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A New Reagent for Stable Thiol-Specific Conjugation

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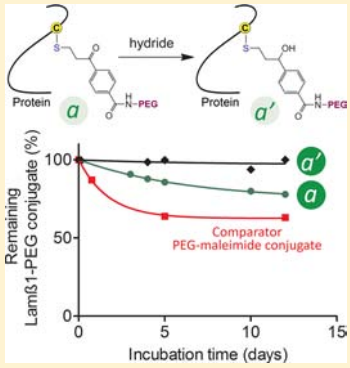
Supporting Information

ABSTRACT: Many clinically used protein therapeutics are modified to increase their efficacy. Example modifications include the conjugation of cytotoxic drugs to monoclonal antibodies or poly(ethylene glycol) (PEG) to proteins and peptides. Monothiol-specific conjugation can be efficient and is often accomplished using maleimide-based reagents. However, maleimide derived conjugates are known to be susceptible to exchange reactions with endogenous proteins. To address this limitation in stability, we have developed PEG−mono-sulfone **3**, which is a latently reactive, monothiol selective conjugation reagent. Comparative reactions with PEG−maleimide and other common thiol-selective PEGylation reagents including vinyl sulfone, acrylate, and halo-acetamides show that PEG−mono-sulfone **3** undergoes more efficient conjugation under mild reaction conditions. Due to the latent reactivity of PEG−mono-sulfone **3**, its reactivity can be tailored and, once conjugated, the electron-withdrawing ketone is easily reduced under mild conditions to prevent undesirable deconjugation and exchange reactions from occurring. We describe a comparative stability study demonstrating a PEG−maleimide conjugate to be more labile to deconjugation than the corresponding conjugate obtained using PEG−mono-sulfone **3**.



INTRODUCTION

Protein therapeutics are being developed for a wide range of medical indications. In an increasing number of cases, these proteins are chemically modified to improve their physico-chemical properties, efficacy, and/or safety. Notable modifications that are employed in products approved for clinical use include the conjugation of (i) cytotoxic drugs to monoclonal antibodies to make antibody drug conjugates (ADCs)[1] and (ii) poly(ethylene glycol) (PEG) to proteins and peptides (i.e., protein PEGylation) to reduce dosing frequency.[2−6]

The chemical modification of proteins with PEG has been investigated and developed over many decades.[7−10] Historically, most PEGylation reagents have been developed to undergo reaction with accessible amines on proteins because these nucleophilic groups are the most prevalent. These efforts have resulted in the development of clinically approved PEG−protein products,[11] some of which are now first line treatments.[12,13] Many amine-specific reagents are often inefficient due to competitive hydrolysis reactions of the reagent (e.g., PEG−*N*-hydroxysuccinimide ester) or unfavorable equilibria during imine formation,[14] which is necessary for conjugation by reductive amination. Because most proteins have several amine moieties that are available for reaction, most of these PEGylation reagents are not site-specific, resulting in heterogeneous mixtures of different PEG−protein isomers in the final products.[5,15]

Different PEG−protein isomers are known to have different properties,[16,17] so having a conjugation process that produces a more homogeneous product is desirable. Efficient strategies for conjugation will also result in more cost-effective manufacture. To address regulatory and economic issues, technologies used to produce protein-based medicines, including those related to protein modification, are constantly improving. To meet the challenges for clinical use, protein conjugation technologies are evolving to better (i) achieve site-specific and efficient conjugation and (ii) produce stable products.

It is possible to reduce the number of amine sites on a protein for conjugation[18] or possibly conjugate lysines site specifically.[19] Many other sites on proteins have also been evaluated for site-specific PEGylation including arginine,[20] glutamic acid,[21] methionine,[22] the C-terminal amino acid,[23,24] histidine tags,[25] non-native amino acids,[26−29] and glycosyl moieties.[30,31] Historically, a widely used modification strategy was the site-specific conjugation of a free thiol on a protein, which can be accomplished efficiently in mild reaction conditions. Although free thiols on a protein are less common than amine moieties, thiol-specific conjugation reagents have also been developed for about the same period of time as have amine-specific reagents.

In general, monothiol-specific reagents are more efficient than amine-specific reagents for conjugation, especially for protein PEGylation. In particular, reagents have been described that undergo alkylation to generate a thio-ether bond between the protein and PEG. Although alkyl halides, ester acrylates,

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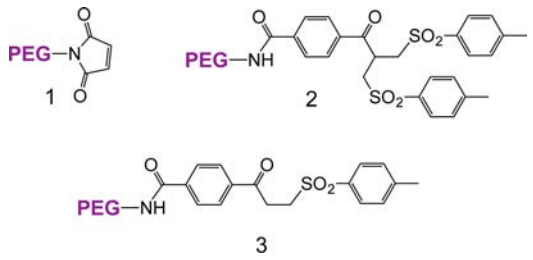
and vinyl sulfone reagents[32] have been developed,[9] the most widely used thiol-specific PEGylation reagents have been derived from maleimides **1**. Maleimide reagents undergo efficient conjugation in mild reaction conditions (e.g., neutral pH), reducing the risk of denaturation of the protein. Although maleimide conjugation reagents have been used to develop two clinically approved ADCs[33−35] and PEGylated products,[36] maleimide-based conjugates can undergo exchange reactions[37,38] including with endogenous proteins.[39,40] In the case of PEGylation, exchange reactions result in deconjugation of the protein from the PEG, which will lead to rapid clearance of the protein and a reduction in efficacy. However, in the case of ADCs, such exchange reactions may result in systemic toxicity due to premature release of the cytotoxic drug from the antibody and reduced efficacy as a result of competitive binding of the unconjugated antibody to the target antigen.[40] Recent work describes alternative thiol selective reagents,[41] some of which are of similar reactivity to maleimide, and strategies to stabilize maleimide reagents[42] to minimize exchange reactions in vivo.



Until recently,[43−45] thiol-specific PEGylation reagents were limited by the difficulties inherent in developing proteins with a free unpaired cysteine thiol for conjugation. If a protein has a native disulfide bond, then an engineered unpaired cysteine may cause disulfide-scrambling. Aggregation also occurs during downstream processing due to intermolecular disulfide formation between protein molecules,[46] which can reduce protein yields. In spite of these considerable limitations, there has been much effort to engineer a free cysteine into a protein for conjugation[47−50] because there are significant advantages for thiol-specific conjugation.

Our approach has been to use thiol-selective PEG−bis-sulfone reagents **2** that are able to undergo site-specific bis-alkylation to conjugate both thiols derived from the two cysteines in a reduced native disulfide bond.[43−45] Following conjugation, the original disulfide bond is rebridged with the reagent. Because there are novel proteins without disulfides in development,[51,52] we have developed the PEG−mono-sulfone **3** as an alternative to maleimide reagents.

Analogous with the bis-alkylating reagents **2**, the elimination of toluene sulfinic acid anion **5** is thought necessary to generate the reactive aryl enone **4**, which undergoes facile conjugation (Scheme 1). Controlling the rate of elimination of the sulfinic acid leaving group **5** would allow in principle to have some control over the rate of conjugation. When necessary, the keto moiety in the reagent can be reduced by hydride (Scheme 3) to completely lock the conjugate to avoid the possibility of the retro Michael reaction.[25] Herein we describe the synthesis and conjugation properties of PEG−mono-sulfone **3**.

EXPERIMENTAL SECTION

Materials. Fab antibody fragment (donkey Fab polyclonal secondary antibody to goat IgG) was supplied by Abcam (AB6520), Lamβ1$_{925-933}$ peptide and HSA were purchased from Sigma Aldrich (C0668), and anti Her2-Affibody was purchased from Affibody AB (10.0817.01.0005). *N*-Acetyl-L-cysteine and *N*-acetyl-L-lysine were both purchased from Sigma Aldrich (code A7250-5G and A2010-1G). *O*-(2-Aminoethyl)-*O*′-methyl-poly(ethylene glycol) polymers were purchased from BioVectra (MeO-PEG-amine 10 kDa BioVectra code 6232). Thiol reactive PEG reagents were purchased as follows: PEG (5 kDa) maleimide from Biochemika Fluka (63187), methoxy PEG (5 kDa) vinylsulfone from JenKem Technology (M-VS-5000), PEG (5 kDa) acrylate from Sigma Aldrich (730289), PEG (5 kDa), PEG-bromoacetamide and PEG-iodoacteamide from Iris Biotech GMBH (CS-0047.0.500 and CS-0048.0500 respectively). Discrete PEG amine was purchased from IrisBiotech GMBH (2145.58 Da, code PEG3310). PD-10, and NAP-5 desalting columns were purchased from GE Healthcare. SDS-PAGE was conducted with XCell Surelock Mini-Cell (Invitrogen), NuPAGE 4−12% Bis-Tris gels, Novex Sharp protein markers, and MOPS or MES running buffer were all from Invitrogen. The gels were stained with InstantBlue (Expedeon). PEGylated conjugates were purified using an ÄKTAprime system (GE Healthcare). HPLC grade acetonitrile was purchased from Fisher (A/0627/17), and trifluoroacetic acid was from Acros (139721000). The RP-HPLC column was an Ace Excel 2 super C18 (dimensions 75 mm × 2.1 mm id, Agilent; catalogue no. EXL-1011-75024). The LC/MS column was purchased from Waters (XSELECT CSH C18 2.5 μm 3.0 mm × 30 mm column XP (part no. 186006107).

Methods. *Preparation of 4-(3-(p-Tolylthio)propanoyl)-benzoic Acid* ***8***. Under an argon atmosphere, Mannich salt **7**[44] (1.0 g, 3.4 mmol) and 4-methylbenzenethiol (417 mg, 3.4 mmol) were suspended in a mixture of absolute ethanol (7.5 mL) and methanol (5 mL). Piperidine (50 μL) was then added and the suspension heated to reflux with stirring for 6 h. The white precipitate produced after cooling to room temperature was filtered using a sintered glass filter, washed with acetone, and dried under vacuum to give the thiol ether **8**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.27 (s, 3H, phenyl-CH$_3$), 3.24, 3.39 (t, 2 × 2H, CH$_2$), 7.14, 7.26 (d, 2 × 2H, Ar*H* of tolyl moiety), 8.03 (m, 4H, Ar*H* of carboxylic acid moiety). ^{13}C NMR (125 MHz, DMSO-d_6) δ 197.9 ppm (C═O), 166.6 ppm (HO-C═O), 139.4 ppm (Ar*C* of phenyl-CO), 135.4 ppm (Ar*C* of phenyl-CH$_3$), 134.6 ppm, (Ar*C* phenyl-COOH), 132.2 ppm (Ar*C* phenyl-tolyl moiety), 129.8−128.1 ppm (Ar*C*) 38.3 ppm (CH$_2$−C═O), 27.4 ppm (CH$_2$−S−), 20.5 ppm (CH$_3$−Ar).

Scheme 1. PEG−mono-sulfone 3 Is a Latently Reactive, Mono-Thiol-Specific Conjugation Reagent[a]

[a]Facile elimination of toluene sulfinic acid anion **5** generates the reactive aryl enone **4** that can readily undergo conjugation with a protein thiol.

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MS (ES^+): m/z $[M + H]^+$ 301.1591, $[M + Na]^+$ 323.1405, $[M + K]^+$ 339.1335.

Preparation of 4-(Tosylpropanoyl)benzoic Acid **9**. OXONE (720 mg, 1.17 mmol) was added to a suspension of the benzoic acid derivative **8** (160 mg, 0.53 mmol) in 20 mL of $MeOH/H_2O$ (v/v: 1/1) in an ice bath. The reaction mixture was then warmed to room temperature, and the reaction was allowed to continue with stirring overnight (15 h). The resulting suspension was diluted with water (40 mL) so that it became nearly homogeneous, and the mixture was then extracted three times with chloroform (total 100 mL). The pooled chloroform extracts were washed with brine and then dried with $MgSO_4$. Evaporation of volatiles under vacuum at 30 °C afforded **9** as a white solid (149 mg). ^1H NMR (400 MHz, DMSO-d_6) 2.41 (s, 3H, phenyl-CH_3), 3.64 (t, 2H, $COCH_2$), 3.74 (t, 2H, CH_2), 7.46, 7.82 (d, 2 × 2H, Ar*H* of tolyl moiety), 8.03 (m, 4H, Ar*H* of carboxylic acid moiety). ^{13}C NMR (125 MHz, DMSO-d_6) δ 195.6 ppm (C═O), 166.6 ppm (HO-C═O), 144.5 ppm (Ar*C* of phenyl-CO), 138.9 ppm (Ar*C* of phenyl-CH_3), 135.9 ppm, (Ar*C* phenyl-COOH), 134.8 ppm (Ar*C* phenyl-tolyl moiety), 129.9 −127.8 ppm (Ar*C*), 50.1 ppm (CH_2−SO_2−), 31.9 ppm (CH_2−C═O), 27.4 20.5 ppm (CH_3−Ar). MS (ES^+): m/z $[M + H]^+$ 333.1621, $[M + Na]^+$ 355.1559; calculated, 332.37; found, 332.1621.

Preparation of PEG−mono-sulfone **3** *(10 kDa PEG As Example)*. The carboxylic acid sulfone **9** (133 mg, 0.4 mmol) and *O*-(2-aminoethyl)-*O*′-methyl-PEG (MW 10 kDa, 502 mg, 0.05 mmol) were dissolved in anhydrous dichloromethane (15 mL) under argon. The resulting solution was cooled in an ice bath, then diisopropylcarbodiimide (DIPC, 50.5 mg, 0.40 mmol) was slowly added. The reaction mixture was then allowed to warm to room temperature and the reaction continued with stirring for 15 h. Volatiles were then removed under vacuum to afford a solid residue that was redissolved in acetone (20 mL). The solution was filtered through a sintered filter to remove insoluble material. The solution was then cooled in a dry ice bath to give a white precipitate, which was separated by centrifugation (4566 *g*, 30 min). The resulting off-white solid was dried under vacuum to afford the PEG−mono-sulfone reagent **3** (437 mg). ^1H NMR (400 Hz, $CDCl_3$) 2.46 (s, 3H, phenyl-CH_3), 3.38 (s, 3H, PEG-OCH_3), 3.45−3.83 (br, m, PEG), 7.37, 7.39 (d, 2H Ar*H* of tolyl moiety), 7.82, 7.84 (d, 2H, Ar*H* of tolyl moiety), 7.92−7.99 (m, 4H, Ar*H* of carboxylic acid moiety).

PEGylation of Human Serum Albumin (HSA). A stock solution of PEG reagent (0.5 mM) was prepared by dissolving 40 kDa PEG−mono-sulfone reagent **3** (9.6 mg) in 50 mM sodium phosphate, 150 mM NaCl, 40 mM EDTA, pH 7.3 (0.48 mL). An aliquot of the PEG solution (0.92 μL, 0.46 nmol) was added to a solution of HSA (20 μL, 14.9 μM). The resulting mixture was placed in the fridge and the conjugation reaction allowed to continue overnight (17 h). Reaction mixtures were then analyzed by SDS-PAGE.

PEGylation of Anti-Her2 Affibody. Lyophilized anti-HER2 Affibody (0.5 mg) was resuspended in a reaction buffer of 50 mM sodium phosphate, pH 7.4, containing 150 mM NaCl and 10 mM EDTA (0.5 mL). DTT (10 μL, 1.0 mM stock solution) was added to the anti-HER2 Affibody solution to give a final DTT concentration of 20 mM. The resulting solution was mixed gently and then incubated at room temperature for 1 h. The solution of reduced anti-HER2 Affibody was buffer exchanged into fresh reaction buffer using an illustra NAP-5 column. The concentration of reduced Affibody solution was determined by measuring UV absorbance at 280 nm. The reduced Affibody solution was diluted with further reaction buffer to a concentration of 0.1 mg/mL, and then it was split equally into seven portions (0.2 mL, 20 μg). Seven PEG reagents: PEG−mono-sulfone **3**, activated PEG−mono-sulfone **4**, PEG−maleimide **1**, PEG−vinyl sulfone **10**, PEG−acrylate **11**, PEG−bromoacetamide **12**, and PEG−iodoacetamide **13**, each with a molecular weight of 5 kDa, were dissolved in ultra pure water (18.2 MΩcm) at a concentration of 1 mg/mL. Each PEG solution (7.1 μL, 1 mol equiv per Affibody) was then added to one of the aliquots of the reduced Affibody solution. The Affibody reaction solutions were mixed gently and then incubated at 25 °C for 30 min and 4 h. After each time point, the crude reaction mixtures were analyzed by SDS-PAGE. The gels were stained with InstantBlue and imaged using an IMAGEQUANT LAS 4010 instrument.

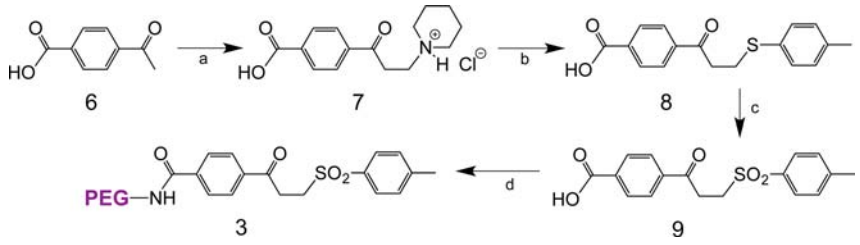
PEGylation of a Fab Antibody Fragment. DTT (5 μL, 100 mM in deionized water, 5 × 10^{-4} mmol) was added to a solution of Fab antibody fragment (0.2 mL, 0.5 mg/mL, 2 × 10^{-6} mmol) in pH 7.8, 50 mM phosphate buffer containing 150 mM NaCl and 10 mM EDTA (buffer A). The resulting solution was allowed to stand at room temperature for 30 min. The solution was then loaded onto an illustra NAP-5 column, pre-equilibrated with buffer A. The NAP-5 column was eluted with 5 × 300 μL of fresh buffer A. The fraction containing reduced Fab was identified by measuring UV absorbance at 280 nm. This showed that reduced Fab was mainly in fraction **3** and had an estimated concentration of 0.23 mg/mL. PEG reagent **3** derived from PEG with a molecular weight 10 kDa was dissolved in buffer A. The PEG reagent solution (0.42 μL, 2 mol equiv per Fab) was then added to an aliquot of the reduced Fab solution (5 μL, 0.23 mg/mL). The Fab reaction solutions were diluted in buffer A (4.6 μL) and then incubated at 4 °C for 12 h. Reaction mixtures were analyzed by SDS-PAGE.

PEGylation of Laminin-β Peptide. A stock solution of Lam$\beta 1_{925-933}$ peptide (1 mg) was prepared by dissolving into deionized water (500 μL, 2.0 mM). A stock solution of 5 kDa PEG reagent **3** (5 mg) was prepared by dissolving into deionized water (200 μL, 5 mM). Buffer stock solutions were prepared containing either 1 M sodium phosphate buffer for the pH 6.0−8.0 range or 1 M sodium carbonate−bicarbonate buffer for the pH 8.5−10.0 range. For all pH values, the buffer stock solution also contained 10 mM EDTA and 0.38 mM hydroquinone.

PEGylation reactions were then allowed to occur by adding together an aliquot of the Lam$\beta 1_{925-933}$ stock solution (5 μL, 10 μmol), buffer solution (1 μL), PEG reagent solution (2 μL, 10 μmol), and deionized water (2 μL). Reaction solutions were mixed using a vortex mixer then centrifuged briefly (30 s at 5000 *g*) to collect the solution at the bottom of the tube, and the reaction mixtures were incubated standing at room temperature for 0.25, 0.5, 1, 2, 4, 16, and 24 h. After incubation at room temperature, the tubes containing the reaction mixtures were placed at −80 °C and stored until analysis by reverse-phase chromatography. Reduction of the Lam$\beta 1_{925-933}$ conjugate derived from PEG−mono-sulfone **3** (1 mg/mL) was accomplished in 20 mM sodium phosphate pH 8.0 that was treated with sodium borohydride (20 mM) for 30 min at room temperature.

Competitive Conjugation of N-Acetyl-L-cysteine and N-Acetyl-L-lysine. Stock solutions of *N*-acetyl-L-cysteine (6.5 mM) and *N*-acetyl-L-lysine (3.4 mM) in 50 mM sodium phosphate buffer, pH 7.3, 20 mM EDTA, 150 mM NaCl were

Scheme 2. Synthesis of PEG−mono-sulfone 3[a]



[a](a) Mannich reaction, piperidine, paraformaldehyde, cat. HCl, ethanol, reflux; (b) elimination-thiol addition, 4-methylbenzenethiol, cat. piperidine, ethanol−methanol, reflux; (c) oxidation, Oxone, aqueous methanol; (d) imide coupling to PEG, DIPC, *O*-(2-aminoethyl)-*O*′-methyl-PEG, anhydrous dichloromethane.

prepared separately. *N*-Acetyl-L-cysteine (0.69 mL, 0.0045 mmol, 1.1 mol equiv) and *N*-acetyl-L-lysine (1.31 mL, 0.0045 mmol, 1.1 mol equiv) were added to a 20 mL reaction vial and allowed to mix. The discrete molecular weight (2460 g/mol) PEG−mono-sulfone **3** (10 mg, 0.0041 mmol, 1 mol equiv) was added to the reaction solution, and then 10.5 mL of reaction buffer (50 mM sodium phosphate buffer, pH 7.3, 20 mM EDTA, 150 mM NaCl) was added to give a total volume of 12.5 mL. The conjugation reaction was monitored by LC/MS and RP-HPLC (214 and 280 nm) at 1 h intervals. A second reaction in a separate 20 mL vial was conducted with a 30 equiv excess of *N*-acetyl-L-lysine (23 mg, 0.122 mmol, 30 mol equiv) that was added to *N*-acetyl-L-cysteine (0.69 mL, 0.0045 mmol, 1.1 mol equiv) and reaction buffer (11.8 mL). The discrete PEG−mono-sulfone **3** (10 mg, 0.0041 mmol, 1 mol equiv) was then added to the solution, and the conjugation reaction was monitored by LC/MS and RP-HPLC (214 and 280 nm) at 1 h intervals. A third reaction in a 20 mL vial was conducted using 30 equiv excess of *N*-acetyl-L-Lysine (23 mg, 0.122 mmol, 30 mol equiv) and discrete PEG−mono-sulfone **3** (10 mg, 0.0041 mmol, 1 mol equiv) in 12.5 mL of the reaction buffer (50 mM sodium phosphate buffer, pH 7.3, 20 mM EDTA, 150 mM NaCl). The reaction was allowed to incubate at room temperature for 20 h.

Reverse Phase HPLC Analysis of Lamβ1$_{925-933}$ PEGylation Reactions. A Source-5 RPC ST 4.6/150 (GE Healthcare, 17-5116-01) column was connected to a Jasco HPLC system comprising a Jasco PU-980 Intelligent HPLC pump, a Jasco LG-980-02 ternary gradient unit, a Jasco Degassys Populaire degassing unit, a Jasco UV-970 4-λ intelligent UV detector, a Jasco LC-NetII/ADC interface for connection to a PC, and a Rheodyne 7725i manual injector valve. The HPLC system was controlled through a computer using the EZchrom SI version 3.2.1 Build 3.2.1.34 chromatography software package (Agilent Technologies). Chromatogram analysis and data export were also performed using the EZchrom SI chromatography data system.

A three-eluent system was used. Eluent A contained 5% acetonitrile and 0.065% trifluoroacetic acid (TFA) in deionized water. Eluent B contained 0.075% TFA in acetonitrile, while eluent C was 100% acetonitrile. Eluents were degassed by sonication before use. The elution program involved a 0−64% B gradient in 20 min, followed by wash in 100% acetonitrile (buffer C) for 3.8 min then re-equilibration in eluent A. A constant flow rate of 1 mL/min was maintained throughout the run. The absorbance at 214 nm was recorded. Each sample (10 μL) was thawed and briefly centrifuged (1 min at 14000 *g*) immediately before 5 μL of supernatant were injected onto the reverse phase chromatography column.

The identity of each peak in the chromatograms was confirmed by running standard samples (PEG reagent, reduced and oxidized unreacted peptide) and by analytical size exclusion chromatography (SEC) for relative size estimation (for PEGylation product: peptide−PEG conjugate). The column used for analytical SEC was a BioSep-SEC-S3000 (300 mm × 7.8 mm) analytical column (Phenomenex, cat. no. 00H-2146-KO). The running eluent used was 10 mM sodium phosphate buffer (pH 7.0) containing 10% (v/v) acetonitrile and the flow rate was kept constant at 2 mL/min.

RESULTS AND DISCUSSION

Synthesis of PEG−mono-sulfone 3. The monothiol conjugating reagent **3** was prepared (Scheme 2) from the *p*-carboxylic acid acetophenone **6** by conducting a Mannich reaction to instil the necessary enone β-carbon. Elimination of the piperidine ammonium salt from **7** in the presence of 4-methylbenzenethiol gave the β-thiol ether **8** as a solid (60% yield). This was then oxidized using OXONE to give carboxylic acid sulfone **9** as a white crystalline solid. Yields ranged from 80% to 90%. Carbodiimide mediated coupling of the acid sulfone **9** using diisopropylcarbodiimide and *O*-(2-aminoethyl)-*O*′-methyl-PEG gave the desired PEG−mono-sulfone **3**. Different molecular weights of PEG ranging from 2 to 40 kDa were used to prepare the reagent **3**, which was simply purified by precipitation in acetone chilled in dry ice in yields ranging from 80 to 90%. The coupling reaction can also be accomplished with the monothiol ether **8** and then the resulting PEG−mono-sulfide treated with OXONE to give the desired PEG−mono-sulfone **3**. This method avoids the precursor sulfone **9** undergoing sulfinic acid elimination during addition of the PEG amine. The purity of the PEG−mono-sulfone **3** was established by H NMR and was usually found to be ≥90%. More recently purities above 95% are routinely achieved as determined from RP-HPLC analysis.

Conjugation Reactions. The PEG−mono-sulfone **3** must undergo elimination of toluene sulfinic acid **5** (Scheme 1) to generate the reactive α,β-unsaturated PEG reagent **4** to allow conjugation to proceed. The PEG−mono-sulfone **3** is a latently reactive molecule, which allows for ease of handling of the reagent. Conjugation reactions were conducted in aqueous conditions using an equivalent or slight molar excess of the PEG−mono-sulfone **3** to the free cysteine thiol from the model protein (1−1.5 equiv). The reagent was added to the protein solution as the PEG−mono-sulfone **3** and generation to the reactive PEG−α,β-unsaturated carbonyl **4** occurred in situ.

Human serum albumin (HSA) has a free cysteine at position Cys 34 which is often used for conjugation.[53,54] HSA was readily conjugated with the PEG reagent **3** (40 kDa) to give the

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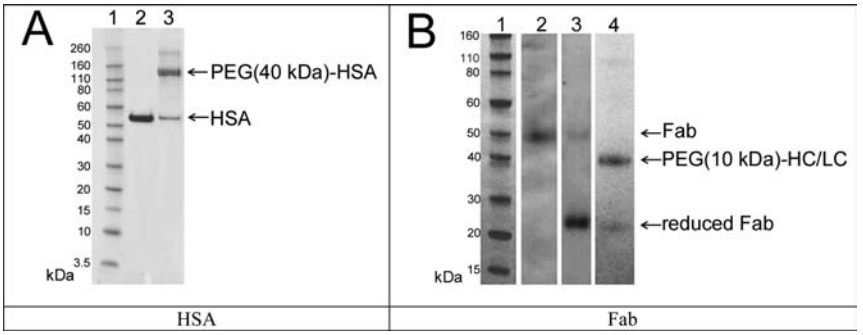

Figure 1. SDS-PAGE analysis of conjugation reactions between PEG−mono-sulfone reagent **3** and either (A) HSA or a (B) partially reduced Fab antibody fragment. NuPAGE: 4−12% Bis-Tris Gel, MES running buffer, 200 V, 35 min. (A) Gel for the PEGylation of HSA using 40 kDa PEG−mono-sulfone reagent **3**: lane 1, protein standard marker; lane 2, HSA; lane 3 reaction mixture for conjugation of HSA with 1.5 equiv PEG reagent **3**. (B) Gel for the PEGylation of a Fab using 10 kDa PEG−mono-sulfone reagent **3**; lane 1, protein standard marker; lane 2, oxidized Fab; lane 3, reduced Fab; lane 4, reaction mixture for conjugation of Fab using 1.5 equiv PEG reagent **3**.

mono-PEG−HSA conjugate (Figure 1A). High conversion (~80%) to the PEG−HSA conjugate was observed as estimated by analysis using ImageQuant (GE healthcare) using 1.5 equiv of the PEG reagent **3**. A Fab antibody can be partially reduced with dithothreitol (DTT) to give two free cysteine thiols, which were efficiently conjugated with PEG−mono-sulfone **3** (5 kDa) (Figure 1B). An anti-Her2 Affibody dimer after reduction with DTT was also conjugated with PEG−mono-sulfone **3** (10 kDa).

N-Acetyl-L-cysteine and *N*-acetyl-L-lysine were used to evaluate if competitive amine conjugation occurred in the conditions that are used for thiol-specific PEGylation. A well-defined, exact molecular weight PEG amine precursor was used to prepare a discrete PEG−mono-sulfone **3** with a molecular weight of 2460 g/mol (Supporting Information Figure S1) so that LC/MS could be used to confirm conjugate identity. Using a slight excess (1.1 equiv) of each of the two acylated amino acids to one equiv of the discrete PEG−mono-sulfone **3**, it was found that the PEG−cysteine conjugate exclusively formed at high conversion within 1−2 h as observed by RP-HPLC at both 214 and 280 nm (Supporting Information Figure S2). Sole formation of the PEG−cysteine conjugate was also confirmed by LC/MS (Supporting Information Figure S3). The PEG−cysteine conjugate was again exclusively formed at high conversion within 1−2 h when the relative molar ratio of *N*-acetyl-L-lysine was increased to 30 in the presence of a 1.1 ratio of *N*-acetyl-L-cysteine and PEG−mono-sulfone **3** (Supporting Information Figure S4). Although the PEG−lysine conjugate could be formed in the absence of *N*-acetyl-L-cysteine (Figures S5−S6), conversion to the conjugate was only about ~30% when a 30:1 ratio of the *N*-acetyl-L-lysine to PEG−mono-sulfone **3** was used.

We also examined a commercially available peptide with a free cysteine thiol as an additional exemplar. The conjugation of PEG−mono-sulfone **3** was examined in some detail with Lam$\beta_{925-933}$, which is a synthetic linear nona-peptide corresponding to residues 925−933 of the laminin β1 chain.[55] Lam$\beta_{925-933}$ has a single cysteine residue at the C-terminus. Conjugation reactions were conducted at a range of pH values from 6.0 to 8.0 and were followed with analytical RP-HPLC. Chromatographic peaks corresponded to Lamβ1$_{925-933}$ monomer, Lamβ1$_{925-933}$ dimer (formed by oxidation to yield intermolecular disulfide bond), toluene sulfinic acid leaving group **5**, PEG$_{5\ kDa}$−Lamβ1$_{925-933}$ product, PEG−α,β-unsaturated carbonyl **4**, and the starting PEG−mono-sulfone reagent **3** (Figure 2).

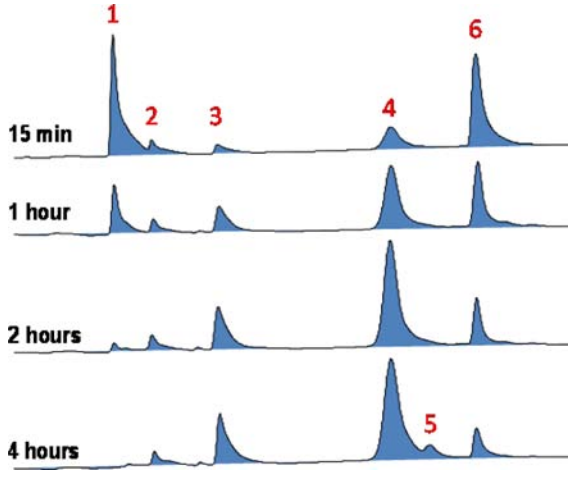

Figure 2. Typical reverse-phase chromatograms (λ = 214 nm) for a time-course PEGylation experiment using PEG−mono-sulfone **3** (5 kDa PEG) at pH 8.0. The peaks were identified as follows: peak 1, reduced Lamβ1$_{925-933}$; peak 2, oxidized Lamβ1$_{925-933}$; peak 3, toluene sulfinic acid leaving group **5**; peak 4, PEG−Lamβ1$_{925-933}$ conjugate (Lamβ1$_{925-933}$−PEG5 kDa conjugate); peak 5, PEG−α,β-unsaturated carbonyl **4**; peak 6, PEG−mono-sulfone **3**. The identity of each peak was confirmed by running standard samples (PEG reagent, reduced and oxidized unreacted peptide) and by analytical size exclusion chromatography (SEC) for relative size estimation.

As the reaction proceeds, the reduced Lamβ1$_{925-933}$ (peak 1) can undergo two competitive reactions: (i) oxidation to the Lamβ1$_{925-933}$ dimer (peak 2) and (ii) formation of the desired PEG−Lamβ1$_{925-933}$ conjugate (peak 4). As seen in Figure 2, the formation of the PEG−Lamβ1$_{925-933}$ conjugate occurred without much change in the amount of the starting Lamβ1$_{925-933}$ dimer. The percentage conversion of Lamβ1$_{925-933}$ into the PEG−Lamβ1$_{925-933}$ conjugate was estimated by monitoring the amount of unconjugated Lamβ1$_{925-933}$ (peak 1). The PEG−Lamβ1$_{925-933}$ conjugate appeared at 15 min, and there was a time-dependent increase in the PEG−Lamβ1 conjugate until all the starting Lamβ1$_{925-933}$ was consumed after approximately 2 h. There was also a corresponding time-dependent increase in the amount of the leaving group, toluene sulfinic acid **5** (peak 3), that was

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observed. Consumption of the starting PEG−mono-sulfone **3** (peak 6) occurred as the conjugation progressed. The presence of the activated PEG reagent **4** (peak 5) was only observed after the conjugation with the free thiol on Lamβ1$_{925-933}$ had gone to completion. There did not appear to be any reaction of the oxidized Lamβ1$_{925-933}$ dimer and the activated PEG reagent **4**, suggesting that this reactive molecule **4** did not readily undergo reaction with the Lamβ1$_{925-933}$ dimer in these conditions (e.g., at the terminal amines).

Conjugation reactions of PEG−mono-sulfone **3** and Lamβ1$_{925-933}$ in the pH range investigated (6−8) were compared by plotting conversion against time (Figure 3).

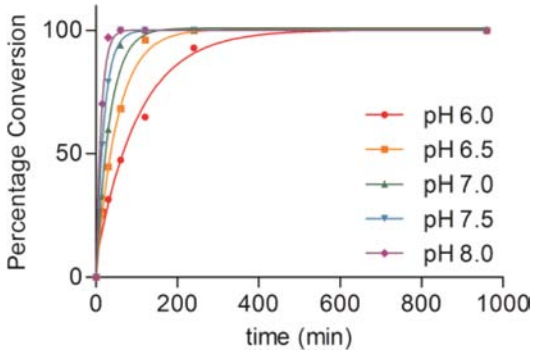
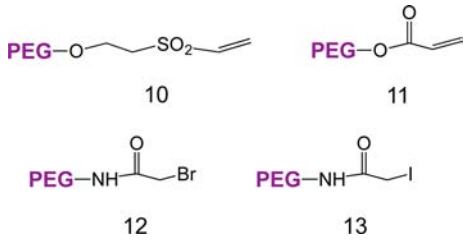


Figure 3. The effect of pH on the rate of PEGylation of Lamβ1$_{925-933}$ using the PEG−mono-sulfone **3** (5 kDa) as determined by RP-HPLC. The data points corresponding to each pH value were fitted to a monophasic exponential function using GraphPad Prism v 5.01 data analysis software.

Conducting the PEGylation reaction at pH 6.0 resulted in the slowest conversion rate with completion after approximately 7 h. The conjugation was completed within 30 min when conducted at pH 8.0. These differences in reaction rates are primarily due to the elimination rate of toluene sulfinic acid **5** to generate the active PEG reagent **4**. This hypothesis was confirmed by conducting the conjugation directly with the reactive α,β-unsaturated PEG reagent **4**. When the activated reagent **4** was used directly, the PEGylation reaction reached completion after just 15 min in all values tested in the pH 6−10 range.

Both the PEG−mono-sulfone **3** and the reactive PEG−enone **4** were then compared for the conjugation of Lamβ1$_{925-933}$ at different pH values (Figure 4A). One equivalent of each reagent was added per equivalent of peptide. The conjugation reactions were conducted at ambient temperature, and conversion to the PEG 5 kDa−Lamβ1$_{925-933}$ conjugate was determined by RP-HPLC after 15 min (Figure 4A). Conjugations using the PEG−enone reagent **4** were conducted at pH values of 6.0−8.0, while reactions with the PEG−mono-sulfone **3** were conducted at a range of pH values from 6.0 to 10.0. Starting with the PEG−mono-sulfone reagent **3**, conversion increased from essentially 0 to 100% with the elimination of toluene sulfinic acid **5** to give the reactive reagent **4** as the pH was increased from acidic to weakly basic values (Figure 4A). The PEG−enone **4** displayed similar conjugation conversion to PEG−maleimide **1** (Figure 4B).

While maleimide reagents are the most common monothiol PEG reagents described in the literature, several other thiol-specific PEGylation reagents have also been described.[9] Comparative conjugations were conducted with the anti-Her2 Affibody with the PEG−mono-sulfone **3**, α,β-unsaturated activated PEG **4**, PEG−maleimide **1**, PEG−vinylsulfone **10**, PEG−acrylate **11**, PEG−bromoacetamide **12**, and PEG−iodoacetamide **13**. Each experiment was conducted using the reagent derived from 5 kDa PEG and 1 mol equiv of the corresponding PEG reagent was used with respect to the Affibody. Conjugations were conducted at pH 7.4 and ambient temperature, then analyzed by SDS-PAGE after different reaction times (30 min and 4 h) (Figure 5). Significant conjugations were only observed with PEG−mono-sulfone reagent **3**, its activated version **4**, and PEG−maleimide **1**. The other Michael acceptor reagents, PEG−vinylsulfone **10** and PEG−acrylate **11**, were relatively less reactive.



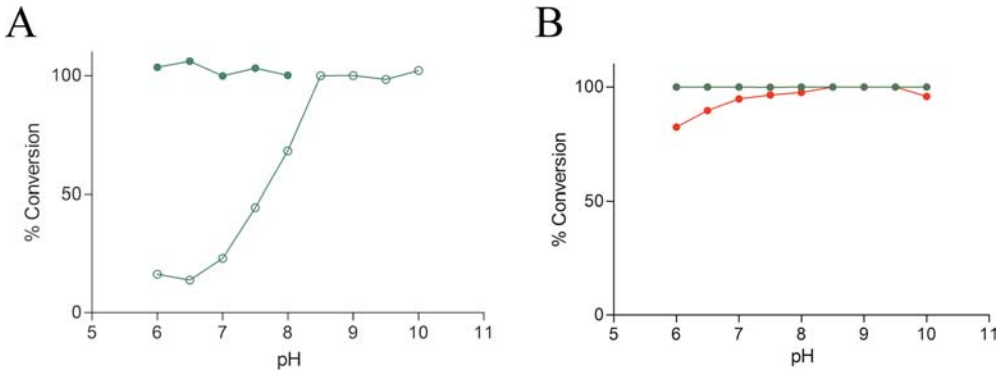


Figure 4. Conjugation of thiol reactive PEG reagents toward Lamβ1$_{925-933}$ peptide at different pH values and analyzed by RP-HPLC. (A) Reactions were incubated for 15 min and were conducted with 5 kDa PEG−mono-sulfone **3** (line with green open circles) and 5 kDa PEG−enone **4** (line with green solid circles). The conjugation conversion of PEG−mono-sulfone **3** increased from pH 7 to 8 as this reagent underwent elimination of toluene sulfinic acid **5** to give the reactive PEG−enone 4. (B) Conjugation reactions incubated overnight (16 h) displayed similar conversion for 5 kDa PEG−enone **4** (line with green solid circles) and 5 kDa PEG−maleimide **1** (line with red solid circles).

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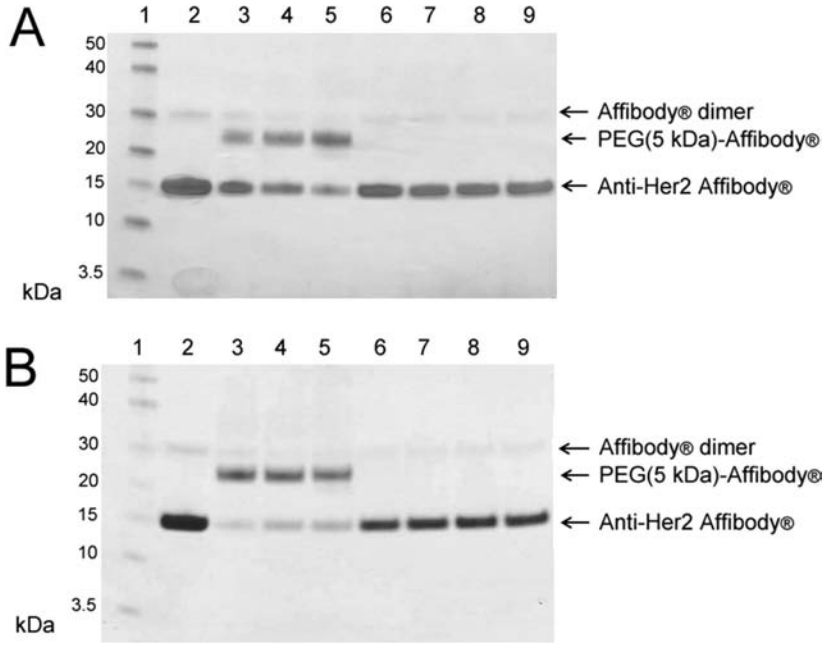
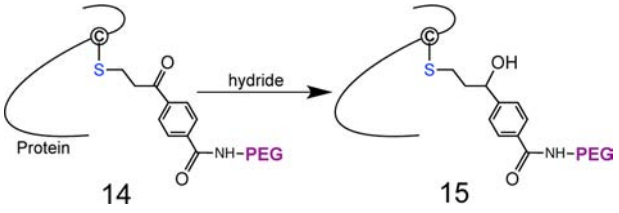


Figure 5. SDS-PAGE analysis of reactions of anti-Her2 Affibody with thiol reactive PEG reagents (5 kDa PEG) at pH 7.4. Gels show reactions after 30 min (A) and 4 h (B). In each gel, the samples are as follows: lane 1, protein standard markers; lane 2, anti-Her2 Affibody; lane 3, reaction with PEG–mono-sulfone **3**; lane 4, reaction with activated PEG reagent **4**; lane 5, reaction with PEG–maleimide **1**; lane 6, reaction with PEG–vinyl sulfone **10**; lane 7, reaction with PEG–acrylate **11**; lane 8, reaction with PEG–bromoacetamide **12**; lane 9, reaction with PEG–iodoacetamide **13**.

Conjugate Stability. Thiol selective PEGylation reagents that undergo conjugation by a Michael addition reaction are susceptible to retro Michael reactions leading to deconjugation of the PEG from the protein. The deconjugated PEG moiety will be reactive and available for an exchange reaction with other thiols in circulation, for example the free cysteine thiol in albumin.

Once the PEG–mono-sulfone **3** undergoes conjugation with a peptide or protein, the conjugate **14** can be "locked" to prevent the retro Michael reaction leading to deconjugation by simple reduction of the electron-withdrawing carbonyl with a mild borohydride reagent to give the corresponding conjugate **15** (Scheme 3).[25,44] Mild reduction conditions are required with proteins to ensure their structure is maintained, for example, to avoid racemization and disulfide reduction. As a process, a mild reducing step is used in reductive amination[56–60] and is used in the production of pegfilgrastim,[61] which is widely used to treat neutropenia during chemotherapy. Preventing deconjugation reactions by the mild reduction of the cyclic imide carbonyl is not readily possible with maleimide derived conjugates in conditions that would preserve protein function.[62]

Scheme 3. Deconjugation Can Be Avoided by Simple Treatment of the Conjugate 14 with a Hydride Reagent to Reduce the Electron Withdrawing Carbonyl Group to an Alcohol[a]



[a]Conjugate **15** is not labile to elimination at neutral pH.

The in vitro stability of PEG–Lamβ1$_{925–933}$ conjugates prepared with 5 kDA versions of PEG–maleimide **1** and PEG–mono-sulfone **3** were compared over 12 days (Figure 6).

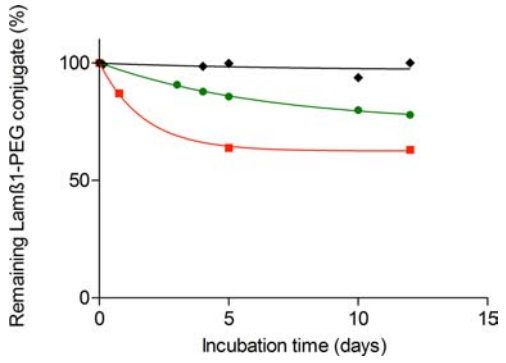


Figure 6. Stability of Lamβ1$_{925–933}$ peptide PEG conjugates prepared with thiol reactive PEG reagents at room temperature in pH 7.5 phosphate buffer over 12 days. Conjugates prepared with 5 kDa PEG–mono-sulfone **3** (line with green solid circles), 5 kDa PEG–mono-sulfone **3** then reduced with sodium borohydride (line with black solid tilted squares) and 5 kDa PEG–maleimide **1** (line with red solid squares) were tested for stability and samples were analyzed by RP-HPLC.

Samples were incubated in a pH 7.5 sodium phosphate buffer at room temperature. The Lamβ1$_{925–933}$ conjugate derived from PEG–mono-sulfone **3** was treated with sodium borohydride prior to commencing the stability study to reduce the keto carbonyl. If milder conditions are required, other borohydrides such as sodium triacetoxyborohydride can be used. The stability of the conjugates was determined by monitoring the area of the PEG–Lamβ1$_{925–933}$ peak using reverse phase chromatography.

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Both conjugates derived from PEG−sulfone **3** and PEG−maleimide **1** were incubated at room temperature in pH 7.5 phosphate buffer for 12 days with evidence that deconjugation had occurred. The conjugate derived from PEG reagent **3** when treated with sodium borohydride remained stable to deconjugation in these conditions.

CONCLUSION

PEG mono-sulfone reagent **3** is easily prepared and is latently reactive so that conversion to the active form during conjugation can be tailored in the mild conditions that are often necessary to preserve protein function. Once reagent **3** is conjugated to a protein or peptide, exchange reactions leading to deconjugation can be easily avoided by treating the conjugate with a mild reducing agent in a way analogous to the process used for conjugation reactions involving reductive amination. We believe that PEG−mono-sulfone **3** should be considered as a viable alternative to PEG−maleimide reagents.

ASSOCIATED CONTENT

Supporting Information

RP-HPLC and mass spectral data are provided for the competitive conjugation experiments of *N*-acetyl-L-lysine and *N*-acetyl-L-cysteine with PEG−mono-sulfone **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): SB a full-time academic employee of the UCL School of Pharmacy. SB was an academic co-founder of PolyTherics Ltd in 2000 and he has equity in the company. He was a non-paid director and part-time CSO until Dec 2012. His current interaction with the company is primarily to supervise PhD students registered at UCL that are partially funded by the company. The other co-authors are all employees of PolyTherics Ltd. Drs Cong and Rumpf have since left the company and their current email addresses were used for the online submission.

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■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on February 11, 2014, with incorrect labels in the Abstract graphic. The corrected version was reposted on February 13, 2014.