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PEGylation of Human Serum Albumin: Reaction of PEG-Phenyl-Isothiocyanate with Protein

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Successful and cost-effective PEGylation protocols require pure functionalized PEG reagents, which can be synthesized by simple and efficient procedures, exhibit high stability against hydrolysis, and maintain a level of reactivity with protein functional groups under mild reaction conditions. PEG-phenyl-isothiocyanate (PIT-PEG) is a new functionalized PEG having these characteristics, and has been synthesized by condensation of the bifunctional reagent 4-isothiocyanato phenyl isocyanate with monomethoxy PEG (mPEG). The data of ¹H NMR and colorimetric analysis of the new PEG reagent establish that the mPEG has been quantitatively functionalized. The $t_{1/4}$ values for the hydrolysis of PIT-PEG5K in 100 mM phosphate solution at pH 6.5 and 9.2 are about 95 and 40 h, respectively. Incubation of human serum albumin (HSA, 0.5 mM) with a 10-fold molar excess of PIT-PEG (3K or 5K) at pH 6.5 and 9.2 generated PEG-HSA conjugates with average of 3.5 and 6.0 PEG chains per HSA molecule, respectively. The circular dichroism spectra of the conjugates showed that PEGylation of HSA has little influence on the secondary structure of HSA. The hexaPEGylated HSA, (TCP-PEG5K)₆-HSA, exhibited very high hydrodynamic volume, and the molecular radius of HSA increased from 3.95 to 6.57 nm on hexaPEGylation. The hexaPEGylation also increased the viscosity of 4% HSA from 1.05 to 2.10 cP, and the colloid osmotic pressure from 15.2 to 48.0 mmHg. The large increase in the hydrodynamic volume and the solution properties of (TCP-PEG5K)₆-HSA suggest that it could be a potential candidate as a plasma volume expander. PIT-PEG is a useful addition to the spectrum of functionalized PEG reagents available for surface decoration of proteins with PEG.

INTRODUCTION

Advances in biotechnology, in particular recombinant DNA technology, have made a large number of proteins available for application as novel drugs. Most of these therapeutic proteins suffer from short circulating life due to their proteolytic degradation and/or rapid clearance. Immunogenic reaction of the therapeutic proteins is another major concern in developing protein-based drugs. This in effect decreases their therapeutic efficacy (1). Covalent attachment of natural or synthetic polymers to therapeutic proteins with the purpose of generation of hybrid biomacromolecules with unique and improved molecular properties has become a popular approach to overcome these limitations of therapeutic proteins (2). Many polymers have been studied for these applications, and the most popular one is poly(ethylene glycol) (PEG), which is nontoxic, nonimmunogenic, highly soluble in water, and FDA approved. Since the pioneering experiments on PEGylation of proteins by Abuchowski et al. (3, 4), the application of this strategy to improve the therapeutic efficacy of protein drugs has been very clearly demonstrated (1, 5, 6).

PEGylation of a protein, peptide, or nonpeptide molecule involves the covalent attachment of one or more copies of PEG chains to the desired molecule. Functionalization of the PEG

chain with a choice reactive functional group is the first step in carrying out PEGylation of macromolecules. Several PEG reagents, which facilitate the formation of bioconjugates (7–9), have been developed over the years, and are commercially available now.

Active esters of PEG, in particular the *N*-hydroxysuccinimide (NHS) esters of PEG acids are the most commonly used PEG reagents to target PEGylation to surface amino groups of protein. The half-life of the NHS esters of PEG acids for hydrolysis is in the range from 1 min (or less) to about 45 min at pH 8.0 and 25 °C (10, 11). As the reactivity of PEG-NHS toward amino groups of proteins goes up, the half-life of the PEG reagent in aqueous buffer decreases. Accordingly, a large excess of PEG reagents over protein have to be used for achieving desired levels of PEGylation.¹

In contrast to the NHS ester of PEG acid, a maleimide-activated PEG, which reacts with the thiols of cysteine residues of protein to form stable thioether linkage with the protein, exhibits a much higher stability against hydrolysis, and accordingly PEG-maleimide reagents have been one of most desired PEGylation reagents. The maleimide moiety reacts rapidly with the thiol group without hydrolysis around neutral pH. The reaction is very selective as well as nearly quantitative. Accordingly, we have developed a new strategy that takes advantage of the high selectivity and efficiency of the thiol-

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¹ Abbreviations: CD, circular dichroism; COP, colloid osmotic pressure; Hb, hemoglobin; HSA, human serum albumin; PBS, phosphate buffer saline; PEG, poly(ethylene glycol); PEG5K, PEG with Mw of 5000 dalton; mPEG, monomethoxy PEG; PEGylation, conjugation with PEG; PIT-PEG, PEG-phenyl-isothiocyanate; SEC, size exclusion chromatography; (TCP-PEG5K)_n-HSA, thiocarbonyl phenyl PEG-HSA conjugate with *n* copies of PEG5K chains.

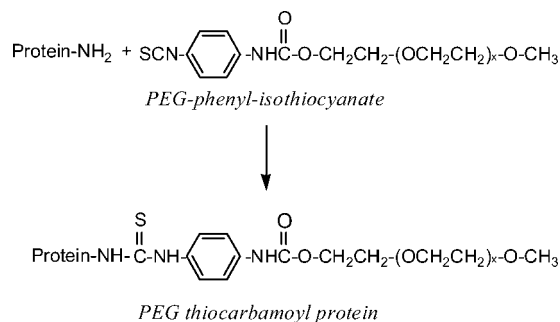


Figure 1. Schematic representation of the reaction of PEG-phenyl-isothiocyanate with protein amino groups.

maleimide reaction to develop a new PEGylation platform, referred to as the extension arm facilitated (EAF) PEGylation. This procedure targets the PEGylation reaction to the reactive surface amino groups of the proteins. Hemoglobin, albumin, and red blood cell have been surface-decorated with multiple copies of PEG chains very efficiently using this simple and cost-effective PEGylation protocol (12–15). In this PEGylation protocol, protein can be incubated with both the thiolating reagent (2-iminothiolane) and PEG-maleimide simultaneously. As new thiol groups are generated *in situ* as a result of reaction of iminothiolane with protein amino group, they are trapped by the PEG-maleimide. The application of this protocol for surface decoration of protein and cells is further facilitated by the development of a single-step procedure for functionalization of the mPEG as PEG-maleimide. An easy and nearly quantitative reaction of aromatic isocyanates with aliphatic hydroxyl that generates a carbamate linkage has been used to functionalize the PEG (16, 17). This approach of using a low molecular weight bifunctional reagent, phenyl isocyanate, carrying the desired functional group at the *p*-position is advanced as a modular approach for functionalizing PEG.

Phenyl isothiocyanate is a well-known reagent for carrying out Edman degradation (18) and amino acid analyses by HPLC (19). Some isothiocyanates were used to modify hemoglobin (Hb) in an attempt to correlate the structure with function (20, 21) and to introduce cross-links into Hb (22, 23). In all these reactions, isothiocyanates showed a high reactivity with the N-terminal valine residues of Hb. Isothiocyanate reacts with nucleophiles such as amines, sulfhydryls, and the phenolate ion of tyrosine side chains. The only stable product of these reactions, however, is with the primary amino groups (24). Therefore, isothiocyanate compounds are very selective reagents for modifying ϵ - and N-terminal amines in proteins. Besides, phenyl isothiocyanate is a stable reagent that can be stored at room temperature. Accordingly, it is reasonable to expect that PEG activated with a phenyl isothiocyanate group will exhibit high stability against hydrolysis and also a good level of reactivity with the α -amino groups and the low pK_a amino groups of proteins to generate thiocarbamoyl protein conjugate (Figure 1). Under the conditions where the NHS esters of PEG acids readily undergo hydrolysis, PEG-isothiocyanate could serve as an alternate reagent for PEGylation.

To functionalize PEG with isothiocyanato moiety, we have translated the one-step modular approach that we had developed earlier to functionalize PEG with a maleimide moiety, i.e., condensation of *p*-maleimide phenyl isocyanate with mPEG (16, 17). Accordingly, for functionalizing mPEG with an isothiocyanato moiety, the synthesis of *p*-isothiocyanato phenyl isocyanate and the condensation of this reagent with mPEG have been pursued (25). The application of this reagent for PEGylation of protein has been investigated using human serum albumin (HSA) as a model protein.

HSA is an abundant negatively charged plasma protein, with ligand binding and transport functions, antioxidant functions, and enzymatic activities (26, 27). We choose HSA as a model protein for PEGylation with multiple copies of PEG chains using this new reagent, in view of our interest in the application of PEGylated albumin as a plasma volume expander to improve microcirculatory function in various clinical settings (28, 29).

Here, we describe the functionalization of PEG as PEG-phenyl-isothiocyanate (PIT-PEG), its stability against hydrolysis, its reactivity with HSA, and optimization of its reaction with HSA for generation of hexaPEGylated albumin. To assess the feasibility of using these new PEG-HSA conjugates as plasma volume expanders, the solution properties (including hydrodynamic molecular radius, viscosity, and colloid osmotic pressure) of the new PEG-HSA conjugates, influence of PEGylation on the secondary structure, and overall conformation of HSA have also been studied.

MATERIALS AND METHODS

Synthesis of PEG-Phenyl-Isothiocyanate. Monomethoxy PEG-phenyl-isothiocyanate, namely, PIT-PEG, was synthesized as described earlier (25). As shown in Figure 2A, the synthesis of PIT-PEG can be achieved in a one-step reaction, activating the mPEG with a small molecular weight bifunctional reagent, 4-isothiocyanato-phenyl isocyanate. The synthesis of the bifunctional reagent can be divided into three steps as schematically represented in Figure 2B: (i) synthesis of 4-carboxy phenyl isothiocyanate from *p*-amino benzoic acid; (ii) the conversion of 4-carboxy phenyl isothiocyanate to 4-isothiocyanato benzoyl azide; (iii) the synthesis of 4-isothiocyanato phenyl isocyanate.

(i) *Synthesis of 4-Carboxy Phenyl Isothiocyanate.* Para-aminobenzoic acid (0.2 mol; 26 g) was dissolved in acetone (400 mL) at room temperature. Activated carbon, (about 5 g, Darco, G60) was added and the mixture was stirred (using magnetic stir bar) for 5 to 10 min. The entire solution was filtered, yielding a much lighter colored solution of *p*-aminobenzoic acid. Sodium acetate (0.3 mol, 26 g) was dissolved in about 200 mL of deionized water, and added to the above filtrate of *p*-aminobenzoic acid, now contained in a 4 L vacuum flask. A vacuum was applied to the flask with an intermediate dry ice/acetone trap connected between the flask and the vacuum pump.

Thiophosgene (about 40 g of neat red liquid) was added to the cooled acetone stripped slurry of *p*-aminobenzoic acid, while stirring rapidly with an overhead paddle stirrer. A tan precipitate formed almost immediately upon the addition of the thiophosgene along with considerable foaming. After the foaming subsided (about 10 min), the insoluble precipitate was filtered and dried under vacuum to get a powdery material. The crude product was recrystallized from hot (80 °C) glacial acetic acid to yield yellow needles of 4-carboxy phenyl isothiocyanate (about 16 g) after drying under vacuum.

Elemental analysis of the yellow crystals yielded: Carbon (found 53.38%, theory 53.63%); hydrogen (found 2.76%, theory 2.79%); nitrogen (found 7.58%, theory 7.82%). The crystals darkened but did not melt at 220 °C.

(ii) *Synthesis of 4-Isothiocyanato Benzoyl Azide.* 15 g of the 4-carboxyphenyl isothiocyanate was suspended in 200 mL of dry methylene chloride in a 1 L sidearm vacuum flask, along with 16 g of pyridine (0.2 M), phenyl dichlorophosphate (0.1 M), and 6.5 g of sodium azide (0.1 M). This mixture was stirred overnight at room temperature. The stirred mixture was then washed in a separatory funnel with 200 mL of water and then with 200 mL of 0.05 M sulfuric acid. The acid-washed methylene chloride layer was dried with anhydrous magnesium sulfate. The dried methylene chloride reaction solution was evaporated under vacuum in a rotary evaporator at room temperature. The resulting light tan crystals were dissolved in

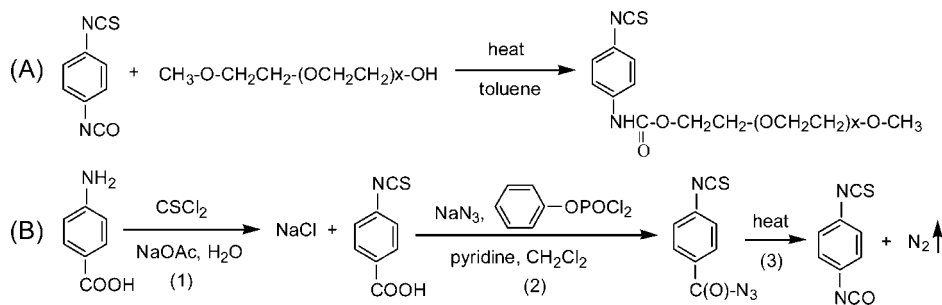


Figure 2. Schematic representation functionalization of mPEG as PEG-phenyl-isothiocyanate. (A) The one-step functionalization procedure. (B) The synthesis of 4-isothiocyanato phenyl isocyanate.

a minimal amount of ethyl ether at room temperature. The recrystallization solution was treated with activated carbon (Darco G 60) and filtered. The resulting light-colored solution was evaporated to dryness at room temperature. White crystals were obtained which melted at 68 to 72 °C with the evolution of nitrogen consistent with azide decomposition.

The elemental analysis of the 4-isothiocyanato benzoyl azide crystals gave the following results: Carbon (found 46.85%, theory 47.05%); hydrogen (found 2.09%, theory 1.96%); nitrogen (found 26.87%, theory 27.45%). The infrared spectrum of the crystals (FTIR) conformed to 4-isothiocyanatobenzoyl azide (e.g., strong broadband from about 2000 to about 2200 cm^{-1} is from $\text{N}=\text{C}=\text{O}$ and $\text{N}=\text{C}=\text{S}$ stretching modes; and a strong absorption due to azide at about 980–1000 cm^{-1}). The compound is stable at room temperature but was stored in the freezer.

(iii) *Synthesis of 4-Isothiocyanato Phenyl Isocyanate.* The 4-isothiocyanatobenzoyl azide was thermally decomposed at about 75–104 °C (Curtius rearrangement) smoothly and quantitatively as a solution in dry refluxing of a toluene solution to 4-isothiocyanato phenyl isocyanate. The 4-isothiocyanato phenyl isocyanate was not isolated, but reacted as formed *in situ* with the hydroxyl-containing PEG according to the following description. An infrared spectrum of the composition containing 4-isothiocyanato phenyl isocyanate showed the decrease in the azide band at about 980–1000 cm^{-1} along with increased complexity of the absorbance in the $\text{N}=\text{C}=\text{O}$ and $\text{N}=\text{C}=\text{S}$ region of the spectrum from about 2000 to about 2300 cm^{-1} .

(iv) *Functionalization of mPEG to PIT-PEG.* 50 g of mPEG5K having a low diol content (NOF Corporation, Japan) was dissolved in 500 mL of toluene in a 2 L, three-necked flask equipped with a thermometer, overhead stirrer with motorized drive, and a Dean–Stark trap and condenser. The solution was stirred and heated to reflux (about 104 °C) and any water present was azeotropically removed as it accumulated in Dean–Stark trap. When no more additional water could be removed, the refluxing was discontinued and the contents were cooled under a dry nitrogen blanket to about 60 °C. To this reaction mixture, *p*-isothiocyanato benzoyl azide (0.5 g) was added and heating was resumed. Nitrogen was evolved during the initial 15 to 30 min after the heating was resumed. Heating was continued (reflux) for an additional hour. Heating was discontinued and the contents of the flask was allowed to cool to room temperature, and the reaction mixture now contained the desired product, PEG5K-phenyl-isothiocyanate. The reaction mixture was concentrated under vacuum using a rotary evaporator into viscous oil. This was treated with anhydrous ethyl ether to induce the crystallization of the activated mPEG5K. Ethyl ether also extracted the unreacted *p*-isothiocyanatophenyl isocyanate.

The crude PIT-PEG was purified with filtration in water, extraction with methylene chloride twice, then recrystallization in mixed solvent of methylene chloride/anhydrous ether twice. Finally, the purified white solid (48 g) was dried at room

temperature under vacuum overnight. The purity of PIT-PEG was estimated as described below.

Estimation of Phenyl Isothiocyanato Moiety in Functionalized PEG. The concentration of phenyl isothiocyanato moiety in PIT-PEG was assayed by a colorimetric method developed by Zhang et al. (30). The principle of the method is that phenyl isothiocyanate reacts with 1,2-benzenedithiol to yield 1,3-benzodithiole-2-thione ($\lambda_{\text{max}} = 365 \text{ nm}$ and $\epsilon_{\text{m}} = 23\,000 \text{ M}^{-1} \text{ cm}^{-1}$). A final volume of 2.0 mL reaction mixture contains 900 μL of 100 mM potassium phosphate solution (pH 8.5), 900 μL of methanol, 100 μL of a solution of the isothiocyanate to be determined (0.5 mM), and 100 μL of 80 mM 1,2-benzenedithiol in methanol. The reactions were carried out in 5 mL of screwtop glass vials. The vials containing the reaction mixture were heated at 65 °C for 1 h and then cooled to room temperature. The absorbance at 365 nm was determined against a solvent blank. Each sample to be analyzed was accompanied by a paired blank containing all ingredients, except 1,2-benzenedithiol. Each set of determinations also included a control vessel that contained the 1,2-benzenedithiol but no isothiocyanate. A typical calibration curve for 0.1–1.0 mM phenyl isothiocyanate was generated with each testing of samples.

Hydrolysis Kinetics of Isothiocyanate of PIT-PEG5K. 25.9 mg of PIT-PEG5K dissolved into 1 mL (5 mM) of 100 mM potassium phosphate solution at different pH values, i.e., 5.5, 6.5, 7.4, and 9.2. The PIT-PEG5K solutions were incubated at room temperature. 40 μL of aliquots were pipetted out from the 5 mM of PIT-PEG5K solution at regular time intervals, and the concentrations of isothiocyanate in the incubation mixtures were estimated by colorimetric method as described above.

PEGylation of HSA with PIT-PEG. 0.5 mM HSA (Sigma, 99.9% free from acid) was incubated with a desired concentration of PIT-PEG reagent in 0.5 mL of 10 mM phosphate solution, at pH 8.5, at room temperature. The unreacted PIT-PEG was removed by centrifugal filtration using Centricon (50 KD cutoff membrane, Millipore Corp.) at 5000 rpm for 30 min four times. To study the kinetics of PEGylation (pH 9.2), 40 μL of reaction mixture was pipetted out at regular time intervals and diluted to 300 μL , and then analyzed by size exclusion chromatography (SEC) to measure the amount of unreacted PIT-PEG. For large preparation of PEGylated HSA, 1 g of HSA was incubated with 0.48 g of PIT-PEG3K or 0.78 g of PIT-PEG5K (HSA: PIT-PEG = 0.5:5 mM) in 30 mL of 10 mM phosphate solution, pH 6.5 or 9.2, at room temperature for 6 h. The unreacted PIT-PEG was removed by tangential flow ultrafiltration against phosphate buffer serum (PBS), using Minim from PALL Corporation and a 70 KD cutoff membrane. The progress of the removing of unreacted PIT-PEG from the products was monitored by the absorbance at 280 nm using a SEC.

Analytical Methods. SEC was performed on a Pharmacia FPLC system, at 25 °C using two HR10/30 Superose 12 columns connected in series with a 100 μL loop. The columns

were equilibrated and eluted with PBS, pH 7.4, at a flow rate of 0.5 mL/min. The effluent was monitored at 280 nm. SDS-PAGE analysis was carried out using a 6% Tris-glycine gel from Invitrogen Corp. as described previously (31).

HSA Content Assay. The concentration of HSA was determined from the absorbance at 279 nm (1 mg/mL, OD = 0.531; $\epsilon_m = 35\,300\text{ M}^{-1}\text{ cm}^{-1}$). The concentrations of PEG-HSA conjugates were determined by the standard procedure of Bio-Rad protein assay (32).

^1H NMR Analysis. ^1H NMR spectra were recorded using D_2O as the solvent and trimethylsiloxypropionate as the internal standard on a Bruker DRX 300 NMR spectrometer at 300 MHz. 10 mg samples (either PIT-PEG reagents or PEG-HSA conjugates) were dissolved in 0.1 mL of D_2O and their ^1H NMR spectra were scanned for 1.3 s. Pure sample of mPEG (10 mg) in D_2O (0.1 mL) served as standard. The integrated signal characteristic for mPEG was recorded for all samples, and by reference to the standard, the amount of functionalized group in PIT-PEG or the amount of mPEG in the conjugates was calculated (33).

Solution Properties of PEGylated HSA. The hydrodynamic molecular radius of the PEGylated HSA was determined at 22 °C at 1 mg/mL by dynamic light scattering measurements on a DynaPro molecular sizing instrument (Protein Solutions, Charlottesville, VA). The viscosity of the PEGylated HSA was measured with a cone and plate rheometer (Brookfield, Middleboro, MA) at 37 °C in PBS. The instrument was calibrated with water prior to measurements of the viscosity of the HSA samples. The colloid osmotic pressure (COP) of the PEGylated HSA was determined using a Wescor 4420 Colloidal Osmometer (Wescor, Logan, UT, USA) with a 30 KD Mw cutoff membrane at room temperature in PBS. The instrument was calibrated with Osmocoll reference standard prior to measurements of the samples. Each sample was measured at least twice and the average of the measurements is presented.

Spectroscopic Studies of PEGylated HSA. CD (circular dichroism) spectra for HSA and PEGylated HSA were recorded on a JASCO-720 spectropolarimeter (Tokyo, Japan) at 25 °C using a 0.2 cm light path cuvette (310 μL). All the samples (3 μM) were scanned in the range 200–250 nm in PBS, pH 7.4. The CD results were expressed in terms of mean residue ellipticity (MRE) [θ] in $\text{deg}\cdot\text{cm}^2/\text{dmol}$. The fluorescence measurements of HSA and PEGylated HSA samples (3 μM) were performed using a Shimadzu RF-5301 spectrofluorimeter at room temperature. The emission spectra were recorded from 305 to 400 nm using an excitation wavelength of 295 nm. A cuvette with a 1 cm path length was used in all these studies.

RESULTS

Isolation and characterization of PIT-PEG. PIT-PEG was isolated by recrystallization as described in Methods and Materials. The ^1H NMR spectrum of mPEG5K functionalized with 4-isothiocyanato phenyl isocyanate is compared with that of mPEG5K in Figure 3. The ^1H NMR spectrum of the functionalized PEG clearly “fingerprints” the signature of phenyl ring introduced, and the methylene groups of PEG that are connected to the phenyl ring through a carbamate linkage. The NMR data confirmed the expected molecular structure of PIT-PEG. The chemical shifts and assignments of the protons in the PEG reagent are as follows: δ 3.3 (s, 3H, mPEG, CH_3O); δ 3.5–3.8 (s, PEG backbone, $-\text{CH}_2\text{CH}_2\text{O}$); δ 4.3 (t, 2H, carbamate PEG linker, $-\text{CH}_2\text{OC}(\text{O})-$); and δ 7.2–7.5 (q, 4H, phenyl ring, $-\text{C}_6\text{H}_4-$).

Purity of PIT-PEG. The purities of PIT-PEGs are calculated from the estimation of isothiocyanato moiety in the functionalized PEG by colorimetric method and by ^1H NMR method (34), and presented in Table 1. The colorimetric method

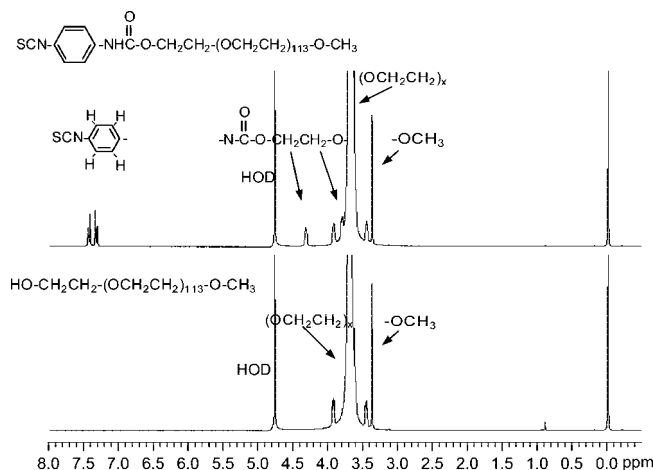


Figure 3. ^1H NMR spectra (D_2O , 300 MHz) of mPEG5K and PIT-PEG5K.

Table 1. Extent of Functionalization of PEG by Isothiocyanato Phenyl Isocyanate

	extent of functionalization	
	colorimetry method	^1H NMR method
PIT-PEG3K	109%	93%
PIT-PEG5K	112%	96%

estimates the purity higher than 100%. ^1H NMR spectroscopy estimates the purity of PIT-PEG3K and PIT-PEG5K as about 93% and 96%, respectively. The purity of PIT-PEG5K was obtained by quantitating the integrated areas of hydrogen of backbone PEG ($113 \times 4\text{H}$) with that of phenyl ring (4H). The purities estimated by both methods indicate that the mPEG appears to be essentially fully functionalized.

Stability of PIT-PEG. The PEG-isothiocyanate is an electrophilic reagent, which can react with a weak nucleophile like hydroxyl ion. In neutral aqueous solution, the hydrolysis of PIT-PEG is relatively very slow, but proceeds more rapidly in aqueous alkaline solutions. The hydrolysis of PEG-isothiocyanate forms corresponding PEG-aniline, carbon dioxide, and hydrogen sulfide (35). The kinetics of hydrolysis of PIT-PEG5K (5 mM) in 100 mM phosphate solution as a function of pH is shown in Figure 4. The hydrolysis rate of PIT-PEG5K increases with the increase of pH. It may be noted that only a negligible amount of PIT-PEG5K hydrolyzed at all the pH within the first 12 h. The $t_{1/4}$ for the hydrolysis of PIT-PEG5K in different buffers of pH 5.5, 6.5, 7.4, and 9.2 (curve a, b, c, and d, respectively) are approximately 105, 95, 70, and 40 h, respectively.

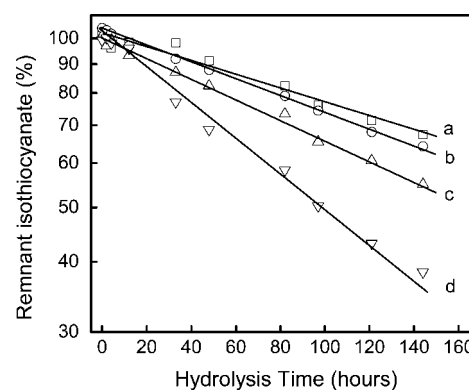


Figure 4. Hydrolysis kinetics of PIT-PEG5K (5 mM) in 100 mM phosphate solutions at different pH (pH 5.5, 6.5, 7.4, and 9.2; curves a–d) at room temperature.

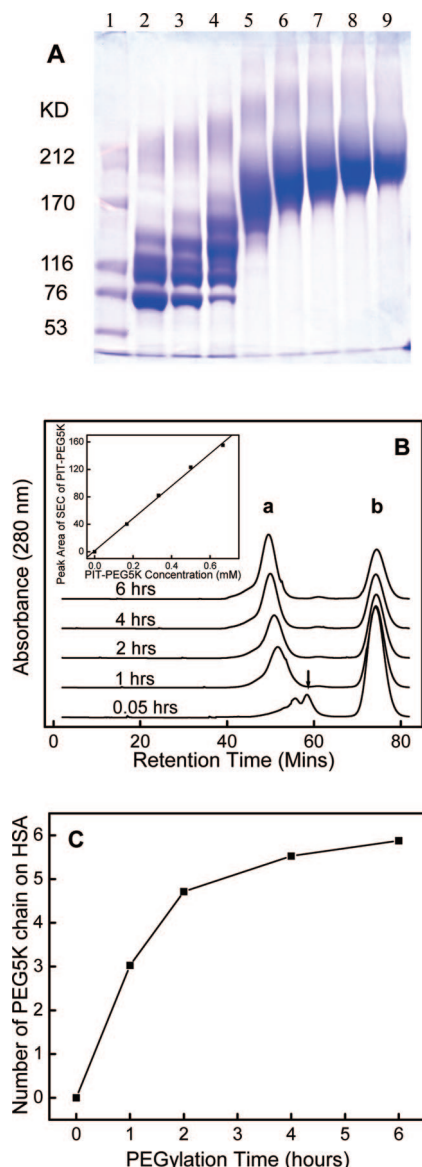


Figure 5. (A) The SDS-PAGE analysis of HSA and PEG-HSA conjugates prepared at the different ratio in 10 mM phosphate solution at pH 8.5 at room temperature for 6 h. Lane 1, marker; Lanes 2 to 9, 0.5 mM albumin reacted with 0.25, 0.5, 1.0, 4.0, 8.0, 10.0, 15.0, and 20.0 mM PIT-PEG5K, respectively. (B) SEC analysis of HSA (0.5 mM) reacted with PIT-PEG5K (5 mM) in 10 mM phosphate solution (pH 9.2) at room temperature. 20 μ L of reaction mixture was diluted to 150 μ L and uploaded into SEC (100 μ L of loop) at 0.05, 1, 2, 4, and 6 h. The arrow points at the unmodified HSA. Inset is the standard curve of the peak area in SEC as a function of concentration of PIT-PEG5K. (C) The kinetic curve of PEGylation of HSA with PIT-PEG5K (0.5:5 mM) reacted in 10 mM phosphate solution (pH 9.2) at room temperature.

Reactivity of PIT-PEG. (i) *Modification of HSA by PIT-PEG5K as a Function of Molar Excess of the Reagent.* The reactivity of the isothiocyanato moiety of PIT-PEG with a protein amino group has been investigated using HSA as the model protein. SDS-PAGE pattern of HSA (0.5 mM) incubated with 0.5- to 40-fold molar equivalents of PIT-PEG5K (0.25 to 20 mM) in 10 mM phosphate solution, pH 8.5, at room temperature for 6 h is shown in Figure 5A. As the molar equivalents of PIT-PEG5K to HSA increase, HSA with an apparent molecular size of approximately 70 kD is converted into higher molecular weight species. Apparently, the new protein bands generated in these reactions represent PEG-HSA conjugates with number of PEG chains. When the PIT-PEG is

only 0.5 equiv (0.25 mM, lane 2 in Figure 5A), most of the HSA remains unPEGylated, but three well-resolved bands are present besides the unmodified HSA. When the PIT-PEG is increased to 1 equiv (lane 3 in Figure 5A), the amount of the HSA band decreases with a concomitant increase in the other three bands and the generation of the fourth band. As the PIT-PEG5K increased further to 2 equiv (lane 4 in Figure 5A), the HSA band becomes a minor species, with generation of the new fifth band (diffused) of higher molecular size. This reflects the high reactivity of PEG-isothiocyanate with the amino groups of HSA. When the PIT-PEG is increased to 8 equiv (lane 5 in Figure 5A), PEGylated HSA moves as a diffused band in the apparent molecular weight range of 130 to 170 kD. With 16-fold molar equiv (lane 6 in Figure 5A) of PIT-PEG, almost all modified HSA appears as a diffused band in the molecular weight range 170–212 kD. Further increase in the amount of PIT-PEG does not increase the apparent molecular mass of the modified PEG significantly, but the product moves as a more compact band.

(ii) *Kinetics of PEGylation of HSA by PIT-PEG.* The kinetics of PEGylation of HSA with PIT-PEG5K (0.5:5 mM) in 10 mM phosphate solution (pH 9.2) at room temperature is studied by SEC and shown in Figure 5B. The decrease in the retention time and the increasing peak area of albumin as reflect by the absorbance at 280 nm (peak a in Figure 5B), suggest that the molecular size of albumin and its absorbance at 280 nm increase as PEG is coupled to HSA gradually with the incubation time increasing (the linkage between the PEG and HSA has a strong UV absorption due to presence of the phenyl ring and thiourea bond). Meanwhile, the amount of the PIT-PEG5K (peak b in Figure 5B) decreases as the reaction proceeds.

In view of the high stability of PIT-PEG (Figure 4), peak b represents the unreacted PIT-PEG in the reaction mixture, and it is conceivable that quantitation of this peak can be used to estimate the PEGylation of the protein. The inset curve in the Figure 5B depicts the linear correlation of that the peak area of PIT-PEG5K in SEC as a function of its concentration loaded into SEC (100 μ L of loop). From the standard curve and the integrated area of unreacted PIT-PEG5K (peak b in Figure 5B), the amount of PIT-PEG5K coupled to albumin (the number of PEG5K chains on HSA) in each of the PEG-HSA conjugate can be calculated.

The kinetic curve of PEGylation of HSA with PIT-PEG5K is shown in Figure 5C. The PEGylation proceeds very fast in the first 2 h, and is almost complete with about 5 copies of PEG chains coupled to albumin molecule. As the reaction is going on, the rate of PEGylation slows down, and only about 1 copy of additional PEG chain is coupled on each molecule in the following 4 h. After 6 h of reaction, there are about 5.9 copies of PEG5K chains attached on HSA molecule. In view of the high stability of the reagent, and the fact that only 50% of the reagent is used by 2 h, the second phase of the kinetics of PEGylation represents an increasing resistance of PEG-HSA conjugate as the number of PEG chains on the protein molecule increases.

(iii) *Influence of pH and Length of PEG Chain on the PEGylation of HSA.* The influences of reaction pH and length of PEG chain on the PEGylation of HSA have been studied by incubating HSA with PIT-PEG3K and PIT-PEG5K at pH 6.5 and 9.2. Incubation of 0.5 mM HSA with 5 mM PIT-PEG3K or PIT-PEG5K in 10 mM phosphate solution, at pH 6.5 and 9.2, at room temperature for 6 h, resulted in a quantitative modification of HSA as anticipated, and a concomitant significant enhancement in apparent molecular size of HSA as shown by SEC pattern (Figure 6A). The HSA modified with PIT-PEG3K at pH 6.5 (curve 2) elutes close to the position of HSA dimer, while the PEG-HSA generated at pH 9.2 with PIT-

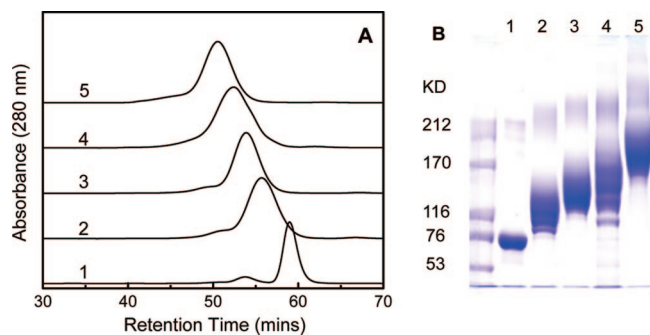


Figure 6. SEC analysis (A) and SDS-PAGE analysis (B) of native HSA (1), PEG3K-HSA generated at pH 6.5 (2), PEG3K-HSA generated at pH 9.2 (3), PEG5K-HSA generated at pH 6.5 (4), and PEG5K-HSA generated at pH 9.2 (5). The reaction condition is described in context. The unreacted PIT-PEG were removed using tangential flow ultrafiltration.

Table 2. Influence of Reaction pH and PEG Chain Length of PIT-PEG on the PEGylation of HSA

pH	ITP-PEGMw	average copies of PEG chains on HSA molecule	
		SEC method	¹ H NMR method
6.5	PEG3K	3.3	3.6
6.5	PEG5K	3.7	3.4
9.2	PEG3K	5.5	5.9
9.2	PEG5K	6.0	6.0

PEG3K (curve 3) is slightly larger than the HSA dimer. Similarly, the hydrodynamic molecular size of PEG5K-HSA conjugate generated at pH 6.5 (curve 4) is smaller than that of PEG5K-HSA conjugate generated at pH 9.2 (curve 5). The numbers of PEG chains on the four PEGylated samples are calculated from their SEC results (data not shown) before removing unreacted PIT-PEG and presented in Table 2. In the reactions at pH 9.2, approximately 6 copies of PEG chains (either PEG3K or 5K) are conjugated to HSA, whereas in the reactions at pH 6.5, about 3.5 copies of PEG chains are linked to HSA. Therefore, it is clear that modulation of pH of the reaction mixture will be a good approach to control the extent of PEGylation, and that the PEG chain length of PIT-PEG has little influence on the PEGylation of HSA.

The SDS-PAGE analysis of the four PEG-HSA conjugates are shown in Figure 6B. From the gel, it is confirmed that the PEG-HSA conjugates generated at pH 9.2 (Lanes 3 and 5) exhibit higher level of PEGylation than the PEG-HSA conjugates generated at pH 6.5 (Lanes 2 and 4). The distribution of the PEGylated materials (based on the number of PEG chains) of Lanes 2 and 4 (conjugates generated at pH 6.5) is wider as compared to the distribution of the PEGylated material in Lanes 3 and 5 (conjugates generated at pH 9.2). Therefore, the PEG-HSA conjugates generated at pH 6.5 appear to be much more heterogeneous as compared to the products generated at pH 9.2.

The SEC and SDS-PAGE results suggest that the level of PEGylation increases with the increase of the pH of the PEGylation reaction, as more ϵ -amino groups of HSA are deprotonated at higher pH.

Solution Properties of PEG-HSA Conjugates. Four PEG-albumin conjugates were prepared in large scale (1 g HSA) and purified using tangential flow ultrafiltration. Two PEG-HSA conjugates have been generated at pH 6.5 using PIT-PEG3K and PIT-PEG5K, respectively, and the another two conjugates have been generated at pH 9.2 again using the same two PEG reagents.

(i) *Molecular Mass of PEG Conjugated to Albumin.* The amount of PEG conjugated to albumin in the four PEG-HSA conjugates have been calculated by the ¹H NMR approach (33)

Table 3. Comparison of Solution Properties of PEG-HSA Conjugates

sample HSA	hydrodynamic radius/nm	PEG mass ^b /kD (n PEG)	viscosity ^c /cP	COP ^d /mmHg
HSA	3.95	0	1.05	15.2
(TCP-PEG3K) ₄ -HSA	4.83	10.8 (3.6)	1.12	21.3
(TCP-PEG5K) ₄ -HSA	5.79	17.0 (3.4)	1.34	33.2
(TCP-PEG3K) ₆ -HSA	5.57	17.7 (5.9)	1.46	26.9
(TCP-PEG5K) ₆ -HSA	6.57	30.0 (6.0)	2.03	46.8

^a Measured by DynaPro, concentration = 1.0 mg/mL, 22 °C. ^b Calculated by ¹H NMR. ^c Albumin concentration = 4.0 g/100 mL, 37 °C. ^d Albumin concentration = 4.0 g/100 mL, 25 °C.

and compared with the amount calculated by spectrophotometric/SEC approach in Table 2. The PEGylation at pH 6.5 couples an average of 3.5 PEG chains on each albumin molecule. Comparatively, the PEGylation at pH 9.2 couples 6 copies of PEG chains. According to the number of PEG chains attached on HSA molecule, the PEG-HSA conjugates generated at pH 6.5 are referred to as tetraPEGylated HSA [named (TCP-PEG3K)₄-HSA or (TCP-PEG5K)₄-HSA], and those generated at pH 9.2 as hexaPEGylated HSA [named (TCP-PEG3K)₆-HSA or (TCP-PEG5K)₆-HSA].

(ii) *Solution Properties of PEGylated Albumins. Molecular Radius of the PEGylated Albumins.* The molecular radius of native HSA is around 3.95 nm (Table 3). The hexaPEGylated HSA with PEG5K chains, (TCP-PEG5K)₆-HSA, has the largest radius of 6.57 nm. The tetraPEGylated HSA with the least amount of PEG, (TCP-PEG3K)₄-HSA exhibits the smallest molecular radius, i.e., 4.83 nm. (TCP-PEG3K)₆-HSA has a molecular radius of 5.57 nm, while (TCP-PEG5K)₄-HSA exhibits a radius of 5.79 nm (Table 3). It may be noted that, although (TCP-PEG3K)₆-HSA carries a little more amount of PEG on HSA than that on (TCP-PEG5K)₄-HSA (17.7 kD vs 17.0 kD, Table 3), the former conjugate exhibits a smaller radius than the latter conjugate (5.57 nm vs 5.79 nm in Table 3, and Curve 3 vs 4 in Figure 6A). This is probably because, although (TCP-PEG3K)₆-HSA carries more PEG chains than (TCP-PEG5K)₄-HSA does, the PEG shell of the former conjugate is fixed on the albumin surface through 6 anchors and the PEG shell of the latter one is fixed through fewer locating sites, 4 anchors. The PEG shell of (TCP-PEG3K)₆-HSA, therefore, appears more compact than that of (TCP-PEG5K)₄-HSA. This result is different from that reported by Fee et al. (36), who suggested that the hydrodynamic radii of PEGylated proteins depend on the total weight of grafted PEG but not on PEG molecular weight, or PEG-to-protein molar grafting ratio.

Colloid Osmotic Pressure of PEGylated Albumins. The COP of PEGylated albumins has been studied as a function of protein concentration and compared with that of unmodified albumin (Figure 7A). The COP of albumin shows only a marginal increase when measured as a function of the protein concentration, and this small increase is linear over the range of the protein concentration studied. On the other hand, the COP values of PEGylated albumin samples are higher than that of native HSA and exhibit an exponential increase as a function of the protein concentration. The increased COP resulting from PEGylation as a function of PEG mass conjugated to HSA at different protein concentration is shown in the inset of Figure 7A. The increase in COP as a function of PEG mass conjugated is also exponential.

Viscosity of PEGylated Albumins. The viscosities of PEGylated albumins are compared with that of unmodified albumin as a function of the protein concentration (Figure 7B). The viscosity of HSA does not increase significantly upon increasing the protein concentration. On the other hand, the viscosities of the four PEGylated albumins exhibit an exponential increase as a function of the protein concentration. The correlation of

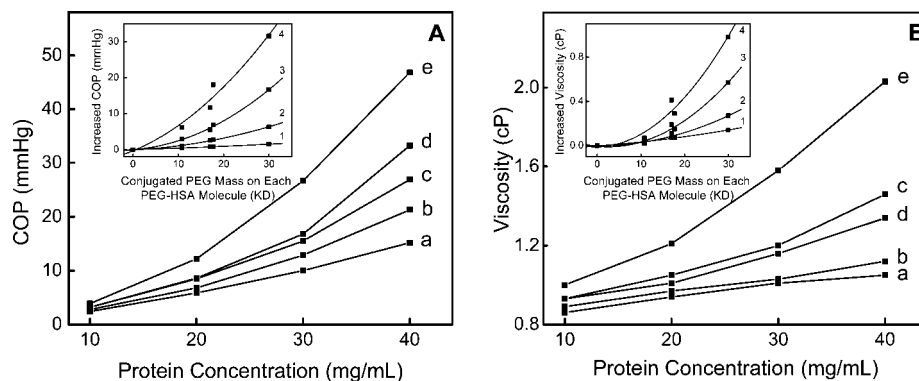


Figure 7. Colloid osmotic pressure (A) and viscosity (B) of HSA (a) and PEG-HSA conjugates, (TCP-PEG3K)₄-HSA (b), (TCP-PEG5K)₄-HSA (c), (TCP-PEG3K)₆-HSA (d), and (TCP-PEG5K)₆-HSA (e) as a function of the protein concentration. Inset A, relationships of conjugated PEG masses on PEG-HSA conjugates and the increased COP at different protein concentrations. Inset B, relationships of conjugated PEG masses on PEG-HSA conjugates and the increased viscosity at different protein concentrations. Curve 1, 1 g/dL; curve 2, 2 g/dL; curve 3, 3 g/dL; curve 4, 4 g/dL.

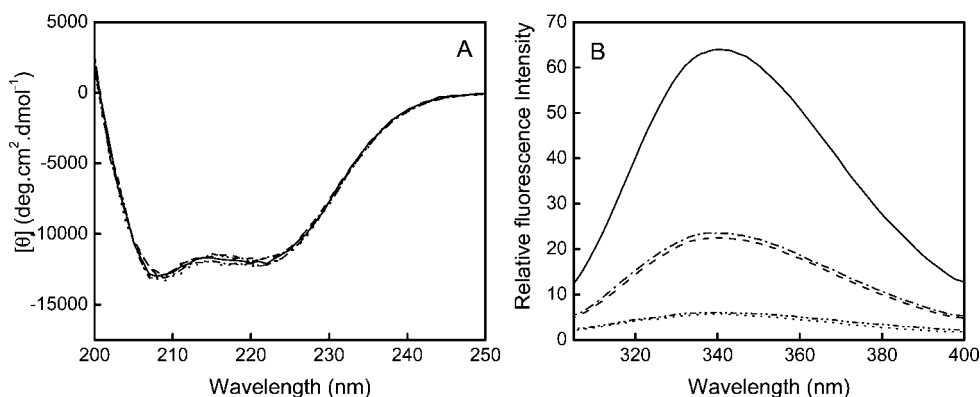


Figure 8. Circular dichroism (A) and intrinsic fluorescence emission (B) spectra of HSA (solid line) and conjugates (TCP-PEG3K)₄-HSA (dash), (TCP-PEG3K)₆-HSA (dot), (TCP-PEG5K)₄-HSA (dash dot), and (TCP-PEG5K)₆-HSA (dash dot dot).

increased viscosity as a function of PEG mass conjugated to HSA is presented in the inset of Figure 7B. Again, the viscosity as a function of the molecular mass of PEG in the conjugate increased exponentially, especially at high protein concentration.

(iii) *Influence of PEGylation on the Conformation of HSA: Influence of PEGylation on the Secondary Structure of HSA.* The CD spectra of PEGylated HSA in the far-UV region (200 to 250 nm) is compared with that of the unmodified HSA (Figure 8A). The CD spectra of PEG-HSA conjugates and albumin in the far ultraviolet region are essentially identical. Only very few changes are seen in the CD spectra, particularly in terms of the values of mean residue ellipticity [θ] at 208 and 222 nm, as a consequence of PEGylation. The results suggest that the α -helical conformation of HSA is not influenced by the PEGylation. Thus, the secondary structure of HSA is not adversely influenced on its PEGylation with PIT-PEG.

Intrinsic Fluorescence Measurement. The intrinsic fluorescence spectra of the PEG-HSA conjugates are analyzed to evaluate the conformational changes around the tryptophan residue (Trp 214) in HSA. When excited at 295 nm, all the PEGylated samples and HSA showed maximum emission intensity at the same wavelength (340 nm) (Figure 8B). The fluorescence intensity of PEGylated HSA, however, showed a significant decrease as compared to that of HSA, indicating that attachment of PIT-PEG on HSA quenched the intrinsic fluorescence of the Trp 214 residue of HSA. The fluorescence intensities of the two conjugates generated at pH 6.5 (using PIT-PEG3K and PIT-PEG5K) are similar; the PEG-HSA adducts generated at pH 9.2 also have a similar level of fluorescence quenching. Thus, the total amount of coupled PEG on the surface of HSA molecule does not dictate fluorescence of

PEGylated HSA. The level of quenching of fluorescence is dictated by the pH of PEGylation reaction, i.e., by the extent of PEGylation. The quenching effect is apparently a consequence of the specific formation of thiocarbonyl-phenyl linker between protein amino group and PEG chain. The hydrophobic phenyl linker section binds near the Trp 214 or its proximity and acts like some small hydrophobic molecules quenching the fluorescence of Trp 214 (37, 38). The quenching of the Trp 214 fluorescence clearly indicates that the conformation of the hydrophobic binding pocket in the second α -helix domain is affected.

DISCUSSION

Functionalizing PEG chains by quantitative modification of the hydroxyl group of methoxy PEG is a very crucial step in achieving a high-purity, reliable, and reproducible PEGylation reagent. If mPEG is subjected to multiple steps of organic synthetic reactions during the process of functionalization, the yield and/or purity of the final PEG reagent will decrease. A one-step functionalization process using a small molecular weight heterobifunctional reagent will minimize the exposure of mPEG to a multistep synthetic protocol. A quantitative modification of mPEG by the heterobifunctional reagent will significantly simplify the subsequent purification of the functionalized PEG. We have now developed such a one-step protocol for functionalizing the mPEG with a desired functional group. The reaction of phenyl isocyanate with hydroxyl group of mPEG is simple and quantitative and can serve as a prototype reaction for attaching a bifunctional reagent that carries the desired functional group at its distal end to the mPEG. The one-step functionalization of PEG by isothiocyanato phenyl isocyanate

anate as PEG-phenyl-isothiocyanate proceeds very efficiently. The functionalization of both mPEG3K and mPEG5K are nearly quantitative as assayed by the colorimetric method and ^1H NMR method (Table 1).

The isolation of the small molecular bifunctional reagent, isothiocyanato phenyl isocyanate, is not required in the synthesis of PIT-PEG, because its precursor, an azide, can be quantitatively converted *in situ* before its reaction with mPEG. However, in the case of synthesis of PEG-maleimides by this approach, the isolation of the small molecular weight bifunctional reagent, *p*-maleimido phenyl isocyanate, is preferred, as it gives a better preparation of PEG-maleimide (39).

The PIT-PEG exhibits a high stability against hydrolysis at room temperature. When PIT-PEG was dissolved in phosphate solution of different pH values (from pH 5.5 to 9.2) at room temperature, only a negligible level of hydrolysis occurred within the first 12 h. The high stability of the PIT-PEG permits the use of the high pH as well as room temperature for PEGylation, as far as the protein is stable under these conditions. The high stability of the PIT-PEG has also made it possible to develop an analytical procedure to calculate the amount of consumed PEG in reaction (i.e., conjugated to protein) using a SEC, that facilitates the easy calculation of the extent of PEGylation.

Generally, when a protein chemical modification reagent exhibits a high stability against hydrolysis, it will exhibit a low reactivity for PEGylation of protein. In spite of the high stability of the PEG-isothiocyanate against hydrolysis, it displays an acceptable level of reactivity to modify HSA at room temperature. Incubation of 0.5 mM HSA and a 10-fold molar excess of PIT-PEG at room temperature for 6 h at pH 9.2 generates hexaPEGylated HSA, i.e., 60% of PEG reagent is utilized and conjugated to HSA. The reaction of PEG-isothiocyanate with an amino group in albumin involves the nucleophilic attack of the amino group and the generation of a stable linkage, thiocarbamoyl linkage, between the PEG and the albumin. PIT-PEG reacts better at slightly alkaline pH, as more amino groups on proteins will be unprotonated. Nearly identical levels of PEGylation of HSA by PIT-PEG3K and PIT-PEG5K at both pH 6.5 and 9.2 reflect that the PEGylation reaction is correlated to the pH and the length of PEG chain of PIT-PEG has limited influence on the PEGylation reaction under the present reaction condition.

PEGylation of HSA with PIT-PEG proceeds very fast in beginning, and then slows down after 2 h reaction (Figure 5C). The PEGylation is expected to target the single α -amino group of amino-terminal Asp and the ϵ -amino groups of internal Lys residues. HSA has a single free SH group, which is a nucleophile. To establish whether the thiol has been conjugated with PEG, we measured the number of SH group on HSA (lyophilized powder, the thiol group was blocked to prevent dimerization) by reaction with 4-PDS (40), and it is 0.32. After hexaPEGylation, the number of SH groups on modified HSA is about 0.31. The results suggested that the PIT-PEG does not react with the thiol group on HSA. The observation that PEGylation of HSA proceeds very fast in the beginning and then slows down after 2 h reaction can be a reflection of one or both of these aspects: (i) the reaction rate slows down as the low pK_a amino groups have been modified; and (ii) PEG chains that are already conjugated to albumin molecules make it difficult to conjugate new PEG chains to the PEGylated albumin molecule. As a combination of these molecular aspects of the PEGylation of HSA with PIT-PEG, at pH 9.2 the hexaPEGylation of HSA appears to be complete under the present experimental condition. Increasing the concentration of PEG reagent from 20- to 40-fold molar excess of HSA has very little influence on the extent of PEGylation (Lanes 7 to 9, Figure

5A), which is consistent with the above conclusion. This suggests that the strong rule of steric factors is slowing down the PEGylation reaction on hexaPEGylation of proteins of molecular size of 65 KD. A similar conclusion has been made with regard to the hexaPEGylation of Hb (41).

The PEGylation studies of Hb exposed that the unique molecular properties could be engineered into proteins by the conjugation of PEG chains, namely, unusually enhanced hydrodynamic volume, high viscosity, and high COP (12, 14, 15, 40–42). These molecular properties, which are also the unique properties of plasma volume expanders that endow unique microvascular properties to the molecules, have been attributed to the nonhypertensive property of PEGylated Hb (12). Accordingly, we have considered the PEGylated albumin generated by extension arm facilitated protocol as plasma volume expanders; and the studies of both dodecaPEGylated and hexaPEGylated albumin with animal models have indeed established that PEGylated albumin is a new therapeutic protein with some unique potential as a plasma volume expander (28, 29). Therefore, we suggest that (TCP-PEG5K)₆-HSA is also potential therapeutic protein as a plasma volume expanders.

PEGylation of HSA has little influence on the secondary structure of HSA. On the other hand, the fluorescence of HSA (Trp 214) is influenced by PEGylation. The extent of quenching of fluorescence is dependent on the extent of PEGylation, that is, the number of copies of PEG chains conjugated, however, not on the size of PEG chain conjugated. Apparently, it is the phenyl linker that quenched the fluorescence of Trp-214 on HSA, and clearly suggests the proximity of the conjugated chains to the Trp residue.

In summary, we have designed and developed a novel PEGylation reagent, PEG-phenyl-isothiocyanate. The new PEG reagent displays a high stability against hydrolysis around neutral pH, and also displays a good level of chemical reactivity with protein amino groups.

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