Site-Specific PEGylation of Protein Disulfide Bonds Using a Three-Carbon Bridge

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The covalent conjugation of a functionalized poly(ethylene glycol) (PEG) to multiple nucleophilic amine residues results in a heterogeneous mixture of PEG positional isomers. Their physicochemical, biological, and pharmaceutical properties vary with the site of conjugation of PEG. Yields are low because of inefficient conjugation chemistry and production costs high because of complex purification procedures. Our solution to these fundamental problems in PEGylating proteins has been to exploit the latent conjugation selectivity of the two sulfur atoms that are derived from the ubiquitous disulfide bonds of proteins. This approach to PEGylation involves two steps: (1) disulfide reduction to release the two cysteine thiols and (2) re-forming the disulfide by bis-alkylation via a three-carbon bridge to which PEG was covalently attached. During this process, irreversible denaturation of the protein did not occur. Mechanistically, the conjugation is conducted by a sequential, interactive bis-alkylation using α,β-unsaturated β′-monosulfone functionalized PEG reagents. The combination of (a) maintaining the protein's tertiary structure after disulfide reduction, (b) the mechanism for bis-thiol selectivity of the PEG reagent, and (c) the steric shielding of PEG ensure that only one PEG molecule is conjugated at each disulfide bond. PEG was site-specifically conjugated via a three-carbon bridge to 2 equiv of the tripeptide glutathione, the cyclic peptide hormone somatostatin, the tetrameric protein L-asparaginase, and to the disulfides in interferon α-2b (IFN). SDS-PAGE, mass spectral, and NMR analyses were used to confirm conjugation, thiol selectivity, and connectivity. The biological activity of the L-asparaginase did not change after the attachment of four PEG molecules. In the case of IFN, a small reduction in biological activity was seen with the single-bridged IFN (without PEG attached). A significantly larger reduction in biological activity was seen with the three-carbon disulfide single-bridged PEG-IFNs and with the double-bridged IFN (without PEG attached). The reduction of the PEG-IFN's in vitro biological activity was a consequence of the steric shielding caused by PEG, and it was comparable to that seen with all other forms of PEG-IFNs reported. However, when a three-carbon bridge was used to attach PEG, our PEG-IFN's biological activity was found to be independent of the length of the PEG. This property has not previously been described for PEG-IFNs. Our studies therefore suggest that peptides, proteins, enzymes, and antibody fragments can be site-specifically PEGylated across a native disulfide bond using three-carbon bridges without destroying their tertiary structure or abolishing their biological activity. The stoichiometric efficiency of this approach also enables recycling of any unreacted protein. It therefore offers the potential to make PEGylated biopharmaceuticals as cost-effective medicines for global use.

INTRODUCTION

Covalent conjugates of poly(ethylene glycol) (PEG) with amino residues of native proteins are typically mixtures of positional isomers (*1*−*5*). The relative amount of each PEG−protein isomer depends upon the PEG's terminal chemical functionality, its molecular weight, its morphology, and the specific reaction conditions used for conjugation (*6*). Some amine-specific PEG reagents (e.g., active esters, carbonates, isocyanates) also have a short half-life in aqueous solution, while others are inefficient for conjugating proteins (e.g., aldehydes). Typically, a large stoichiometric excess of the PEG reagent is required. These characteristics often lead to complex purification procedures for removing the excess PEG and the unwanted protein side products.

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Conjugation strategies and PEG reagents have been developed to address the important issue of site specificity (*7*−*18*). Of these, thiol-specific reagents are notable because some of them can lead to efficient conjugation reactions. However, in order to utilize thiol-specific PEG reagents, it has become necessary to recombinantly engineer a new and free cysteine into the protein (*19*). This is technically demanding and expensive, and the presence of an accessible free thiol often leads to disulfide scrambling, protein misfolding, and an increased propensity for irreversible protein aggregatation during its purification.

Our approach is particularly relevant to those therapeutic proteins whose mode of action is via cell surface receptors because they often have paired disulfides but seldom have a free cysteine residue (*20*). Typically, these proteins have an even number of cysteines that pair up as disulfides (*21*−*23*). Our strategy exploits the chemical reactivity of *both* of the sulfur atoms in an accessible disulfide bond. While disulfides influence a protein's properties in subtle and complex ways (*24*), the accessible disulfides typically contribute to the stability of the protein rather than to its structure or its function (*21*). In contrast, the disulfides that are present in a protein's hydrophobic interior

10.1021/bc0601471 CCC: $37.00
Published on Web 11/29/2006

(*21*, *25*) typically contribute to the maintenance of its tight packing and its function. Although there are examples where chemical modification of a disulfide results in a major loss of tertiary structure (*26*) and biological activity (*27*−*29*), there are also examples where chemical modification, especially of accessible disulfides, does not lead to a loss of protein structure or activity (*30*, *31*).

We have found that mild reduction of an accessible native disulfide bond to liberate the cysteine thiols can be followed by PEGylation with a bis(thiol)-specific reagent. This leads to the bridging of the two cysteine thiols with PEG attached. We have recently shown that (i) a single intrachain three-carbon bridge (with and without PEG attached) can be inserted across a native disulfide bond in interferon α-2b (IFN), and (ii) a single interchain three-carbon disulfide bridge with PEG attached can be inserted into an anti-CD4 antibody fragment using PEG monosulfone. There is no disruption of the tertiary structure or the biological activity of either protein (*32*, *33*). We now report further details of the synthesis of the disulfide bridging PEGylation reagents and their use with (i) reduced glutathione, (ii) somatostatin, (iii) the multimeric protein L-asparaginase, and (iv) interferon α-2b (IFN).

EXPERIMENTAL SECTION

Materials. Reduced (GSH) and oxidized (GSSG) glutathione, somatostatin, dithiothreitol (DTT), tris(2-carboxyethyl) phosphine (TCEP-HCl), *O*-(2-aminoalkyl)-*O*′-methyl poly(ethylene glycol) 5 kDa, *E. coli*-derived L-asparaginase (L-asparagine amidohydrolase, E.C. 3.5.1.1) and the commercial PEG−L-asparaginase were from Sigma-Aldrich. The IFN is in clinical use as Shanferon (Shantha Biotechnics, India). It is manufactured using a yeast-based protein expression system, and it shows chemical and biological equivalence to the NIBSC (U.K.) international biological standard (*34*). *O*-(2-aminoethyl)-*O*′-methyl poly(ethylene glycol) (10 and 20 kDa) were purchased from NOF Corporation, Japan. Superdex 200 prep grade, Sephadex G-25 desalting (PD-10), HiTrap SP FF cation exchange, and HiTrap Q FF anion exchange columns were from GE Healthcare. All other reagents and solvents were purchased from Sigma-Aldrich and used as received.

Preparation of 4-[2,2-bis[(*p*-tolylsulfonyl)methyl]acetyl) Benzoic Acid−NHS Ester 2. Under an argon atmosphere, a stirred mixture of 4-(2,2-bis[(*p*-tolylsulfonyl)methyl]acetyl) benzoic acid **1** (2.00 g, 4.0 mmol) prepared as described (*35*, *36*), *N*-Hydroxysuccinimide (0.483 g, 4.2 mmol, NHS) and anhydrous dichloromethane (5 mL) were cooled using an ice bath. Neat 1,3-diisopropylcarbodiimide (657 μL, 4.2 mmol, DIPC) was then added dropwise. A further 60 μL of DIPC was added after 1.5 h. After 3 h, the reaction mixture was passed through a nonabsorbent cotton wool filter. The homogeneous filtrate was diluted with dichloromethane (∼30 mL), washed with water (2 × 15 mL), and dried with magnesium sulfate. Gravity filtration followed by removal of volatiles under vacuum gave the desired active NHS ester **2** as a solid product (1.89 g, 79% yield).

^{1}H NMR: ($CDCl_3$, 400 MHz) δ 2.48 (s, 6H, CH_3), 2.94 (s, 4H, CH_2CH_2), 3.56 (A2B2X, 4H, CH_2C), 4.38 (quintet, 1H, C*H*, J = 6.3 MHz), 7.37, 7.70 (AB q, SO_2Ar, 8H, J = 8.0 and 8.3 MHz, respectively), 7.76, 8.14 (AB q, CO*Ar*, 4H, J = 8.6 MHz).

^{13}C NMR: ($CDCl_3$, 400 MHz) δ 195.29, 168.92, 160.95, 145.63, 139.11, 135.29, 130.92, 130.24, 129.59, 128.73, 128.28, 55.67, 35.89, 25.70, 21.70.

MALDI-TOF-MS: m/z = 620.3070 ± 0.43 RSD $[M + H]^+$.

Preparation of PEG Bis(sulfone) 3. To anhydrous dichloromethane (4 mL) in an argon atmosphere was added 4-(2,2-bis[(*p*-tolylsulfonyl)methyl]acetyl) benzoic acid−NHS ester **2** (300 mg, 503 μmol). This solution was cooled with an ice bath, and to this was added an anhydrous dichloromethane (5 mL) solution of *O*-(2-aminoethyl)-*O*′-methyl poly(ethylene glycol) (5000 g/mol) (0.5 g, 100 μmol). The reaction solution was stirred for 48 h. Volatiles were removed under vacuum, and the crude solid PEG bis(sulfone) **3** was redissolved in acetone (15 mL) with gentle warming. The stirred solution was then cooled in an ice bath to precipitate the desired product **3**, which was isolated using a no 3 sintered glass funnel and washed with chilled acetone (∼30 mL). Drying in vacuo gave the PEG bis-(sulfone) **3** as a white solid product (0.46 g, 84% yield).

^{1}H NMR: ($CDCl_3$, 400 MHz) δ 2.49 (s, 6H, CH_3Ar), 3.38 (s, 3H, CH_3OPEG), 3.44−3.84 (m, PEG + 4H, CH_2SO_2), 4.34 C*H*CO (qn, 1H, C*H*CO), 7.36, 7.69 (AB q, SO_2Ar, 4H, J = 8.3 MHz), 7.64, 7.81 (AB q, CO*Ar*, 4H, J = 8.3 MHz).

^{13}C NMR: ($CDCl_3$, 500 MHz) δ 195.11, 166.14, 145.51, 139.43, 136.19, 135.37, 130.19, 128.63, 128.31, 127.68, 71.94, 70.57, 69.59, 59.01, 55.56, 40.01, 35.61, 21.72.

Preparation of PEG Bis(sulfone) 3 Directly from Bis-(sulfone) Carboxylic Acid 1. To a Schlenk flask containing *O*-(2-aminoethyl)-*O*′-methyl poly(ethylene glycol) (5000 g/mol, 0.5 g, 0.33 equiv, 100 μmol) was added 4-(2,2-bis[(*p*-tolylsulfonyl)methyl]acetyl) benzoic acid **1** (150 mg, 1 equiv, 300 μmol) and *N*,*N*′-dicyclohexylcarbodiimide (62 mg, 1 equiv, 300 μmol). The flask was sealed with a rubber septum and then subjected to high vacuum for 30 min through the side arm. Argon was introduced into the flask, which was then placed in an ice bath to cool the solution. Anhydrous dichloromethane (10 mL) was injected and the contents stirred to form a homogeneous solution that was allowed to warm slowly to room temperature. The reaction mixture was then stirred for 3 h and filtered through a sintered glass filter no 3 to remove insoluble dicyclohexylurea. Dichloromethane was removed from the filtrate by rotoevaporation, and the viscous crude product residue was redissolved in acetone (20 mL) with gentle warming. The flask was then placed in a dry ice bath for 5 min to precipitate the product, which was isolated by filtration (no 3 sintered glass) and dried in vacuo to afford the desired PEG bis(sulfone) **3** as a white solid product (0.4 g, 73%).

^{1}H NMR: ($CDCl_3$, 400 MHz) δ 2.49 (s, 6H, CH_3Ar), 3.38 (s, 3H, CH_3OPEG), 3.44−3.84 (m, PEG + 4H, CH_2SO_2), 4.34 C*H*CO (qn, 1H, C*H*CO), 7.36, 7.69 (AB q, SO_2Ar, 4H, J = 8.3 MHz), 7.64, 7.81 (AB q, CO*Ar*, 4H, J = 8.3 MHz).

^{13}C NMR: ($CDCl_3$, 500 MHz) δ 195.11, 166.14, 145.51, 139.43, 136.19, 135.37, 130.19, 128.63, 128.31, 127.68, 71.94, 70.57, 69.59, 59.01, 55.56, 40.01, 35.61, 21.72.

Preparation of PEG Monosulfone 4. The PEG bis(sulfone) **3** (∼5500 g/mol, 0.20 g, 36 μmol) was dissolved in 10 mL of sodium phosphate buffer, pH 7.8 (20 mg/mL), and incubated overnight at ambient temperature. The reaction solution was added to a reverse-phase (C-18) packed sintered glass funnel that had been pre-equilibrated with deionized water. Water was removed from the filter by vacuum. The column was then washed with 25% aqueous acetonitrile to remove the buffer salts and the eliminated sulfinic acid. The reaction mixture was eluted with 75% aqueous acetonitrile and the filtrate freeze-dried to obtain the PEG monosulfone **4** (which was 80% pure by ^{1}H NMR with the remainder being the starting PEG bis(sulfone) **3**) as a white solid (0.16 g, 82% yield).

^{1}H NMR: ($CDCl_3$, 400 MHz) δ 2.35 (s, 3H, CH_3Ar), 3.31 (s, 3H, CH_3OPEG), 3.39−3.76 (m, PEG), 4.28 (s, 2H, CH_2-SO_2), 5.93, 6.22 (s, 2H, CH_2═C), 7.26, 7.64 (AB q, SO_2Ar, 4H, J = 8.3 and 8.6 MHz, respectively), 7.72, 7.81 (AB q, CO*Ar*, 4H, J = 8.1 and 8.3 MHz, respectively).

Conjugation of PEG to Reduced Glutathione (GSH). A microfuge tube was charged with PEG bis(sulfone) **3** (16 mg, 2.91 μmol, 1 equiv, 5500 g/mol) and GSH (2 mg, 2.24 equiv,

6.51 μmol) followed by 900 μL of 50 mM sodium phosphate buffer, pH 7.8, containing 10 mM EDTA. The tube was gently shaken to dissolve the solids and left for 18 h at ambient temperature. To ensure complete reaction, 100 μL of 1 N NaOH was added. The solution (1 mL) was loaded onto a PD-10 desalting column pre-equilibrated with 90:10 (v/v) H_2O/D_2O and the elution (1 mL) discarded. Additions of 1 mL each of fresh 90:10 (v/v) H_2O/D_2O to the PD-10 column were made while collecting the eluting 1 mL fractions separately. Each eluted 1 mL fraction was analyzed by UV absorbance at 215 nm. Typically, the initial two fractions which did not contain the conjugate (wash volume 2 mL) were discarded. The third fraction with the maximum amount of reaction product was analyzed by ^{1}H NMR with water suppression, 2D [^{1}H,^{1}H]-TOCSY, and 2D [^{1}H,^{1}H] NOESY.

PEGylation of Somatostatin. The PEG bis(sulfone) **3** (25 mg, 4.55 μmol, 5500 g/mol**)** was incubated in 50 mM sodium phosphate buffer (1 mL), pH 7.8, for 20 h and purified by reverse-phase (C-18) HPLC using a gradient of 30−60% acetonitrile in 30 min at a flow rate of 1 mL/min with UV detection at 215 nm. The collected fraction was subjected to freeze-drying to obtain the PEG monosulfone **4** (0.7 mg). Somatostatin (0.25 mg/mL in 50 mM sodium phosphate buffer, 0.15 μmol, pH 6.2) was disulfide-reduced with 1 equiv of TCEP-HCl (*37*) for 1 h at ambient temperature. PEG monosulfone **4** (0.7 mg, 0.13 μmol, 0.86 equiv) was added to the reduced peptide. The reaction solution was gently mixed and left overnight at 4 °C. The solution was then loaded onto a PD-10 desalting column pre-equilibrated with deionized water and the elution (1 mL) discarded. Additions of 1 mL each of fresh deionized water were made to the PD-10 column while collecting the eluting 1 mL fractions separately. Each eluted 1 mL fraction was analyzed by UV absorbance at 215 nm. Typically, the initial two elution fractions, which did not contain the conjugate (wash volume 2 mL), were discarded. The third fraction, which had the maximum amount of conjugate, was analyzed by MALDI-TOF-MS. A control reaction was conducted with somatostatin that was not previously incubated with TCEP-HCl and was followed by PD-10 desalting. MALDI-TOF-MS analysis showed only the presence of the PEG reagent, indicating that the conjugation reaction had not occurred. Unreacted somatostatin was removed during the desalting step.

Preparation of a Three-Carbon Disulfide-Bridged Somatostatin. Carboxylic acid bis(sulfone) **1** (5.7 mg) was incubated in 2:3 (v/v) acetonitrile/50 mM sodium phosphate buffer, pH 8.0 (1 mL), for 24 h. The rate of formation of the corresponding monosulfone was monitored by ^{1}H NMR (data not shown), and the pH was then adjusted to 6.2 using 1 N HCl. Somatostatin (0.25 mg/mL, 1 mL) in 2:3 (v/v) acetonitrile/50 mM sodium phosphate buffer, pH 6.2, was disulfide-reduced using 2 equiv of TCEP−HCl for 1 h at ambient temperature. The solution containing ∼1.4 equiv of the monosulfone moiety was added to the reduced peptide. The reaction solution was gently shaken and left overnight at 4 °C. It was then subjected to freeze-drying to remove volatiles. The solids were resolubilized in water and filtered (0.2 μm). The filtrate was subjected to reverse-phase (C-18) HPLC using acetonitrile/water solvent system (gradient: 20−40% acetonitrile in 10 min) containing 0.05% trifluoroacetic acid (TFA) with UV detection at 215 nm. The peptide peak (retention time 8.03 min; chromatogram not shown) was collected and mass-analyzed.

PEGylation of L-Asparaginase. To a solution of L-asparaginase (0.5 mg/mL, 1 mL) in 50 mM sodium phosphate buffer, pH 7.8, containing 10 mM EDTA was added DTT (100 mM, 15.4 mg). The solution was incubated for 30 min at ambient temperature and then loaded onto a PD-10 desalting column pre-equilibrated with 50 mM sodium phosphate buffer, pH 7.8, containing 10 mM EDTA and the elution (1 mL) discarded. Fresh 2 mL buffer (wash volume) was then added to the PD-10 and the elution discarded again. More fresh 2.5 mL buffer was added while collecting the elution for PEGylation. To the reduced protein solution (∼0.19 mg/mL, 2.5 mL; yield was 96% of the starting protein concentration) was added PEG monosulfone **4** (1.3 equiv for each disulfide) as a freshly prepared 20 mg/mL solution in 50 mM sodium phosphate buffer, and the solution was allowed to react for 16 h at 4 °C (approximately 5, 10, and 20 μL PEG monosulfone solution for 5, 10, and 20 kDa PEGylation reactions, respectively). The reaction solution (2.5 mL) was buffer-exchanged to 50 mM Tris buffer, pH 8.6, using a PD-10 desalting column (giving 0.13 mg/mL, 3.5 mL; yield 91% of the starting concentration). The solution was then loaded onto a HiTrap Q FF 1 mL column aided by a peristaltic pump. The column was washed manually using a syringe with 5 mL (2 column volumes) of fresh 50 mM Tris buffer, pH 8.6. The pure conjugate eluted on washing the column with 1 M sodium chloride in 50 mM Tris buffer (2.0 mL). The conjugates were further characterized by size exclusion chromatography (SEC) using a Superdex 200 prep grade column.

PEGylation of IFN. To a solution of IFN (0.5 mg/mL, 1 mL) in 50 mM sodium phosphate buffer, pH 7.8, containing 10 mM EDTA was added DTT (100 mM, 15.4 mg). The solution after incubation for 30 min at ambient temperature was loaded onto a PD-10 desalting column pre-equilibrated with 50 mM sodium phosphate buffer, pH 7.8, containing 10 mM EDTA and the elution (1 mL) discarded. Fresh 2 mL buffer (wash volume) was then added to the PD-10 and the elution discarded again. Further fresh buffer (2.5 mL) was added while collecting the elution for PEGylation. To the reduced protein solution (∼0.19 mg/mL, 2.5 mL; yield 96% of protein remaining) was added 1 equiv of 10 kDa PEG monosulfone **4** or 10 kDa PEG bis(sulfone) **3** (13 μL of a freshly prepared 20 mg/mL solution in 50 mM sodium phosphate buffer, pH 7.8), and the solution was allowed to react for 16 h at 4 °C. Glutathione refolding solution (50 μL of 50 mM GSH/50 mM GSSG solution to yield a reaction concentration of 1 mM GSH/1 mM GSSG) (*38*) was added to the reaction mixture and allowed to incubate for 24 h at 4 °C. The reaction solution (2.5 mL) was buffer-exchanged to 20 mM sodium acetate, pH 4.0, using a PD-10 desalting column. The resulting 3.5 mL solution had a protein concentration of 0.13 mg/mL (91% of protein remaining). The solution was then loaded onto a HiTrap SP FF 1 mL column aided by a peristaltic pump. The column was washed manually using a syringe with 5 mL (2 column volumes) of fresh 20 mM sodium acetate buffer, pH 4.0. The pure conjugate eluted on washing the column with 1 M sodium chloride in 20 mM sodium acetate buffer (0.185 mg/mL, 2 mL, yield 74%). The conjugates were further isolated by size exclusion chromatography (SEC) using a Superdex 200 prep grade column, and the yields with PEG monosulfone **4** (10 kDa, 1 equiv) were as follows: three-carbon disulfide single-bridged 10 kDa PEG−IFN, 64.3%; three-carbon disulfide double-bridged 10 kDa PEG−IFN, 17.4%; IFN, 13.4%; and aggregated IFN, 4.8%. The yields with PEG bis(sulfone) **3** (10 kDa, 1 equiv) were as follows: three-carbon disulfide single-bridged 10 kDa PEG−IFN, 56.69%; three-carbon disulfide double-bridged 10 kDa PEG−IFN, 8.69%; IFN, 32.36%; and aggregated IFN, 2.25%.

Preparation of Three-Carbon Disulfide Double-Bridged IFNs. To a solution of IFN (0.2 mg/mL, 1 mL) in 50 mM sodium phosphate buffer at pH 7.8 containing 10 mM EDTA was added dithiothreitol (DTT, 100 mM, 15.4 mg). The solution after incubation for 30 min at ambient temperature was loaded onto a PD-10 desalting column pre-equilibrated with 50 mM sodium phosphate buffer, pH 7.8, containing 10 mM EDTA and the elution (1 mL) discarded. Fresh 2 mL buffer (wash

volume) was then added to the PD-10 and the elution discarded again. More fresh 2 mL buffer was added while collecting the elution for reaction. To the solution containing the reduced IFN (~75 μg/mL, 2 mL) was then added 8 equiv of the carboxylic acid bis(sulfone) **1** as a solution in acetonitrile (50 μL of 0.625 mg/mL). The mixture was gently shaken and incubated for 2 h at 4 °C. The reaction solution was made up to 2.5 mL and then buffer-exchanged to 20 mM sodium acetate, pH 4.0, using a PD-10 desalting column to obtain the three-carbon double-bridged IFN (3.5 mL, ~40 μg/mL).

MALDI-TOF-MS. Mass spectra were acquired using an Applied Biosystems Voyager System DE PRO MALDI-TOF mass spectrometer using a nitrogen laser. The matrix was a saturated solution of α-cyano-4-hydroxycinnamic acid in a 50:50 mixture of acetonitrile and water containing 0.1% trifluoroacetic acid. Sample and matrix were mixed 1:1, and 1 μL was spotted onto a 100-well sample plate. All spectra were acquired in positive mode over the range 600−2500 Da under reflectron conditions (20 kV accelerating voltage, 350 ns extraction delay time) and 2−100 kDa under linear conditions (25 kV accelerating voltage, 750 ns extraction delay time).

Tryptic Digestion and MALDI-TOF Analysis. IFN and the bridged IFN solutions (1 mL each of 0.2 mg/mL in 10 mM sodium phosphate buffer, pH 8.0) were concentrated to 200 μL using Vivaspin 6 mL concentrators (Sartorius). Sequencing grade modified trypsin (50 μL of 0.2 mg/mL, Promega) was added to each of the samples followed by incubation at 37 °C for 14 h. To each solution was added acetonitrile (30 μL), and the incubation continued at 37 °C for another 4 h. Finally, each digest was diluted with 220 μL of acetonitrile and the resulting samples analyzed using a Voyager MALDI-TOF-MS. High-resolution reflectron mode was used to observe low molecular masses. Low-resolution linear mode was used to observe high molecular masses.

NMR Spectroscopy. NMR spectra of all reactants were obtained on a Bruker Avance 400 MHz spectrometer, while the spectra of glutathione and conjugates were acquired using a Bruker Avance 500 MHz. Both instruments were equipped with a 5 mm BBO probe including *Z*-axis pulse field gradients. Assignments for the ^{1}H spectra of peptides were achieved using standard sequential assignment procedures of protein spectra (*39*). 2D [^{1}H,^{1}H]TOCSY (*40*) were acquired employing 60 ms mixing time and 2D [^{1}H,^{1}H] NOESY (*41*) employing mixing times from 100 to 300 ms. NMR spectra were processed using Bruker *NMR Suite 3.5* and Amix *Viewer 3.1.5* (Bruker Biospin GmbH). Chemical shifts were referenced to the residual solvent signals when the sample was dissolved in an organic solvent and to sodium trimethylsilyl-(2*H*4)-propionate (TSP) when it was dissolved in D_2O/H_2O.

Enzymatic Assay for L-Asparaginase. The concentration of the PEG−L-asparaginase was determined by microBCA protein assay using an L-asparaginase standard curve. The enzymatic activities of L-asparaginase, our PEGylated L-asparaginase, and the commercially available PEGylated L-asparaginase (15 μg/mL) were determined by measuring ammonia formation (i.e., hydrolysis of L-asparagine to L-aspartic acid and ammonia) after incubation with 10 mM L-asparagine in 50 mM Tris-HCl buffer (pH 8.6) for 30 min at 37 °C (*42*, *43*). The reaction was stopped by the addition of 1.5 M trichloroacetic acid and Nessler's reagent. The ammonia that was produced was determined using ammonium sulfate as a standard.

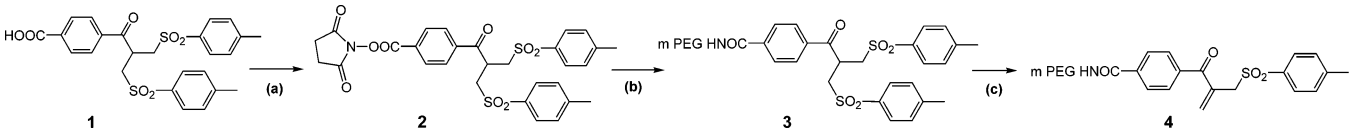
Antigenicity Assay for L-Asparaginase. The concentration of PEGylated (20 kDa) L-asparaginase was determined with a microBCA protein assay using an L-asparaginase standard curve. Enzyme immunoassay plates (96-well) were coated overnight at 4 °C with 10 μg/mL each of L-asparaginase (Sigma-Aldrich), PEG−L-asparaginase, and commercial PEG−L-asparaginase conjugate (Sigma-Aldrich) in 0.2 M carbonate coating buffer solution, pH 9.6. The plate wells were then washed four times with 0.5% v/v solution of Tween20 in PBS (PBS-T). A 0.5% BSA in PBS (200 μL) solution was added to the wells and incubated at 37 °C for 1 h. The wells were then washed with PBS-T. Rabbit anti-asparaginase polyclonal antibody (Chemicon Int., U.S.A.) (100 μL of a 1:1000 dilution) was added and incubated for 1 h at 37 °C. After washing with PBS-T, an anti-rabbit IgG−horseradish peroxidase conjugate (Sigma-Aldrich), which had been diluted 1500-fold in PBS-T, was added and incubated for 1 h at 37 °C. After washing, 100 μL of tetramethylbenzidine, 0.03% v/v hydrogen peroxide solution in 0.1 M sodium acetate buffer, pH 6.0, was added and incubated for 5 min at ambient temperature. The reaction was stopped using 100 μL of 2.5 M sulfuric acid and absorbance measured at 405 nm.

In Vitro IFN Antiviral Assay. The human lung fibroblast cell line A549 was maintained in DMEM media supplemented with 10% fetal calf serum (FCS), 50 units/mL penicillin, and 50 μg/mL streptomycin. For the antiviral assay, A549 cells were resuspended at 0.3×10^6 cells in assay media and then aliquotted at 50 μL/well into 96-well microtiter plates. Twelve hours later, 50 μL of each protein sample was prepared in assay media and added to the test wells in serial doubling dilutions. Each protein sample was tested in quadruplicate. Control wells contained either cells (negative control) or cells and virus (positive control). The plates were then incubated overnight at 37 °C. The media was then removed and the cells infected with encephalomyocarditis virus (EMCV) for 1 h after resuspending the virus in 50 μL/well of DMEM supplemented with 2% FCS, penicillin, and streptomycin. Assay media containing EMCV was then removed and 100 μL of fresh assay media added to each well. On the next day, the positive control wells were monitored until there was cell death in 80% of the wells. Each well was then washed twice with PBS and 50 μL 4% formaldehyde/0.1% methyl violet 2B solution added for 30 min. The plates were then washed twice with PBS and air-dried. The dye used was solubilized by agitation with 10% SDS (50 μL/well) and the OD measured at 570 nm.

2′,5′-Oligoadenylate Synthetase mRNA Assay. Molt-4 cells were grown in RPMI 1640 media supplemented with 10% FCS, penicillin, and streptomycin, and resuspended at 10^6 cells/mL. They were then aliquoted into a 48-well tissue culture plate at 1 mL/well. Protein samples were added in serial doubling dilutions and the plates incubated for 24 h. The cells were then washed and the mRNA (200 ng) extracted using a total mRNA extraction kit (Nucleospin RNA II, Machery-Nagel, Germany) in accordance with the manufacturer's instructions. Fifty microliters of the total RNA extract was then reverse-transcribed for 2 h to generate the cDNA. Quantitative PCR was performed on 60 ng of this cDNA for 2′,5′-oligoadenylate synthetase (2′,5′-OAS) using a RotorGene 3000 real-time PCR machine (Corbett Research, U.K.). The forward and reverse primers used were GGC TAT AAA CCT AAC CCC CAA ATC and AGC TTC CCA AGC TTC TTC TTA CAA, respectively. The PCR amplification conditions used were denaturation at 95 °C for 3 s, annealing at 59 °C for 5 s, extension of 72 °C for 8 s, and fluorescence acquisition at 83 °C for 5 s. Melting curve analysis was performed over the range 72−95 °C. mRNA quantitation was achieved by coamplifying with an in-house plasmid containing the 2′5′-OAS gene sequence.

Molecular Modeling Studies. All computational studies were carried out using integrated molecular modeling package *Maestro v6.5* (Maestro) and *Macromodel v9.1* (*44*). The OPLS-2005 force field (*45*) was used with extended cutoff values for nonbonded interactions (Van der Waals, 8 Å; electrostatic, 20

Scheme 1. Preparation of PEG Monosulfone 4[a]



[a] (a) DIPC, NHS; (b) methoxy-PEG-NH_2; (c) 50 mM sodium phosphate buffer, pH 7.8.

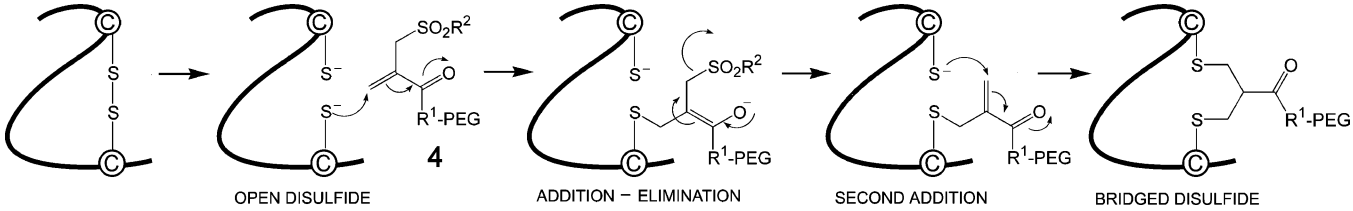


Figure 1. Site-specific PEGylation of a protein disulfide bond. The disulfide was reduced, and this was followed by reaction with a monofunctionalized PEG that was capable of sequential, interactive bis-alkylation. Mechanistically, PEGylation involves a first thiol addition to a PEG monosulfone followed by sulfinic acid elimination to generate a second double bond, which then undergoes a second thiol addition to complete the conjugation.

Å; and hydrogen bond, 4 Å). Solvent effects were considered as an aqueous generalized Born/surface area (GB/SA) solvent model (*46*). All protein and peptide models were subjected to the modified stochastic simulation protocol at 300 K used for proteins with chemically modified disulfides (*32*): 1500 steps of conjugated gradient minimization, time step of 1 ps, equilibration time of 10 ps, and 2000 ps of simulation time using the SHAKE algorithm for all bonds to hydrogen atoms. Snapshots of molecular structures were recorded at every 20 ps during simulations and energy minimized.

PEG with linker subunit was constructed as a linear chain comprising 230 monomers (10 kDa) and the simulation performed using the parameters defined above—except that molecular dynamics was used instead of stochastic dynamics. The 3D structure of the IFN was obtained as described (*33*) and imported using the *Maestro* program. Hydrogen atoms were added and the structure subjected to 1500 steps of gradient minimization using the AMBER force field and *Macromodel v8.5* (*44*). Water was defined as an aqueous generalized Born/surface area (GB/SA) solvent model to consider the solvent effect. The IFN with a three-carbon bridge across Cys1-Cys98 and across Cys29-Cys138 was built and subjected to the modified stochastic simulation protocol used for proteins with chemically modified disulfides (*32*). The initial 100 steps of conjugate gradient minimization using OPLS-2005 force field and GB/SA solvent model were used to remove clashes between added atoms and the rest of the protein. This was followed by equilibrating models at 300 K for 1 ps and stochastic simulation of 2000 ps in 1.0 fs steps at 300 K. The SHAKE algorithm was applied to bonds containing hydrogen atoms. Snapshots of the molecular structures were recorded every 2 ps during simulations and energy minimized. The modeled structure was compared to IFN α-2a by calculating the root-mean-square displacement (rmsd) values. They were determined using the McLachlan algorithm (*47*) as implemented in the program *ProFit v1.8* (*48*). The molecular model of IFN with both disulfide bonds reduced was subjected to the molecular simulation protocol, and the final snapshot structure was used to build the IFN model with a PEG attached at Cys1-Cys98 and at Cys29-Cys138. This model was subjected to the stochastic dynamics protocol after removing initial clashes between atoms.

RESULTS AND DISCUSSION

Thiol-Specific Bis-Alkylation. Our approach to protein PEGylation involves the mild reduction of a protein disulfide to free the two cysteine sulfur atoms followed by reaction with the bis-thiol-specific PEG reagent **4** (*33*). The conjugating moiety of the PEG reagent **4** (Scheme 1) is a substituted propenyl system. It contains a thiol-specific bis-alkylating group which comprises a carbonyl electron withdrawing group, an α,β-unsaturated double bond, and a β′ leaving sulfonyl group. The latter is susceptible to elimination. This juxtaposition of chemical functionality allows for a sequence of interactive and sequential addition−elimination reactions (Figure 1) that can occur at neutral or slightly acidic pH with the two free thiols that are derived from a reduced disulfide bond (*33*). Lawton et al. (*35, 36, 49, 50*) first demonstrated that this type of chemical functionality could undergo bis-thiol alkylation to cross-link proteins (e.g., ribonuclease (*49*) and antibodies (*35, 36*)). Wilbur et al. subsequently examined this functionality with antibody fragments (*51*). Other substituted propenyl systems capable of bis-alkylation with nucleophiles have also been used in organic synthesis (e.g., refs 50, 52−56 and references therein).

Our results show that appending the bis-alkylating functionality to the terminus of PEG (*33*), as shown in structure **4**, results in a water-soluble reagent that is more susceptible to an intramolecular protein thiol-specific reaction. Competitive intermolecular alkylation reactions are unlikely to occur, because the conjugation reaction occurs in aqueous conditions. This ensures that the protein's tertiary structure is maintained after the gentle reduction of a disulfide. It is also important to keep the free thiols close to each other because (a) this ensures that irreversible denaturation or aggregation of the protein does not occur during the conjugation reaction with the PEG monosulfone **4** (*33*) and (b) disulfide scrambling reactions do not occur when more than one disulfide is reduced.

The PEG monosulfone **4** is fundamentally different from the other PEGylation regents that can undergo conjugation reactions with two thiols. The dithiol reactive PEGylation reagents that have been described have two separate thiol-accepting moieties (e.g., two maleimides (*10, 57*)), which are chemically independent and which are not capable of the controlled sequential reactions of PEG monosulfone **4**. In addition, the distance between the sites for addition of the two sulfur atoms with these other reagents is much greater than the distance for our three-carbon bridge. Therefore, these reagents are unable to rebridge reduced disulfides in the way that our PEG monosulfone **4** can bridge the reduced disulfide bond.

Synthesis of PEG Conjugation Reagents. The PEG monosulfone **4** was prepared from the known carboxylic acid bis(sulfone) acid **1** (*35, 36, 50*) (Scheme 1). Coupling of methoxyamino-terminated PEG was routinely accomplished using the active ester bis(sulfone) **2** or by direct carbodiimide-mediated coupling with acid bis(sulfone) **1**. The synthesis of PEG monosulfone **4** was accomplished by incubation of the PEG bis(sulfone) **3** (25 mg/mL) in 50 mM sodium phosphate buffer

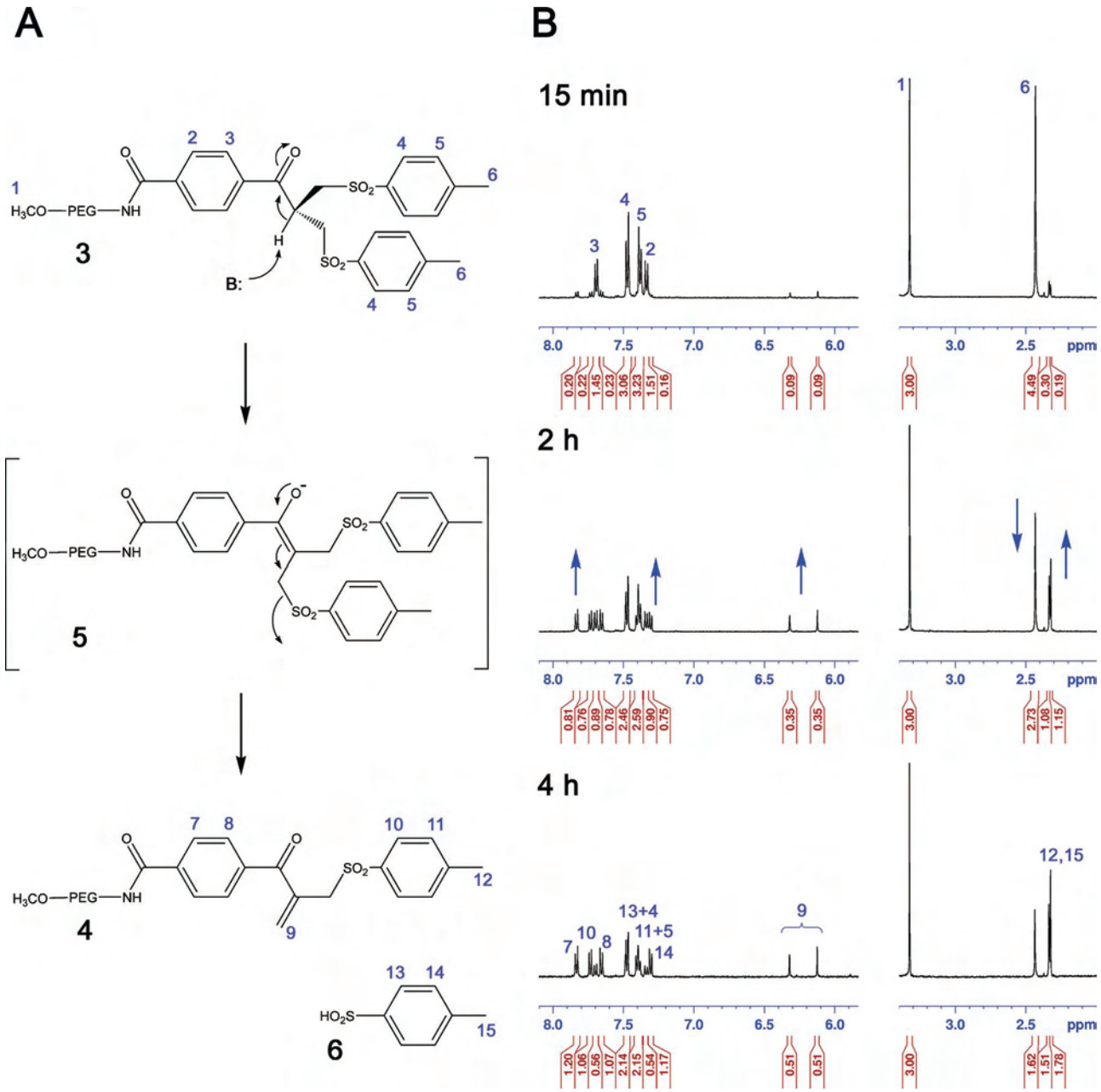


Figure 2. A. Elimination of toluene sulfinic acid **6** from PEG bis(sulfone) **3** via the enolate **5** to generate PEG monosulfone **4** is required for the first thiol addition reaction to occur. B. The elimination reaction was monitored by ^{1}H NMR in 50 mM phosphate buffer in D_2O (pH 7.2) with 51% conversion to PEG monosulfone **4** observed after 4 h as calculated by integration of the geminal vinylic protons. Typically, overnight incubation at ambient temperature was required for maximum conversion of the reagent.

(pH 7.8) and purified by RP-HPLC. PEG bis(sulfone) **3** undergoes elimination of toluene sulfinic acid **6** to give the PEG monosulfone **4** at pH values of 7.0 or higher. The elimination reaction was monitored by ^{1}H NMR (Figure 2) and reverse-phase RP-HPLC (Figure 3). The rate at which this elimination reaction occurred from the PEG bis(sulfone) **3** was dependent on pH and temperature (Figure 4). Although a decreased rate of elimination was observed at pH 6.0, this suggested that PEGylation could also be conducted at a slightly acidic pH value as was seen for somatostatin at pH 6.3 (see below).

These observations indicate that the rate to generate the double bond in the PEG monosulfone **4** can be tailored to the protein thiol's reactivity after disulfide reduction. Therefore, PEGylation was usually conducted using the PEG bis(sulfone) **3** which gave the monosulfone **4** in situ. This means that the optimized conjugation conditions can be tailored to balance the rate of the first elimination with the rate of the first thiolate addition reaction and thereby avoid the competitive conjugation of PEG to each of the thiols that are derived from a disulfide. When it is necessary to conduct PEGylation at an acidic pH value (because of issues related to protein solubility or stability, such as those described for somatostatin below), it is best to isolate and use the PEG monosulfone **4** directly in the conjugation reaction.

Bis-Thiol Selectivity of the Reagents. The tripeptide GSH was used to study the thiol reactivity and selectivity of the PEG bis(sulfone) **3** in the aqueous conditions that would typically be used for proteins. The conjugate, $PEG(SG)_2$ (Figure 5A) was derived from 2 equiv of GSH (each possessing one cysteine thiol), and it was purified by desalting column. The two glutathione molecules were conjugated to PEG via a three-carbon bridge. The conjugate structure (Figure 5A) was characterized and compared to oxidized glutathione (GSSG) by 1D and 2D ^{1}H NMR analysis. The set of 1D and 2D ^{1}H NMR spectra of GSSG was acquired and assigned using the standard procedure for proteins. The amide region of the 1D spectrum

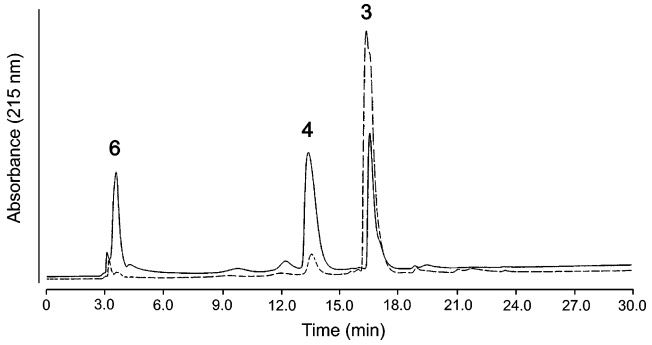


Figure 3. Reverse-phase HPLC chromatograms at $t = 15$ min (broken line) and $t = 5$ h (solid line) for the elimination of toluene sulfinic acid **6** from PEG bis(sulfone) **3** to give PEG monosulfone **4**. The elimination reaction was conducted in 50 mM phosphate buffer, pH 7.8, at ambient temperature.

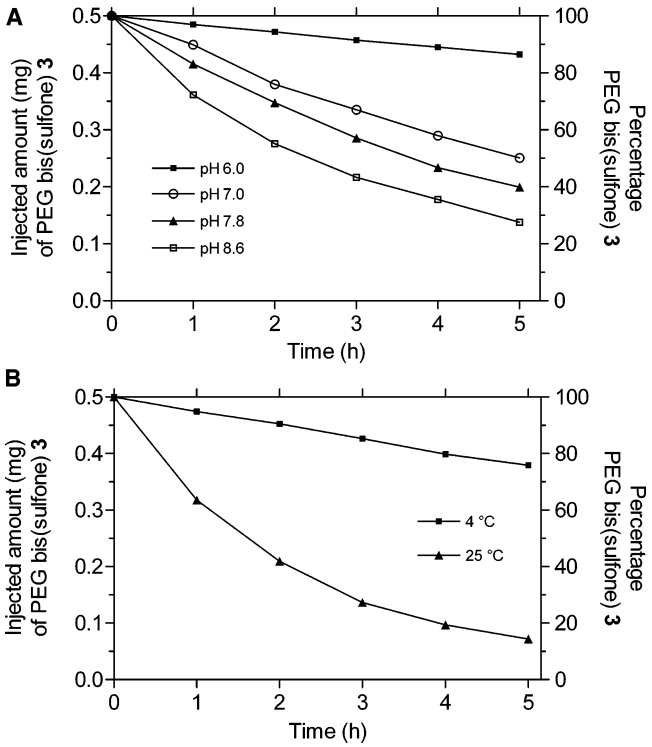


Figure 4. Effect of (**A**) pH on elimination of PEG bis-sulfone **3** (25 mg/mL) at 25 °C and (**B**) temperature on elimination of PEG bis-sulfone **3** (10 mg/mL) as monitored by reversed phase-HPLC using a C_{18} Phenomenex column (5 μm, 250 × 4.6 mm). Solvent A: acetonitrile containing 0.05% CF_3COOH (TFA), Solvent B: water containing 0.05% TFA. The gradient used was 30−60% B in 30 min with a flow rate of 1 mL/min at room temperature. UV detection was at 215 nm. The sample injection volumes for the temperature experiments were adjusted to give PEG bis(sulfone) **3** in the amount of 0.5 mg per injection.

had only two peaks: the doublet for NH of Cys and the triplet for the NH of Gly. The presence of only one set of peaks in both regions of the NMR spectra (amide and side chain) indicated that the two peptide chains of the tripeptide were connected by thiol ether bonds with a similar conformation. The two β-methylene protons of Cys are nonequivalent (2.8−3.25 ppm) in GSSG due to the restricted rotation of the disulfide bone. These methylene protons are the same in GSH where there is free rotation of the Cys side chain.

The assignment of NMR spectra for PEGylated glutathione ($PEG(SG)_2$) was achieved by observing spin system patterns in TOCSY (Figure 5B) for Cys and Gly. These corresponded to the NH doublet of Cys at 8.3 ppm and the NH triplet of Gly 8.05 ppm. The NOESY cross-peak at 8.39 and 4.45 ppm (Figure 5C) confirmed the sequential connectivity between the α-hydrogen of Cys (*) and NH of Gly. The triplet for the NH of the amide in the PEG reagent was at 8.7 ppm. This signal had a cross-peak in the TOCSY spectrum to the methylene hydrogen atoms of PEG at 3.7 ppm. This confirmed that the two GSH tripeptides and the PEG were in solution, while the 1:2 ratio for the integration of amide hydrogen atoms of the PEG reagent and the amide hydrogen atoms of the peptides was consistent with that expected for conjugation. Furthermore, the Cys β-methylene hydrogen atoms were nonequivalent, indicating the loss of free rotation seen in GSH and that the sulfur atoms were no longer in the form of free thiols. These β-hydrogen atoms from the two Cys residues are diasterotropic and without free rotation. They are therefore not magnetically equivalent, and they display slightly different chemical shifts. The possibility of conjugation on the NH_2 of the N-terminal can be ruled out, as no new peak for the hydrogen atom as a consequence of amine alkylation was observed and the keto α-proton was at ∼4.1 ppm.

The reaction of PEG bis(sulfone) **3** with reduced glutathione after overnight incubation and purification showed only the presence of the PEG−$(GS)_2$ conjugate. No unreacted PEG bis or monosulfone (**3** or **4**) was seen. If the reaction had been stopped earlier (e.g., 5 h), there was a mixture of $PEG(SG)_2$ conjugate and PEG bis(sulfone) **3**. There was no evidence of a monoglutathione adduct or of the PEG monosulfone **4**. This indicated that the second elimination−addition reaction to give a PEG alkenyl monoglutathione intermediate was thermodynamically driven and that this compound was consumed to give the final $PEG(SG)_2$ conjugate.

Mono-PEGylation of the Cyclic Peptide Somatostatin. Somatostatin is a naturally occurring cyclic peptide hormone with 14 amino acid residues (molecular mass 1637.9 Da) and a disulfide bond between Cys3 and Cys14. The disulfide reduction of somatostatin was accomplished with equimolar TCEP−HCl, pH 6.2, at ambient temperature. Reduction at higher pH values caused the peptide to precipitate out of solution. TCEP−HCl (*37*) was ideal for disulfide reduction at acidic pH values, and it could be used at stoichiometric equivalence. Once oxidized, TCEP was not nucleophilic, and it did not react with PEG monosulfone **4**; therefore, its removal (*58*) before conjugation was unnecessary.

The disulfide-bridging PEGylation of the reduced somatostatin was performed using an equimolar amount of the PEG monosulfone **4**. Further purification by simple desalting and MALDI-TOF-MS analysis indicated the presence of PEG−somatostatin (Figure 6C). To determine whether the three-carbon linker without PEG attached could be inserted, the conjugation was also performed with the carboxylic acid monosulfone, which was obtained from the carboxylic acid bis(sulfone) **1**. As removal of PEG from the reagent led to a loss of water solubility, this conjugation reaction was performed in a 2:3 (v/v) acetonitrile and 50 mM sodium phosphate buffer mixed-solvent system at pH 6.2. The three-carbon bridged somatostatin was analyzed by MALDI-TOF. The increase in mass of 190 Da confirmed the presence of 4-[2,2-bis(methylene acetyl)]-benzoic acid (Figure 6B). This suggested that the bis-alkylation reaction of the monosulfone moiety with somatostatin could occur at a slightly acidic pH. These experiments also showed that the PEG monosulfone **4** could be used for site-specific PEGylation in acidic pH conditions.

PEGylation of the Multimeric Protein L-Asparaginase. *E. coli* derived L-asparaginase is a protein (MW 133−138 kDa) that consists of four identical subunits, each of which contains 326 amino acids and a single disulfide bond between Cys77-Cys105 (*59*, *60*). L-Asparaginase must exist in its tetrameric

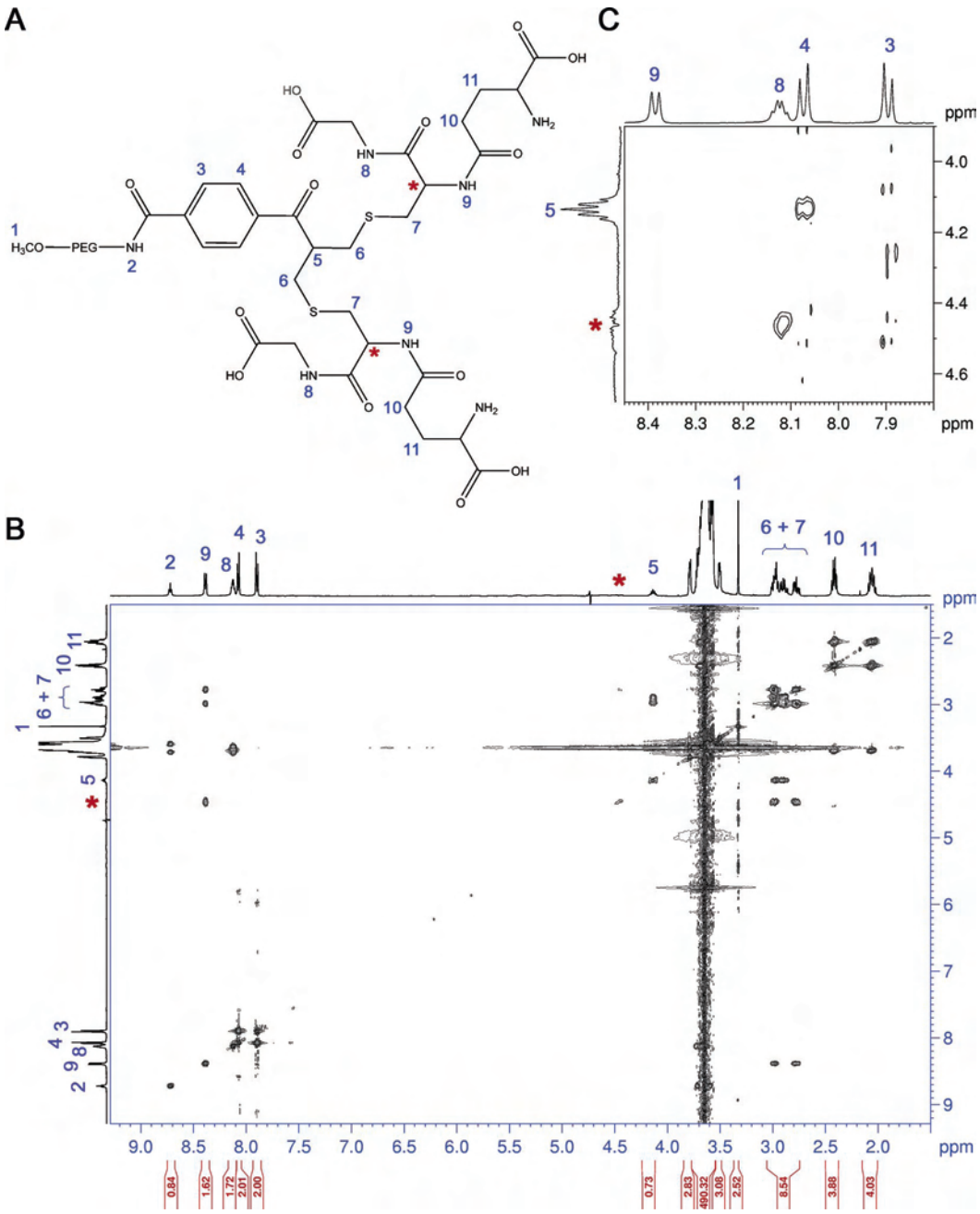


Figure 5. Thiol selectivity of PEGylation was demonstrated with reduced glutathione (GSH) to give PEG−$(SG)_2$. A. Chemical structure of PEG−$(SG)_2$; bond connectivity of the cysteine thiols was confirmed by ^{1}H-TOCSY as shown in B and ^{1}H-NOESY as shown in C.

form to display its biological activity. Structurally, the disulfide is accessible, because it lies on the outer edge of the protein and it is distant from the protein's enzymatic site (*61*). This explains why the L-asparaginase tetramer remains biologically active after reduction and carboxymethylation of its disulfides (*31*). We have PEGylated asparaginase to determine the biological effect of inserting a three-carbon bridge with PEG attached into each of the four subunits of this multimeric protein.

PEGylation of reduced L-asparaginase was examined with PEG monosulfones **4** of varying molecular weight (i.e., 5, 10, and 20 kDa as shown in Figure 7A, lanes 4, 5, and 6). The native L-asparaginase was seen at ~35 kDa on SDS-PAGE gels because of dissociation of the tetramer into the four monomers (Figure 7A, lane 2). The reduced protein displayed an apparently larger size than the unreduced protein (Figure 7A, lane 3). PEGylation only occurred if the L-asparaginase was first incubated with DTT (Figure 7A, lanes 7−9). Conjugation with 1.3 equiv of the PEG monosulfone **4** to each disulfide gave nearly quantitative conversion to the mono-PEGylated L-asparaginase monomer (Figure 7A, lanes 4−6).

Anion exchange chromatography followed by SEC−HPLC was used to purify PEG−L-asparaginase. Single peaks eluted with different retention times depending upon the size of the PEG (Figure 7B). MALDI-TOF-MS (Figure 7C,D) was used to confirm the mass of the PEG−L-asparaginase. The enzymatic activity of L-asparaginase and our PEG−L-asparaginase were similar irrespective of the size of PEG attached (Figure 8A). In an enzyme immunoassay (Figure 8B) that was based upon the use of a rabbit anti-asparaginase polyclonal antibody, the in vitro antigenicity of the PEG−L-asparaginase was similar to that of L-asparaginase. A representative, computationally calculated structure of PEG−L-asparaginase is shown in Figure 8C. These results are consistent with the site-selective conjugation of PEG to a site that is distant to the enzyme's antigenic site.

E. coli derived L-asparaginase cannot be used as a safe therapeutic drug in man, because it causes severe hypersensitiv-

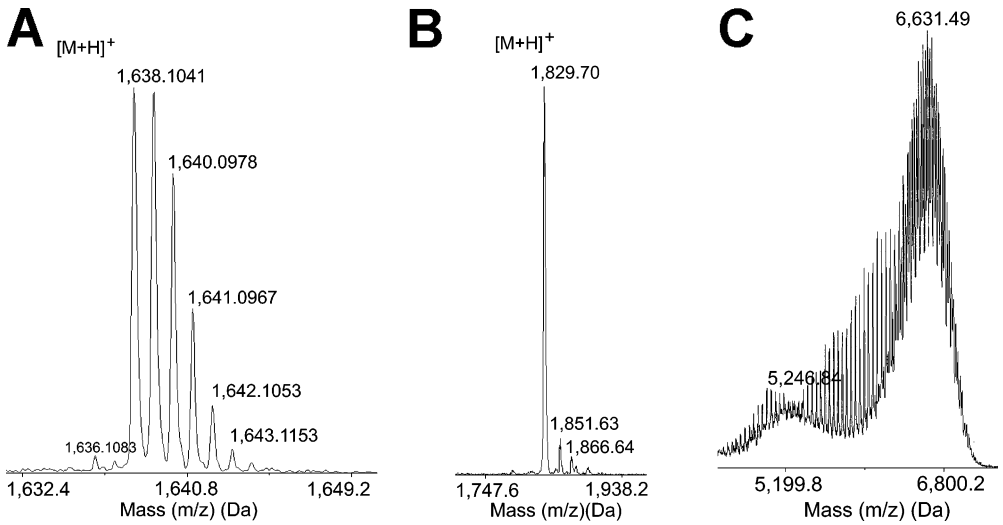


Figure 6. MALDI-TOF-MS: A. Native somatostatin showing its isotope signals. B. Three-carbon disulfide bridged somatostatin with its salt adducts. C. Reaction mixture of PEGylated somatostatin. Also seen is the unreacted PEG in the region of 5246 Da.

ity reactions. This is due to its multiple antigenic sites (*62*). PEGylation has been used to reduce this antigenicity and thereby enable its use for the treatment of acute lymphoblastic leukemia and of lymphomas (*63*, *64*). We have found that, after disulfide site-specific PEGylation, our PEGylated L-asparaginase retained its enzymatic activity and its antigenicity, irrespective of the size of PEG attached. In contrast, commercially available amine PEGylated L-asparaginase had lower enzymatic activity and was less antigenic (Figure 8B). This could be due either to the amine-based site of PEGylation of the commercially available PEG−L-asparaginase and/or to it having ∼40 molecules of 5 kDa PEGs per protein molecule. Our results show that it is possible to attach four PEG molecules to sites on a protein that are distant to its biologically active site using disulfide site-specific PEGylation without disrupting its biological activity. In this context, we have also illustrated the biological benefits of PEGylating at sites that are distant to a protein's receptor binding site by PEGylating the interchain disulfide of an antibody fragment to CD4. Both the anti-HIV-1 activity and the antigenicity of the PEGylated antibody fragment were preserved (*33*).

Three-Carbon Disulfide Single- and Double-Bridged PEGylation of IFN. We have already shown that a single native disulfide in IFN can be modified by site-specific bis-alkylation of two cysteine sulfur atoms to form a three-carbon bridged PEGylated IFN (*33*). The yield of three-carbon disulfide single-bridged PEG−IFN was high (65%). It was a mixture of the two isomers Cys1-CC[PEG]C-Cys98 and Cys29-CC[PEG]C-Cys138. The tertiary structure of IFN was retained. The stability of the three-carbon bridge was maintained under reducing conditions, at temperatures up to 90 °C, and in human serum for 24 h at 4 °C. It also had a prolonged circulation half-life in vivo of 12 h compared to 1 h for IFN.

In contrast to the multimeric L-asparaginase described above, IFN also provided an opportunity to determine the effect of introducing two PEGylated bridges into a monomeric protein. In molecular modeling studies, both disulfide bonds were replaced with three-carbon bridges linked by thioether bonds from the respective cysteines and the modified structure subjected to simulations of 2000 ps. A C-2 substituted *p*-benzoic acid acetophenone was also placed in the carbon bridge to mimic the entire linking moiety that was used for PEGylation. The conformations were recorded every 20 ps, and they resulted in a trajectory that was analyzed by calculating the rmsd values for the backbones of the starting model of IFN and for each conformation of the modified protein at the different time frames. A plot of the calculated rmsd values versus time indicated that the overall structures deviated by <4 Å from the native conformation (Figure 9A). All of the conformations were then superimposed and a ribbon plot created with the positions of the linkers at different time points shown in CPK representation (Figure 9B). These results showed that the double-bridged IFN retained its primary, secondary (i.e., helices), and tertiary structure.

The carbon bridge between Cys29-Cys138 had a lower conformational flexibility than the bridge between Cys1-Cys98 because of the restricted movements of the interhelical connecting loops. The aromatic moiety at C-2 of the carbon bridge between Cys1-Cys98 had greater freedom to move, because the N-terminal was not constrained by interactions. This resulted in greater conformational space sampling by the C-2 benzoic acid causing different interactions with the surface of the protein. These results show that the insertion of a carbon bridge that was linked by thioether bonds displayed greater conformational mobility than the native disulfide bond, and they could explain how the three-carbon bridge modified IFN was able to retain its tertiary structure.

As an example of reagent efficiency, IFN was then conjugated with 2 equiv of PEG monosulfone **4** to give the three-carbon disulfide double-bridged PEG−IFN. In order to define the structural and biological effects of the three-carbon bridges (i.e., without PEG attached) and to aid the mass-spectral analysis, a double-bridged IFN adduct was also prepared from the carboxylic acid bis(sulfone) **1**. This reaction had to be conducted in a mixed organic−aqueous solvent system to solubilize the carboxylic acid sulfone **1**. In the case of the bis(sulfone) **1**, the SDS-PAGE gel showed that the disulfide-reduced IFN had a larger apparent size, which was a consequence of the open disulfides (Figure 10A, lane 3) when compared to (a) IFN (Figure 10A, lane 2) and (b) the double-bridged IFN in which conjugation and formation of the two bridges had occurred (Figure 10A, lane 4). MALDI-TOF-MS analysis showed that the mass of the IFN had increased by 380 Da, thereby confirming the insertion of two bridges of 190 Da each (Figure 10B). Tryptic digestion of the IFN and the double-bridged IFN followed by MALDI-TOF-MS of the fragments confirmed the site-specific insertion of a carbon bridge at Cys1-Cys98 and at Cys29-Cys138 (Figure 10C,D).

Stoichiometrically efficient PEGylation reactions could be conducted with either PEG monosulfone **4** or PEG bis(sulfone) **3**. When 1 equiv of the PEG monosulfone 20 kDa **4** was used for the conjugation, a mixture of the three-carbon disulfide

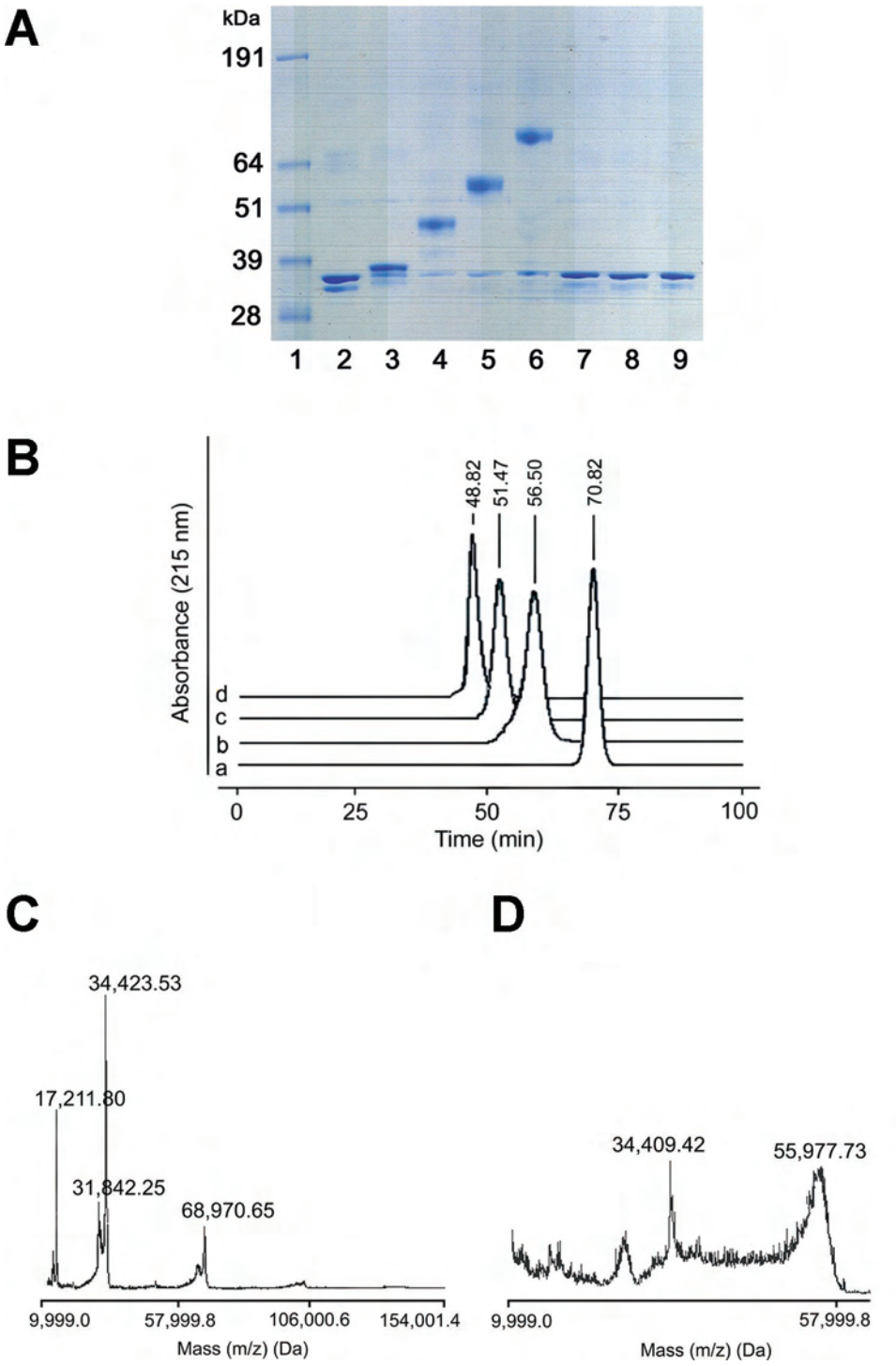


Figure 7. A. SDS-PAGE gel stained with colloidal blue to show the L-asparaginase three-carbon disulfide-bridged PEGylation reactions. Lane 1: prestained markers. Lane 2: L-asparaginase. Lane 3L disulfide-reduced L-asparaginase. Lane 4: 5 kDa PEGylation reaction. Lane 5: 10 kDa PEGylation reaction. Lane 6: 20 kDa PEGylation reaction. Lanes 7−9: control reactions with 5 kDa, 10 kDa, and 20 kDa PEG reagents **4**, respectively, in which the L-asparaginase was not reduced. Dissociation of the tetramer on SDS-PAGE resulted in monomeric L-asparaginase, and it was observed as two bands. UnPEGylated monomers of L-asparaginase (16% by densitometry) were seen in lanes 4−6. B. Size exclusion chromatography of L-asparaginase and the three-carbon disulfide-bridged PEG−L-asparaginase on a Superdex 200 prep grade column. The protein elutes in a tetrameric form: (a) L-asparaginase at 70.57 min; (b) 5 kDa PEG−L-asparaginase at 56.50 min; (c) 10 kDa PEG−L-asparaginase at 51.47 min; (d) 20 kDa PEG−L-asparaginase at 48.82 min. C. MALDI-TOF-MS of L-asparaginase ionized as a monomer with a MW range of 31 842−34 423 Da. D. MALDI-TOF-MS of the three-carbon disulfide-bridged 20 kDa PEG−L-asparaginase.

single-bridged PEG−IFN (65%), three-carbon disulfide double-bridged PEG−IFN (23.5%), IFN (4.9%), and aggregated IFN (6.6%) was obtained (*33*). When 1 equiv of the 10 kDa PEG monosulfone **4** was used for the conjugation, a mixture of the three-carbon disulfide single-bridged PEG−IFN (64.3%), three-carbon disulfide double-bridged PEG−IFN (17.4%), IFN (13.4%), and aggregated IFN (4.8%) was obtained (Figure 11B). When 1.5 equiv of PEG monosulfone **4** was used, a mixture of the three-carbon disulfide double-bridged PEG−IFN (61%) and the three-carbon disulfide single-bridged PEG−IFN (38%) was obtained (Figure 11C). There was no residual IFN, and only 1% of the IFN was aggregated.

When 1 equiv of PEG bis(sulfone) **3** was used, the conjugation reaction was similar and the three-carbon disulfide single-

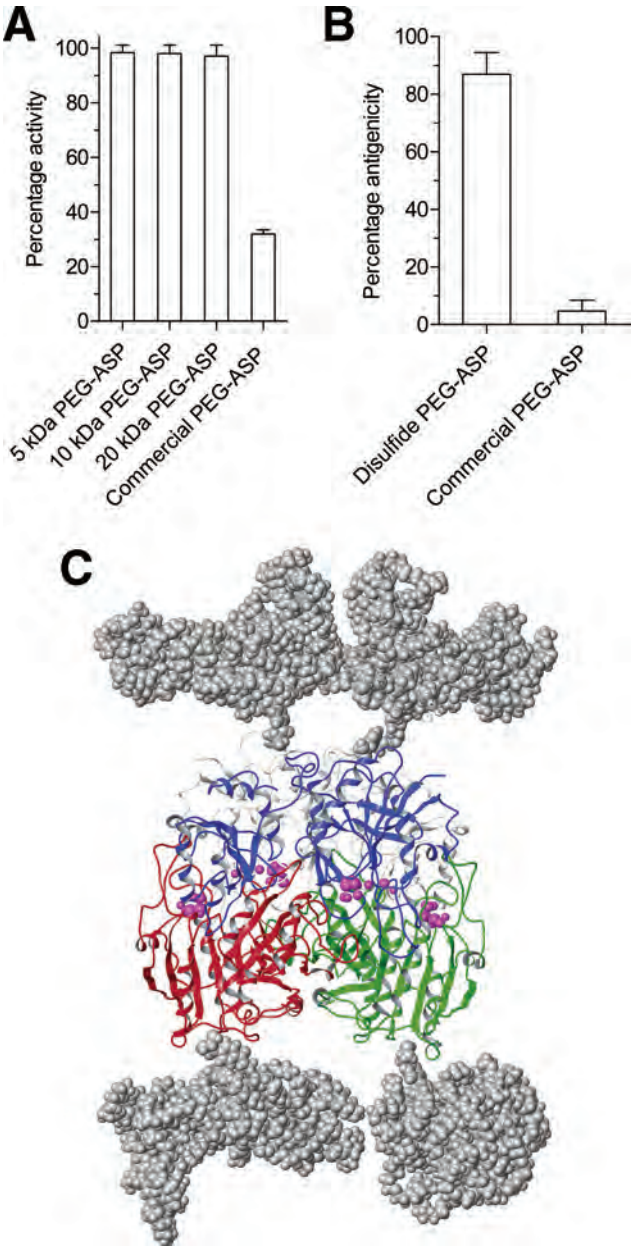


Figure 8. Evaluation of the in vitro biological properties of PEG−L-asparaginase: A. Enzymatic activity. B. Antigenicity. C. Tetrameric 10 kDa PEG−L-asparaginase (PDB 4ECA) was stochastically modeled with implicit solvent. A PEG molecule was conjugated at each of the four disulfides. The substrate aspartic acid is shown in pink.

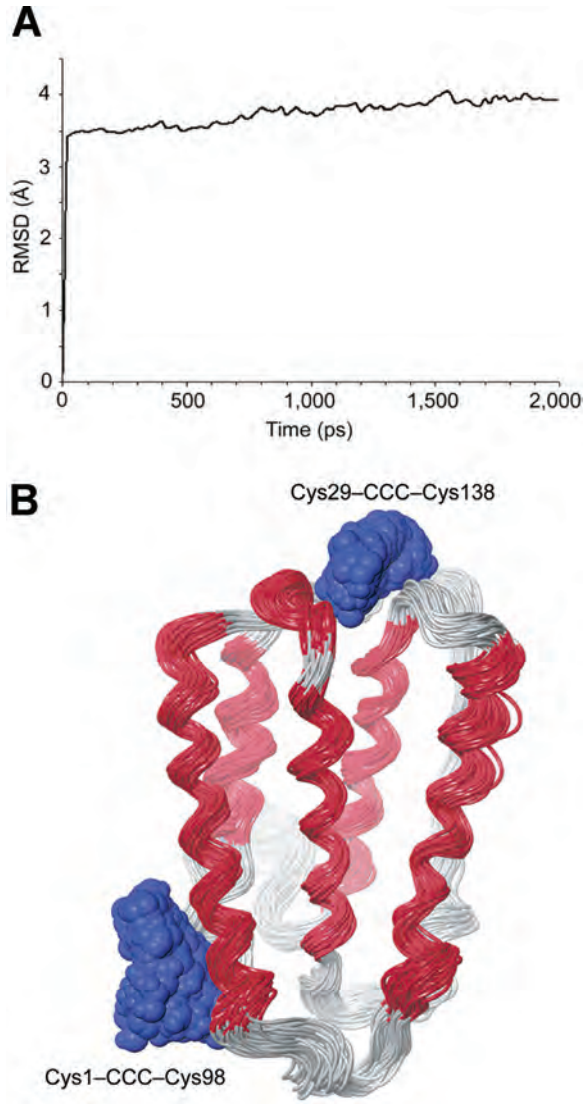


Figure 9. A. Stochastic simulation of the double-bridged IFN (2000 ps). The rmsd plot of the backbone molecules of the modified IFN was calculated using the native conformation of IFN α-2a (PDB 1ITF). B. Backbone plots of the chemically modified IFN α-2b in CPK representation. The three-carbon disulfide bridges are shown in blue.

bridged PEG-IFN was the main product (Figure 11A, lane 4). When 2 equiv of the PEG bis(sulfone) **3** was used (Figure 11A, lane 5), the three-carbon disulfide double-bridged PEG−IFN was the main product. In order to produce the three-carbon disulfide double-bridged PEG−IFN, the disulfide-reduced IFN was allowed to react with the PEG bis(sulfone) **3** immediately after the protein had been separated from the reducing agent and stored overnight at 4 °C. The PEG−IFN conjugates were purified by cation exchange chromatography followed by size exclusion chromatography and then analyzed by MALDI-TOF-MS (Figure 12A,B with the reference MALDI of IFN shown in Figure 12C). The presence of IFN in the PEG−IFN was confirmed by silver stain and by Western immunoblotting with an anti-IFN antibody (Figure 13). This study also confirmed the absence of un-PEGylated IFN in these particular fractions. As both disulfides were reduced for the mono-PEGylation of IFN, it was also necessary to reoxidize any cysteines that had not been PEGylated with glutathione. This led to increased amounts of the three-carbon disulfide single-bridged PEG−IFN and of IFN. Aggregation of the reduced IFN was not observed in 50 mM sodium phosphate buffer, pH 7.8, containing 10 mM EDTA. However, aggregation was found after buffer exchange of the non-glutathione-treated reaction mixture to 20 mM sodium acetate buffer, pH 4.0. Reannealing of the open disulfides with glutathione (1 mM GSH/1 mM GSSG) decreased the formation of aggregates while buffer exchanged to 20 mM sodium acetate buffer, pH 4.0 (Figure 14).

The biological effects of modified IFNs were studied using the A549-EMC antiviral assay and the 2′,5′-OAS mRNA induction assay (Figure 15A−D). The unreacted IFN after purification had an ED_{50} of 17 (antiviral) to 38 (2′,5′-OAS mRNA) pg/mL. The addition of a single three-carbon bridge (i.e., single-bridged IFN) reduced this slightly (given the doubling dilution format of the assays) to 41 (antiviral ED_{50}) to 97 (2′,5′-OAS mRNA ED_{50}) pg/mL. This is a remarkably small change in antiviral activity, given our expectation at the outset of our studies that manipulation of a disulfide bond would severely disrupt the biological activity of IFN. The addition of a single PEG molecule (i.e., three-carbon disulfide single-bridged PEG−IFN) reduced it significantly to 175−265 pg/mL (antiviral assay ED_{50} for the 20 and 10 kDa PEGs) and

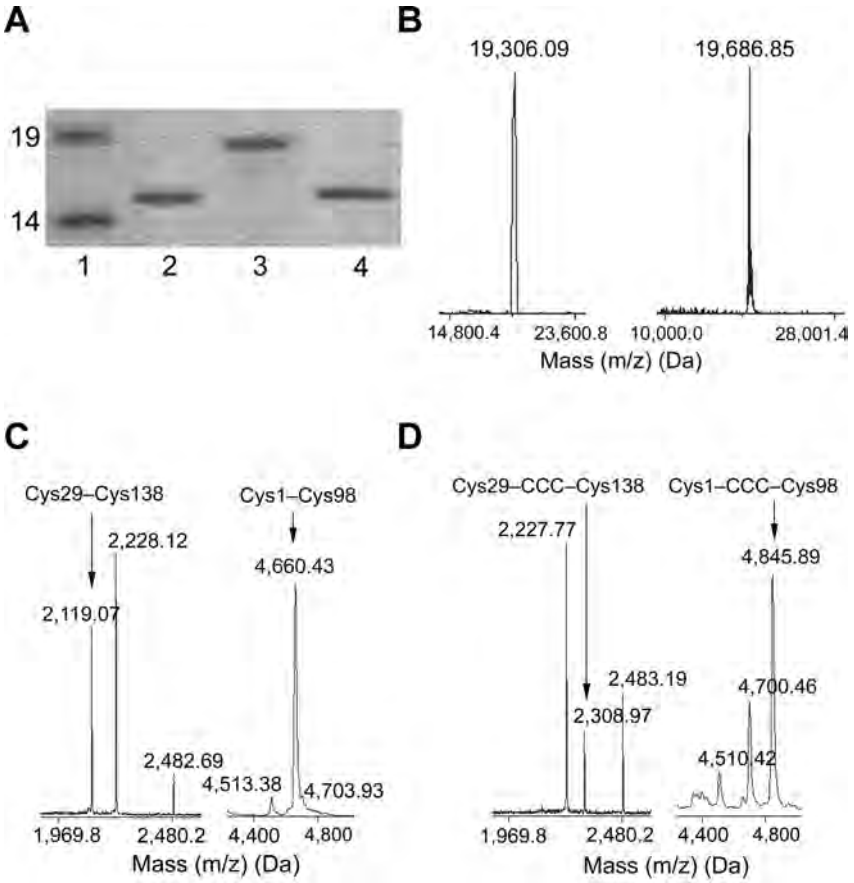


Figure 10. A. Silver stain of a 12% bis−tris SDS-PAGE gel of the double-bridged IFN reaction mixture to which PEG was not attached. Lane 1: prestained standards. Lane 2: IFN. Lane 3: disulfide reduced IFN (control for 2 h). Lane 4: double-bridged IFN after reaction with carboxylic acid bis(sulfone) **1** for 2 h. B. MALDI-TOF-MS of IFN (19 306 Da, left) and the double-bridged IFN (19 686 Da, right) showing an increase in MW of 380 Da that was due to the insertion of 2 three-carbon bridges of 190 Da each. C. MALDI-TOF-MS of trypsin-digested IFN showing the peptide fragments containing Cys29-Cys138 and Cys1-Cys98. D. MALDI-TOF-MS of the trypsin-digested double-bridged IFN showing the peptide fragments containing Cys29-CCC-Cys138 and Cys1-CCC-Cys98.

338−441 pg/mL (2′,5′-OAS mRNA ED_{50} for the 10 and 20 kDa PEGs, respectively). The length of the PEG had no effect on the biological activity of the PEG−IFN. Taken together, these results show that the insertion of a single three-carbon disulfide bridge into IFN contributed ∼11%, and the addition of PEG to that bridge contributed ∼89% to the reduction in the in vitro biological activities of the single-bridged PEG−IFNs. Therefore, the antiviral activity of our three-carbon disulfide single-bridged PEG−IFN was ∼8% of IFN and, as such, is the same as the reported activity of the mono-PEGylated IFN (∼7% of IFN) in clinical use (*65*−*69*).

The insertion of two three-carbon bridges in IFN (i.e., double-bridged IFN) caused a significant reduction in the biological activity of IFN (104 pg/mL (antiviral assay ED_{50}) and 414 pg/mL (2′,5′-OAS mRNA ED_{50}); Figure 15A−D). This was a surprising biological result, given that our molecular modeling studies had shown that the double-bridged IFN retained its primary, secondary (i.e., helices), and tertiary structure. It is possible that, as the experiment to add the two three-carbon linkers to IFN (without PEG attached) was conducted in the presence of acetonitrile (in order to solubilize the conjugation reagent shown as structure **1**), the IFNs receptor binding domains might have been significantly disrupted. This study was necessary in order to complete our mass spectral analysis (Figure 10 and (*33*)). The reduction in the biological activity of the double-bridged IFN was similar to that of adding a PEG molecule to a single-bridged IFN (175−265 pg/mL (antiviral assay ED_{50}) and 338−441 pg/mL (2′,5′-OAS mRNA ED_{50})). While the three-carbon disulfide double-bridged PEG−IFNs had only ∼2% of the activity of IFN, once again, we found that the length of the PEG had no effect on the biological activity of the PEG−IFN; the three-carbon disulfide double-bridged 10 kDa PEG−IFN had an antiviral ED_{50} of 639 pg/mL and the three-carbon disulfide single-bridged 20 kDa PEG−IFN had an antiviral ED_{50} of 697 pg/mL. Therefore, in the case of IFN, it was better to increase the MW of a single PEG molecule to optimize the molecule's pharmacokinetic profile rather than attach a second PEG molecule to the protein. This property has not previously been described for PEG−IFNs, presumably because each PEG reagent used for amine conjugation of IFN gives a different mixture of positional PEG−IFN isomers.

The reduction of the in vitro biological activity of the PEG−IFNs is a consequence of the steric shielding caused by PEG, and it is seen with all the PEG−IFNs described in the literature that have been biologically evaluated (*6*, *65*−*69*). The complex nature of the binding of IFN to its receptor (i.e., at least two receptor−ligand interactions occur) is disrupted by the presence of PEG, which is at least as large in solution as the IFN (*70*). When amine PEGylation is used, reduction of the antiviral activity of the IFN can be minimized by attaching a very short PEG to certain residues, but this is at the expense of a considerably reduced in vivo half-life. In the clinical context, the increase in the in vivo half-life of IFN to at least 12 h (*68*) is more important than the reduction in its in vitro antiviral activity, because the markedly enhanced in vivo therapeutic efficacy of IFN more than compensates for the reduced biological activity seen in vitro. This is the case for all of the IFNs that have been described in the literature (*65*−*69*). Therefore, the decrease in the in vitro activity and the decrease

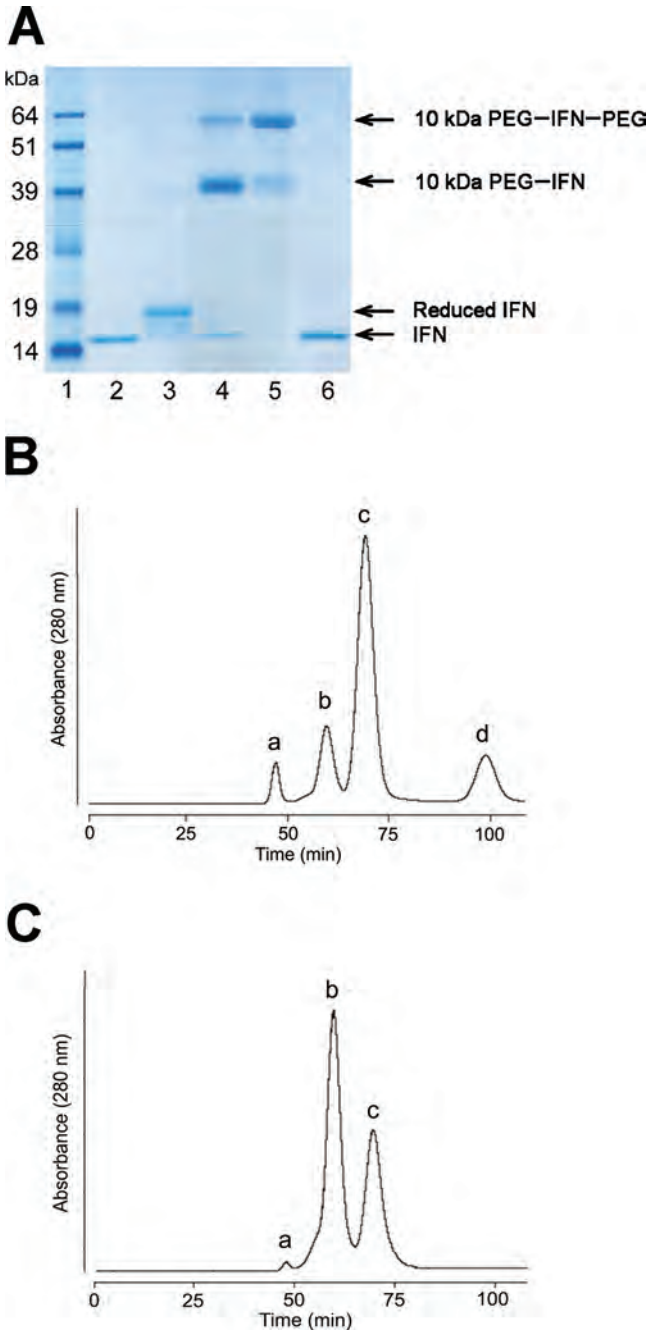


Figure 11. A. Colloidal blue stain of 12% bis−tris SDS-PAGE gel of the three-carbon disulfide double-bridged PEG−IFN. Lane 1: prestained standards. Lane 2: IFN. Lane 3: disulfide reduced IFN. Lane 4: disulfide reduced IFN with 1 equiv of PEG monosulfone **4** (10 kDa). Glutathione was used to refold the IFN. Lane 5: disulfide reduced IFN with 2 equiv of PEG monosulfone **4**. Lane 6: control reaction of IFN without prior disulfide reduction with 2 equiv of PEG monosulfone **4**. B. Size exclusion chromatography of the IFN PEGylation reaction with 1 equiv of PEG monosulfone **4**. C. Size exclusion chromatography of the IFN PEGylation reaction with 1.5 equiv of PEG monosulfone **4**. Eluting peaks are (a) aggregates, (b) three-carbon disulfide double-bridged PEG-IFN, (c) three-carbon disulfide single-bridged PEG-IFN, and (d) IFN.

in the in vivo clearance of a PEG−IFN are a direct consequence of the presence of PEG.

Using BIAcore measurements and cell binding assays, Kubetzko et al. (*71*) have recently shown that attachment of a 20 kDa PEG to an antibody fragment distal to the binding region results in a 5-fold reduction in the apparent affinity of the PEGylated antibody fragment for its receptor. This was due to a reduction in the association rate of the PEGylated molecule

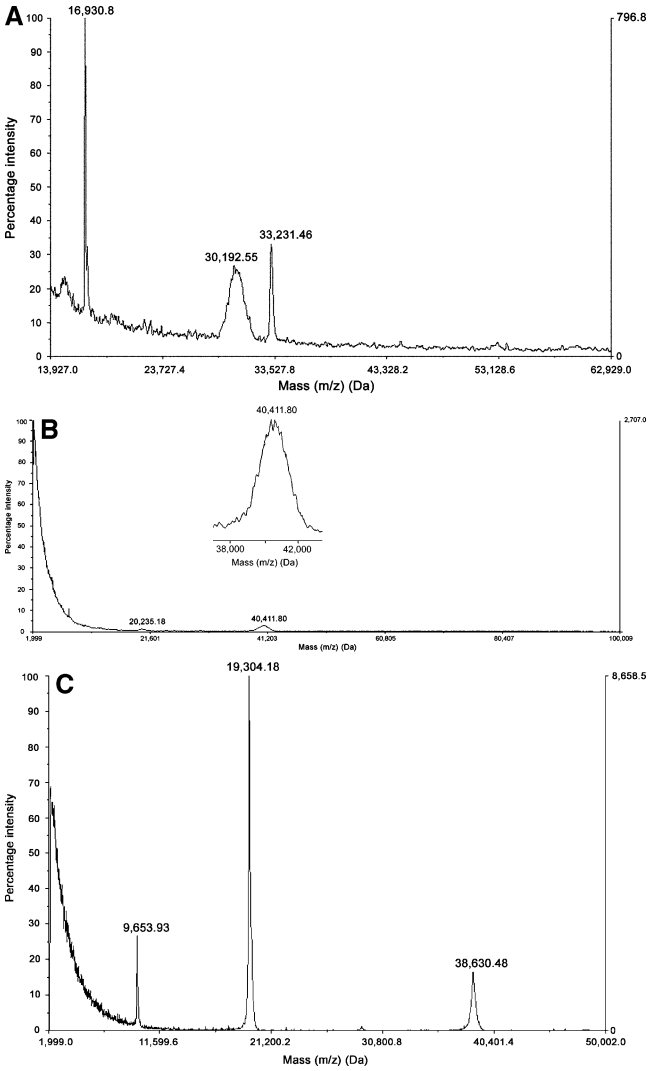


Figure 12. A. MALDI-TOF-MS of the three-carbon disulfide single-bridged 10 kDa PEG−IFN with the reference protein of doubly charged BSA seen at 33 231 Da. B. MALDI-TOF-MS of the three-carbon disulfide double-bridged 10 kDa PEG−IFN. The expanded region indicates the signal for the three-carbon disulfide double-bridged PEG−IFN. F. MALDI-TOF-MS of IFN.

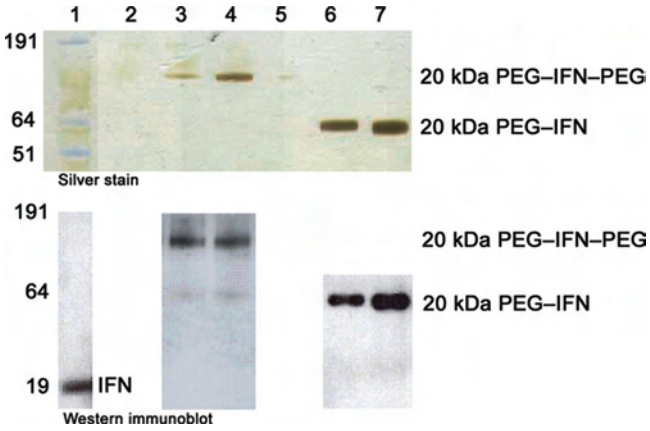


Figure 13. Silver stain and Western immunoblot with an anti-IFN antibody to confirm the presence of IFN in the PEGylated fractions, and the absence of un-PEGylated IFN (at 19 kDa) in these fractions. Lanes 1: MW markers in kDa. SEC−HPLC fractions (lane 2) 29−31 min; (lane 3) 31−33 min; (lane 4) 33−35 min; (lane 5) 35−37 min; (lane 6) 37−39 min; (lane 7) 39−41 min; (lane 8) 41−43 min.

for its receptor with no change in the dissociation rate. There was also a strong effect of the 20 kDa PEG on the hydrodynamic

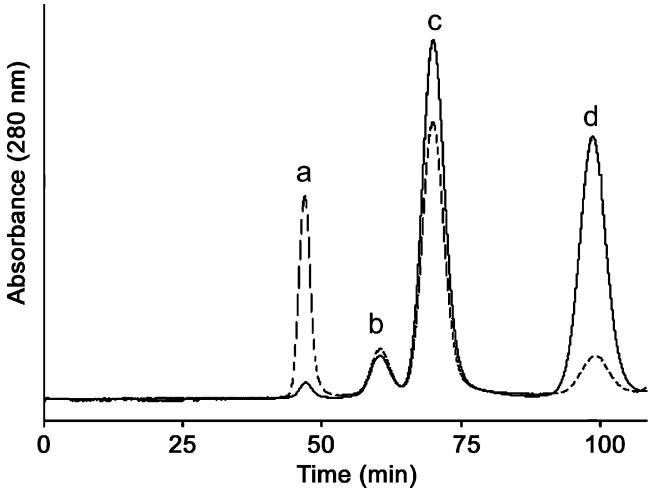


Figure 14. The addition of glutathione (solid line) to the reaction mixture after incubation of the fully reduced IFN with the PEG bis-(sulfone) **3**. Glutathione was used to reoxidize the unreacted cysteine thiols. When glutathione was not added, there was increased aggregation of the IFN (dotted line). Eluting peaks are (a) Aggregates, (b) three-carbon disulfide double-bridged PEG-IFN, (c) three-carbon disulfide single-bridged PEG-IFN and (d) IFN.

properties of the conjugated protein with its hydrodynamic radius becoming much larger than would be expected for the PEG-associated increase in molecular mass. While only a modest effect was seen with a 5 kDa PEG, conjugates modified with a 20 kDa PEG displayed a 100-fold reduction in the association rate. Their modeling studies also suggested that PEGylation results in <7% of a PEGylated molecule being available for binding to its receptor at any moment in time. The remainder has intramolecularly blocked binding interfaces and is in rapid equilibrium with the functional molecule. Although this reduces the observed association rate, all of the PEGylated molecules can eventually bind to the receptor. Similar and related observations have been made for PEG−IFN (*6*, *69*). In addition, the PEGylated molecules intermolecularly block the attachment of neighboring molecules, causing an apparent reduction in the number of binding sites, minimizing protein aggregation, and increasing protein stability (*72*). This masking of proteins by PEG also explains their lower immunogenicity, lower toxicity, and longer circulation half-life.

CONCLUSION

PEGylation reagents that undergo sequential bis-alkylation with two thiols are described and shown to undergo site-specific conjugation with the cysteine sulfur atoms that are liberated by

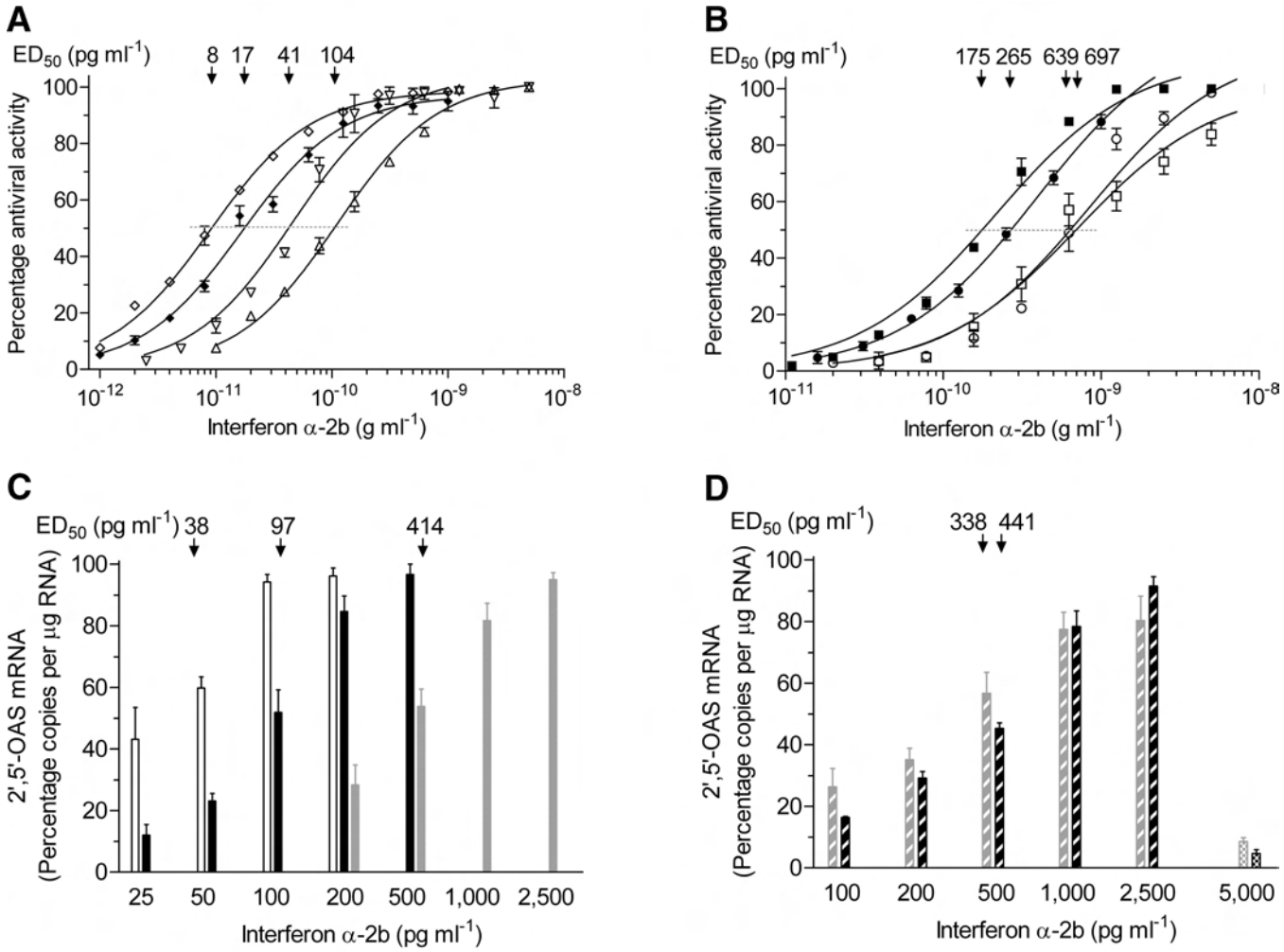


Figure 15. A,B. Antiviral activity in A549 cells infected with EMC virus with the ED_{50} shown ($n = 6$). Symbols: IFN (◇), unreacted IFN (◆), single-bridged IFN (▽), double-bridged IFN (△), three-carbon disulfide single-bridged 10 kDa PEG−IFN (●), three-carbon disulfide single-bridged 20 kDa PEG−IFN (■), three-carbon disulfide double-bridged 10 kDa PEG−IFN (○), three-carbon disulfide double-bridged 20 kDa PEG−IFN (□). C,D. 2′,5′-OAS mRNA expression in Molt-4 cells ($n = 3$), Symbols: IFN (white bars), single-bridged IFN (black bars), double-bridged IFN (gray bars), three-carbon disulfide single-bridged 10 kDa PEG−IFN (gray striped bars), three-carbon disulfide single-bridged 20 kDa PEG−IFN (black striped bars).

the mild reduction of a protein's disulfide bond. Most therapeutic proteins, antibody fragments, and enzymes have at least one disulfide bond which can be modified using disulfide site-specific PEGylation without disrupting either their tertiary structure or their biological activity (*33*). This approach does not require the incorporation of a new cysteine into the protein to exploit selective thiol conjugation chemistry. As our approach is stoichiometrically very efficient and enables the recycling of any unreacted protein, it offers the potential to make cost-effective PEGylated biopharmaceuticals as global medicines. In this context, clinical trials are now in progress with our three-carbon disulfide single-bridged PEG−IFN as a cost-effective treatment for the epidemic of hepatitis C in developing countries.

ACKNOWLEDGMENT

We are grateful to the Wellcome Trust (068309), BBSRC-UK (BB/D003636/1) and PolyTherics for funding, and to The School of Pharmacy and Avecia for contributing towards the costs of S. Balan's Ph.D. studies.

LITERATURE CITED

(1) Harris, J., and Chess, R. (2003) Effect of Pegylation on pharmaceuticals. *Nat. Rev. Drug Discovery 2* (3), 214−221.

(2) Zalipsky, S. (1995) Chemistry of poly(ethylene glycol) conjugates with biologically active molecules. *Adv. Drug Delivery Rev. 16*, 157−182.

(3) Greenwald, R., Choe, Y., McGuire, J., and Conover, C. (2003) Effective drug delivery by PEGylated drug conjugates. *Adv. Drug Delivery Rev. 55*, 217−250.

(4) Pasut, G., Guiotto, A., and Veronese F. (2004) Protein, peptide and non-peptide drug PEGylation for therapeutic application. *Expert Opin. Ther. Pat. 14* (5), 1−36.

(5) Veronese, F., and Pasut G. (2005) PEGylation, successful approach to drug delivery. *Drug Discovery Today 10* (21), 1451−1458.

(6) Grace, M., Lee, S., Bradshaw, S., Chapman, J., Spond, J., Cox, S., DeLorenzo, M., Brassard, D., Wylie, D., Cannon-Carlson, S., Cullen, C., Indelicato, S., Voloch, M., and Bordens, R. (2005) Site of pegylation and polyethylene glycol molecule size attenuate interferon-a antiviral and antiproliferative activities through the JAK/STAT signaling pathway. *J. Biol. Chem. 280* (8), 6327−6336.

(7) Woghiren, C., Sharma, B., and Stein S. (1993) Protected thio-poly(ethylene glycol): A new activated polymer for reversible protein modification. *Bioconjugate Chem. 4* (5), 314−318.

(8) Kinstler, O., Brems, D., Lauren, S., Paige, A., Hamburger, J., and Treuheit, M. (1996) Characterization and stability of N-terminally PEGylated rhG-CSF. *Pharm. Res. 13* (7), 996−1002.

(9) Kinstler, O., Molineux, G., Treuheit, M., Ladd, D., and Gegg, C. (2002) Mono-N-terminal poly(ethylene glycol)-protein conjugates. *Adv. Drug Delivery Rev. 54* (4), 477−485.

(10) Chapman, A. (2002) PEGylated antibodies and antibody fragments for improved therapy: a review. *Adv. Drug Delivery Rev. 54*, 531−545.

(11) Wylie, D., Voloch, M., Lee, S., Liu, Y., Cannon−Carlson, S., Cutler, C., and Pramanik, B. (2001) Carboxyalkylated histidine is a pH-dependent product of pegylation with SC-PEG. *Pharm. Res. 18*, 1354−1360.

(12) Manjula, B., Tsai, A., Upadhya, R., Perumalsamy, K., Smith, P., Malavalli, A., Vandegriff, I., Winslow, R., Intaglietta, I., Prabhakaran, M., Friedman, J., and Acharya, A. (2003) Site-specific PEGylation of hemoglobin at cys-93: Correlation between the colligative properties of the PEGylated protein and the length of the conjugated PEG chain. *Bioconjugate Chem. 14*, 464−472.

(13) Lee, H., Jang, H., Ryu, S., and Park, T. (2003) N-terminal site-specific mono-PEGylation of epidermal growth factor. *Pharm. Res. 20* (5), 818−825.

(14) Yamamoto, Y., Tsutsumi, Y., Yoshioka, Y., Nishibata, T., Kobayashi, K., Okamoto, T., Mukai, Y., Shimizu, T., Nakagawa, S., Nagata, S., and Mayumi, T. (2003) Site-specific PEGylation of a lysine-deficient TNF-alpha with full bioactivity. *Nat. Biotechnol. 21*, 546−552.

(15) Gentle, I., DeSouza, D., and Baca, M. (2004) Direct production of proteins with N-terminal cysteine for site-specific conjugation. *Bioconjugate Chem. 15*, 658−663.

(16) Edwards, C., Martin, S., Seely, J., Kinstler, O., Buckel, S., Bendele, A., Cosenza, M., Feige, U., and Kohno, T. (2003) Design of PEGylated soluble tumor necrosis factor receptor type I (PEG sTNF-RI) for chronic inflammatory diseases. *Adv. Drug Delivery Rev. 55* (10), 1315−1336.

(17) Sato, H., Yamamoto, K., Hayashi, E., and Takahara, Y. (2000) Transflutaminase-mediated dual and site-specific incorporation of poly(ethylene glycol) derivatives into a chimeric interleukin-2. *Bioconjugate Chem. 11*, 502−509.

(18) Sato, H. (2002) Enzymatic procedure for site-specific pegylation of proteins. *Adv. Drug Delivery Rev. 54*, 487−504.

(19) Rosendahl, M., Doherty, D., Smith, S., Carlson, S., Chlipala, E., and Cox, G. (2005) A long-acting, highly potent interferon a-2 conjugate created using site-specific PEGylation. *Bioconjugate Chem. 16*, 200−207.

(20) Pavlou, A., and Reichert, J. (2004) Recombinant protein therapeutics- success rates, market trends and values to 2010. *Nat. Biotechnol. 22*, 1513−1519.

(21) Thornton, J. (1981) Disulphide bridges in globular proteins. *J. Mol. Biol. 151*, 261−287.

(22) Petersen, M., Jonson, P., and Petersen S. (1999) Amino acid neighbours and detailed conformational analysis of cysteines in proteins. *Protein Eng. 12* (7), 535−548.

(23) Leung, H., Xu, G., Narayan, M., and Scheraga, H. (2005) Impact of an easily reducible disulfide bond on the oxidative folding rate of multi-disulfide-containing proteins. *J. Peptide Res. 65*, 47−54.

(24) Betz, S. (1993) Disulfide bonds and the stability of globular proteins. *Protein Sci. 2*, 1551−1558.

(25) Saunders, A., Young, G., and Pielak, G. (1993) Polarity of disulfide bonds. *Protein Sci. 2*, 1183−1184.

(26) Bewley, T., Brovetto-Cruz, J., and Li, C. (1969) Human pituitary growth hormone. Physicochemical investigations of the native and reduced-alkylated protein. *Biochemistry 8* (12), 4701−4708.

(27) Wedemeyer, W., Welker, E., Narayan, M., and Scheraga, H. (2000) Disulfide bonds and protein folding. *Biochemistry 39*, 4207−4216.

(28) Rajarathnam, K., Sykes, B., Dewald, B., Baggiolini, M., and Clark-Lewis, I. (1999) Disulfide bridges in interleukin-8 probed using non-natural disulfide analogues: dissociation of roles in structure from function. *Biochemistry 38*, 7653−7658.

(29) Zhu, H., Dupureur, C., Zhang, B., and Tsai, M. (1995) Phospholipase A2 engineering. The roles of disulfide bonds in structure, conformational stability, and catalytic function? *Biochemistry 34*, 15307−15314.

(30) Morehead, H., Johnston, P., and Wetzel, R. (1984) Roles of the 29-138 disulfide bond of subtype A of human a interferon in its antiviral activity and conformational stability. *Biochemistry 23*, 2500−2507.

(31) Todokoro, K., Saito, T., Obata, M., Yamazaki, S., and Tamaura, Y. (1975) Studies on conformation and antigenicity of reduced S-methylated asparaginase in comparison with asparaginase. *FEBS Lett. 60*, 259−262.

(32) Godwin, A., Choi, J., Pedone, E., Balan, S., Jumnah, R., Shaunak, S., Brocchini, S., and Zloh, M. (2006) Molecular dyamics simulations of proteins with chemically modified disulfide bonds. *Theor. Chem. Acc.* DOI: 10.1007/s00214-006-0134-0.

(33) Shaunak, S., Godwin, A., Choi, J., Balan, S., Pedone, E., Vijayarangam, D., Heidelberger, S., Teo, I., Zloh, M., and Brocchini, S. (2006) Site-specific PEGylation of native disulfide bonds in therapeutic proteins. *Nat. Chem. Biol 2*, 312−313.

(34) Meager, A., Gaines, D., Zoon, K., and Mire-Sluis, A. (2001) Establishment of new and replacement World Health Organization international biological standards for human interferon alpha and omega. *J. Immunol. Methods 257*, 17−33.

(35) Liberatore, F., Comeau, R., McKearin, J., Pearson, D., Belonga, B., Brocchini, S., Kath, J., Phillips, T., Oswell, K., and Lawton, R. (1990) Site directed modification and cross-linking of a monoclonal antibody with equilibrium transfer alkylating cross-link reagents. *Bioconjugate Chem. 1*, 36−50.

(36) de Rosario, R., Brocchini, S., Lawton, R., Wahl, R., and Smith, R. (1990) Sulfydral site-specific cross-linking of a monoclonal

antibody by a fluorescent equilibrium transfer alkylating cross-link reagent. *Bioconjugate Chem. 1*, 51−65.
(37) Burns, J., Butler, J., Moran, J., and Whitesides, G. (1991) Selective reduction of disulfides by tris(2-carboxyethy1)phosphine. *J. Org. Chem. 56*, 2648−2650.
(38) Fahey, E., Chaudhuri, J., and Binding, P. (2000) Refolding of low molecular weight urokinase plasminogen activator by dilution and size exlusion chromatography. A comparative study. *Sep. Sci. Technol. 35* (11), 1743−1760.
(39) Wüthrich, K. (1986) *NMR of the Proteins and Nucleic Acids*, Wiley, New York.
(40) Bax, A., and Davis, D. (1985) MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy. *J. Magn. Reson. 65*, 355−360.
(41) Jeener, J., Meier, B., Ernst, B., and Ernst, R. (1979) Investigation of exchange processes by 2-dimensional NMR-spectroscopy. *J. Chem. Phys. 71*, 4546−4553.
(42) Mashburn, L., and Wriston, J. (1964) Tumor inhibitory effect of L-asparaginase from *Escherichia coli*. *Arch. Biochem. Biophys. 105*, 450−452.
(43) Soares, A., Guimaraes, G., Polakiewicz, B., Pitombo, R., and Abrahao-Neto, J. (2002) Effects of polyethylene glycol attachment on physicochemical and biological stability of *E. coli* L-asparaginase. *Int. J. Pharm. 237*, 163−170.
(44) Mohamadi, F., Richards, N., Guida, W., Liskamp, R., Lipton, M., Caufield, C., Chang, G., Hendrickson, T., and Still, W. (1990) Macromodel - an integrated software system for modeling organic and bioorganic molecules using molecular mechanics. *J. Comput. Chem. 11*, 440−467.
(45) Jorgensen, W., Maxwell D., and Tirado-Rives (1996) Development and testing of the OPLS all-atom force field on conformational energetics and properties of organic liquids. *J. Am. Chem. Soc. 118*, 11225−11236.
(46) Still, W., Tempczyk, A., Hawley, R., and Hendrickson, T. (1990) Semianalytical treatment of solvation for molecular mechanics and dynamics. *J. Am. Chem. Soc. 112* (16), 6127−6129.
(47) McLachlan, A. (1982) Rapid comparison of protein structures. *Acta Crystallogr., Sect. A 38*, 871−873.
(48) Martin A. (2005) *Profit*, version 1.8; http://www.bioinf.org.uk/software/profit/.
(49) Mitra, S., and Lawton, R. (1979) Reagents for the cross-linking of proteins by equilibrium transfer alkylation. *J. Am. Chem. Soc. 101* (11), 3097−3110.
(50) Brocchini, S., Eberle, M., and Lawton, R. (1988) Molecular yardsticks. Synthesis of extended equilibrium transfer alkylating cross-link reagents and their use in the formation of macrocycles. *J. Am. Chem. Soc. 110*, 5211.
(51) Wilbur, D., Stray, J., Hamlin, D., Curtis, D., and Vessella, R. (1994) Monoclonal antibody Fab′ fragment cross-linking using equilibrium transfer alkylation reagents. A strategy for site-specific conjugation of diagnostic and therapeutic agents with F(ab′)2 fragments. *Bioconjugate Chem. 5*, 220−235.
(52) Kim, S., and Lim, C. (2002) Tin-free radical-mediated C-C bond formations with alkyl allyl sulfones as radical precursors. *Angew. Chem., Int. Ed. 41* (17), 3265−3267.
(53) Pan, Y., Hutchinson, D., Nantz, M., and Fuchs, P. (1989) Synthesis via vinyl sulfones. 34. Sn2′ additions of cuprates to sulfone and ester-polarized cyclopentenylic systems. *Tetrahedron 45* (2), 467−478.
(54) Seebach, D., and Knochel, P. (1984) 2′-nitro-2′-propen-1-yl 2, 2-dimethylpropanoate (NPP), a multiple coupling reagent. *Helv. Chim. Acta 67* (1), 261−283.
(55) Nelson, R., and Lawton, R. (1966) On the α,α′ annelation of cyclic ketones. *J. Am. Chem. Soc. 88* (16), 3884−3885.
(56) Padwa, A., Kline, D., Murphree, S., and Yeske, P. (1992) Use of 2,3-bis(phenylsulfonyl)-l-propene as a multicoupling reagent. *J. Org. Chem. 57*, 298−306.
(57) Casey, J., Pedley, R., King, D., Boden, R., Chapman, A., Yarranton, G., and Begent, R. (2000) Improved tumour targeting of di-Fab′ fragments modified with polyethylene glycol. *Tumor Targeting 4*, 235−244.
(58) Shafer, D., Inman, J., and Lees, A. (2000) Reaction of Tris(2-carboxyethyl)phosphine (TCEP) with Maleimide and a-Haloacyl Groups: Anomalous Elution of TCEP by Gel Filtration. *Anal. Biochem. 282*, 161−164.
(59) Ho, P., Milikin, E., Bobbitt, J., Grinnan, E., Burck, P., Frank, B., Boeck, L., and Squires, R. (1970) Crystalline L-asparaginase from *Escherichia coli* B. I. Purification and chemical characterisation. *J. Biol. Chem. 245*, 3708−3715.
(60) Swain, A., Jaskolski, M., Housset, D., Rao, J., and Wlodawer, A. (1993) Crystal-structure of *Escherichia coli* L-asparaginase, an enzyme used in cancer-therapy. *Proc. Natl. Acad. Sci. U.S.A. 90*, 1474−1478.
(61) Palm, G., Lubkowski, J., Derst, C., Schleper, S., Rohm, K., and Wlodawer, A. (1996) A covalently bound catalytic intermediate in *Escherichia coli* asparaginase: crystal structure of a Thr-89-Val mutant. *FEBS Lett. 390*, 211−216.
(62) Killander, D., Dohlwitz, A., Engstedt, L., Franzen, S., Gahrton, G., Gullbring, B., Holm, G., Holmgren, A., Hoglund, S., Killander, A., Lockner, D., Mellstedt, H., Moe, P., Palmblad, J., Reizenstein, P., Skarberg, K., Swedberg, B., Uden, A., Wadman, B., Wide, L., and Ahstrom, L. (1976) Hypersensitive reactions and antibody formation during L-asparaginase treatment of children and adults with acute leukemia. *Cancer 37*, 220−228.
(63) Kamisaki, Y., Wada, H., Yagura, T., Matsushima, A., and Inada, Y. (1981) Reduction in immunogenicity and clearance rate of *Escherichia coli* L-asparaginase by modification with monomethoxy-polyethylene glycol. *J. Pharmacol. Exp. Ther. 216*, 410−414.
(64) Yoshimoto, T., Nishimura, H., Saito, Y., Sakurai, K., Kamisaki, Y., Wada, H., Sako, M., Tsujino, G., and Inada, Y. (1986) Characterization of Polyethylene Glycol-Modified L-Asparaginase from *Escherichia coli* and Its Application to Therapy of Leukemia. *Jpn. J. Cancer Res. 77*, 1264−1270.
(65) Bailon, P., Palleroni, A., Schaffer, C., Spence, C., Fung, W., Porter, J. E., Ehrlich, G., Pan, W., Xu, Z., Modi, M., Farid, A., and Berthold, W. (2001) Rational design of a potent, long-lasting form of interferon: A 40 kDa branched polyethylene glycol-conjugated interferon α-2a for the treatment of Hepatitis C. *Bioconjugate Chem. 12*, 195−202.
(66) Grace, M., Youngster, S., Gitlin, G., Sydor, W., Xie, L., Westreich, L., Jacobs, S., Brassard, D., Bausch, J., and Bordens, R. (2001) Structural and biologic characterization of pegylated recombinant IFN-a2b. *J. Interferon Cytokine Res. 21*, 1103−1115.
(67) Rajender, R., Modi, M., and Pedder, S. (2002) Use of peginterferon alfa-2a (40 KD) (Pegasys) for the treatment of Hepatitis C. *Adv. Drug Delivery Rev. 54*, 571−586.
(68) Youngster, S., Wang, Y., Grace, M., Bausch, J., Bordens, R., and Wyss, D. (2002) Structure, biology, and therapeutic implications of pegylated interferon alpha-2b. *Curr. Pharm. Des. 8*, 2139−2157.
(69) Dhalluin, C., Ross, A., Huber, W., Gerber, P., Brugger, D., Gsell, B., and Senn, H. (2005) Structural, kinetic, and thermodynamic analysis of the binding of the 40 kDa PEG-interferon-alpha 2a and its individual positional isomers to the extracellular domain of the receptor IFNAR2. *Bioconjugate Chem. 16*, 518−527.
(70) Piehler, J., Roisman, L., and Schreiber, G. (2000) New structural and functional aspects of the type I interferon-receptor interaction revealed by comprehensive mutational analysis of the binding interface. *J. Biol. Chem. 275*, 40425−40433.
(71) Kubetzko, S., Sarkar, C., and Pluckthun, A. (2005) Protein PEGylation decreases observed target association rates via a dual blocking mechanism. *Mol. Pharmacol. 68*, 1439−1454.
(72) Dhalluin, C., Ross, A., Leuthold, L., Foser, S., Gsell, B., Muller, F., and Senn, H. (2005) Structural and biophysical characterization of the 40 kDa PEG-interferon-α2a and its individual positional isomers. *Bioconjugate Chem. 16*, 504−517.

BC0601471