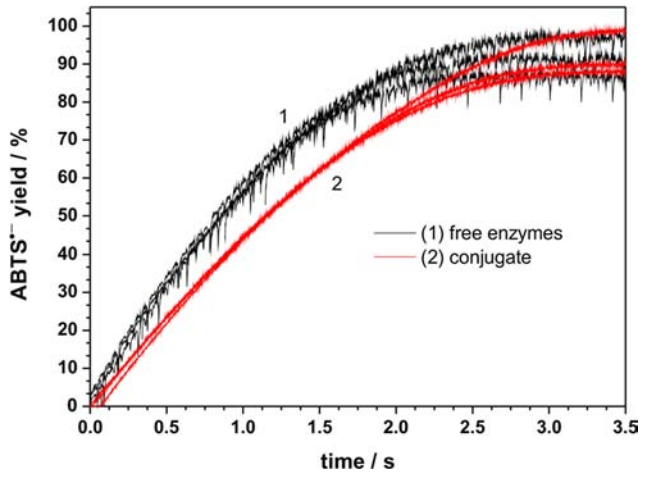

Figure 4. Time-course measurements of the two-step enzymatic cascade reaction: comparison of (1) free and (2) polymer-bound SOD and HRP. $O_2^{\bullet-}$ was produced by pulse radiolysis, formation of $ABTS^{\bullet-}$ was followed spectrophotometrically at λ= 415 nm; for calculation of the $ABTS^{\bullet-}$ yield, see SI. Conditions: $[ABTS^{2-}]_0$ = 0.25 mM, [HRP] = 0.4 μM, [SOD] = 0.2 μM, 100 mM phosphate buffer pH 7.2, 25 °C.

copies of the same protein,[16,21−23] this is, to the best of our knowledge, the first report on the preparation of a polymer−enzyme conjugate containing *multiple copies of two different types of enzymes on one and the same polymer chain*. The conjugation reaction could be quantified spectrophotometrically and occurred under mild conditions and therefore under complete retention of the catalytic activity of the two enzymes. The reason why the two enzymes could be linked in two consecutive steps to the dendronized polymer by using the *same* conjugation chemistry is probably due to the different reaction behaviors of the two enzymes (see SI). Reaction of *de*-$PG1_{2000}$-BAH-(FL)-HyNic with HRP-4FB resulted in a polymer−HRP conjugate which still had reactive HyNic groups that could not react with HRP-4FB but reacted with the added SOD-4FB instead. This behavior is probably related to the smaller size of SOD as compared to HRP (radii of gyration: R_G(HRP) = 2.65 nm,[24] R_G(SOD) = 1.82 nm)[25] and to the differences in the isoelectric points, pI(HRP isoenzyme C) = 9.0[26] and pI(SOD) = 4.95.[27] Whether this methodology is applicable to other polymer−enzyme systems is currently under investigation. Furthermore, we are elaborating experimental conditions for a localized enzymatic cascade reaction, i.e. encapsulated inside lipid vesicles or immobilized on solid surfaces, for mechanistic studies and bioanalytical applications.[28−30] Our findings open the fascinating opportunity to eventually perform cascade reactions on single hybrid molecules in confined geometries.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental protocols including polymer, linker and conjugate synthesis as well as enzymatic activity measurements for SOD and HRP, separately and together using pulse radiolysis, and additional UV/vis-spectra. This material is available free of charge *via* the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

peter.walde@mat.ethz.ch

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Prof. Dr. Donald Hilvert and Dr. An Vandemeulebroucke (ETH Zürich) for fruitful discussions and Dr. Thomas Schweizer (ETH Zürich) for GPC measurements. Moreover, the help from the following undergraduate students is highly appreciated: Darija Dedic, Karoline Herget, and Juri Regli.

■ REFERENCES

(1) Broyer, R. M.; Grover, G. N.; Maynard, H. D. *Chem. Commun.* **2011**, *47*, 2212.
(2) Klok, H. A. *Macromolecules* **2009**, *42*, 7990.
(3) Lutz, J. F.; Börner, H. G. *Prog. Polym. Sci.* **2008**, *33*, 1.
(4) Hoffman, A. S.; Stayton, P. S. *Prog. Polym. Sci.* **2007**, *32*, 922.
(5) Grover, G. N.; Maynard, H. D. *Curr. Opin. Chem. Biol.* **2010**, *14*, 818.
(6) Gauthier, M. A.; Klok, H. A. *Chem. Commun.* **2008**, 2591.
(7) Heredia, K. L.; Maynard, H. D. *Org. Biomol. Chem.* **2007**, *5*, 45.
(8) Gauthier, M. A.; Klok, H. A. *Polym. Chem.* **2010**, *1*, 1352.
(9) Duncan, R. *Curr. Opin. Biotechnol.* **2011**, *22*, 492.
(10) Veronese, F. M.; Mero, A.; Pasut, G. *Protein PEGylation, basic science and biological applications*; Birkhauser Verlag AG: Basel, Switzerland, 2009.
(11) Zhu, G.; Wang, P. *Nanoscale Biocatalysis: Methods and Protocols*; Humana Press Inc.: Totowa, NJ, 2011; Vol. *743*, p 27.
(12) Sheldon, R. A. *Adv. Synth. Catal.* **2007**, *349*, 1289.
(13) Schoffelen, S.; van Hest, J. C. M. *Soft Matter* **2012**, *8*, 1736.
(14) Pereira de Souza, T.; Stano, P.; Luisi, P. L. *ChemBioChem* **2009**, *10*, 1056.
(15) Guo, Y. F.; van Beek, J. D.; Zhang, B. Z.; Colussi, M.; Walde, P.; Zhang, A.; Kröger, M.; Halperin, A.; Schlüter, A. D. *J. Am. Chem. Soc.* **2009**, *131*, 11841.
(16) Grotzky, A.; Manaka, Y.; Kojima, T.; Walde, P. *Biomacromolecules* **2011**, *12*, 134.
(17) Solulink Company, San Diego, USA, *Catalog & Reference Manual*, 2008.
(18) Childs, R. E.; Bardsley, W. G. *Biochem. J.* **1975**, *145*, 93.
(19) Michel, E.; Nauser, T.; Sutter, B.; Bounds, P. L.; Koppenol, W. H. *Arch. Biochem. Biophys.* **2005**, *439*, 234.
(20) Heredia, K. L.; Grover, G. N.; Tao, L.; Maynard, H. D. *Macromolecules* **2009**, *42*, 2360.
(21) Tao, L.; Kaddis, C. S.; Loo, R. R. O.; Grover, G. N.; Loo, J. A.; Maynard, H. D. *Macromolecules* **2009**, *42*, 8028.
(22) Chen, J. P.; Yang, H. J.; Hoffman, A. S. *Biomaterials* **1990**, *11*, 625.
(23) Altikatoglu, M.; Arioz, C.; Basaran, Y.; Kuzu, H. *Cent. Eur. J. Chem* **2009**, *7*, 423.
(24) Laberge, M.; Huang, Q.; Schweitzer-Stenner, R.; Fidy, J. *Biophys. J.* **2003**, *84*, 2542.
(25) Martel, P.; Powell, B. M.; Zepp Johnston, R. A.; Petkau, A. *Biochim. Biophys. Acta* **1983**, *747*, 78.
(26) Aibara, S.; Yamashita, H.; Mori, E.; Kato, M.; Morita, Y. *J. Biochem.* **1982**, *92*, 531.
(27) Bannister, J.; Bannister, W.; Wood, E. *Eur. J. Biochem.* **1971**, *18*, 178.
(28) Gorris, H. H.; Walt, D. R. *J. Am. Chem. Soc.* **2009**, *131*, 6277.
(29) Gorris, H. H.; Walt, D. R. *Angew. Chem., Int. Ed.* **2010**, *49*, 3880.
(30) Logan, T. C.; Clark, D. S.; Stachowiak, T. B.; Svec, F.; Frechet, J. M. J. *Anal. Chem.* **2007**, *79*, 6592.