

## Synthesis of PEGylated Human Serum Albumin by Maleimide-Thiol Click Chemistry and Histopathological Evaluation in Mice Model of Carrageenan-Induced Inflammation

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### Abstract

Occasional incompetence of human serum albumin (HSA) as a plasma expander is linked to severity of the underlying endothelial cell injury so that HSA can readily extravasate if structural integrity of the endothelial is compromised. Consequently, the leaking HSA may exacerbate the oncotic plasma gradient and results in capillary leak syndrome; therefore, we hypothesized that HSA modification by the covalently attaching multiple PEG (polyethylene glycol) groups would result in enhanced retention of HSA in blood circulation in animal model of acute inflammation. PEGylation of HSA was performed by maleimide-thiol chemistry. The products were characterized by polyacrylamide gel electrophoresis and size-exclusion chromatography. Extravasation of PEGylated HSA was compared to the native protein in BALB/C mice model of carrageenan-induced inflammation by histopathological evaluations. HSA was thiolated by either reaction of 2-iminothiolane (Traut's reagent) with lysine side chains or DTT reduction of disulfide bridges for subsequent reaction with methoxyPEG-maleimide (mPEG-Mal). The PEGylation reaction was optimized in terms of pH and mPEG/HSA molar ratio, producing high conjugation yield. mPEG 5 KDa-HSA demonstrated higher osmotic pressure and more homogeneous weight distribution than mPEG 20 KDa-HSA. According to the histopathological findings, mPEG 5 KDa-HSA showed lower extravasation in comparison to native HSA in carrageenan-induced inflammation model. Conclusively, PEGylated form of HSA might lessen the need of frequent HSA administration via decreasing the capillary leakage and endow a product with improved intravascular retention.

**Keywords:** Acute Inflammation, Human Serum Albumin, Maleimide-Thiol, Click Chemistry, Mice Model, PEGylation.

### 1. Introduction

Human serum albumin (HSA) is among the most abundant protein and approximately constitutes 60% of serum proteins. It presents a heart-shape molecule with molecular weight of 66463

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Da. HSA molecule consists of 17 disulfide bridges and 35 cysteine residues and a free thiol group at cysteine 34 (1, 2). It plays a major role in body, including normalizing water distribution and maintenance of plasma colloid oncotic pressure (COP). It is indicated in hypovolemic conditions secondary to burns, hemorrhagic shock, traumatic shock, cirrhosis and hypoalbuminemia.

There are many challenges, according to production of albumin, as it is mainly produced by plasma fractionation. Gene manipulation techniques also have many shortcomings including costly production. Also, in diseases such as burns and shock, increasing microvascular permeability results in trans-capillary albumin leakage and finally the patient will suffer from interstitial edema as the result of poor intravascular retention. Unfortunately, the patient will be in a repetitive demand of albumin administration (3, 4).

Protein PEGylation, covalent attachment of polyethylene glycol (PEG), is a breakthrough research field in biological chemistry and pharmaceutical sciences (5, 6) that can make protein drugs more hydrophilic, non-aggregating, and non-immunogenic and more stable to enzymatic digestion (7). It can provide molecules with higher hydrodynamic diameters so that it may reduce extravasation (8, 9). Therefore, we hypothesized if a PEGylated form of HSA might lessen the demand for frequent albumin administration via decreasing the capillary leakage in inflamed paw of mice model. Various chemistries are generally employed for HSA PEGylation including N-terminal mono-PEGylation by aldehyde-amine Schiff base reaction (10), poly-PEGylation by maleimide-phenyl reagent (11) and solid-phase synthesis by acylation chemistry (12). In present study, synthesis of PEGylated HSA was performed by a highly specific maleimide-thiol chemistry. In the first step, thiolation of HSA was carried out by either through DTT reduction of natural disulfide bridges or thiolation of lysine residues in HSA protein. Then, the thiolated HSA was served for the reaction with mPEG-maleimide (mPEG-Mal), producing PEGylated HSA (mPEG-HSA). Regarding that PEG molecular weight determines the reaction yield, homogeneity and biological function of the PEGylated protein, the reaction products were compared for mPEG-Mal 5 kDa and 20 kDa. *In-vivo* study was also conducted to compare the extravasation pattern of FITC-labeled HSA and mPEG-HSA in inflamed tissues.

## 2. Materials and methods

### 2.1. Materials

MethoxyPEG-Maleimide (mPEG-Mal)

5 kDa and 20 kDa was purchased from Jenkem, USA. HSA was obtained from CSL Behring, Australia. Fluorescein isothiocyanate isomer I (FITC) was supplied by Sigma-Aldrich, USA. HPLC size exclusion column TSK-GEL G5000 PWXL was purchased from Tosoh, Japan. Other reagents and chemicals were of commercially available analytical grade and used without further purification. Carrageenan was kindly received as a gift from Dr. J. Khoshnood, Shiraz School of Pharmacy.

### 2.2. Protein PEGylation

HSA (1 mM) in HEPES/EDTA buffer (20 mmol/L HEPES buffer contains 87.5 mM diamine tetraacetic acid, pH 7.5) was incubated with different concentrations of Traut's reagent in range of 20-80 mM or 5 mM DTT's reagent in pH 7.5 and 30 °C for one hour. The mixtures were ultra-filtered three times with the same buffer using Ultracel Microcon YM-10 (Millipore, USA) at 10,000×g to remove the residual thiolation reagents. The Ellman's assay was done to determine the molar concentration of free thiol in each sample with reference to reduced glutathione by determining the light absorbance at 412nm by ELISA reader (BioTek, USA). In the second step, the thiolated HSA was incubated with Mal-PEG (5 or 20 kDa) at PEG/protein molar ratio (1:1, 2:1, and 4:1) in 30 °C for one hour. The reaction was stopped by adding excess amount of reduced glutathione and then transferring the mixtures to -20 °C freezer (Table 1).

### 2.3. Purification and characterization of PEGylated HSA

The reaction products were fractionated using Knauer HPLC equipped with TSK-GEL G5000 PWXL column. The mobile phase consisted of double deionized water and 87.5 mM EDTA was pumped at the flow rate of 0.5 ml/min. HSA or mPEG-HSA was detected by V7605 UV-visible detector at 280 nm. All the fractions were collected and vacuum concentrated. The physical mixtures and the resulting fractions were analyzed by SDS-PAGE. Accordingly, the samples were incubated at 80 °C for 5 min in the loading buffer, and then loaded into 10% polyacrylamide Tris-glycine gel. Electrophoresis was programmed using a one-step

**Table 1.** Different reaction conditions for PEGylation of human serum albumin (HSA) and related percentages of the products.

HSA thiolation reagent	PEG molecular weight	PEG/HSA molar ratio	Mono-mPEG HSA (%)	Poly-mPEG HSA (%)	Residual HSA (%)	Poly- / mono-mPEG HSA ratio
60mM Traut's reagent	5KDa	1	25.14	43.09	31.77	1.71
60mM Traut's reagent	5KDa	2	24.91	60.86	12.23	2.44
60mM Traut's reagent	5KDa	4	2.17	97.63	ND	44.99
60mM Traut's reagent	20KDa	1	12.03	45.43	32.44	3.77
60mM Traut's reagent	20KDa	2	11.64	68.17	19.19	5.85
60mM Traut's reagent	20KDa	4	1.50	98.50	ND	65.55
5 mM DTT	5KDa	1	13.11	26.94	59.77	2.05
5 mM DTT	5KDa	2	19.57	41.93	38.5	2.14
5 mM DTT	5KDa	4	10.70	89.30	ND	8.34
5 mM DTT	20KDa	1	17.85	38.89	42.02	2.17
5 mM DTT	20KDa	2	5.57	73.47	20.96	13.19
5 mM DTT	20KDa	4	6.82	93.18	ND	13.66

\*Not Detected.

mode applying constant current (12 mA). The gels were stained with Coomassie Brilliant Blue R-250 for one hour and then de-stained overnight. Gel images were scanned and analyzed by UN-SCAN IT software v6.0.1 to quantitate the resulting protein bands. The PEGylation reaction was optimized for the method of HSA thiolation, the reagents molar ratio and Mal-PEG molecular weight (5 vs. 20 kDa) to obtain high degree of HSA PEGylation as determined by SDS-PAGE.

#### 2.4. Osmotic pressure determination

Colloidal osmotic pressure (COP) of the purified PEGylated HSA product was determined by Osmomat 010 freezing point osmometer (Gonotec GmbH). The measurements were done in triplicates for 50  $\mu$ l of 1mM solutions of native HSA or PEG-HSA purified by Ultracell centrifugal device of 10 and 30 kDa for the respective Mal-PEG reagents of 5 and 20 kDa. Deionized water was used as blank.

#### 2.5. Fluorophore labeling of HSA or mPEG-HSA

HSA or the purified mPEG-HSA was FITC-labeled according to the published methods (5). The protein samples (1 mg in carbonate buffer 0.1 M at pH 9) were incubated with 10  $\mu$ l of FITC solution in DMSO (10 mg/ml) for 4 hours at 8 °C with gentle stirring. Then, the reactions were left in refrigerator for 24 hours to complete the reaction. The mixture was filter-washed three times with 10 kDa Ultracell filters in order to remove residual FITC. The pure mixtures were collected in clean tubes. The fluorescent absorption was read after substantial dilution with TECAN fluorescent plate-reader (Infinite200M, Austria). The labeling ratio (D/P) was calculated from Eq. 1.

$$D/P = \frac{A_{\text{max}} \cdot \epsilon_{280}}{(A_{280} \epsilon_{\text{max}} - A_{\text{max}} \cdot \epsilon'_{280})} \quad (\text{Eq. 1})$$

Where  $A_{280}$  and  $\epsilon_{280}$  are the absorbance and extinction coefficient of HSA at 280 nm, respectively.  $A_{\text{max}}$  and  $\epsilon_{\text{max}}$  are the absorbance of FITC-labeled protein and the extinction coefficient

of free FITC at 480 nm, and  $\epsilon'_{280}$  is the extinction coefficient of FITC at 280 nm.

### 2.6. Histopathological evaluation in carrageenan induced inflammation in mice paw

The vascular permeability of HSA and mPEG-HSA were compared in Carrageenan-induced capillary leak model (7). Three to four weeks-old male BALB/c mice weighing 22-25 g were supplied by the Comparative Medicine Center of Shiraz University of Medical Sciences (SUMS). The animals were housed and cared according to the SUMS guidelines for Animal Care and Use. Animals were temperature- ( $23 \pm 2$  °C) and humidity-controlled ( $56 \pm 4$  %), under a 14:10 h light/dark cycle. Inflammation was produced in left hind paw by direct injection of 0.1 ml of 1% carrageenan solution in normal saline. Mice were anesthetized, shaved thoroughly and randomly allocated to two experimental groups: HSA-treated model group (Carrageenan + FITC-HSA), and mPEG-HSA-treated model group (Carrageenan + FITC-mPEG-HSA). Paw swelling was confirmed by the subsequent change in the paw diameter as measured by Vernier caliper. Then, fluorescent-labeled HSA or mPEG-HSA solutions (0.21 mg/kg) were administered as a single slow I.V. injection in normal saline via the tail vein of BALB/c mice 4 hours after the model induction. Left and right hind paw were amputated, then the flushed paw tissue was removed and fixed in 10% formalin. The fluorescence intensities of the fixed sections of mice paw were examined with Eclipse E-600

fluorescence microscope (Nikon, Japan) using 40× objectives.

### 2.7. Statistics

Effects of Mal-PEG/HSA molar ratio and the Mal-PEG molecular weight on degree of HSA PEGylation and the ratio of poly-/mono-PEGylated products were analyzed by two-way analysis of variance (ANOVA). Levels of each significant factor were compared by LSD post-hoc test with Bonferroni correction for  $\alpha$  error of multiple testing. P values less than 0.05 were considered statistically significant. Statistical analysis was done by Minitab software ver. 16.0.1.

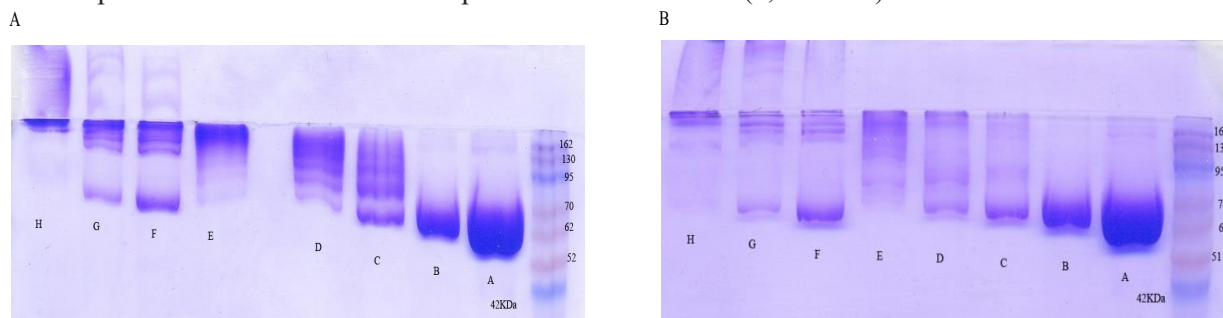
## 3. Results

### 3.1. HSA Thiolation

Mal-PEG is a thiol-reactive PEG derivative that can be used to selectively modify protein, peptide or any other surfaces with available free thiol groups. It was found the degree of thiol modification increases at higher concentrations of Traut's reagent. Comparing free thiol concentration using Ellman's assay ( $\epsilon=0.0077$ ,  $R^2=0.9996$ ), it was revealed that 60 mM Traut's reagent produced 2 moles of free thiol per 1 mole of HSA as similarly achieved for 5 mM DTT's reagent.

### 3.2. HSA PEGylation

The PEGylation reaction was carried out for different molecular weights of Mal-PEG (5 and 20 kDa), various molar ratios of Mal-PEG to HSA (1, 2 and 4) and HSA thiolation methods



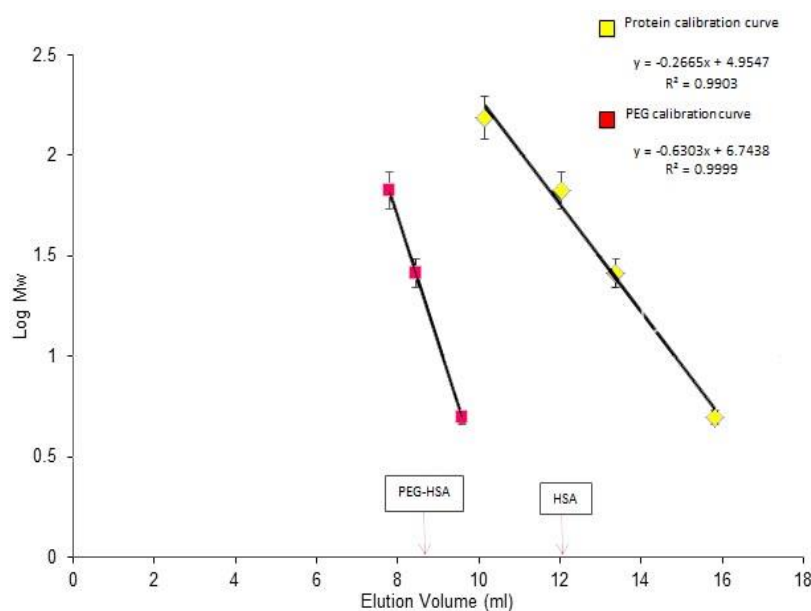
**Figure 1.** SDS-PAGE (10%) for the PEGylation of thiolated human serum albumin (HSA) by Traut's reagent (A) or DTT (B); Lane A: thiolated HSA, Lane B: physical mixture of HSA and PEG, Lane C, D, E: thiolated HSA reaction with Mal-PEG 5 kDa at the respective molar ratio 1, 2 and 4, Lane F, G, H: thiolated HSA reaction with Mal-PEG 20 kDa at the respective molar ratio 1, 2 and 4.

(Traut's and DTT reagents). Two-way ANOVA showed that the degree of PEGylation doesn't differ for Mal-PEG 5 and 20 kDa (respective P values of 0.225 and 0.282); however, the effect of the reagents ratio was determined significant (P value=0.022). Moreover, the ratio of poly- to mono-PEGylated product changed with the reagent ratio (P value=0.029). As shown in Figure 1A, the degree of PEGylation was only determined statistically different for the reagent ratio = 4. In a parallel experiment, the statistical analysis was similarly conducted for the thiolated HSA by DTT's reagent (Figure 1B). No significant effect of the investigated factors was determined on the degree of HSA PEGylation and the ratio of poly-/mono-PEGylated product (respective P values of 0.224 and 0.270). Therefore, the protein thiolation method plays an important role in the subsequent PEGylation by maleimide-thiol chemistry.

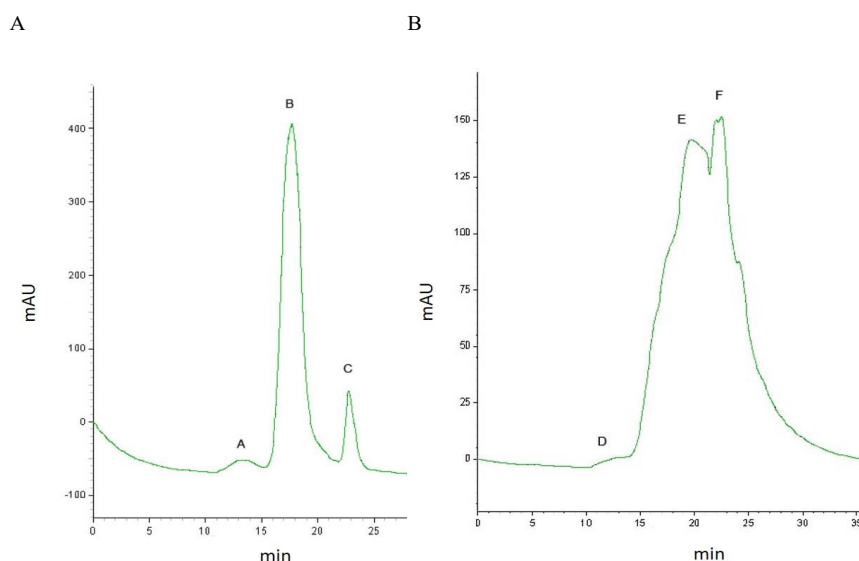
### 3.3. Characterization of mPEG-HSA

Regarding that higher degree of HSA PEGylation results in larger molecular weight of mPEG-HSA and possibly lower chance of vascular extravasation, it was aimed to obtain poly-PEGylated HSA product. As shown in Figure 1, higher degree of HSA PEGylation was achieved for the thiolated HSA by the Traut's reagent that was chosen for the optimization of the reaction condi-

tion. Regardless of which m Mal-PEG molecular weight was chosen, the optimum condition was 60 mM Traut's reagent and the reagents molar ratio of 4 at pH=7.5 and 30 °C. The protein products were analyzed by size-exclusion column chromatography calibrated for standard proteins and PEGs of different molecular weights (Figure 2). In size-exclusion chromatograms (Figure 3A and Figure 3B), there are three well-resolved peaks for 5 kDa Mal-PEG while several interfering peaks related to 20 kDa Mal-PEG appears. To identify the protein fractions in the size-exclusion chromatograms, further characterization of the collected fractions was performed by SDS-PAGE (Figure 4), showing that the peak C is related to residual native HSA, the peak B or E correspond to the poly-PEGylated HSA product, though the fraction E contains also the native protein. Fraction A and D are related to traces of protein aggregates which have been formed during HSA processing. It was revealed that the PEG 5kDa-HSA conjugate contained only about 6.7% native HSA (fraction C), demonstrating HSA PEGylation of about 93.3%. In contrast, residual HSA was clearly observed in fraction F of PEG 20 kDa-HSA (Figure 4). As shown in Figure 2, the elution volume of native HSA (67 KDa) was shifted from 12.15 ml to 8.81 ml for the fraction B (poly-PEGylated HSA), indicating successful PEGylation. Accordingly, total weight of the conju-



**Figure 2.** Elution volume versus logarithm of molecular weight (kDa) for standard proteins and poly ethylene glycols using the TSKgel 5000 PWXL column.



**Figure 3.** Size exclusion chromatograms for the reaction mixtures of thiolated human serum albumin (HSA) and Mal-PEG 5 kDa (A) and 20 kDa (B) using the TSKgel 5000 PWXL column.

gated PEG 5 kDa was estimated about 19 kDa that equals to the conjugation yield ~90%.

COP of 1 mM native HSA, PEG 5kDa-HSA, PEG 20kDa-HSA were determined  $50 \pm 3$ ,  $115 \pm 1$  and  $244 \pm 6$  mmHg, respectively, confirming increased COP of HSA following the PEGylation reaction.

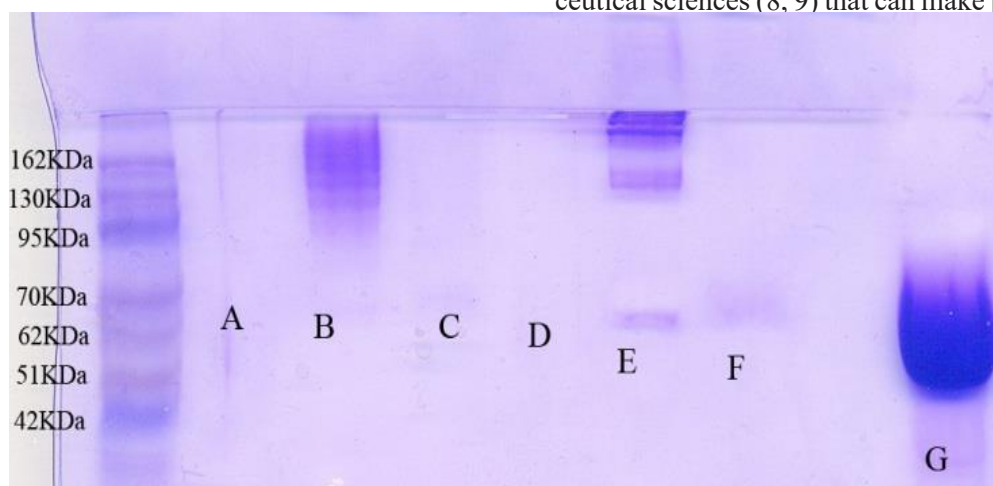
### 3.4. Animal Study

In order to examine capillary leak of mPEG-HSA in comparison with HSA, the proteins were fluorescently labeled with FITC. Average

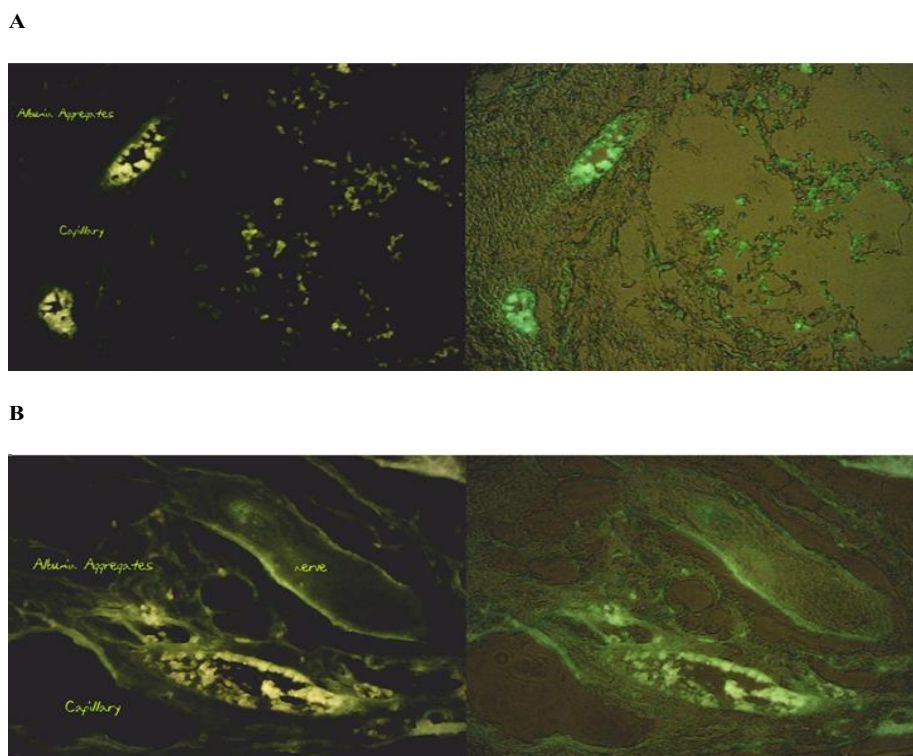
numbers of the conjugated FITC were calculated  $7 \pm 1$  and  $9 \pm 1$  molecules per HSA or mPEG-HSA, respectively. Fluorescent microscope images (Figure 5A) showed the extravasation of I.V injected native HSA (FITC-labeled) in left paw section in mice. In comparison, no or a little extravasation into interstitial space was noticed for the mPEG-HSA injected mice (Figure 5B).

### 4. Discussion

Protein PEGylation is a breakthrough research field in biological chemistry and pharmaceutical sciences (8, 9) that can make protein drugs



**Figure 4.** SDS-PAGE of different size exclusion chromatography fractions (A-F); A, D: possible residual HSA aggregate; B, E: poly PEGylated HSA products; C, F: residual (unreacted) HSA; G: native HSA.



**Figure 5.** Typical light (left) and fluorescent micrographs (right) of the inflamed mice paw induced by subcutaneous injection of carrageenan. After 4 hours, the mice were received slow I.V. injection of native HSA (A) and PEG 5 kDa-HSA solution (B), scale 10 $\mu$ m.

more hydrophilic, non-aggregating, and non-immunogenic and more stable to enzymatic digestion (10). PEGylated HSA has shown a higher biological half-life and lower vascular permeability (11). Various chemistries are generally employed for HSA PEGylation including N-terminal mono-PEGylation by aldehyde-amine Schiff base reaction (12), poly-PEGylation by maleimide-phenyl reagent (13) and solid-phase synthesis by acylation chemistry (14). Methoxy PEG maleimide (Mal-PEG) 5 kDa and 20 kDa reagents were chosen since they site-specifically react with HSA molecules through Michael addition (15, 16). It happens either via thiolation of HSA by DTT reduction of core disulfide bonds (17) or transformation of primary amines of peripheral lysine to free thiol groups with Traut's reagent (18). In order to optimize the PEGylation reaction condition, as series of possible factors such as pH (data not shown), the reagents molar ratio, PEG molecular weight and the method of HSA thiolation were taken into

account (Table 1). As shown in Fig. 1, to achieve high degree of PEGylation (or at the same time high proportion of poly-/mono-PEGylated product), relatively high ratio of Mal-PEG/HSA (4:1) is required. Moreover, the reaction was performed at near neutral pH to avoid non-specific reaction with primary amines (19). The method of thiolation using Traut's reagent resulted in a better product in comparison with DTT (Fig. 1). HSA disulfide bridges are buried in the core of protein and any reduction by DTT can promote formation of amorphous aggregates (20). In contrast, thiolation of superficial HSA lysine amino acids by Traut's reagent provides less steric hindrance and more accessible sites for reacting with Mal-PEG. Purification of mPEG-HSA product can be performed by various techniques such as ion-exchange chromatography (14), size exclusion chromatography (21), hydrophobic interaction membrane chromatography (12) and non-chromatographic methods (22) such as ultrafiltration as has been done

in the present study. The resulting mPEG-HSA product was then characterized by size exclusion chromatography that shows a remarkable change in the elution volume of HSA (Fig. 3), confirming successful PEGylation of HSA. Although it was assumed that higher molecular weight of the conjugated PEG relative to HSA can be achieved by Mal-PEG 20 kDa than Mal-PEG 5 kDa at the same reaction condition, Mal-PEG 5 kDa demonstrated more uniform poly-PEGylated products without detectable residues of native HSA in the chromatogram (Fig. 3A and 3B). Similarly, it has been shown that by increasing molecular weight of PEG in range of 2-10 kDa, the PEGylation degree of lysozyme decreases (23). Further characterization of mPEG-HSA by MALDI-TOF-MS spectroscopy is required to calculate the average number of PEG chains per HSA molecule.

To demonstrate therapeutic value of mPEG-HSA, COP was first determined at the protein concentration similar to HSA. The results indicated that the COP of native HSA increases about 2.3 and 4.9 times with 5 and 20 kDa mPEG-HSA, respectively. Similarly, Cabrales *et al.* showed that PEGylated HSA presents more than 2 folds higher COP than native HSA. Also, 4% PEGylated HSA provided long-lasting resuscitation and a volume expansion similar to 10% HSA at early times (13). From in-vivo evaluation of carrageenan induced acute inflammation in the model mice, it was found that unlike native HSA, to a much less extent the mPEG-HSA product leaked out of inflamed endothelium into interstitial space of left mice paw (Fig. 5). This issue can be explained by the growing size of HSA molecules following protein PEGylation that can retain HSA in the vas-

cular space in a moderate to severe capillary leak syndrome (24, 25). Similarly, Zhao *et al.* carried out synthesis of mPEG-HSA by Mal-PEG 20 kDa through site-specific reaction with cysteine 34 and found that the product exhibits high efficiency in vivo (26).

## 5. Conclusion

By increasing the molecular size of HSA by PEG modification, the protein flow can be reduced across capillary membranes under conditions with increased capillary leakage. The present study shows the advantageous use of HSA thiolation by Traut's reagent and a rapid conjugation by maleimide-thiol click reaction to synthesize mPEG-HSA with high purity. Increased intravascular COP as well as reduction in vascular permeability of the infused albumin can consequently promote intravascular fluid retention. For future study, it is suggested to investigate any change in HSA secondary structure and colloidal dispersion by circular dichroism and light scattering, respectively; Furthermore, extensive characterizations of the product safety and efficiency and in-vivo bio-distribution are required.

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## Conflict of Interest

None declared.

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