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Squaric Acid Mediated Synthesis and Biological Activity of a Library of Linear and Hyperbranched Poly(Glycerol)−Protein Conjugates

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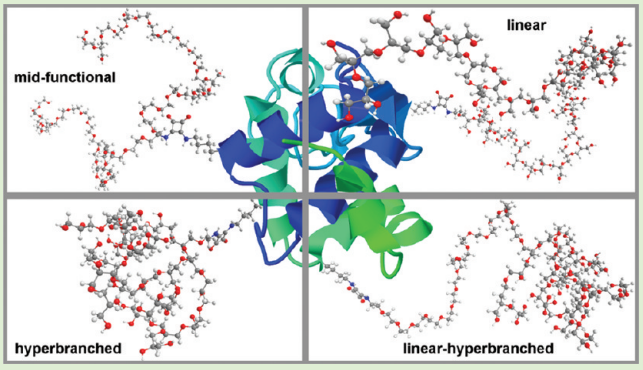
Supporting Information

ABSTRACT: Polymer−protein conjugates generated from side chain functional synthetic polymers are attractive because they can be easily further modified with, for example, labeling groups or targeting ligands. The residue specific modification of proteins with side chain functional synthetic polymers using the traditional coupling strategies may be compromised due to the nonorthogonality of the side-chain and chain-end functional groups of the synthetic polymer, which may lead to side reactions. This study explores the feasibility of the squaric acid diethyl ester mediated coupling as an amine selective, hydroxyl tolerant, and hydrolysis insensitive route for the preparation of side-chain functional, hydroxyl-containing, polymer−protein conjugates. The hydroxyl side chain functional polymers selected for this study are a library of amine end-functional, linear, midfunctional, hyperbranched, and linear-block-hyperbranched polyglycerol (PG) copolymers. These synthetic polymers have been used to prepare a diverse library of BSA and lysozyme polymer conjugates. In addition to exploring the scope and limitations of the squaric acid diethylester-mediated coupling strategy, the use of the library of polyglycerol copolymers also allows to systematically study the influence of molecular weight and architecture of the synthetic polymer on the biological activity of the protein. Comparison of the activity of PG−lysozyme conjugates generated from relatively low molecular weight PG copolymers did not reveal any obvious structure−activity relationships. Evaluation of the activity of conjugates composed of PG copolymers with molecular weights of 10000 or 20000 g/mol, however, indicated significantly higher activities of conjugates prepared from midfunctional synthetic polymers as compared to linear polymers of similar molecular weight.



INTRODUCTION

In recent years, with the discovery of novel pharmaceutically active peptides and proteins, a novel field of human therapy is rapidly evolving based on complex biopolymers.[1] A drawback of protein-based "biotherapeutics" is their fast degradation in the human body by both the digestive system and the circulatory system, that is, they are rapidly removed by proteolytic digestion and renal excretion.[2,3] In the last decades, the covalent attachment of synthetic macromolecules has proven to be an effective strategy to improve protein stability, reduce immunogenicity, extend plasma half-life times, and increase solubility.[4]

Seminal work has been conducted in the 1970s by Davis and Abuchowski, who found that covalent attachment of poly(ethylene glycol) (PEG) to bovine serum albumin (BSA) and bovine liver catalase resulted in reduced immunogenicity and increased blood circulation times.[5,6] To date, among a variety of other water-soluble and biocompatible polymers, PEG is still the most frequently used for the modification of peptides and proteins. In more recent work, it has been demonstrated that the use of "branched" PEG-structures, that is, PEG derivatives with a single branching point bearing the protein-reactive group in the center of a linear polymer chain leads to improved circulation times as compared to conjugates based on linear PEG chains.[7] Today, several FDA-approved PEGylated proteins are used for the treatment of cancer, hepatitis C, anemia, and diabetes.[8,9] A commercial therapeutic for the treatment of hepatitis C ("PEGASYS, Roche") is available that relies on a midfunctional PEG coupled to interferon α-2a.[10]

Although it is a highly valuable concept, protein PEGylation also has several limitations: (i) linear PEG only possesses two functional groups at the polymer chain ends, which restricts the possibilities for further functionalization and the preparation of multiprotein conjugates, and (ii) PEGylation often leads to a loss of protein activity.[11] The development of strategies to overcome these limitations and that allow access to protein−polymer

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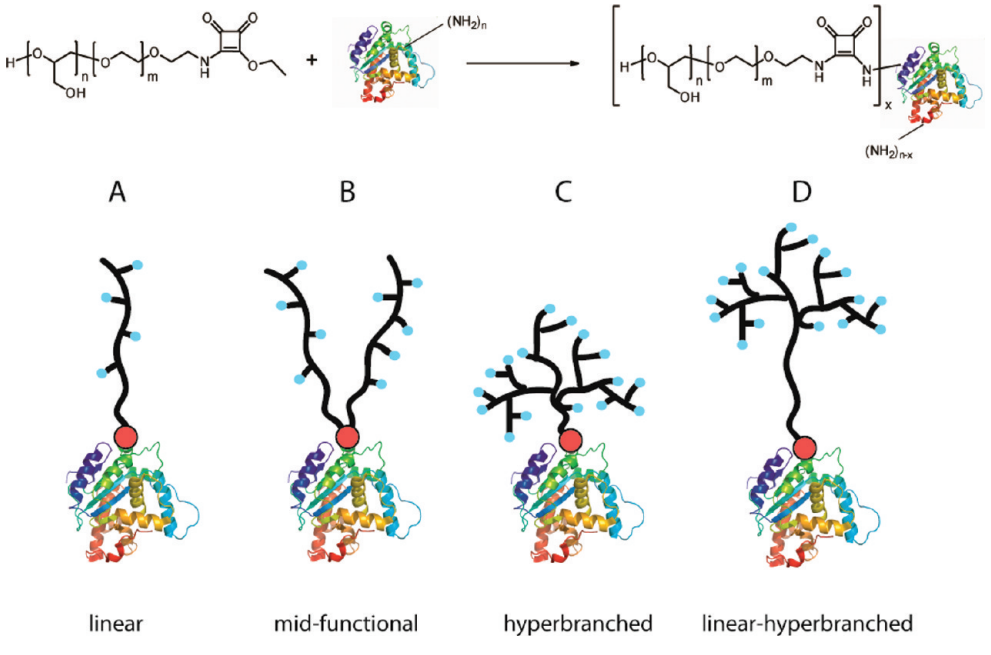
conjugates with superior properties is a very topical and active field of research.

One approach to generate "PEG-like" polymers with a large number of functional groups is based on the controlled radical polymerization of appropriate side-chain functional (meth)-acrylates, such as 2-hydroxyethyl(meth)acrylate, poly(ethylene glycol)(meth)acrylate, or 2-hydroxypropylmethacrylamide.[12−14] These polymers can be conjugated to the peptide or protein of interest via a "grafting from" or a "grafting to" approach to the respective amino acid residues.[14,15] Another interesting class of PEG-like polymers, which, however, so far has been barely explored for the synthesis of protein−polymer conjugates, are polyglycerols (PGs).[16] PGs are attractive because they are biocompatible and nontoxic,[17,18] as well as more protein-resistant and thermally and oxidatively stable as compared to PEG.[19] PG can be obtained via ring-opening polymerization of glycidol or glycidyl ethers in a controlled manner.[18−22] Depending on the type of monomer used and the polymerization conditions applied, both linear and (hyper)branched PGs can be synthesized. This is attractive as it allows access to libraries of chemically identical but structurally different PG analogues, which may be valuable to investigate the influence of polymer architecture on the properties of polymer−protein conjugates.

In many instances, PEGylation is aimed at modifying amine groups of the peptide/protein of interest. Traditionally, site-selective modification of amine groups is accomplished by using, for example, *N*-hydroxysuccinimide (NHS), 4-nitrophenyl chloroformate, or similar end-functionalized PEG derivatives.[15,23] While these approaches work well for a large variety of synthetic polymers, they cannot be applied to side-chain (hydroxy) functional polymers such as PG, because the classical active esters are susceptible to concurrent transesterification and hydrolysis. Lysine-selective protein modification with PG (co)polymers requires protein-reactive groups that are tolerant toward hydroxy groups and have extended hydrolysis half-lives. One example of an amine-reactive, hydroxy-tolerant activation group is the thiazolidine-2-thione group. This activating group, which has been successfully used by Tao et al. for the synthesis of PHPMA−protein conjugates,[12] has a hydrolysis half-life of several hours as compared to minutes for NHS esters.[24,25] The thiazolidine-2-thione group, however, has been reported to undergo side reactions with thiol groups.[25] Another potentially interesting class of reagents to allow amine-selective modification of proteins are squaric acid dialkyl esters, such as squaric acid diethyl ester (SADE). While SADE has found widespread use for glycosylation[26−28] or other coupling reactions between low molecular weight compounds,[29,30] it has not been explored for the preparation of synthetic polymer−protein conjugates. Squaric acid dialkyl esters react selectively with amine groups at pH 7 and room temperature without interference of transesterification or hydrolysis reactions.[30] Due to the lower reactivity of the reaction product as compared to the starting material, only squaric acid ester monoamides are formed, which can be isolated and characterized. Under more basic reaction conditions (pH 9), the ester amide intermediates can then be conjugated to another amine functional compound in a second amine-selective and hydroxy-tolerant reaction,[26] which makes the squaric acid mediated coupling a potentially attractive approach to synthesize PG and other side chain functional (co)polymer−protein conjugates.

In a recent study, we have explored the one-step activation of amino poly(ethylene glycol)s (PEGs) with squaric acid diethyl ester and investigated the reactivity and chemoselectivity of these PEG derivatives toward the amine-selective modification of α-amino acids and proteins.[31] The present study is aimed at further exploring and expanding the scope of this approach and (i) explores the feasibility of the squaric acid mediated coupling for the synthesis of side-chain functional synthetic polymer (specifically, linear and hyperbranched polyglycerol)−protein conjugates; (ii) uses the versatility of the glycidol ring-opening polymerization to generate an architecturally diverse library of PG−protein conjugates; and (iii) evaluates the biological activity of these polymer−protein hybrids (Scheme 1).

Scheme 1. (Top) Squaric Acid Mediated Coupling of PG (Co)Polymers to Proteins; (Bottom) Schematic Representation of the Architecturally Different Protein−Polymer Conjugates Prepared and Investigated in This Study



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EXPERIMENTAL SECTION

Materials. Ethylene oxide (Fluka 99%), glycidol (Acros 96%), cesium hydroxide monohydrate (99.95% Aldrich), ethyl vinyl ether (98% Aldrich), ethanolamine (99% Acros), serinol (2-amino-1,3-propanediol, 98%, Acros), *p*-methoxybenzylalcohol (98% Acros), phosphorus tribromide (phosphorus tribromide, 1.0 M solution in dichloromethane, AcroSeal), potassium carbonate (99+% Acros), palladium on activated charcoal (10%Pd, Aldrich), benzene (99% Acros), lysozyme (90%), bovine serum albumin (98%), *Micrococcus lysodeikticus* (lyophilized cells), and ninhydrin reagent were purchased from Aldrich and used as received. Squaric acid diethyl ester was purchased from VWR and used as received. α-Amino-ω-methoxy-poly(ethylene glycol)s were purchased from IRIS Biotech and used as received. DMSO-d_6 and $CDCl_3$ were purchased from Deutero GmbH. Ethoxy ethyl glycidyl ether was prepared as described by Fitton et al.,[32] dried over CaH_2, and freshly distilled before use. Ethylene oxide and glycidol were distilled from CaH_2 before use. *p*-Methoxybenzylbromide was synthesized as described previously.[33] All other reagents and solvents were purchased from Aldrich and used as received, if not otherwise mentioned.

Methods. ^{1}H NMR spectra were recorded using either a Bruker AC 300 (300 MHz spectra) or a Bruker AMX 400 instrument (400 MHz spectra). All spectra were referenced internally to the residual proton signals of the deuterated solvent. SEC analysis in water was carried out on a Viscotek TDA 300 instrument equipped with a MetaChem degasser, a Visotek VE 1121 SEC solvent pump, and a VE 5200 SEC autosampler. A 9:1 mixture of phosphate buffer (0.1 M, pH = 6.5)/methanol was used as a mobile phase. Samples were eluted at 25 °C and at a flow rate of 0.5 mL·min^{-1} over Shodex OHpak 804 and 805 columns. Sample elution was monitored using a triple detection setup and absolute molecular weights were determined via light scattering. For SEC measurements in DMF (containing 0.25 g/L of lithium bromide as an additive), an Agilent 1100 Series instrument equipped with a PSS HEMA column (106/105/104 g/mol), a UV (275 nm), and a RI detector was used. Calibration was carried out using poly(ethylene oxide) standards provided by Polymer Standards Service. MALDI-ToF mass spectrometry was performed on a Shimadzu Axima CFR MALDI-ToF MS mass spectrometer equipped with a nitrogen laser delivering 3 ns laser pulses at 337 nm. α-Cyano-hydroxycinnamic acid (CHCA) was used as a matrix and potassium triflate was added to facilitate ionization of polymer samples. SDS-PAGE was carried out with 4−20% Tris-HCl gels (Biorad, 0.75 mm, 10 well). Reverse phase HPLC was performed on a Grace Vydac C4-protein column using a modular setup from JASCO equipped with a quaternary gradient pump PU-2089plus, autosampler AS-2055plus, UV-detector UV-2075plus, and a column oven (25 °C) CO-2060plus. Gradient elution was carried out at a flow rate of 0.5 mL/min with a mobile phase A (99.9% H_2O, 0.01% TFA) and a mobile phase B (99.9% acetonitrile, 0.01% TFA). The gradient sequence (B) was 5−100% from 0−60 min, 100% from 60−80 min, 100−5% from 80−110 min. Sample elution was monitored at a UV absorbance of 280 nm. Ninhydrin test: A solution of the analyte was dropped onto a silica gel TLC plate, dipped into the solution, and dried with a heatgun; a positive test was indicated by a color change.

Procedures. *N,N-Di(p-methoxybenzyl)aminoethanol (1).* Freshly distilled *p*-methoxybenzyl bromide (7.5 g, 37.5 mmol), ethanolamine (1.15 g, 18.5 mmol), and potassium carbonate (7 g, 50 mmol) were mixed in about 80 mL of DMF and refluxed for 24 h. After the reaction mixture was allowed to cool to room temperature, the solution was filtered and diethyl ether (ca. 200 mL) was added. The organic phase was then washed with water and a saturated $NaHCO_3$ solution and dried with $MgSO_4$. The organic phase was dried and concentrated in vacuo to afford a highly viscous liquid. The crude product was purified by column chromatography using ethyl acetate and petrol ether (6:4) as eluent. Yield: 4.5 g (80%). ^{1}H NMR (300 MHz, DMSO): δ (ppm) 7.26−6.85 (8H, aromatic), 4.33 (t, J = 6 Hz 1H, OH), 3.72 (s, 6H, OCH_3), 3.47 (s, 4H, NCH_2Ph), 3.44 (t, J = 6 Hz, 2H, CH_2OH), 2.65 (t, J = 6 Hz, 2H, NCH_2). ESI-MS: 302.5 (MH^+), 324.8 (MNa^+).

N,N-Di(p-methoxybenzyl)serinol (2). Compound **2** was synthesized as above with the following ratios: serinol (1.13 g, 12.4 mmol), *p*-methoxybenzyl bromide (5.03 g, 25 mmol), potassium carbonate (4.8 g, 35 mmol), and 80 mL DMF. Yield: (3.1 g, 78%). ^{1}H NMR (300 MHz, DMSO): δ (ppm) 7.23−6.84 (8H, aromatic), 4.28 (br, 2H, OH), 3.70 (s, 6H, OCH_3), 3.62 (s, 4H, NCH_2Ph), 3.54 (m, 2H, CH_2OH), 2.66 (t, J = 6 Hz, 1H, NCH). ESI-MS: 322.8 (MH^+).

General Procedure for the Synthesis of Linear Poly(ethoxy ethyl glycidyl ether) (Co)Polymers. The appropriate initiator (ca. 100 mg) was placed in a Schlenk flask and dissolved in benzene (ca. 5 mL) under an argon atmosphere. Cesium hydroxide monohydrate was introduced to achieve a degree of deprotonation of 50%. The mixture was stirred at 60 °C over a period of 45 min and then heated to 80−90 °C in vacuo for 2 h to remove the formed water and benzene azeotropically. In a separate setup, first dry THF, and the respective monomers (ethylene oxide or ethoxy ethyl glycidyl ether (EEGE), predried over CaH_2) were distilled into a Schlenk flask equipped with a stir bar, a Teflon tap, and a rubber septum. The flask was closed under vacuum and the cesium salt of the initiator was added dissolved in dry DMSO to afford about a 10 wt % solution of the monomer(s) in a 9:1 mixture of THF and DMSO. The reaction mixture was directly heated to 70 °C in static vacuo over a period of 20 h and quenched by the addition of about 20 mL of methanol and 0.5 g acidic ion-exchange resin. After that, the solution was filtered, concentrated, and precipitated into diethyl ether/acetone (70:30) first and subsequently in pure diethyl ether to yield the final polymer after drying in a yield typically between 90% to quantitative. Note: PEEGE-homopolymers were filtered and precipitated twice into water. ^{1}H NMR (for PEG-*co*-PEEGE and PEG-*b*-PEEGE in DMSO-d_6, 300 MHz): 7.22−6.82 (8H, aromatic signals from the initiator), 4.64 (br s, acetal H), 3.69 (s, 6H, MeO-C_6H_4-), 3.64−3.18 (br, -CH_2-$CH_{(2)}$O- (backbone), and -O-CH_2-CH_3 (side chain)), 1.25−0.9 (-O-CH_2-CH_3 and -CH-CH_3).

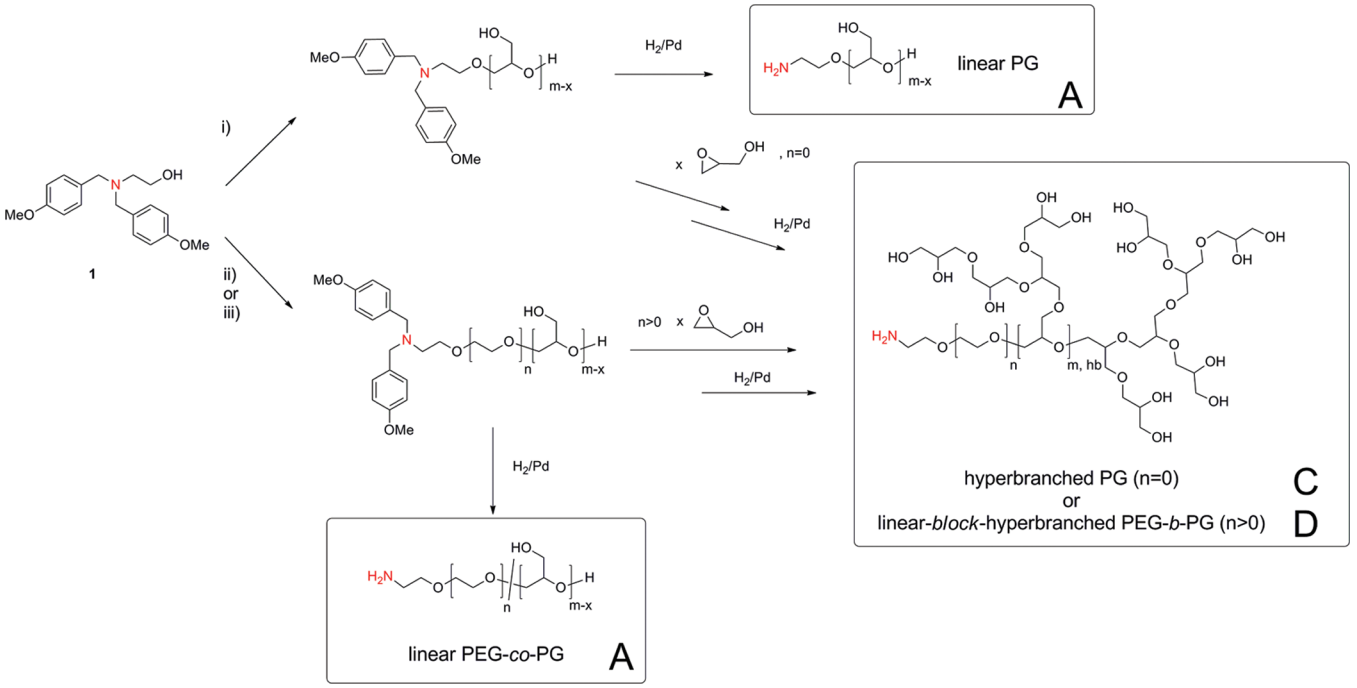
Removal of the Acetal Protective Groups. The PEEGE-containing (co)polymer was dissolved in methanol (ca. 20 wt %) and the same volume of 1 N HCl was added. The mixture was stirred at room temperature overnight, concentrated in vacuo and precipitated three times into diethyl ether. The isolated yields ranged between 80 and 90% in all cases. ^{1}H NMR (DMSO-d_6, 300 MHz): 10.57 (s, 1H, *H*-N^+R_3), 7.54−6.97 (8H, aromatic signals from the initiator), 4.27 (br s, OH, intensity and chemical shift can vary), 3.75 (s, 6H, MeO-C_6H_4-), 3.64−3.18 (br, -CH_2-$CH_{(2)}$O- (backbone)).

Hypergrafting of Glycidol. The linear precursor polymer was dissolved (or suspended) in benzene at a concentration of about 20 wt %. Cesium hydroxide monohydrate was added (to achieve a degree of deprotonation of 20%) and the mixture was allowed to react at 60 °C for 60 min. The formed water and benzene were removed azeotropically at 90 °C over a period of 90 min. After that, the activated macroinitiator was suspended in dry diglyme (ca. 20 wt %), heated to 90 °C, and freshly distilled glycidol (amount depends on the targeted degree of polymerization) in dry diglyme was added slowly with a syringe pump over a period of 5−8 h. The reaction was terminated by the addition of 20 mL methanol and 0.5 g acidic ion-exchange resin, filtered, concentrated in vacuo, and precipitated into a 10-fold excess of diethyl ether to afford the desired polymer in quantitative yield.

Hydrogenation (General Procedure). A hydrogenation vessel was charged with the polymer (ca. 1 g) dissolved in degassed methanol (ca. 20 mL) and palladium on activated charcoal (ca. 100−200 mg) was added under a stream of argon. The vessel was closed, pressurized with hydrogen (8 bar) and stirred at room temperature until no residual aromatic signals could be detected by ^{1}H NMR spectroscopy (typically 24−48 h were necessary to achieve complete conversion). After completion of the reaction, workup was done by purging the reaction vessel with argon and filtration over Celite to remove the catalyst and washed with about 100 mL of methanol. The filtrate was then concentrated to about 5 mL, precipitated into cold diethyl ether and dried. ^{1}H NMR (DMSO-d_6, 300 MHz): 5.0−3.80 (br, OH, intensity and chemical shift can vary), 3.64−3.10 (br, -CH_2-$CH_{(2)}$O- (backbone)).

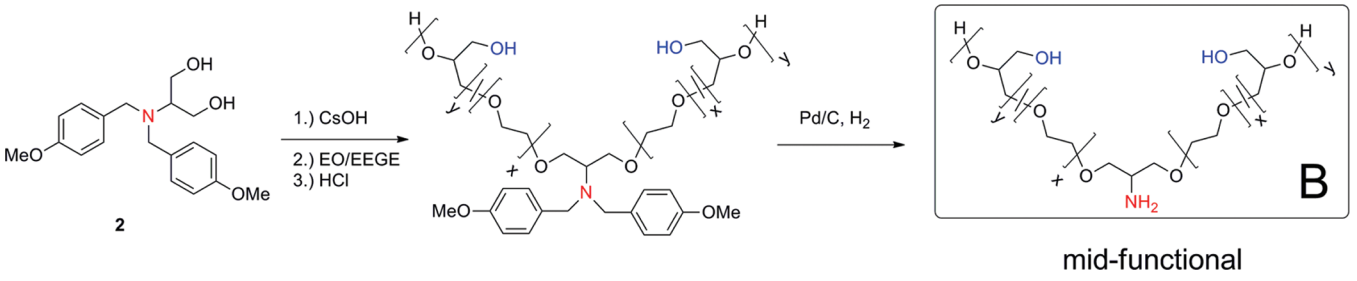
General Procedure for the Polymer Chain End Modification with Squaric Acid Diethyl Ester. The polymer (0.05 mmol) was dissolved in ethanol (15 mL) and pyridine was added (ca. 20 mg). To this mixture a 5-fold molar excess of squaric acid diethyl ester (SADE, 0.25 mmol, 43 mg) was added with a microliter syringe and the mixture was gently shaken at room temperature over a period of 16 h. The solution

Scheme 2. Synthesis of Different Monoamino Polyethers Based on Polyethylene Glycol (PEG) and Polyglycerol (PG) with Varying Architecture[a]



[a](i) 1. $CsOH{\cdot}H_2O$, vacuo; 2. ethoxyethyl glycidyl ether; 3. HCl/MeOH. (ii) 1. $CsOH{\cdot}H_2O$, vacuo; 2. ethylene oxide and ethoxyethyl glycidyl ether; 3. HCl/MeOH. (iii) 1. $CsOH{\cdot}H_2O$, vacuo; 2. ethylene oxide; 3. ethoxyethyl glycidyl ether; 4. HCl/MeOH.

Scheme 3. Synthesis of Mid-Functional Poly(ethylene glycol-*co*-glycerol)s Starting from Di(*p*-methoxybenzyl)serinol (2)[a]



[a]EO: ethylene oxide; EEGE: ethoxyethyl glycidyl ether.

was concentrated in vacuo (taking care to keep the temperature below 60 °C to prevent transesterification) and precipitated four times into diethyl ether. Typical yields are between 85 and 95%. ^{1}H NMR (DMSO-d_6, 300 MHz): 8.7−8.4 (1H *H*-N-, two amide signals), 4.6 (br, 2H, −O-C*H*$_2$-CH$_3$), 3.64−3.18 (br, -C*H*$_2$-C*H*$_{(2)}$O- (backbone)), 1.4 (br, 3H, -O-CH$_2$-C*H*$_3$).

General Procedure for Squaric Acid Mediated Protein Conjugation. BSA or lysozyme was dissolved in a 3:1 mixture of borate buffer (0.01 M, pH = 9.1) and DMSO to yield a final protein concentration of 1 g/L. The calculated amount of polymer (for BSA, 9 equiv, or for lysozyme, 1−7 equiv) was added to the protein solution and the reaction mixture was gently shaken over a period of 20 h at room temperature. Samples were taken from the reaction mixture and dialyzed against deionized water (molecular weight cutoff of 1000 g/mol) to remove salts and DMSO and were analyzed by SDS-PAGE to monitor the conjugation reaction. Purification of the remaining solution was achieved by dialysis against deionized water for 48 h (depending on the molecular weight of the polymer, dialysis membranes with molecular weight cutoff between 15000 and 100000 g/mol were used for lysozyme conjugates, while all BSA conjugates were dialyzed against a molecular weight cutoff of 50000 g/mol), and subsequently freeze-dried. Yield: 95% quantitative (compared to protein).

Lysozyme Activity Measurements. The activity of the lysozyme conjugates was tested using *Micrococcus lysodeikticus* (Ml) cells as substrate. *Micrococcus lysodeikticus* cells (17 mg) were suspended in sodium acetate buffer (45 mL, pH = 5, 50 mM) and an aliquot of 3 mL was transferred into a UV cuvette. The initial absorbance at 450 nm was defined as baseline. Subsequently, 5 μL of a 1 g/L (protein) solution of the appropriate polymer−protein conjugate in borate buffer/DMSO (3:1) was added, the resulting solution mixed, and the absorption at 450 nm was recorded as a function of time during a period of 3 min. The activity of the conjugate is reported as a percentage compared to unmodified lysozyme with activity of 100%. The reported activities are the average of three independent experiments.

RESULTS AND DISCUSSION

Polymer Synthesis. A library of different poly(glycerol) (PG) and poly(ethylene glycol-*co*-glycidol) copolymers was synthesized via anionic polymerization as outlined in Schemes 2 and 3. The library consists of linear PG and linear poly(ethylene glycol-*co*-glycerol) copolymers (series A), midfunctional PEG-*co*-PG copolymers (series B), hyperbranched PG (series C), and linear-*block*-hyperbranched PEG-*b*-*hb*PG copolymers (series D; Scheme 1).

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Di(*p*-methoxybenzyl)aminoethanol (**1**) was used as the initiator for the synthesis of linear, linear-hyperbranched, and hyperbranched polymers,[16,34] while di(*p*-methoxybenzyl)-serinol (**2**) was used as initiator for the preparation of midfunctional polymers. In the random copolymers the glycidol content was adjusted to 10%. The molecular weights were varied by the monomer: initiator ratio and several well-defined polymers (M_w/M_n = 1.05−1.15 for the linear polymers, respectively, 1.20−1.50 for the hyperbranched polymers) with variation in architecture and molecular weight were obtained. For the synthesis of linear PG and poly(ethylene glycol-*co*-glycerol) copolymers, ethoxy ethyl glycidyl ether[32] was used as the monomer, which was deprotected after polymerization by diluted hydrochloric acid to release the hydroxyl groups (see Supporting Information, Figure S1 for a representative MALDI ToF mass spectrum). Hyperbranched PG was synthesized via a slow-monomer addition hypergrafting protocol onto linear PG as reported previously.[16,35] For all synthesized polymers, the di(*p*-methoxybenzyl) protected amino group was released in a final step by catalytic hydrogenation at a hydrogen pressure of about 8 bar with palladium on activated charcoal as a catalyst. The end point of the reaction was determined by ^{1}H NMR spectroscopy monitoring the disappearance of the aromatic signals of the protective group. After completion of the reaction, all polymers showed a positive ninhydrin test. The resulting amino-functionalized PG (co)polymers were characterized by ^{1}H NMR spectroscopy, SEC and MALDI ToF mass spectrometry. ^{1}H NMR spectra of several selected polymers are included in the Supporting Information (Figure S2−S5). Table 1 lists the molecular weights of the different polymers as determined from ^{1}H NMR spectroscopy and SEC. Generally, the experimentally determined molecular weights were in good agreement with those expected based on the monomer: initiator ratio, which illustrates the living nature of the anionic ring-opening polymerization.

Synthesis of Protein-Reactive Poly(glycerol) (Co)-Polymers. To allow protein modification, the PG (co)polymers shown in Schemes 2 and 3 need to be further modified with an appropriate protein reactive functional group. The specific aim of this study was to explore the squaric acid mediated coupling strategy for the synthesis of PG (co)polymer−protein conjugates. The synthesis of the protein-reactive, squaric acid modified PG (co)polymers is outlined in Scheme 4.

In a first series of test reactions, the conditions for the reaction outlined in Scheme 4 were optimized with respect to the molar excess of SADE to minimize the formation of the bisamide analogue. These experiments were carried out with commercially available monoamino poly(ethylene glycol)s with molecular weights of 750, 2000, 5000, and 10000 g/mol. In general, dimerization by the formation of the diamide is unlikely because the reactivity of the diester is much higher compared to the ester amide. However, because during the deprotection of the acetal functionalized prepolymers the corresponding ammonium salts are formed, pyridine was added for neutralization during the SADE modification, which may facilitate dimerization.[36] When 5 equiv of SADE were used, the MALDI-ToF spectra indicated that no coupling takes places for polymers with a molecular weight of 2000 g/mol and higher and that quantitative end-capping is guaranteed. Low molecular weight mPEG$_{17}$ (M_n = 800 g/mol) showed a slight tendency for dimerization (ca. 10% as estimated from MALDI ToF mass spectrometry; see Supporting Information, Figures S6 and S7). However, because they do not participate in the protein modification reaction, dimerized polymer impurities are uncritical and easily removed via dialysis after the conjugation reaction.

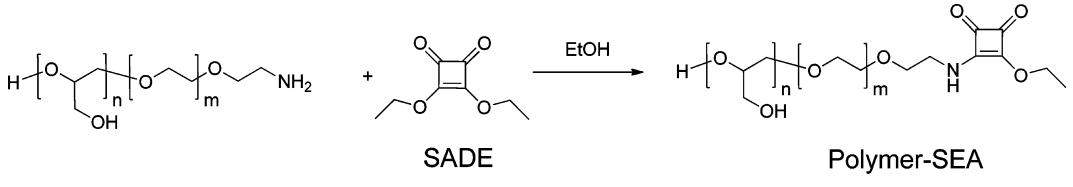
Modification of the amino-functionalized PG (co)polymers using the conditions established above proceeded quantitatively as indicated by a negative ninhydrin test. The resulting SADE-modified PG (co)polymers were further characterized with MALDI-ToF mass spectrometry and ^{1}H NMR spectroscopy. As a typical example, Figures 1 and 2 show the MALDI-ToF mass spectrum and the ^{1}H NMR spectrum, respectively, of a midfunctional poly(ethylene glycol-*co*-glycerol) copolymer (mid-P(EG$_{100}$-*co*-G)$_8$). The MALDI-ToF mass spectrum shows the typical distribution of a copolymer with all possible linear combinations of

Table 1. Molecular Weight Characteristics of the Poly(glycerol) (Co)Polymers Investigated in this Study

series[a]	sample code[b]	$M_{n,th}$[c] (g/mol)	$M_{n,NMR}$[d] (g/mol)	$M_{n,SEC}$[e] (g/mol)	M_w/M_n[e] (−)
A	lin-PG$_{35}$	2500	2600	1900	1.12
	lin-PG$_{74}$	5000	5500	5200	1.18
	lin-P(EG$_{41}$-*co*-G$_3$)	2000	2000	2200	1.05
	lin-P(EG$_{102}$-*co*-G$_7$)	5000	4500	3400	1.05
	lin-P(EG$_{204}$-*co*-G$_{14}$)	10000	10000	7600	1.07
	lin-P(EG$_{407}$-*co*-G$_{28}$)	20000	19400	14800	1.12
B	mid-PG$_{30}$	2000	2200	1700	1.09
	mid-PG$_{68}$	5000	5500	4600	1.11
	mid-P(EG$_{41}$-*co*-G$_3$)	2000	2000	1800	1.04
	mid-P(EG$_{100}$-*co*-G$_8$)	4000	3400	3000	1.09
	mid-P(EG$_{205}$-*co*-G$_{14}$)	10000	9800	6500	1.12
	mid-P(EG$_{367}$-*co*-G$_{24}$)	20000	18000	13700	1.15
C	hb-PG$_{35}$	2500	2600	2500	1.30
	hb-PG$_{65}$	5000	4800	4400	1.28
	hb-PG$_{86}$	8000	6400	6000	1.52
	hb-PG$_{203}$	15000	15000	13200	1.39
D	PEG$_{13}$-*b*-hbPG$_{28}$	3000	2800	2710	1.26
	PEG$_{52}$-*b*-hbPG$_{14}$	4500	3300	2900	1.18
	PEG$_{50}$-*b*-hbPG$_{40}$	5500	5200	5000	1.20

[a]Compare Scheme 1. [b]Subscripts indicate the number-average degree of polymerization determined via ^{1}H NMR. [c]Theoretical number-average molecular weight calculated from the initial monomer/initiator ratio. [d]Experimentally determined number-average molecular weight via ^{1}H NMR. [e]Experimentally determined number-average molecular weight (M_n) and polydispersity (M_w/M_n) via SEC in DMF vs poly(ethylene oxide) standards.

Scheme 4. Polymer Modification with Squaric Acid Diethyl Ester to Form a Protein-Reactive Polymer-Squaric Ethylester-Amide (Polymer-SEA)



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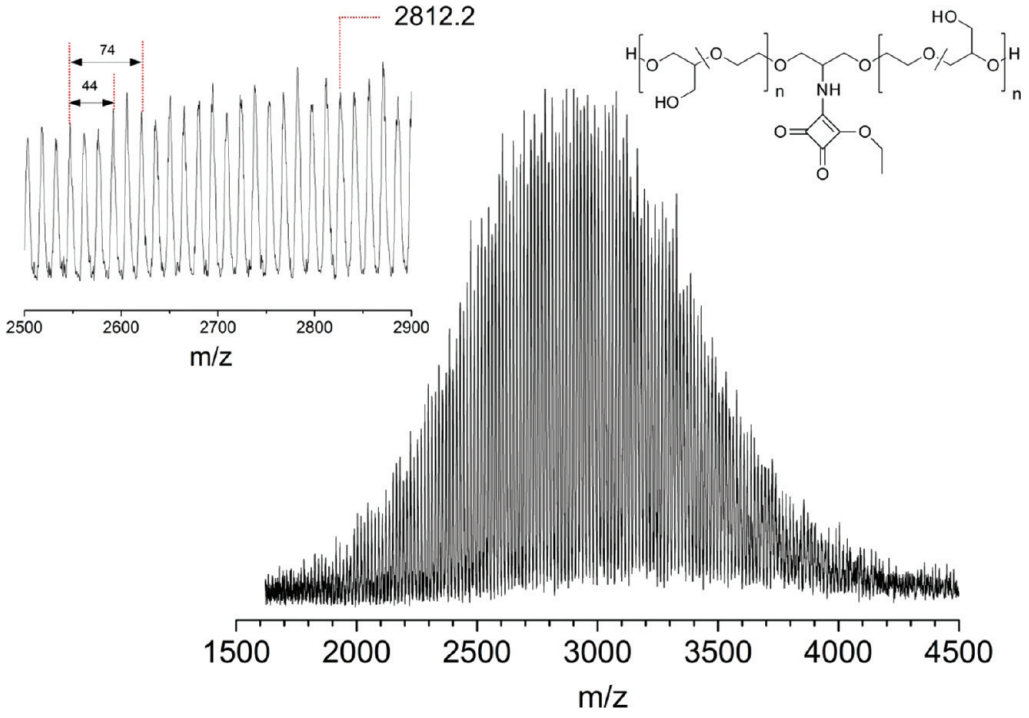

Figure 1. MALDI-ToF mass spectrum of a midfunctional random copolymer of PEG and PG (midP(EG_{100}-*co*-G_8)) modified with squaric acid ethyl ester (M_n = 3400 g/mol (NMR)). The mass indicated in the insert at 2812 corresponds to the sum formula of P(EG_{50}-*co*-G_5).

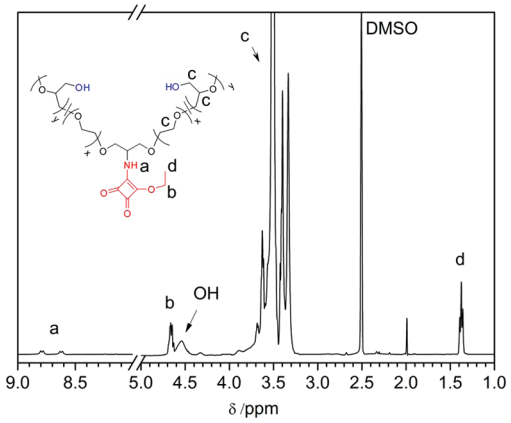

Figure 2. ^{1}H NMR spectrum of a squaric acid activated midfunctional random copolymer of PEG and PG (mid-P(EG_{100}-*co*-G_8)).

both monomer masses (44 for ethylene glycol and 74 for glycerol). The inset in Figure 1 only reveals a single distribution of species, which can be assigned to the SADE-modified polymer.

The ^{1}H NMR spectrum in Figure 2 reveals the characteristic squaric acid ethyl ester resonances at 1.37 (-CH_3) and 4.66 ppm (-CH_2-), respectively (note: the methylene resonance at 4.66 ppm is not visible in every sample due to possible overlap with the OH resonances of the polyol), as well as signals due to the polymer backbone between about 3.3 and 3.8 ppm and an amide resonance at 8.6 and 8.8 ppm. The splitting of the amide signal into two resonances has been reported before for low molecular weight squaric acid derivatives and is a result of the rotational barrier across the rigid cyclobutene carbon nitrogen bond and the different chemical environment of the proton provided by the *syn* rotamer and the corresponding *anti* form.[37]

Protein Conjugation. BSA was selected as a model protein to explore the squaric acid mediated polymer conjugation. BSA contains 59 lysine residues[38] of which 30−35 are accessible for polymer modification.[39] In a first set of experiments, several BSA−polymer conjugates were prepared by dissolving the protein in a 3:1 (v/v) mixture of borate buffer (ca. 1−3 g/L, pH = 9.1, 10 mM) and DMSO and adding an 8-fold excess of the SAE-activated polymer (Scheme 5). The mixture was gently shaken overnight at room temperature and the reaction product purified via dialysis.

The BSA−PG conjugates were first characterized by SEC. The results of these analyses are summarized in Table 2. The number of conjugated polymer chains per protein was calculated from RALS detection after determination of the apparent d*n*/d*c* and by considering the known molecular weight of BSA and the conjugated PG (co)polymer. In addition to the PG conjugates, Table 2 also lists the characteristics of two PEG conjugates that were obtained by reacting BSA with a 10-fold excess of squaric acid monoamide functionalized PEG. For the lower molecular weight P(E)G (co)polymers, Table 2 reveals excellent agreement between the expected and experimentally determined degree of P(E)Gylation. Increasing the molecular weight of the synthetic polymer generally results in a decrease in the experimentally determined degree of P(E)Gylation as compared to the expected value. Similar findings have been reported previously for other systems and were ascribed to decreasing end group reactivity and steric crowding at the protein with increasing molecular weight of the polymer.[13] The SEC experiments further revealed that the Mark−Houwink α-parameter of the PG-protein conjugates was influenced by the polymer architecture. All conjugates with linear polymers showed a value of about α = 0.33, which is an often found value for a star-branched polymer,[40] while conjugates prepared with midfunctional or hyperbranched polymers showed lower α values in the range of 0.19−0.24, that is, are more branched. The lowest α values (0.11−0.13) are observed for the *hb*PG and linear-hyperbranched block copolymer conjugates.

Scheme 5. Conjugation of Squaric Acid Modified PG (Co)Polymers to Lysine Residues of a Protein

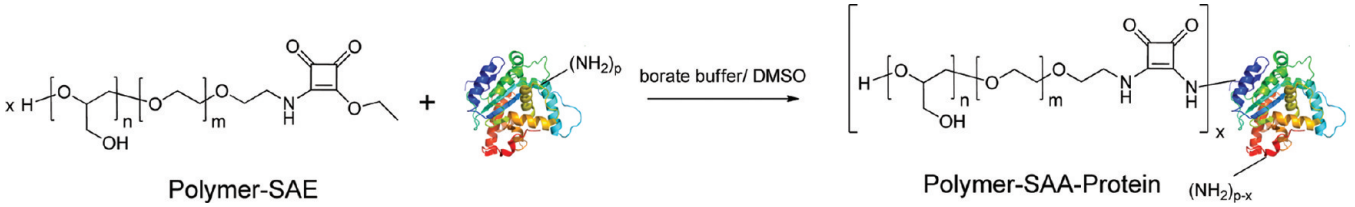

Table 2. Characteristics of the BSA−Polymer Conjugates Prepared in This Study

sample code	M_n[a] (g/mol)	M_w/M_n[a] (−)	dn/dc[a] (mL/g)	α[a] (−)	N_{th}[b]	N_{SEC}[c]
BSA	64400	1.08	0.16	0.16	0	0
BSA-$(PEG_{44})_n$	87000	1.17	0.14	0.36	10	9.8
BSA-$(PEG_{113})_n$	112250	1.12	0.13	0.38	10	9.3
BSA-(lin-P(EG_{41}-*co*-G_3)$_n$	83100	1.11	0.14	0.29	8	8.6
BSA-(lin-P(EG_{102}-*co*-G_7)$_n$	106000	1.22	0.12	0.32	8	8
BSA-(lin-P(EG_{204}-*co*-G_{14})$_n$	123000	1.13	0.10	0.33	8	6
BSA-(lin-P(EG_{407}-*co*-G_{28})$_n$	158 000	1.10	0.10	0.33	8	4.6
BSA-(mid-P(EG_{100}-*co*-G_8)$_n$	97 300	1.08	0.12	0.24	8	6.3
BSA-(mid-P(EG_{205}-*co*-G_{14})$_n$	114 000	1.11	0.12	0.19	8	5
BSA-$(hbPG_{35})_n$	83 000	1.09	0.11	0.11	8	6.8
BSA-(PEG_{13}-*b*-$hbPG_{28}$)$_n$	88 700	1.10	0.13	0.13	8	8.1

[a]Number average molecular weight (M_n), polydispersity index (M_w/M_n), refractive index increment (dn/dc), and Mark−Houwink parameter (α) determined by triple-detection SEC in water (phosphate buffer, pH = 6.5, 0.1 M)/methanol (9:1). [b]Expected number of polymer chains conjugated per protein. [c]Average number of polymer chains per protein as detected from SEC.

The dn/dc values of the conjugates are approaching those of the pure polymers with increasing mass percentage of the polymer in the conjugate (note: the dn/dc for *hb*PG was found to be 0.11 cm^3/g under these conditions and is close to the literature value of 0.12 in aqueous $NaNO_3$[17]).

To further corroborate the GPC analyses, the BSA conjugates were also analyzed by gel electrophoresis. Figure 3 summarizes the results of these experiments. The gels in Figure 3 were run using unpurified conjugation mixtures, which were only dialyzed to remove excess salt and DMSO before electrophoresis. The results of these experiments confirm the absence of free, unmodified BSA, which underlines the efficiency of the conjugation reaction under the investigated reaction conditions. Furthermore, the gel electrophoresis experiments indicate that the apparent molecular weight of the conjugates shifts to higher values with increasing molecular weight of the conjugated synthetic polymer.

In a second series of conjugation experiments, the enzyme lysozyme was chosen as substrate for the modification with the PG−(co)polymers. Lysozyme was selected as it is a convenient model protein to assess the influence of PG-conjugation on biological activity (vide infra). In contrast to the BSA modification experiments discussed above, which were carried out with a large excess of PG, the objective of the lysozyme modification experiments was to control the degree of modification by adjusting the relative quantity of squaric acid activated PG

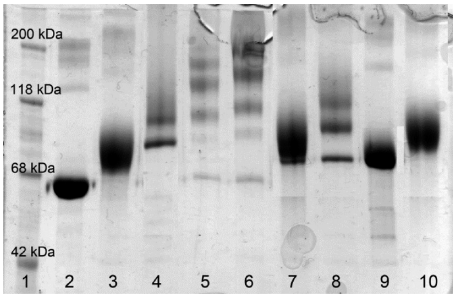

Figure 3. SDS-PAGE (12%) of BSA−polyether conjugates: (Lane 1) molecular weight markers; (Lane 2) BSA; (Lane 3) BSA-(lin-P(EG_{41}-*co*-G_3)$_n$); (Lane 4) BSA-(lin-P(EG_{102}-*co*-G_7)$_n$); (Lane 5) BSA-(lin-P(EG_{204}-*co*-G_{14})$_n$); (Lane 6) BSA-(lin-P(EG_{407}-*co*-G_{28})$_n$); (Lane 7) BSA-(mid-P(EG_{41}-*co*-G_3)$_n$); (Lane 8) BSA-(mid-P(EG_{205}-*co*-G_{14})$_n$); (Lane 9) BSA-$(hbPG_{35})_n$; (Lane 10) BSA-(PEG_{13}-*b*-$hbPG_{28}$)$_n$.

and lysozyme during the conjugation reaction. Lysozyme contains seven amine groups that are available for polymer modification: six lysine residues and the *N*-terminal amine group. To investigate to which extent the degree of polymer modification can be controlled, first a series of screening experiments with squaric acid activated α-methoxy-ω-amino poly(ethylene glycol)s (mPEG) with molecular weights of 750, 2000, 5000, and 10000 g/mol was carried out. In these experiments, varying equivalents of squaric acid activated mPEG were reacted with lysozyme at pH 9.1 in a 9:1 (v/v) borate buffer:DMSO mixture and the degree of PEGylation investigated via MALDI ToF mass spectrometry and electrophoresis (see Supporting Information, Figures S8−S11). In all cases, MALDI ToF mass spectrometry revealed a mixture of conjugates with different degrees of PEGylation. Up to a targeted degree of PEGylation of 4 and using PEG derivatives with molecular weights of 750 or 2000 g/mol, the MALDI ToF mass spectra suggest relatively good control over the number of conjugated PEG chains. Increasing the mPEG/lysozyme ratio from 4:1 to 5:1 for a squaric acid activated mPEG derivative with a molecular weight of 2000 g/mol, in contrast, was not found to result in a significant further improvement in the degree of PEGylation (see Figure S9). Further increasing the PEG molecular weight to 5000 g/mol made it more difficult to generate lysozyme−polymer conjugates where the degree of PEGylation corresponds to the used stoichiometry of amine reactive PEG and lysozyme (Figure S10).

Based on the results of the screening experiments discussed above, a series of polyglycerol (co)polymer−lysozyme conjugates were prepared with a targeted degree of modification of 3. As an example, Figure 4 shows the MALDI-ToF mass spectrum of lysozyme and the corresponding midfunctional P(EG_{100}-*co*-G_8) conjugate. The mass spectrum confirms the successful conjugation and reveals multiple distributions, which are due to multiple charged and multimeric species as is expected for protein analysis by MALDI ToF mass spectrometry.

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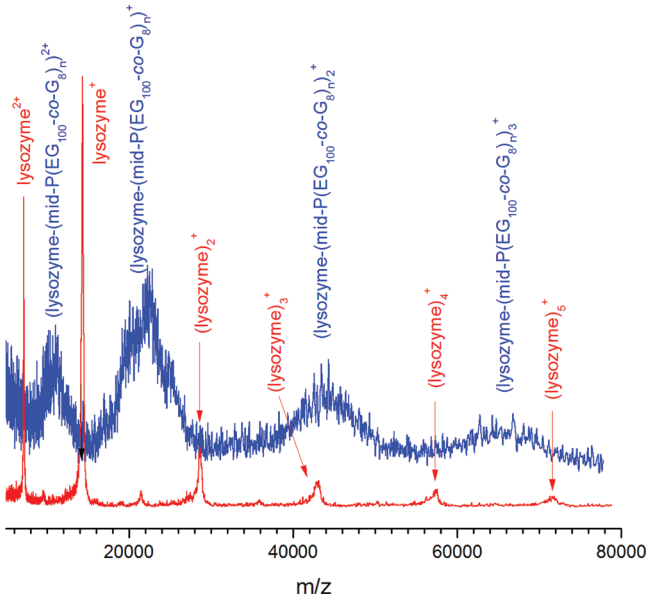

Figure 4. MALDI-ToF mass spectra of lysozyme (red) and a conjugate of lysozyme and midfunctional P(EG_{100}-*co*-G_8) (blue).

Figure 5 shows the results of the SDS-PAGE analysis of a series of lysozyme-polyglycerol (co)polymer conjugates that were prepared using a 3-fold molar excess of the protein-reactive polymer relative to lysozyme. These experiments were carried out with samples taken from the crude, unpurified reaction mixture. Figure 5A,B show the results for different lysozyme conjugates prepared with linear and midfunctional polyglycerol (co)polymers at a polymer/lysozyme ratio of 3:1 and demonstrate the success of the conjugation reaction in all cases. In most cases a small amount of free protein can be detected in the unpurified conjugation mixture together with a distribution of conjugates with a variable number of polymers attached to the protein. If a 3-fold molar excess of two architecturally different copolymers with similar molecular weights is used (cf. lin-P(EG_{204}-*co*-G_{14}; Figure 5A, Lane 5) and mid-P(EG_{205}-*co*-G_{14}) (Figure 5A, Lane 10)), one can clearly see that the apparent molecular weights of the resulting conjugates determined via SDS-PAGE are very similar. Further increasing the molecular weight of the synthetic polymer (see, e.g., lin-P(EG_{407}-*co*-G_{28}), Lane 6) results in a shift to higher apparent molecular weights.

In addition to lysozyme conjugates in which only a part of the available amine groups was modified, another series of conjugation experiments was performed that was aimed at complete modification of the lysozyme amine groups. Figure 6 shows SDS-PAGE analysis of lysozyme that was that was reacted with 10 or 20 equiv of a linear (Lane 3), midfunctional (Lane 2), or linear-hyperbranched polyglycerol derivative (Lane 1) of a molecular weight of about 2000−2700 g/mol as well as different hyperbranched polyglycerol−lysozyme conjugates (Lanes 4−7). As expected for the complete modification of lysozyme, SDS analysis of the $hbPG_{35}$ conjugate (Lane 7) reveals a single broad band. In contrast, for hbPGs with higher molecular weight, that is, 4800, 6400, and 15000 g/mol, conjugation is observed (which is detected by SDS-PAGE (Figure 6, Lanes 4−6)) but the resulting conjugates show a broader distribution of molecular weights. This may be due to steric reasons as the protein-reactive SA ester amide is located at the core of the hyperbranched polymer and probably less accessible compared to linear polymers, so that only low

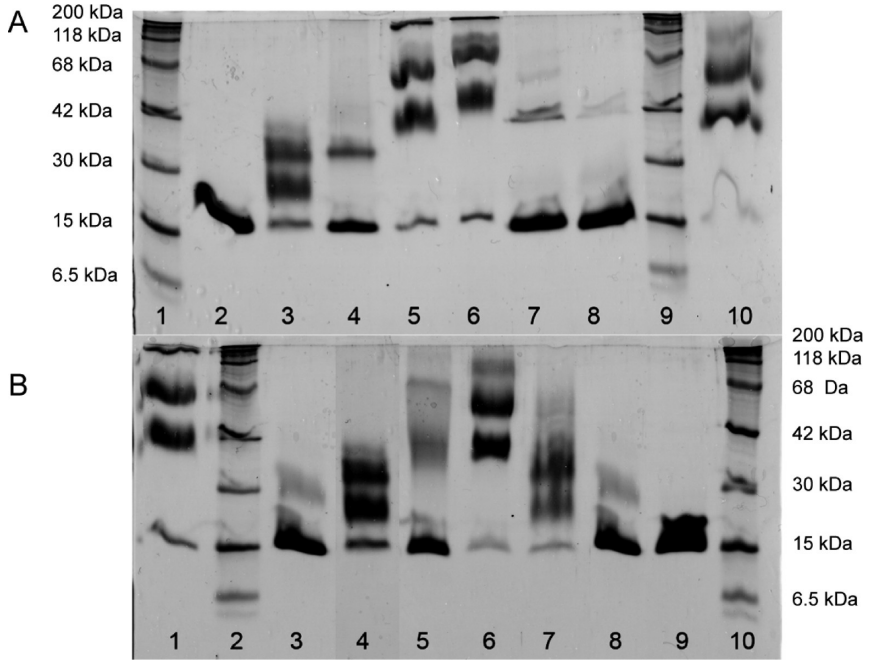

Figure 5. SDS PAGE of (A): (Lane 1) molecular weight markers; (Lane 2) lysozyme; (Lane 3) lin-P(EG_{41}-*co*-G_3) (2000 g/mol, 3 equiv); (Lane 4) lin-P(EG_{102}-*co*-G_7) (4500 g/mol, 3 equiv); (Lane 5) lin-P(EG_{204}-*co*-G_{14}) (10000 g/mol, 3 equiv); (Lane 6) lin-P(EG_{407}-*co*-G_{28}) (19400 g/mol, 3 equiv); (Lane 7) $linPG_{35}$ (2600 g/mol, 2 equiv); (Lane 8) $linPG_{74}$ (5500 g/mol, 2 equiv); (Lane 9) molecular weight markers; and (Lane 10) mid-P(EG_{205}-*co*-G_{14}) (9800 g/mol, 3 equiv); (B): (Lane 1) lin-P(EG_{204}-*co*-G_{14}) (10000 g/mol, 3 equiv); (Lane 2) molecular weight markers; (Lane 3) mid-P(EG_{41}-*co*-G_3) (2000 g/mol, 3 equiv); (Lane 4) mid-P(EG_{100}-*co*-G_8) (3400/mol, 3 equiv); (Lane 5) mid-P(EG_{205}-*co*-G_{14}) (9800 g/mol, 3 equiv); (Lane 6) mid-P(EG_{367}-*co*-G_{24}) (18000 g/mol, 3 equiv); (Lane 7) mid-PG_{68} (5500 g/mol, 3 equiv); (Lane 8) mid-PG_{30} (2200 g/mol, 3 equiv); (Lane 9) lysozyme; and (Lane 10) molecular weight markers.

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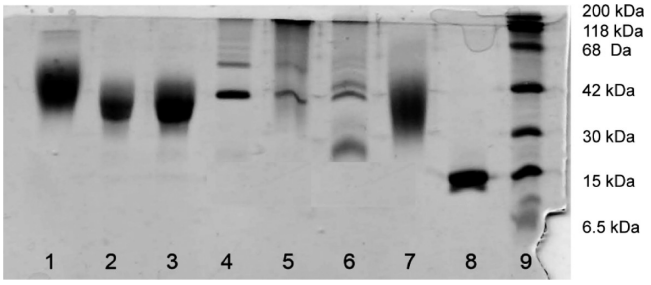

Figure 6. SDS-PAGE analysis of lysozyme conjugates prepared with (Lane 1) PEG_{13}-*b*-$hbPG_{28}$ (2800 g/mol, 20 equiv); (Lane 2) mid-P(EG_{41}-*co*-G_3) (2000 g/mol, 20 equiv); (Lane 3) lin-P(EG_{41}-*co*-G_3) (2000 g/mol, 20 equiv); (Lane 4) $hbPG_{203}$ (15000 g/mol, 10 equiv); (Lane 5) $hbPG_{86}$ (6400 g/mol, 10 equiv); (Lane 6) $hbPG_{65}$ (4800 g/mol, 10 equiv); (Lane 7) $hbPG_{35}$ (2500 g/mol, 10 equiv); (Lane 8) lysozyme; (Lane 9) molecular weight markers.

molecular weight polymers react with lysozyme while the larger ones do not react. Conjugation of $hbPG_{203}$ results in quite sharp bands on the SDS PAGE, which probably represent lysozyme was modified with one and two polymer chains (Lane 4) as the main product. In addition several other conjugates can be detected even with very high molecular weight, which do not penetrate into the gel.

Enzymatic Activity. The activity of a series of PEG and PG (co)polymer-lysozyme conjugates was investigated against *Micrococcus lysodeikticus* (Ml) cells.[41] Prior to the activity measurements, all conjugates were purified by exhaustive dialysis to remove unmodified lysozyme. Gel electrophoresis, HPLC and GPC analysis results of the purified conjugates are included in the Supporting Information (Figures S12−S17). The activity of the conjugates was measured in acetate buffer at pH = 5 (50 mM) as lysozyme has its highest activity at pH = 5.[42] The results are summarized in Table 3.

All polymer−lysozyme conjugates show a loss of activity as compared to unmodified lysozyme as it has been reported in literature for other lysozyme−polymer conjugates.[13,42] From the activity data, three general conclusions can be drawn: (i) the more polymer chains (of a given molecular weight) are conjugated to lysozyme the more the activity is decreased; (ii) at any given degree of modification, the activity of the conjugates (of the same polymer-type) decreases as the molecular weight of the conjugated polymer increases; (iii) only at high PG (co)polymer molecular weights, lysozyme activity appears to be significantly influenced by polymer architecture.

To illustrate the first conclusion, if five PEG_{17} chains (M_n = 800 g/mol) were conjugated, the residual activity was found to be 18 ± 2%. For lysozyme modified with 5 equiv PEG_{44} (M_n = 2100 g/mol), the activity was reduced further to 3.8 ± 1.2% and essentially no activity was observed when in average five chains PEG_{113} (M_n = 5100 g/mol) were conjugated to lysozyme (residual activity 0.5 ± 1%). When all amine groups of lysozyme were P(E)Gylated (use of 20-fold molar excess of the polymer), no residual activity was detected for most conjugates (data not shown), which is also in agreement with other studies that have been reported on various polymers.[11] The influence of the degree modification on enzymatic activity is also obvious from the results on the *hb*PG_{35} conjugates. The activity of these conjugates decreases from 93 to 31% upon increasing the degree of P(E)Gylation from 1 to 5.

The influence of polymer molecular weight at a given degree of P(E)Gylation on the activity is most apparent from the

Table 3. Comparison of the Activities of Different Lysozyme Conjugates

conjugated polymer	$M_{n,polymer}$ (g/mol)[a]	$N_{polymer}$[b]	activity[c]
PEG_{17}	800	3	99 ± 3.0
PEG_{17}	800	5	18 ± 0.6
PEG_{44}	2100	3	71 ± 2.0
PEG_{44}	2100	5	2.8 ± 0.4
PEG_{113}	5100	3	41 ± 1.0
PEG_{113}	5100	5	0.5 ± 0.4
PEG_{226}	10100	3	25 ± 1.9
lin-P(EG_{41}-*co*-G_3)	2000	3	71 ± 2.4
lin-P(EG_{102}-*co*-G_7)	4500	3	62 ± 1.0
lin-P(EG_{204}-*co*-G_{14})	10000	3	28 ± 2.3
lin-P(EG_{407}-*co*-G_{28})	19400	3	23 ± 0.9
lin-PG_{35}	2600	3	67 ± 2.5
lin-PG_{74}	5500	3	52 ± 2.1
mid-P(EG_{41}-*co*-G_3)	2000	3	78 ± 2.0
mid-P(EG_{100}-*co*-G_8)	3400	3	67 ± 1.7
mid-P(EG_{205}-*co*-G_{14})	9800	3	57 ± 2.0
mid-P(EG_{367}-*co*-G_{24})	18000	3	34 ± 1.7
PEG_{13}-*b*-$hbPG_{28}$	2700	3	79 ± 1.1
PEG_{52}-*b*-$hbPG_{14}$	3300	3	58 ± 1.2
PEG_{50}-*b*-$hbPG_{40}$	5200	3	51 ± 1.3
$hbPG_{35}$	2500	1	93 ± 1.0
$hbPG_{35}$	2500	2	85 ± 2.0
$hbPG_{35}$	2500	3	60 ± 1.0
$hbPG_{35}$	2500	5	31 ± 5.0

[a]Number average molecular weight (M_n) determined via ^{1}H NMR. [b]Ratio polymer/protein used in the reaction. [c]Activity measured against *Micrococcus lysodeikticus* cells in percent compared to unmodified lysozyme.

results obtained on the lin-P(EG-*co*-G) and mid-P(EG-*co*-G) lysozyme conjugates. For both families of conjugates, the enzymatic activity was found to decrease from 71% (lin-P(EG-*co*-G)), respectively, 78% (mid-P(EG-*co*-G)) to 23% (lin-P(EG-*co*-G)), and 34% (mid-P(EG-*co*-G)) upon increasing the molecular weight of the conjugated polymer from 2000 to ∼20000 g/mol.

In several studies, the influence of polymer architecture on the biological activity of polymer−protein conjugates has been reported. Tao et al., for example, reported a 2.5 times higher activity of a polymer−protein conjugate consisting of a mid-functional PHPMA (28.5% residual activity) as compared to the linear counterpart (11.5%) of comparable molecular weight (ca. 9000 g/mol).[12] However, the compared protein conjugates differed in the number of attached polymer chains (1.6 for the midfunctional vs 3.1 for the linear), which makes an architectural comparison difficult. Another example was given for uricase, which was PEGylated with a 10000 g/mol linear or midfunctional PEG by Veronese et al.[7] The authors found a residual activity of 29% for linear PEG and 70% for midfunctional PEG. On a first sight, comparison of the enzymatic activities of the conjugates listed in Table 3 that have identical degrees of modification but are generated from architecturally different P(E)G copolymers of comparable molecular weight does not provide any very obvious hints as to possible structure−activity relationships. For example, at a degree of modification of 3 and a polymer molecular weight of ≈2000 g/mol, the PEG_{44} conjugate shows a relative activity of 71% as compared to 71, 78, 79, and 60% for the comparable lin-P(EG_{41}-*co*-G_3), mid-P(EG_{41}-*co*-G_3), PEG_{13}-*b*-$hbPG_{28}$, and $hbPG_{35}$ conjugates. At a similar degree

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of modification but using polymers with a molecular weight of ≈5000 g/mol, the relative activities of the PEG_{113}, lin-P(EG_{102}-*co*-G_7), and PEG_{50}-*b*-hbPG_{40} were determined as 41, 62, and 51%. While possible effects of the P(E)G architecture on the activity of the low molecular weight polymer conjugates seem to be relatively minor, comparison of the activities of the higher molecular weight P(E)G conjugates does reveal some interesting insights. From the three conjugates that contain three copies of a P(E)G copolymer with a molecular weight of ∼10000 g/mol, the residual activity of the mid-P(EG_{205}-*co*-G_{14}) conjugate is two times higher than those of the PEG_{226} and lin-P(EG_{204}-G_{14}) conjugates. Also, comparison of the lin-P(EG_{407}-*co*-G_{28}) and mid-P(EG_{367}-*co*-G_{24}) conjugates, which consists of synthetic polymers of comparable molecular weights, shows a 1.5 times higher residual activity of the mid-functional as compared to the linear P(E)G copolymer conjugate. These observations are in agreement with earlier studies (vide supra)[7,12] and indicate that, at similar molecular weights, the use of a midfunctional, water-soluble polymer is advantageous to minimize the loss of enzymatic activity as compared to the use of the corresponding linear polymer.

CONCLUSIONS

This contribution has successfully demonstrated the feasibility of the squaric acid dialkyl ester mediated amine selective modification of proteins with amine end functionalized, side chain functional synthetic polymers. The synthetic polymers investigated in this study were amine end-functionalized polyglycerol (PG) copolymers obtained via ring-opening polymerization of glycidol derivatives and ethylene oxide. The anionic ring-opening polymerization of glycidol is a versatile synthetic strategy that was used to prepare a library of linear, midfunctional, hyperbranched, and linear-block-hyperbranched PG copolymers. Whereas the efficiency of traditional approaches to couple such hydroxyl side chain functional polymers to proteins can be compromised by concurrent transesterification and hydrolysis reactions, the squaric acid diethylester strategy presented herein combines a high amine selectivity with low susceptibility to hydrolysis, which enables the amine selective protein conjugation of side chain hydroxyl functionalized PG copolymers. For the experiments in this study, BSA and lysozyme were used as model proteins. Varying the relative amounts of squaric acid diethylester activated polymer to protein allowed to control the degree of modification of these proteins. The library of PG–lysozyme conjugates was used to investigate the possible effects of the architecture of the PG copolymers on the enzymatic activity of the PG–lysozyme conjugates. Comparison of the activity of PG–lysozyme conjugates generated from relatively low molecular weight PG copolymers (<5000 g/mol) did not reveal any obvious structure–activity relationships. Evaluation of the activity of conjugates composed of PG copolymers with molecular weights of 10000 or 20000 g/mol, however, indicated significantly higher activities of conjugates prepared from midfunctional synthetic polymers as compared to linear polymers of similar molecular weight. The squaric acid diethyl ester mediated protein conjugation presented here allows unprecedented access to side chain functional synthetic polymer–protein hybrid conjugates and may prove particularly useful for the generation of polymer–protein hybrids modified with labeling groups or targeting ligands for biological or medical applications. Combined with the versatility of the glycidol ring-opening polymerization, this unique coupling strategy opens the way to a broad architectural variety of polymer–protein conjugates and allows to engineer the architecture of the synthetic polymer such as to minimize loss of enzymatic activity in the resulting modified proteins.

ASSOCIATED CONTENT

Supporting Information

Additional NMR, mass spectrometry, and SDS PAGE analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) James, E. T. *Adv. Drug Delivery Rev.* **1993**, *10*, 247–299.
(2) Putney, S. D.; Burke, P. A. *Nat. Biotechnol.* **1998**, *16*, 153–157.
(3) Veronese, F. M.; Pasut, G. *Drug Discovery Today* **2005**, *10*, 1451–1458.
(4) Caliceti, P.; Veronese, F. M. *Adv. Drug Delivery Rev.* **2003**, *55*, 1261–1277.
(5) Abuchowski, A.; van Es, T.; Palczuk, N. C.; Davis, F. F. *J. Biol. Chem.* **1977**, *252*, 3578–3581.
(6) Abuchowski, A.; McCoy, J. R.; Palczuk, N. C.; Es, T. v.; Davis, F. F. *J. Biol. Chem.* **1977**, *252*, 3582–3586.
(7) Veronese, F. M.; Caliceti, P.; Schiavon, O. *J. Bioact. Compat. Polym.* **1997**, *12*, 196–207.
(8) Pasut, G.; Veronese, F. M. *Prog. Polym. Sci.* **2007**, *32*, 933–961.
(9) Alconcel, S. N. S.; Baas, A. S.; Maynard, H. D. *Polym. Chem.* **2011**, *2*, 1442–1448.
(10) Bailon, P.; Palleroni, A.; Schaffer, C. A.; Spence, C. L.; Fung, W.-J.; Porter, J. E.; Ehrlich, G. K.; Pan, W.; Xu, Z.-X.; Modi, M. W.; Farid, A.; Berthold, W.; Graves, M. *Bioconjugate Chem.* **2001**, *12*, 195–202.
(11) Gauthier, M. A.; Klok, H.-A. *Polym. Chem.* **2010**, *1*, 1352–1373.
(12) Tao, L.; Xu, J.; Gell, D.; Davis, T. P. *Macromolecules* **2010**, *43*, 3721–3727.
(13) Tao, L.; Liu, J.; Davis, T. P. *Biomacromolecules* **2009**, *10*, 2847–2851.
(14) Broyer, R. M.; Grover, G. N.; Maynard, H. D. *Chem. Commun.* **2011**, 2212–2226.
(15) Gauthier, M. A.; Klok, H.-A. *Chem. Commun.* **2008**, 2591–2611.
(16) Wurm, F.; Klos, J.; Räder, H. J.; Frey, H. *J. Am. Chem. Soc.* **2009**, *131*, 7954–7955.
(17) Kainthan, R. K.; Brooks, D. E. *Biomaterials* **2007**, *28*, 4779–4787.
(18) Wilms, D.; Stiriba, S.-E.; Frey, H. *Acc. Chem. Res.* **2009**, *43*, 129–141.
(19) Siegers, C.; Biesalski, M.; Haag, R. *Chem.—Eur. J.* **2004**, *10*, 2831–2838.
(20) Dworak, A.; Walach, W.; Trzebicka, B. *Macromol. Chem. Phys.* **1995**, *196*, 1963–1970.
(21) Kainthan, R. K.; Muliawan, E. B.; Hatzikiriakos, S. G.; Brooks, D. E. *Macromolecules* **2006**, *39*, 7708–7717.
(22) Sunder, A.; Hanselmann, R.; Frey, H.; Mulhaupt, R. *Macromolecules* **1999**, *32*, 4240–4246.
(23) Roberts, M. J.; Bentley, M. D.; Harris, J. M. *Adv. Drug Delivery Rev.* **2002**, *54*, 459–476.

(24) Greenwald, R. B.; Pendri, A.; Martinez, A.; Gilbert, C.; Bradley, P. *Bioconjugate Chem.* **1996**, *7*, 638−641.
(25) Subr, V.; Ulbrich, K. *React. Funct. Polym.* **2006**, *66*, 1525−1538.
(26) Hou, S.-j.; Saksena, R.; Kovác, P. *Carbohydr. Res.* **2008**, *343*, 196−210.
(27) Izumi, M.; Okumura, S.; Yuasa, H.; Hashimoto, H. *J. Carbohydr. Chem.* **2003**, *22*, 317−329.
(28) Yan, H.; Aguilar, A. L.; Zhao, Y. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 6535−6538.
(29) Sejwal, P.; Han, Y.; Shah, A.; Luk, Y.-Y. *Org. Lett.* **2007**, *9*, 4897−4900.
(30) Tietze, L. F.; Arlt, M.; Beller, M.; Glüsenkamp, K. H.; Jähde, E.; Rajewsky, M. F. *Chem. Ber.* **1991**, *124*, 1215−1221.
(31) Dingels, C.; Wurm, F.; Klok, H.-A.; Frey, H. submitted for publication.
(32) Fitton, A. O.; Hill, J.; Jane, D. E.; Millar, R. *Synthesis* **1987**, *1140*, 1142.
(33) Zhang, W.; Go, M. L. *Eur. J. Med. Chem.* **2007**, *42*, 841−850.
(34) Mangold, C.; Wurm, F.; Obermeier, B.; Frey, H. *Macromol. Rapid Commun.* **2010**, *31*, 258−264.
(35) Wurm, F.; Nieberle, J.; Frey, H. *Macromolecules* **2008**, *41*, 1184−1188.
(36) Schmidt, A. H. *Synthesis* **1980**, 961−994.
(37) Rotger, M. C.; Pina, M. N.; Frontera, A.; Martorell, G.; Ballester, P.; Deyà, P. M.; Costa, A. *J. Org. Chem.* **2004**, *69*, 2302−2308.
(38) Hirayama, K.; Akashi, S.; Furuya, M.; Fukuhara, K.-I. *Biochem. Biophys. Res. Commun.* **1990**, *173*, 639−646.
(39) Van Regenmortel, M. H. V., Briand, J. P., Müller, S.; Plaué, S. *Synthetic Polypeptides as Antigens*; Elsevier: Amsterdam, 1988; Vol. *19*.
(40) Held, D.; Müller, A. H. E. *Macromol. Symp.* **2000**, *157*, 225−238.
(41) Salton, M. R. J. *Nature* **1952**, *170*, 746−747.
(42) Nodake, Y.; Yamasaki, N. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 767−774.