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Squaric Acid Mediated Chemoselective PEGylation of Proteins: Reactivity of Single-Step-Activated α-Amino Poly(ethylene glycol)s

Carsten Dingels,[a] Frederik Wurm,[b, c] Manfred Wagner,[c] Harm-Anton Klok,[b] and Holger Frey*[a]

Abstract: The covalent attachment of poly(ethylene glycol) (PEG) to therapeutically active proteins (PEGylation) has become an important method to deal with the pharmacological difficulties of these polypeptides, such as short body-residence times and immunogenicity. However, the derivatives of PEG used for PEGylation lack further functional groups that would allow the addition of targeting or labeling moieties. Squaric acid diethyl ester was used for the chemoselective single-step activation of poly(ethylene glycol)s into the respective ester amides. The resultant selective protein-reactive poly(ethylene glycol)s were investigated with respect to their selectivity towards amino acid residues in bovine serum albumin (as a model protein). The presented procedure relies on a robust two-step protocol and was found to be selective towards lysine residues; the activated polyethers are efficient and stoichiometric PEGylation agents with a remarkable hydrolytic stability over a period of several days. By adjusting the pD value of the conjugation mixture, the chemoselectivity of the activated PEGs towards the α- and ε-amino groups of lysine methyl ester was effectively changed.

Keywords: chemoselectivity · kinetics · PEGylation · proteins · squaric acid

Introduction

The application of therapeutically active proteins such as insulin, interferons, and granulocyte-colony-stimulating factor as pharmaceuticals suffers from their immunogenicity and susceptibility towards degradation in vivo. Therefore, different strategies for protein modification have been developed to reduce these effects, such as the addition, removal, substitution, or chemical modification of amino acids and the attachment of synthetic polymers.[1] Abuchowski et al. linked poly(ethylene glycol) (PEG) to bovine serum albumin (BSA) and bovine liver catalase through a cyanuric chloride coupling route[2] and laid the cornerstone for PEGylation, that is, the covalent attachment of PEG to (poly)peptides and proteins. PEG exhibits a unique combination of properties valuable for bioconjugation: It is highly soluble in water, chemically inert, nontoxic, mostly nonimmunogenic, and soluble in many organic solvents.[3a] PEGylation is the most important strategy to reduce immunogenicity and to increase the blood circulation times of drugs. In addition, due to the increased molecular weight and hydrodynamic volume of the PEGylated therapeutic in comparison with those of the native protein, renal excretion is reduced. Furthermore, the polyether chains shield the protein and hinder antibodies from recognizing the determinants on the surface of the therapeutic, thus reducing its immunogenicity. Also, the shielding impedes proteases in catalyzing the hydrolytic degeneration of the polypeptides.[3] Today, a number of PEGylated enzymes, cytokines, antibodies, and growth factors are approved by the US Food and Drug Administration.[4]

In the last decades, a variety of strategies were established to covalently bind PEG to proteins. Commonly, the monomethyl ether of PEG (mPEG) is used for PEGylation after (usually multistep) activation of the hydroxy group with a protein-reactive moiety. Most of the protein-reactive PEG derivatives that have been used so far, however, are susceptible towards a range of functional groups (for example, amines, alcohols, and thiols), and the PEGylation chemistries that have been developed so far are not easily extended towards alternative synthetic polymers that contain functional groups other than the one that is used for the protein conjugation. This obviously impedes the development of synthetic polymer–peptide/protein conjugates in which the synthetic polymer contains functional groups that can be used for further functionalization, for example, to introduce labels or target ligands. A potentially interesting reagent that could help to overcome these problems is diethyl squa-

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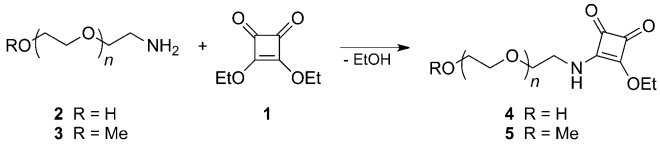
rate (**1**). Diethyl squarate has been previously used for the amine-selective, hydroxy-orthogonal attachment of carbohydrates[5] and glycopeptides[6] to proteins. Diethyl squarate consists of two connected vinylogous carboxylic acid esters sharing a carbon–carbon double bond (Scheme 1). Due to



Scheme 1. One-step activation of α-amino-PEGs with diethyl squarate.

this connection, derivatization of one ester moiety affects the reactivity of the other. The reactivity of the diester towards amidation is high. At neutral pH values, it can be converted selectively into the analogous ester amide, without formation of the coupled product, that is, the diamide (squaramide), because the resulting ester amide has a reduced reactivity.[7] Thus, the ester amide can be isolated, and at higher pH values, usually around pH 9, it can react with a second amine to result in an asymmetric squaric acid di amide. Squaric acid derivatives were calculated to be weakly aromatic,[8] so the amidation of **1** can be viewed as a nucleophilic substitution in an aromatic system. In comparison to **1**, the squaric acid ester amide possesses a higher electron density in the carbon cycle, which makes a second nucleophilic attack less favorable. Moreover, squaramides exhibit high proton affinity and enhanced aromaticity when protonated,[8] which might also apply for the ester amides and explain the increased reactivity in basic media.

Herein, we report the activation of α-amino PEGs with **1** in a single step that tolerates the presence of hydroxy and other functional groups, which allows for subsequent derivatization of the polymer. In addition, we demonstrate efficient PEGylation with the novel activated PEGs under different conditions and their stability towards hydrolysis. Selectivity of the PEGylation towards the α- and ε-amino groups of lysine was investigated in detail, and it was proven that the chemoselectivity can be adjusted selectively towards either of the amines at different pH values.

Results and Discussion

Synthesis of protein-reactive PEGs: α-Amino-ω-hydroxy PEG (**2**) and the respective methoxy-PEGs, **3**, with number-averaged molecular weights (M_n) of 2 and 5 kDa, were modified with **1**, and the properties of the resulting protein-reactive polymers were investigated. The amino mPEGs, synthesized according to a known protocol,[9] were activated with diethyl squarate in ethanol with triethylamine as an additive. The size exclusion chromatography (SEC) elugrams of the squaric acid ester amide mPEGs (SEA-mPEGs, **5**) showed narrow molecular-weight distributions (polydispersity index (PDI) = 1.05), and no dimers, that is, diamides, were detectable, for either the 2.1 kDa sample (Figure 1) or the 5.1 kDa sample (see the Supporting Information, Figure S14). In addition, the presence of the squaramide was also detected by strong UV absorption during the SEC

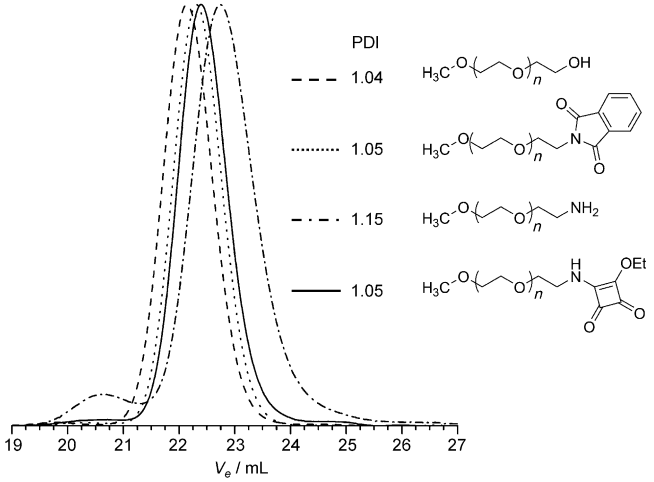


Figure 1. SEC elugrams of **4** (2.1 kDa) and its precursors (eluent: DMF; refractive index (RI) detector signal). The amino-PEGs often revealed a broadening of the mass distribution in the SEC analysis that led to an increase in the M_w/M_n ratio (the polydispersity index). Sometimes, even bimodal SEC traces were observed. This effect was attributed to interactions of the amino moiety with the poly(hydroxyethylmethacrylate) columns of the chromatograph. Upon derivatization, the PDI values decreased significantly again.

measurements. Quantitative end-group functionalization was proven by MALDI-TOF MS and ^{1}H NMR spectroscopy. The MALDI-TOF mass spectra of **5** with molecular weights of 2.1 kDa and 5.1 kDa displayed the expected single mass distribution. No unreacted amino mPEG or coupled products were detected. Besides the distribution of the polymer cationized with potassium, a second distribution with lower intensity was observed in some cases, which was assigned to the sodium-cationized species (Figure 2 and Figure S8 in the Supporting Information). Full conversion was also confirmed by comparison of the ^{1}H NMR integrals of

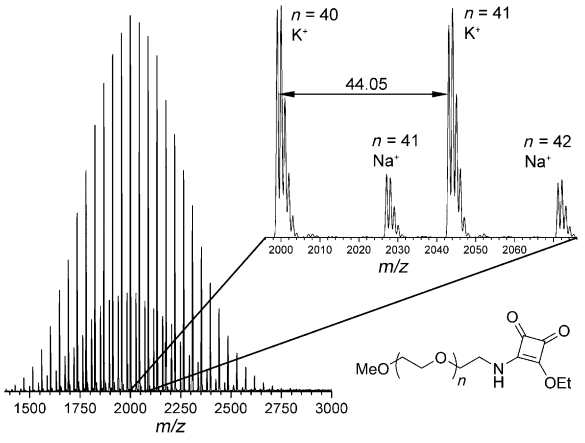
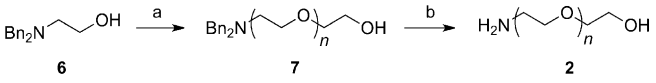


Figure 2. MALDI-TOF mass spectrum of **5** (2.1 kDa).

the peaks assigned to the methyl groups at both polymer termini (Figure S3 and S4 in the Supporting Information). The signal of the single amide proton splits into two broad peaks at approximately $\delta = 6.6$ ppm. This has already been reported for low-molecular-weight compounds[10] and is a result of the rotational barrier along the cyclobutene carbon–nitrogen bond, which leads to the presence of *syn* and *anti* conformers.

The tolerance of the diethyl squarate activation of PEG towards hydroxy groups was further demonstrated by the synthesis of α-squaric acid ester amido ω-hydroxy-PEG (**4**; Scheme 1). The necessary α-amino ω-hydroxy PEG was synthesized by *N*,*N*-dibenzyl amino ethoxide initiated anionic ring-opening polymerization of ethylene oxide (EO) and subsequent cleavage of the protecting groups by hydrogenation (Scheme 2), analogous to protocols for the synthesis of



6 7 2

Scheme 2. Synthetic route to α-amino-ω-hydroxy-PEG. a) 1) C_6H_6, 90 °C, vacuum; 2) $KC_{10}H_8$, THF, *n*EO; 3) MeOH; b) H_2, $Pd(OH)_2$/C, MeOH/THF/water (2:1:1), 80 bar. Bn: benzyl; THF: tetrahydrofuran.

copolymers of EO with different comonomers that were published recently.[11] Successful synthesis of **7** with narrow molecular-weight distributions (PDI ≤ 1.06) was confirmed by NMR spectroscopy, MALDI-TOF MS, and SEC (see the Supporting Information). Removal of the benzyl protecting groups by catalytic hydrogenation[12] was complete after 24 h (at 30–40 bar), as determined by NMR spectroscopy. Conversion of **2** into the analogous squaric acid ester amide **4** was accomplished by the reaction of **1** in ethanol/water (1/1) with triethylamine as an additive (Scheme 1). ^{1}H NMR spectroscopy and MALDI-TOF MS confirmed full derivatization of the amino group and preservation of the hydroxy moiety. As for the SEA-mPEGs, the formation of the corresponding squaramide was suppressed and no coupled product was found by SEC analysis.

Reactivity of the squaric acid ester amide at the polymer terminus: In a next step, the reactivity of the terminal squaric acid ester amide towards the ε-amino group of lysine was investigated by following the reaction kinetics with ^{1}H NMR spectroscopy. As a model substrate, N_α-acetyl-L-lysine methyl ester hydrochloride (Ac-Lys-OMe), a lysine derivative with protected α-amino and carboxylic acid groups, was chosen. The solvent was a 0.1 M sodium borate buffer in deuterium oxide that provided a stable pD value of about 9.6 (0.4 added to the corresponding pH value of 9.2)[13] throughout the reaction, which was sufficiently high to enable the diamide formation. As the resonances of the ester amide's ethyl group were superimposed by other signals and the amide protons were not observable under these conditions, the resonance of the methyl group of ethanol, which is released during the reaction, was monitored at a chemical shift value of $\delta = 1.16$ ppm. Figure 3 shows the intensity of

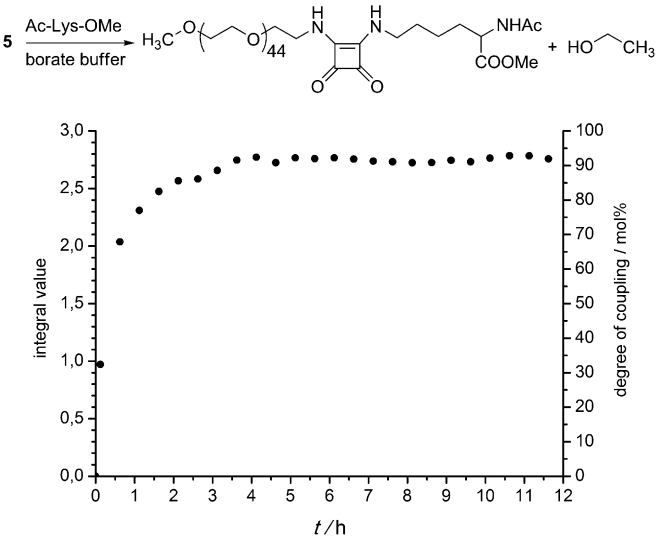


Figure 3. Reaction kinetics of SEA-mPEG (2.1 kDa) with N_α-acetyl-L-lysine methyl ester (2 equiv) in deuterated borate buffer (pD 9.6) monitored by ^{1}H NMR spectroscopy.

the methyl signal of the recorded spectra plotted against time for the reaction of a 2 kDa SEA-mPEG with 1.8 equivalents of Ac-Lys-OMe. All signals were normalized to the singlet resonance of the methoxy group of mPEG at $\delta =$ 3.36 ppm. The resonance of the *N*-acetyl protons of the polymer-bound lysine ($\delta = 2.02$ ppm, Figure 3) was shifted by $\delta = 0.02$ ppm to higher field relative to the analogous signal of the substrate. However, these signals superimposed and, hence, were not suitable for the kinetic data analysis but could qualitatively show the progress of the reaction. As shown in Figure 3, after approximately four hours, the reaction was complete and the signal ratios remained constant. The reaction was carried out at room temperature in an NMR tube and mixing was merely provided by the spinning of the tube. Under the reaction conditions, the lysine methyl ester protecting group was hydrolyzed slowly (18% cleavage after 12 h). Obviously, hydrolysis of the polymer's terminal ethyl ester is a possible side reaction that has to be taken into account. It was monitored under the same conditions as the experiment described above but without the substrate by monitoring the decreasing intensity of the methyl resonance of the ethyl ester (see Figure S18 in the Supporting Information). The half-life of **5** in deuterated borate buffer (pD 9.6) was found to be three days, which evidenced very slow hydrolysis relative to the fast diamide formation. The hydrolysis of **5** was further studied at pD 8.4 in an analogous ^{1}H NMR experiment by using a deuterated phosphate buffer solution. Within three days, 4.5% of the squaric acid ester was degraded (see Figure S20 in the Supporting Information). When it is considered that the reported hydrolytic half-lives of the commonly used PEG carboxylic acid *N*-hydroxysuccinimide esters are in the order of 0.75–23.3 min at pH 8,[14] this hydrolytic stability of **5** is remarkable and underlines the potential of this reagent to enable amino-selective protein conjugation of functional synthetic polymers.

The reaction rate was increased significantly by adding dimethylsulfoxide (DMSO) to the reaction. When 2 equivalents of substrate were used and the solvent consisted of 70% deuterated borate buffer and 30% [D_6]DMSO, the reaction was completed after approximately 40 min (Figure 4).

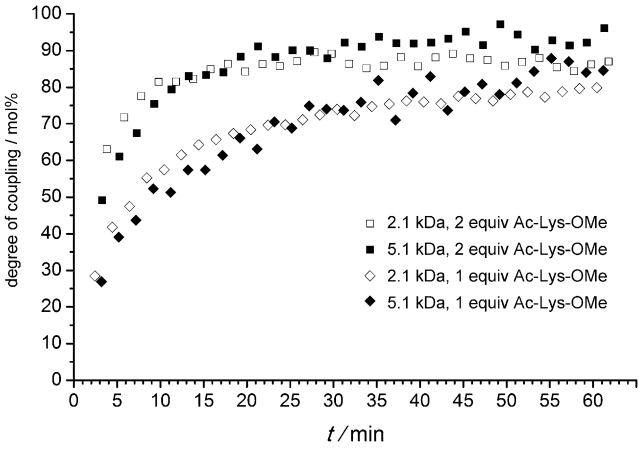


Figure 4. Diamide formation in borate buffer/DMSO (7:3) for two SEA-mPEGs with different molecular weights and two different concentrations of N_α-acetyl-L-lysine.

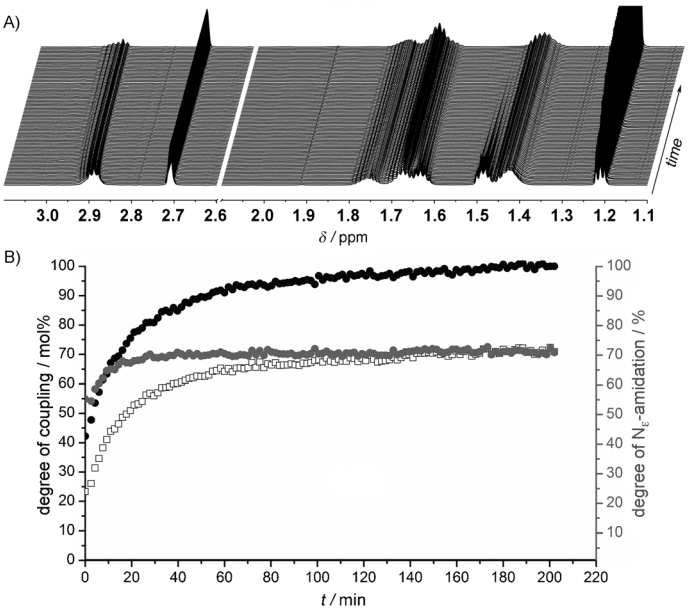


Figure 5. Reaction kinetics of SEA-mPEG (2.1 kDa) with L-lysine methyl ester (1 equiv) in borate buffer (pD 9.6)/[D_6]DMSO (7:3). A) Selected regions of the ^{1}H NMR spectra (700 MHz; T = 294 K). B) Normalized signal intensities (left scale; black circles: EtOH; open squares: N_ε-amide) and the degree of N_ε-amidation (right scale; gray circles) plotted against reaction time.

As before, the increasing signal intensity of the released ethanol was monitored. The more pronounced data-point fluctuation in the case of the larger polymers is attributed to the lower signal-to-noise ratio. Interestingly, the degree of polymerization, that is, 44 or 112, had no considerable influence on the reactivity of the ethyl squarate. The reaction rates of the second amidation step can be influenced by the amount of substrate. As expected, adding equimolar amounts of Ac-Lys-OMe resulted in slightly lower reaction rates than those with a twofold excess (Figure 4). Again, the rates did not depend on the molecular weight of the PEG derivatives that were used. The hydrolysis of **5** in the presence of DMSO was also found to be faster: 50% of the macromolecular ester was hydrolyzed in 1.5 days (see Figure S19 in the Supporting Information). However, in comparison to the timescale of the diamide formation, the hydrolysis can be neglected, which should allow for stoichiometric control over the degree of PEGylation (see below).

Chemoselectivity: The selectivity of SEA-mPEG towards the α- and the ε-amino group of lysine was investigated by following the reaction of **5** with one equivalent of L-lysine methyl ester dihydrochloride (H-Lys-OMe) in detailed ^{1}H NMR spectroscopy experiments. Figure 5A shows selected regions of a series of NMR spectra recorded in a borate buffer (pD 9.6)/[D_6]DMSO solution (the full spectra can be found in Figure S21 in the Supporting Information). The evolving triplet at δ = 1.21 ppm, which can be assigned to the ethanol released during the reaction, was used to monitor the total conversion by amidation and is plotted against the reaction time in Figure 5B (black circles). Full conversion was achieved after approximately three hours, which is also confirmed by the decaying peak of the terminal methyl group of the squaric acid ester amide at δ = 1.5 ppm. The conversion of the lysine ε-amino group was calculated from the decreasing signal of the adjacent methylene group at δ = 2.9 ppm and is also shown in Figure 5B (open squares). After an initial phase, the degree of N_ε-amide formation, which was obtained by dividing the ε-amino group conversion by the total conversion was found to be constantly 70%. Hence, under these conditions, the ε-amino group is more reactive than the sterically more hindered α-amino group, which would suggest preferred PEGylation of the ε-amino groups in a protein. During the reaction time of three hours, the hydrolysis of the polyether's terminal ethyl ester in this solvent is negligible (see above).

When the same reaction was carried out in a deuterated phosphate buffer at pD 8.4, the reaction time increased significantly. The amidation was monitored continuously for the first 28 h and selected regions of the spectra are shown in Figure 6A (the full spectra are shown in Figure S22 in the Supporting Information). As determined from the released ethanol, approximately 70% of the SEA-mPEG undergoes amidation during this period of time; after storage of the reaction mixture for six days, the conversion increased to 92%. Data derived from two spectra recorded after 63 h and 139 h are marked with asterisks in Figure 6B and only can be regarded as a trend because the sample was stored at room temperature in between the measurements, whereas it was kept constantly at 294 K within the spectrometer for the first 28 h.

Most interestingly, the chemoselectivity of the SEA-mPEG changes at this pD value compared with that in the

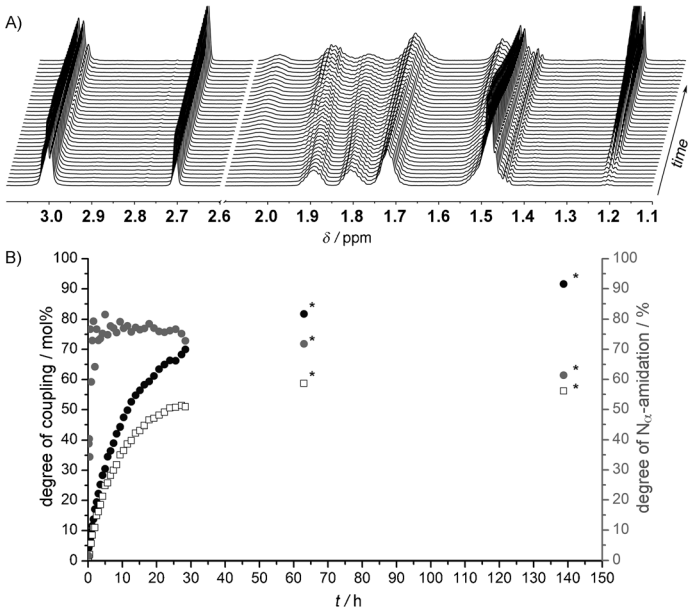


Figure 6. Reaction kinetics of SEA-mPEG (2.1 kDa) with L-lysine methyl ester (1 equiv) in phosphate buffer (pD 8.4)/[D_6]DMSO (7:3). A) Selected regions of ^{1}H NMR spectra (700 MHz; $T=294$ K) obtained in the first 28 h. B) Normalized signal intensities (left scale; black circles: EtOH; open squares: N_α-amide) and the degree of N_α-amidation (right scale; gray circles) plotted against reaction time. Data points marked with asterisks were recorded after resting of the sample at room temperature, whereas the reaction was followed at $T=294$ K for the first 28 h.

experiment at pD 9.6. A major fraction of the amino-reactive PEG reacts with the α-amino group of lysine. To our knowledge, this is the first time that such behavior has been observed for a squaric acid alkyl ester amide. Whereas the signal of the methylene group adjacent to the ε-amino group of the lysine derivative exhibited a significant decrease in the experiments described above, only a slight decrease occurred during the reaction at the lower pD value. Instead, a signal at $\delta=2.05$ ppm was detected and was assigned to one of the diastereotopic protons of the β-carbon atom of lysine, which was PEGylated through the α-amino group. After 28 h, 50% of the α-amino groups had been converted; after 63 h, it was 59%. However, 76 h later, the signal had decreased by 3%. Whether this observation should be assigned to a hydrolytic cleavage of the squaramide or to solvent effects is under current investigation. The assignment of this signal to one of the β-protons of N_α-PEGylated lysine was validated by DOSY (diffusion-ordered spectroscopy) and TOCSY (total correlation spectroscopy) NMR experiments on the sample. The TOCSY spectrum (see Figure S26 in the Supporting Information) revealed the affiliation of the signal at $\delta=2.05$ ppm to the spin system of the lysine's alkyl chain and coupling to a signal superimposed by the HDO signal at $\delta=4.8$ ppm, which was assigned to the α-proton of N_α-PEGylated lysine. According to the DOSY spectrum (see Figure S27 in the Supporting Information), the aforementioned signals belong to a species with the lowest diffusion constant of the system (1×10^{-10} m^{2}s^{-1}), which indicates a PEGylated lysine. The degree of N_α-amidation was found to be approximately 75% within the first 28 h and diminished during the course of the reaction. One of the reasons is the hydrolysis of the SEA-mPEG, which has to be taken into account because the total conversion took longer than 140 h. As the lysine derivative can be seen as a model for the N terminus of a protein, it might be possible to use SEA-PEGs for the selective PEGylation of a protein's N terminus at a specific pH value which is currently under investigation. The reaction of **5** with H-Lys-OMe in the deuterated borate buffer/DMSO solution was also carried out at a lower temperature (283 K). Apart from a prolonged reaction time (total conversion was achieved after 9 h), no significant differences to the experimental results illustrated in Figure 5 were observed. Again, the lysine derivative was PEGylated mainly at the ε-amino group and the degree of N_ε-amidation was around 70% (see Figure S23 in the Supporting Information). These experiments clearly prove the versatility and the superior hydrolytic stability of squaric acid ester amides over the conventional activated esters used in protein PEGylation.

Besides the amino groups of the lysine residues or the N terminus, other nucleophilic amino acids may potentially react with the activated polyethers **4** and **5**, such as the hydroxy group of serine, the guanidine group of arginine, and histidine's imidazole moiety. To evaluate the selectivity of the squaric acid modified PEGs towards amine groups, the reactivity of **5** towards arginine and histidine residues was investigated in ^{1}H NMR experiments analogous to those described earlier. *N-tert*-Butoxycarbonyl-L-histidine methyl ester (Boc-His-OMe) served as the histidine model compound with protected α-amino and carboxylic acid groups, and a solution of 70% deuterated borate buffer with 30% [D_6]DMSO was used as the solvent. As before, the signal intensity of the methyl group of ethanol released in the course of the reaction was monitored for 12 h (1.5 equivalents of protected amino acid were used; see Figure S25 in the Supporting Information). The NMR experiments revealed the release of only 5% ethanol over this time period, which is identical to the loss of ethyl ester groups that was observed during the experiments that were carried out to assess the hydrolytic stability of **5** (see above). This clearly indicates that **5** is essentially nonreactive towards histidine. A similar result was obtained from experiments with N_α-*p*-tosyl-L-arginine methyl ester hydrochloride (Ts-Arg-OMe). Even after 2.5 h, the expected resonance of the methyl group of ethanol was barely visible (see Figure S24 in the Supporting Information). Thus, undesired PEGylation of histidine and arginine residues with **5** under these conditions can be ruled out. In addition to reaction with arginine and histidine residues, another possible side-reaction during protein conjugation could involve transesterification with hydroxy-side-chain functional amino acids, such as serine. In principle, however, the reaction of **4** or **5** with serine, or another hydroxy-containing amino acid, would merely generate another PEG squaric acid monoester derivative, which would still be reactive towards amine groups and, as a consequence, would not be expected to compromise the chemoselectivity

of the SEA-PEG derivatives. To evaluate this hypothesis, a mixture of two equivalents each of Ac-Lys-OMe and *N*-benzyloxycarbonyl-L-serine methyl ester (Z-Ser-OMe) was treated with **5** in a solution of ethanol with triethylamine as an additive. MALDI-TOF MS analysis of the reaction products only indicated the formation of the diamide (see Figure S9 in the Supporting Information). This clearly proves that the novel SEA-PEGs can chemoselectively PEGylate proteins at the amino groups of lysine residues and probably the N terminus without interference by arginine, histidine, or serine moieties. The reaction of squaric acid ester amides with low molecular weights towards cysteine has already been studied and was found to result exclusively in the corresponding squaramide because the thioester formation is reversible.[15] Therefore, in the presence of amino groups, squaric acid mediated PEGylation at cysteine residues is unlikely.

Bioconjugation: The bioconjugation of the novel activated PEGs was investigated with a model protein, namely bovine serum albumin (BSA), the primary structure of which consists of 59 lysine residues,[16] from which 30–35 are available for covalent attachment.[17] 10-, 20-, 50-, and 100-fold excesses of **5** were fed to solutions of the protein in an aqueous borate buffer (pH 10). These amounts correspond to 0.29, 0.57, 1.4, and 2.9 equivalents of the available lysine residues, respectively. Two series of conjugates with varying molecular weights of **5**, that is, a 2.1 kDa polymer (Table 1, conjugates **A**–**D**) and a 5.1 kDa polymer (Table 1, conjugates **E**–**H**), were prepared. All PEGylated proteins were purified by dialysis and isolated after freeze–drying in almost quantitative yields (with respect to the amount of BSA used). Figure 7 shows the sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) results for the obtained conjugates (lanes 3 to 10) and the unmodified BSA (lane 2), as well as a molecular-weight size marker (lane 1). The absence of free unmodified BSA in all of the lanes indicates complete conversion of the native protein. All conjugates exhibit significantly increased molecular weights compared to BSA, which evidences modification of the protein in all cases. The samples prepared by adding a 10- and 20-fold molar excess of the polymer show a clear difference in their apparent size, whereas the conjugates obtained from the batches treated with 50 and 100 equivalents displayed only slight differences. The PEGylated BSA synthesized with 100 equivalents of the 5.1 kDa polymer barely moved through the gel (Figure 7, lane 10). These results were in very good agreement with the results obtained by SEC and measured with a right-angle light-scattering detector (SEC-RALS), which provided a more specific size analysis of the synthesized protein–polymer conjugates. The SEC traces of all samples including the unmodified protein tailed to higher molecular weights, which indicates the presence of a small fraction of dimeric BSA in the commercial protein; this is also visible in the SDS-PAGE result for the unmodified BSA (Figure 7, lane 2). From the absolute number-averaged molecular weights determined by the SEC-RALS analysis, the average number of PEG chains attached to a protein was calculated (Table 1).

Table 1. Sizes and degrees of PEGylation for the mPEG–protein conjugates.

Conjugate	M_{PEG} [kDa]	n_{PEG}/n_{BSA}[a]	M_{con} [kDa][b,c]	n_{PEG}/n_{BSA}[c]
A	2.1	10	87.0	10.0
B	2.1	20	106.5	19.4
C	2.1	50	117.5	24.7
D	2.1	100	136.0	33.5
E	5.1	10	112.3	9.1
F	5.1	20	157.4	18.0
G	5.1	50	226.0	31.5
H	5.1	100	240.0	34.2

[a] Ratio applied. [b] Determined by SEC-RALS analysis. [c] Ratio found.

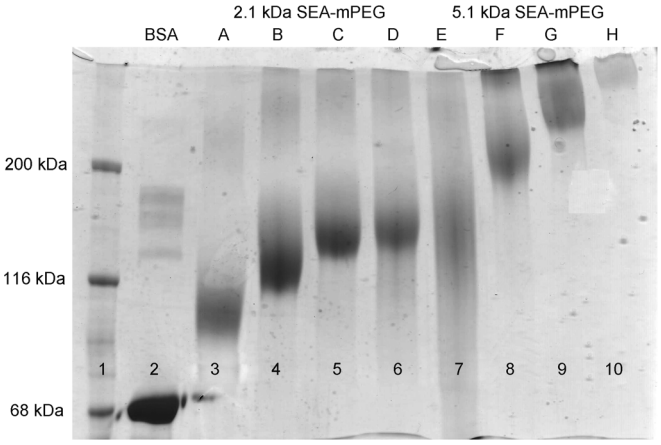


Figure 7. SDS-PAGE (6%) results of BSA (lane 2) and BSA–polymer conjugates prepared by different feeds of either 2.1 kDa SEA-mPEG (lanes 3–6) or 5.1 kDa SEA-mPEG (lanes 7–10) in aqueous borate buffer (pH 10).

For the conjugates synthesized with low feeds of **5** (**A**, **B**, **E**, and **F**), the average number of polymers bound to BSA corresponded well to the targeted ratio of polymer/protein and confirmed stoichiometric PEGylation of BSA without loss of active polymer. This is rationalized by the extreme hydrolytic stability of the reactive ester amide at the polymer chain end. **D** and **H** carry approximately 34 polymer chains, respectively, which indicated quantitative PEGylation of the surface-available lysines. In order to investigate the influence of the PEGylation reaction conditions, especially the elevated pH value, on the protein structure, circular dichroism (CD) spectra of both BSA and the PEGylated BSA samples were recorded. The CD spectroscopy experiments demonstrated that the structure of the model protein BSA was not changed by the squaric acid mediated PEGylation with **5** ($M_n = 2.1$ kDa), regardless of the degree of PEGylation (CD spectra of BSA, **B**, and **D** are provided in Figure S29 in the Supporting Information). The high pH protocol is probably not suitable for all proteins. However, some proteins like rabbit kidney cytokinase and catechol 1,2-dioxygenase exhibit maximal activity at pH 8.5 or 9, for exam-

ple.[18] For the production of PEGASYS, one of the most prominent PEGylated therapeutics on the market, human interferon α-2a is PEGylated in a borate buffer at pH 9 at 4 °C.[19] The results of kinetic studies on the reaction of SEA-mPEGs with L-lysine methyl ester at pD 8.4 suggest a promising outcome for the squaric acid mediated PEGylation under less basic conditions. Furthermore, Luk and coworkers demonstrated the reactivity of low-molecular-weight squaric acid ethyl ester alkyl derivatives towards peptides with N-terminal cysteines under very mild conditions (neutral pH value).[15] The applicability of these procedures to our PEGylation agents is under current investigation.

The hydroxy-functionalized SEA-PEGs, **4**, were found to be equally suitable for the PEGylation of BSA as the methyl ether derivatives (see Figure S28 in the Supporting Information).

Conclusions

We have demonstrated the single-step activation of α-amino PEGs with commercially available diethyl squarate (**1**). This general protocol proved to be highly selective for PEGylation of lysine residues, without side reactions at other nucleophilic amino acid residues, on the model protein BSA in aqueous systems at room temperature. In comparison with standard PEGylation agents, the squaric acid ester amide activated polyethers proved to have prolonged hydrolytic stability (half-life of several days at pD 9.6), which broadens the applicability of PEGylation reactions in biologically interesting media and guarantees efficient PEGylation even over a long reaction time. Furthermore, the squaric acid mediated PEGylation tolerates hydroxy-functionalized PEG derivatives and, as shown recently, even polyglycerol derivatives,[11d] which gives the opportunity for labeling or attachment of targeting functions to the polymer chains in subsequent reactions.

Detailed NMR investigations proved a remarkable change in the chemoselectivity of the squaric acid activated PEG: Whereas mainly the ε-amino group of lysine is PEGylated at elevated pD values, the major product in a less basic environment is the N_α-amide of lysine. This selectivity and high hydrolytic stability renders the squaric acid mediated PEGylation superior over many other activated ester techniques for protein modification. The chemoselectivity of the SEA-PEGs will be explored in further studies.

Experimental Section

Materials: All reagents and solvents were purchased from Acros Organics or Sigma–Aldrich and were used without further purification, unless stated otherwise. Diisopropylazodicarboxylate was purchased from Apollo Scientific, and aqueous borate buffer (pH 10) was obtained from Fisher Scientific. N_α-Acetyl-L-lysine methyl ester hydrochloride and *N*-*tert*-butoxycarbonyl-L-histidine methyl ester were bought from Bachem. The dialysis membranes (Spectra/Por 1, molecular-weight cut off (MWCO) = 6–8 kDa, 40 mm flat width; Spectra/Por 6, MWCO = 50 kDa, 34 mm flat width) were obtained from Spectrum Laboratories. Deuterated solvents were purchased from Deutero GmbH and, except for deuterium oxide, were stored over molecular sieves (3 Å). Dibenzylaminoethanol was synthesized according to a known protocol.[20]

Instrumentation: ^{1}H NMR spectra were recorded at 294 K, unless stated otherwise, either on a Bruker ARX 400 with a 5 mm BBO probe (400 MHz spectra) or a Bruker Avance III 700 (16.4 T) with a 5 mm *z*-gradient BBI inverse ^{1}H/X probe (700 MHz spectra). Spectra recorded on the 700 MHz spectrometer were regulated with a standard ^{1}H methanol NMR sample by using the Topspin 3.0 software (Bruker). The spectra were referenced against the residual HDO signal at $\delta(^1H) = 4.80$ ppm. All 1D spectra were processed with MestReNova 6.1.1-6384 software. The 2D-TOCSY program was run with the MLEV17 sequence for the homonuclear Hartman–Hahn transfer with a "tocsy" mixing time of 100 ms. For the 700 MHz probe, the spectrum was obtained with a π/2-pulse length of 9.3 μs (^{1}H) and a sweep width of 8400 Hz (12 ppm) for both dimensions. A relaxation delay of 3 s was chosen for the 2D experiment. The DOSY experiments were recorded with a 5 mm BBI ^{1}H/X *z*-gradient probe and a gradient strength of 5.516 G mm^{-1} on the 700 MHz spectrometer by using a double-stimulated echo for convection compensation. The relaxation time was 2 s, the diffusion delay was kept at 90 ms, and the gradient pulse had a length of 1600 μs. For the calibration of the gradient strength, a sample of $^2H_2O/^1H_2O$ was measured at a defined temperature and compared with the literature diffusion coefficient for $^2H_2O/^1H_2O$.[21] 2D spectra were processed with Topspin 3.0. For SEC measurements, a 9:1 mixture of phosphate buffer (0.1 M, pH 6.5)/methanol was used at a flow rate of 0.5 mL min^{-1} on Shodex OHpak 804 and 805 columns in a Viscotek TDA 300 instrument with triple detection at 25 °C, equipped with a MetaChem degasser, a Viscotek VE 1121 GPC solvent pump, and a VE 5200 GPC autosampler. SEC measurements in *N*,*N*-dimethylformamide (DMF) containing 0.25 g L^{-1} of lithium bromide as an additive were performed on an Agilent 1100 Series integrated instrument with a Polymer Standards Service (PSS) hydroxyethylmethacrylate column (106/105/104 g mol^{-1}), an RI detector, and a UV detector operating at 275 nm. Calibration was executed by using poly(ethylene oxide) standards from PSS. MALDI-TOF MS measurements of all polymers were recorded on a Shimadzu Axima CFR MALDI-TOF mass spectrometer, equipped with a nitrogen laser delivering 3 ns laser pulses at 337 nm. *a*-Cyanohydroxycinnamic acid was used as a matrix and potassium trifluouroacetate was added for ion formation. SDS-PAGE was carried out with 4–8% tris(hydroxymethyl)aminomethane–HCl (Tris-HCl) gels (Biorad, 0.75 mm, 10 wells). CD spectra were recorded on a JASCO J-815 CD spectrometer equipped with a PTC-423S Peltier type temperature-control system at 20.0 °C in a 1 mm cell in water with analyte concentrations of 1.5 μmol L^{-1} and a band width of 1.0 nm at a scanning speed of 50 nm s^{-1} (data pitch: 0.1 nm). Spectra presented are accumulations of 10 individual measurements each. IR spectra were recorded on a Thermo Scientific Nicolet iS10 FT-IR spectrometer, equipped with a diamond attenuated-total-reflectance unit and were processed with OMNIC 8.1.210 software.

α-Amino-ω-hydroxy-poly(ethylene glycol) (2): Hydrogenation of α-dibenzylamino-ω-hydroxy-PEG (**7**) was carried out analogously to a known protocol.[12] Details are given in the Supporting Information.

α-Squaric acid ester amido ω-hydroxy-poly(ethylene glycol) (4): A solution of **2** (500 mg, 0.222 mmol), diethyl squarate (340 mg, 1.99 mmol), and triethylamine (230 mg, 2.27 mmol) was stirred in a mixture of water (6.3 mL) and ethanol (6.3 mL) for 4 h. After removal of the ethanol with a rotational evaporator, the solution was extracted with dichloromethane three times. The combined organic phases were dried over sodium sulfate, and the product was precipitated several times from methanol in cold diethyl ether. After confirmation of complete removal of the diethyl squarate by thin layer chromatography, the SEA-PEG was dried in vacuo and eventually lyophilized from distilled water to give a colorless powder; ^{1}H NMR (400 MHz, [D$_6$]DMSO, 21 °C): $\delta = 8.81$ (br s, 0.52 H; NH), 8.63 (br s, 0.47 H; NH), 4.65 (q, $^3J(H,H) = 6.9$ Hz, 2 H; CH_2–CH_3), 4.57 (t, $^3J(H,H) = 5.4$ Hz, 1 H; OH) 3.87–3.35 (m, 203 H; ($CH_2CH_2O)_n$), 1.37 ppm (t, $^3J(H,H) = 7.0$ Hz, 3 H; CH_2–CH_3); IR (ATR): $\tilde{\nu} = 3435$, 2882,

1802 (C=O)$_s$, 1706 (C=O)$_{as}$, 1609 (C=C), 1466, 1454, 1359, 1341, 1279, 1241, 1147, 1099, 1059, 960, 841 cm^{-1}.

α-Squaric acid ester amido poly(ethylene glycol) ω-monomethyl ether (5): α-Amino-mPEG[9] (1 mmol), triethylamine (1.02 g, 10.1 mmol), and diethyl squarate (860 mg, 5.05 mmol) were dissolved in ethanol (50 mL). After the mixture had been stirred for 24 h at room temperature, the solvent was removed under reduced pressure. The crude product was precipitated several times from dichloromethane in cold diethyl ether. After confirmation of complete removal of the diethyl squarate by thin layer chromatography, the SEA-mPEG was dried in vacuo and eventually lyophilized from distilled water to give a colorless powder; yield: 57–63%; ^{1}H NMR (400 MHz, [D$_6$]DMSO, 21 °C): δ = 8.80 (br s, 0.56 H; NH), 8.62 (br s, 0.44 H; NH), 4.65 (q, ^3J(H,H) = 7 Hz, 2 H; CH_2–CH$_3$), 3.80–3.25 (m, 215 H; (CH_2CH_2O)$_n$), 3.24 (s, 3 H; O–CH$_3$), 1.37 ppm (t, ^3J(H,H) = 7 Hz, 3 H; CH$_2$–CH_3); IR (ATR): $\tilde{\nu}$ = 2883, 1803 (C=O)$_s$, 1710 (C=O)$_{as}$, 1612 (C=C), 1466, 1454, 1359, 1340, 1279, 1240, 1146, 1103, 1060, 947, 841 cm^{-1}.

α-Dibenzylamino-ω-hydroxy-PEG (7): Under argon, dibenzylaminoethanol (**6**, 603.3 mg, 2.50 mmol) was placed in a dry Schlenk flask and dissolved in benzene (10 mL). Traces of moisture were removed by azeotropic distillation of benzene and subsequent drying at 333 K under high vacuum for 14 h. After dry THF (20 mL) had been cryotransferred into the Schlenk flask, it was flooded with argon and, under vigorous stirring, a 1 M solution of potassium naphthalenide in THF (2 mL; prepared from potassium (235 mg, 6.0 mmol), naphthalene (770 mg, 6.0 mmol), and dry THF (6 mL) in a glove box) was added slowly. The hydrogen released and half of the amount of THF were removed by distillation under reduced pressure. After ethylene oxide (5.0 g, 0.11 mol) had been cryotransferred through a graduated ampule into the initiator solution, the flask was closed tightly and stirred overnight at 323 K. Methanol (2 mL) was added and the polymer was precipitated twice in cold diethyl ether. Filtration and subsequent drying under reduced pressure gave **7** in quantitative yields; ^{1}H NMR (400 MHz, CDCl$_3$, 21 °C): δ = 7.38–7.14 (m, 10 H; CH$_{Ar}$), 3.95–3.25 (m, 219 H; Ph–CH$_2$, (CH_2CH_2O)$_n$), 2.65 ppm (t, ^3J(H,H) = 8.2 Hz, 2 H; CH$_2$–NBn$_2$). Attention must be paid when working with the gaseous, toxic, and flammable ethylene oxide.

Typical protocol for the PEGylation of BSA: Solutions of BSA (25 mg, 379 nmol) in an aqueous borate buffer (250 μL, pH 10) were treated with various amounts of SEA-mPEG (2.1 kDa) and stirred for 2 days. The solutions were dialyzed against distilled water (MWCO = 50 kDa). Upon lyophilization, the conjugates were obtained quantitatively. Further information is available in the Supporting Information.

The reaction of 5 with Ac-Lys-OMe in the presence of Z-Ser-OMe: Ac-Lys-OMe (11.9 mg, 50 μmol), Z-Ser-OMe (12.7 mg, 50 μmol), and **5** (50.0 mg, 24 μmol) were stirred in ethanol (2 mL) with triethylamine (100 μL) as an additive for 12 h. After the solvent had been evaporated, the product was precipitated from dichloromethane in cold diethyl ether three times; yield: 48.3 mg (89%).

Kinetic studies: Typical reaction kinetics protocol for the reaction of **5** with H-Lys-OMe in borate buffer with DMSO. H-Lys-OMe (4.4 mg, 19 μmol) and 5 (40.0 mg, 19 μmol) were dissolved in a 7:3 mixture of a 0.1 M deuterated borate buffer and [D$_6$]DMSO (600 μL) in an NMR tube. After locking and shimming, a ^{1}H NMR spectrum (700 MHz) was recorded with 32 scans and a relaxation delay of 10 s at T = 294 K every 1.69 min for 3.4 h. All spectra were processed (Fourier transformation, phase correction, baseline correction) and referenced to the solvent signal (DMSO). All signals were integrated in the same limits.

Reaction kinetics protocols for all reactions monitored by ^{1}H NMR spectroscopy are described in the Supporting Information.

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