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Mini Review

PEGylated Human Serum Albumin: Review of PEGylation, Purification and Characterization Methods

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Abstract

Human serum albumin (HSA) is a non-glycosylated, negatively charged protein (Mw: about 65-kDa) that has one free cystein residue (Cys 34), and 17 disulfide bridges that these bridges have main role in its stability and longer biological life-time (15 to 19 days). As HSA is a multifunctional protein, it can also bind to other molecules and ions in addition to its role in maintaining colloidal osmotic pressure (COP) in various diseases. In critical illnesses changes in the level of albumin between the intravascular and extravascular compartments and the decrease in its serum concentration need to be compensated using exogenous albumin; but as the size of HSA is an important parameter in retention within the circulation, therefore increasing its molecular size and hydrodynamic radius of HSA by covalent attachment of poly ethylene glycol (PEG), that is known as PEGylation, provides HSA as a superior volume expander that not only can prevent the interstitial edema but also can reduce the infusion frequency. This review focuses on various PEGylation methods of HSA (solid phase and liquid phase), and compares various methods to purifiy and characterize the pegylated form.

Introduction

Human serum albumin (HSA) is a non-glycosylated, negatively charged plasma protein, with a molecular weight of about 65-kDa that is composed of 585 amino acids in a single polypeptide chain with a quaternary helix-line structure. HSA has a ‘‘heart-shaped’’ three-dimensional structure that the binding sites for many ligands are located in the center of the molecule which is made up of hydrophobic radicals, while the outer part of it is composed of hydrophilic ligands.[1-5]

Negative net charge of HSA is due to exceeding number of the acidic amino acid residues to the basic ones, which results in about 15 negative net charges per molecule, at pH 7.0 (pI 4.7). In structure of HSA there is one free cystein residue (Cys 34) and 17 disulfide bridges that these disulfide bridges significantly contribute to the stability of this protein, and make clear its long biological life-time.[3,4]

Hepatocytes produce about 9-12 g of albumin daily that there are no reservoirs to store it, and the changes in colloid osmotic pressure and the osmolality of the extravascular liver space are involved in controlling the production rate.[5] Albumin is the most abundant protein in human plasma with a physiological concentrations of 40 - 50 g/L, (3.5 to 5 g/dL), that constitutes 50% of the total plasma protein content and accounts for 70% of plasma's oncotic pressure (25 mmHg). The total body albumin content is 4 to 5 g/kg of which one third is in the intravascular space and two thirds is in the extravascular compartment. Albumin is exchanged in a constant process between plasma and interstitial, and its half-life averages 15 to 19 days.[1,2,5]

In treatment of several diseases, including hypovolemia, shock, burns, surgical blood loss, trauma, hemorrhage, cardiopulmonary bypass, acute respiratory distress syndrome, hemodialysis, acute liver failure, chronic liver disease, nutrition support, resuscitation, and hypoalbuminemia, this protein has wide clinical application.[3] Recently, it has identified that hypoalbuminemia has an association with pulmonary edema and even in heart failure as a potent prognostic biomarker.[1]

HSA is a multifunctional protein that in addition to maintaining colloidal osmotic pressure (COP) plays other roles related to its capacity to bind other molecules and ions. These include buffering through binding hydrogen ions; transporting hormones; and neutralizing toxins such as bilirubin as well as contributing to the redox potential of plasma and binding and transporting drugs.[2] Albumin is a free radical scavenger and is a major source of reduced sulphydryl groups, the thiols, which are involved in nitric oxide metabolism.[2-5] Also it has a role in the maintenance of membrane permeability.[5]

HSA solution is commercially available as 5% and 20% albumin. 5% HSA is formulated to have the same oncotic pressure as normal plasma. Their manufacture is through

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fractionation of human plasma in ethanol (the Cohn fractionation scheme) and heat treatment for several hours. The yield of this method is at least 95%. Using ion exchange and/or gel filtration chromatographic steps increasing purity to 99% is achievable at the cost of a further reduction in recovery.[6]

As has mentioned, HSA is a monomeric multi-domain macromolecule, representing the main determinant of plasma oncotic pressure and the main modulator of fluid distribution between body compartments.[3] On the other hand we know that critical illnesses alter the distribution of albumin between the intravascular and extravascular compartments, and the decrease in its serum concentration will not compensate until recovery phase of the illness. So using exogenous albumin at this situation to increase its serum concentration is beneficial. But as the altered distribution of albumin in critical illnesses is due to increase in capillary leakages,[7] and as the Size of protein has always been deemed to be an important consideration in retention within the circulation,[5] therefore increasing the molecular size and the hydrodynamic radius of HSA may be a potential approach to achieve a superior volume expander that not only can prevent the interstitial edema but also can reduce the infusion frequency. Covalent attachment of poly ethylene glycol (PEG) to the protein that is known as PEGylation, in addition to its wide advantages such as increasing thermal and physical stability, protection against enzymatic degradation, and decreasing the immunogenicity, antigenicity and toxicity of protein,[8-14] can increase the apparent size of protein, thus increasing its half life by reducing renal filtration and altering biodistribution.[12,15-17] Various factors that can affect the properties of PEGylated protein and PEGylation reaction include: the molecular weight, structure and the number of PEG chains attached to the protein; the PEGylation site on the protein; PEGylation chemistry; pH, temperature and time of reaction; protein to PEG molar ratio; and even purification methods.[13,18] This review will focus on various PEGylation methods have done on HSA and also will compare different purification and characterization methods have used to separate and characterize the PEGylated HSA.

PEGylation of HSA

Liquid-phase PEGylation

This type of PEGylation is the usual method and can be done in a tank as a batch reaction. At this method the protein is reacted with the PEG reagent in a stirred tank reactor in the presence of other reagents (if required).

Site specific PEGylation

PEGylation at cystein 34: Ting Zhao et.al[17] has pegylated HSA specifically in Cys34 with a 20kDa PEG-maleimide. To do this reaction they benefited from the advantages of the reactivity of the sulfhydryl group of free Cys34 of HSA and the high specificity of 20kDa PEG-Mal for that group. They examined various protein/polymer molar ratios and different pH values of reaction buffer which was 50mmol/L phosphate, 10 mmol/L ethylene diaminetetraacetic acid (EDTA). Also they incubated the reaction mixture at 37°C for various durations. In order to quench the reaction, after the incubation period has passed, they diluted the reaction mixture with reaction buffer to 10 fold.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has applied to investigate the modification rates and homogenicity of the final reaction mixtures, and results indicated that the mono-pegylated HSA is chemically well-defined and molecularly homogeneous.

After characterizing the purity using SDS-PAGE, PEGylated site and the secondary structure of PEGylated protein were analysed using, thiol blockage method and circular dichroism (CD) measurement respectively. The pharmacokinetic of modified protein was investigated in LPS induced lung injurey mouse, LPS induced sepsis model with systemic capillary leakage and normal mouse. They inferred that the chemically well defined and molecularly homogeneous PEGylated HSA is superior to native HSA in capillary permeability increase related illness because of following reasons:

- higher biological half-life of PEG-HSA (2.3 times)
- lower vascular permeability of PEG-HSA
- Better recovery in blood pressure and haemodilution observed in rats treated with PEG-HSA, compared with its native form

Also even this monopegylated HSA may be useful in preventing dimerisation.

N – Terminal PEGylation

N-Terminal PEGylation is a site-specific attachment that takes advantage of the differences between the pKa values of the α-amino group of the N-terminal amino acid residue and the ε-amino groups of the Lys residues in the protein backbone. HSA has 58 Lysine residues[19] that its ε-amino group has a significant high pK_a value of about 10.8, which is more basic than the N-Terminal α-amino group (pK_a value of 8.0).[20] As Kinstler et al. has disclosed,[21-23] mPEGpropionaldehyde, such as other aldehydes, under acidic conditions (approximately pH 5) is largely selective for the N-terminal α-amine because of its lower pK_a compared to other nucleophiles.

To achieve the N-terminally monoPEGylated HSA, as has shown schematically at Figure 1, mPEG-propionaldehyde should be coupled to free α-amino group of the N-terminal amino acid residue of protein through a reductive alkylation via Schiff base reaction, using sodium cyanoborohydride, a mild reducing agent, to give a stable secondary amine linkage.[13]

At 2012 Ting Zhao et al have conjugated HSA with 20kDa mPEG-propionaldehyde at free α-amino group of the N-terminal amino acid residue to obtain monoPEGylated HAS.[16] After Incubation time is elapsed they diluted the mixture to 10 fold with 10 mM phosphate buffer, pH 6.5 to stop the reaction. The reaction condition which they have used is summarized in Table 1.

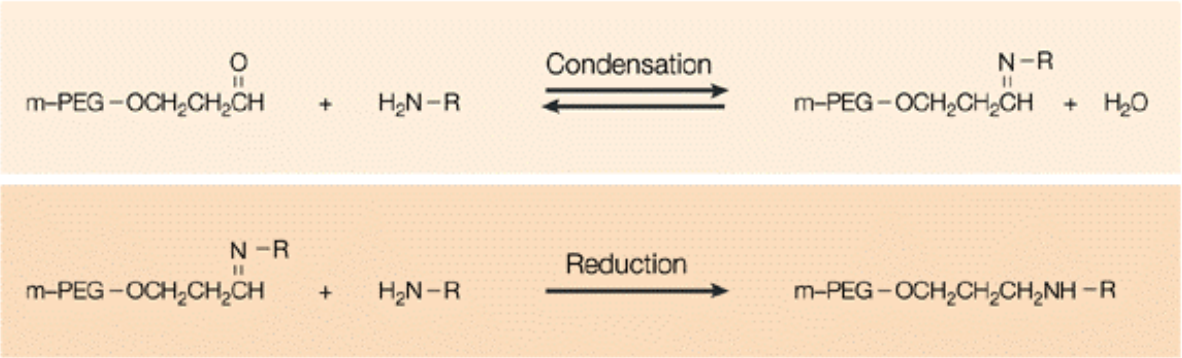

Figure 1. Reductive amination using PEG-propionaldehyde[24]

Table 1. Reaction condition for N-terminal PEGylation of HSA

Concentration of HSA	Concentration of 20kDa mPEG-propionaldehyde	Molar ratio of PEG/protein	Buffer & pH	Temperature	Incubation time
25 mg freeze-dried HSA at a 5 mg/mL concentration	23 mg	3:1	10 mM sodium phosphate buffer, pH 6.0, containing 20 mM sodium cyanoborohydride	4°C	For 36h while stirring

Mono-PEGylated HSA is separated on DEAE sepharose FF column using ion-exchange chromatography by linear gradient. The fraction of monoPEGylated HSA is desalted by ultrafiltration (30 kDa cut-off membrane), and its concentration was determined using bicinchoninic acid (BCA) assay method.[16]

The following analysis (Table 2) has done on native and PEGylated HSA and the results showed 2.2 time higher biological half-life and a lower vascular permeability for PEG-HSA.

The same conclusion as previous studies of this group can be propose here; that molecularly homogeneous PEG-HSA could be a superior candidate as a plasma volume expander in capillary permeability increase related illness.

Table 2. Comparison of hydrodynamic radius between Native and hexa PEGylated HSA $(TCP\text{-}PEG5K)_6$-HSA

-	Native HSA	$(TCP\text{-}PEG5K)_6$-HSA
Molecular radius (nm)	3.95	6.57
Viscosity (cP)	1.05	2.10
Solution colloidal osmotic pressure (mmHg)	15.2	48.0

Later at 2013 X Shang et,al have PEGylated HSA at N-terminal amine using P1PAL-10 (mPEG-propionaldehyde) in 100 mM sodium acetate buffer (pH 5.0) containing 10 mM sodium cyanoborohydrid at room temperature for 20 h in small shaken flasks and the molar ratio of polymer to protein was 4:1. They used hydrophobic interaction membrane chromatography on stack of hydrophilized polyvinylidene fluoride membrane discs for separation of unmodified and PEGylated HSA. The hydrophobicity difference between the different fractionated species was induced by the addition of a lyotropic salt into HSA PEGylation reaction mixture that caused phase transition of PEG (hydrophilic under normal condition) to a mildly hydrophobic form. As a result mono-PEGylated HSA was eluted first by reducing the salt concentration. SDS-PAGE, dynamic light scattering, and SEC combined with multi-angle light scattering have used to analyze the presence of species having molar mass consistent with mono-PEGylated HAS.[19]

Multi PEGylation

Multi PEGylation of HSA at 6 free generated thiol sites**:** Pedro Cabrales et al[24] have compared the effect of restoring intravascular volume with HexaPEGylated HSA (PEG-Alb). To produce the HexaPEGylated HSA, they reacted HSA for overnight with 2-Iminothiolane (2-IT) and Maleimide Phenyl PEG5K (MPPEG5K) in cold, which is known as thiolation mediated maleimide chemistry based protocol, Figure 2.

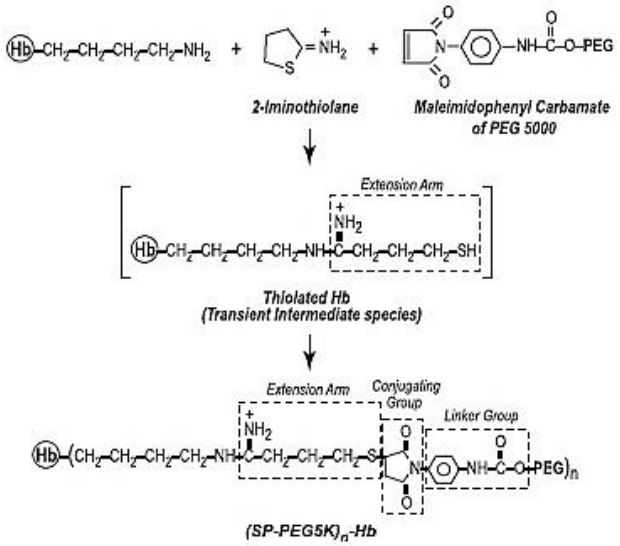

Figure 2. Schematic representation of the iminothiolane dependent thiolation mediated maleimide chemistry based PEGylation of Alb[25].

As a result of this reaction and due to standardized ratio of concentration of HSA to that of 2-IT, 6 free thiols generated on the surface of HSA that were estimated by 4-PDS method. They reacted this ratio of HSA and 2-IT with 3 fold molar excess of MPPEG5K over the thiols.

In order to remove the excess of 2-IT and MPPEG5K they dialyzed the reaction product through 50K MW cutoff membranes against PBS (pH 7.4) using a Minim™ Tangential Flow Filtration instrument, and confirmed their complete removal via monitoring the retentate by size exclusion chromatography at regular intervals. Then after concentrating the final product in the retentate with an Amicon concentrator using pressurized nitrogen 4 % and sterilizing it by filtration through 0.22 μM Millipore filters, they determined the concentration of PEGylated HSA using a Bradford protein assay method.

NMR and MALDI-TOF-MS results confirmed attachment of an average of 6 PEG5K chains to each HSA molecule. Colloidal oncotic pressure (COP) of HexaPEGylated HSA, HSA at 5% (HSA5) and HSA at 10% (HSA10) was about 48 mmHg, 22 mmHg and 46 mmHg; and their viscosity was about 2.08 cp at 150 s^{-1}, 0.9 cp at 150 s^{-1} and 1.0 cp at 150 s^{-1} respectively.

Authors conclude that PEG-Alb could be a better resuscitation solution because of its hemodynamic functional benefits and also the potential disadvantages of HSA.

Multi PEGylation of HSA at reactive primary amino groups: Fantao Meng et al at 2009[25] have generated PEGylated human serum albumin (PEG-HSA) with average of 3.5 and 6.0 PEG chains per HSA molecule by conjugating 10-fold molar excess of PEG- Phenyl-Isothiocyanate (PIT-PEG) 3kDa and 5kDa respectively to 0.5 mM HSA at pH 6.5 and 9.2. PIT-PEG has been synthesized by condensation of the bifunctional reagent 4-isothiocyanato phenyl isocyanate with monomethoxy PEG (mPEG). This kind of PEG reacts readily with amine groups at a pH of from almost 6.5 to 9.5.

Circular dichroism (CD) spectra of conjugates showed that effect of PEGylation on the secondary structure of HSA is not significant. The hydrodynamic radius (molecular radius), Viscosity and solution colloid osmotic pressure of 4% HSA increased on hexaPEGlation which occurred at pH 9.2 ($(TCP\text{-}PEG5K)_6$-HSA) (Table 2). Based on these results PEG-HSA conjugates could be a potential candidate as a plasma volume expander.

Solid phase PEGylation

Solid-phase adsorption method for PEGylation has developed by Xiaoyan Suo, et. al[26] in order to prepare homogeneous PEGylated proteins. Controlling the PEGylation process and so producing homogenous monoPEGylated proteins due to the steric hindrance of the solid-phase to the PEGylation is an advantage of solid-phase PEGylation. On the other hand as a result of this steric hindrance effect, and also lack of multi-PEGylated products, the yield of PEGylation is lower at this method. However above mentioned advantage is considerable point because in purification steps the interference of overlapped peaks due to varied PEG number and position of attachment has prevented.

Xiaoyan Suo‘s group used HSA (pI 4.7) as a model protein in their experiments. They adsorbed proteins on an anion-exchange chromatography media (AEC media), DEAE Sepharose Fast Flow, as adsorption solid-phase (mixture of 5 ml of AEC media and 1 ml HSA (1 mg ml-1) in 10 mM PBS buffer, pH 7.0), and then acylation chemistry-based PEGylation of absorbed proteins has done with incubation of column that is loaded by succinimidyl carbonate (SC)-mPEG5K (molar ratio of 1:10), for 4 h at room temperature. In Elution step linear gradient of 0–1 M NaCl in buffer A over 60 min is used for elution of PEGylated HSA.

They analyzed the peak was corresponding to the mixture of unreacted protein and PEGylated protein with Superdex 200 or 75 column, and achieved one more peak eluting earlier than native one on chromatogram while two additional major peaks were eluted earlier using the sample from liquid phase PEGylation.

SDS-PAGE and Size exclusion chromatography studies demonstrated that solid phase adsorption method circumvent the heterogeneity of the PEGylated products (monoPEGylated protein with the production yield of 35-47% compared to 70% of PEGlated product including mono-, di-, or multi-PEGylated proteinsin liquid-phase PEGylation method). Also little conformational changes of PEGylated protein have shown using Circular dichroism and intrinsic fluorescence studies.

Purification and analysis of PEGylated HSA

All analysis methods and the aim of study are summarized at Table 3.

Table 3. Analyzing the characteristics of N-terminally monoPEGylated and native HSA

Characteristics of N-terminally monoPEGylated and native HSA	Method of analysis	Result
purity	sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)	As it was expected homogeneous conjugate was obtained (the yield of the purified mono-PEGylated HSA was about 32%)
secondary structure	Circular dichroism measurements (CD)	-
hydrodynamic radius	dynamic light scattering	The Rh of PEG-HAS (50.7 nm), was increased about 6.5 times as compared to that of the HSA molecule (7.8 nm)
pharmacokinetic studies	Iodine-125 (125I) isotope tracing technique	PEG-HSA has longer retention in blood circulation
vascular permeability studies	In lipopolysaccharide (LPS) induced acute lung injury mice model	PEGylation of HSA could reduce extravasation into lung parenchyma

Determination of PEGylation Site

To determine the site of PEGylation following the conjugation of mPEG maleimide to free thiol group of Cyst 34 at HSA, Ting Zhao et.al[17] have conjugated a reagent (N-ethylmaleimide (NEM)) that can specifically and covalently bind to thiol residues before PEGylation reaction (5 h incubation with HSA at 37°C). Then they started optimized PEGylation reaction by mPEG-maleimide. Analyzing the reaction mixture by SDS-PAGE showed that NEM-treated HSA mPEG maleimide was not able to attach to free thiol at Cys34 due to blocking the free thiol of Cys34 by NEM.

Purification of PEGylated HSA

For separation and purification of PEGylated proteins various chromatographic methods including ion exchange chromatography (IEC),[26] size exclusion chromatography (SEC),[27] reversed phase chromatography (RP- HPLC),[28] hydrophobic interaction chromatography (HIC),[29] and hydrophobic interaction membrane chromatography (HIMC)[19] because of their reproducible results are used.

Purifying the reaction mixture by using HPLC will result multiple peaks representing different species in applied sample. To achieve a good resolution in chromatogram, properties of the protein and PEG should be considered.

Among above mentioned chromatographic methods IEC is preferred as a result of charge shielding effect of PEGylation on surface charge density and the number of binding sites of protein to the column's matrix.[30]

HSA is negatively charged at pH values around 6 – 7 due to its pI value that is 4.7, and anion exchange chromatography is suitable method to separate its native and PEGylated forms based on their difference in surface charge density that is smaller in PEGylated ones than the native HSA.

Ting Zhao et.al[16,17] have purified PEGylated HSA from reaction mixture (unreacted HSA and PEG) by anion exchange chromatography on a DEAE Sepharose FF column (2.6 cm×20 cm) at room temperature. Before applying the sample, they exchanged reaction buffer to 10 mmol/L phosphate buffer, pH 6.5 (buffer A). In elution step a linear salt gradient of 0.1—0.3 mol/L NaCl in buffer A was used during 5 column volumes as gradient volume.

As PEGylation significantly increases the size of protein,[12,15-17] size exclusion chromatography (SEC) can apply to separate native and PEGylated proteins based on their difference in size. Pedro Cabrales et al[24] have used this method to separate hexa-PEGyaletd HSA, and Xiaoyan Suo, et. el[31] have applied SEC on Superdex 200 or 75 column for analysis of the solid-phase and liquid phase PEGylated proteins.

Higher resolution in separating species of pegylated HSA have achieved using HIMC as a chromatographic technique. Ten-disc membrane module were used with 1.25 M ammonium sulfate in binding buffer while the flow rate was 0.8 mL/min and the feed sample was containing ~50 _g of total protein. In order to elute out the bound proteins, step and linear gradients (10 and 20 and 40 mL) were used.[19]

Purity identification

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is very effective method for protein analysis due to its availability and reproducibility, and is also have used for characterizing both the purity of PEGylated proteins after separation and purification steps and the extent of PEGylation by studying the molecular weight of appeared bands on gel.[17,20,31-33]

Ting Zhao et.al[16,17] have employed discontinuous system of SDS-PAGE to analyze the reaction mixtures under different reaction conditions and the purity of fractions after purification steps. In their experiments the separating and stacking gels were containing 10% and 3% (w/v) acrylamide respectively. Composition of running buffer was 0.1% SDS, 0.05 mol/L Tris, 0.38 mol/L glycine buffer, pH8.3 and before electrophoresis the samples were incubated at 100°C for 5 min in loading buffer (2% SDS, 5% mercaptoethanol, 25% glycerol, 0.01% bromophenol blue, 0.01 mol/L Tris–HCl, pH 8.8).

After running a two –step programmed mode (80 V in stacking gel, 130 V in the separation gel), first they did barium iodide staining to determine the free and conjugated PEG molecules in gel (incubation for 10 min, at 40 mL of 2 mol/L perchloric acid and then adding 5% BaCl2 and 2 mL of 0.1 mol/L iodine solution) and after rinsing the gel by distilled water they continued staining with Coomassie brilliant blue to determine the free and conjugated HSA in gel.

Also Xiaoyan Suo et al[31] have used this method to characterize the PEGylated products and to compare the extent of attached PEG after solid phase and liquid phase PEGylation based on the number of appeared bonds on the gel.

Chunyang Y. Zheng et al at 2007 have conducted Native PAGE[32] that depends on both size and charge of the protein. PEGylation increases the size and reduces the pI value of proteins that will results reduces and increase in mobility respectively.

They concluded that when the interaction between PEG chains either free or conjugated and SDS micelles interfere with migration property of PEGylated protein,[34,35] the Native PAGE can offer a higher resolution than SDS-PAGE especially for complicated proteins having more reaction sites such as serum albumin.

As the apparent hydrophobicity of a PEGylated protein increases with the number of attached PEG chains , X Shang et al[19] used duplicated SDS-PAGE (12.5% nonreducing) by Coomassie blue staining and PEG-staining to detect the purified pegylated species.

Protein concentration

Since PEG itself is invisible to UV at 280 nm and typical amine reactive groups such as succinimide do not contain UV active chromophores, UV adsorption at 280 nm may be used to quantify the concentration of PEGylated species. However, some chromophores, such as maleimide or fmoc, do have UV active components. For conjugates with these types of linkers, it may be necessary to determine the extinction coefficient after a non-UV

method, such as amino acid analysis, has been used to determine the protein concentration.[36]
In addition to concentration assay using HPLC method, by now the concentration of PEGylated HSA has been measured by using Lowry method,[17] bicinchoninic acid assay[17] and Bradford protein assay.[24]

Secondary structure identification

Circular dichroism measurement (CD) is a usuall method to analyze the secondary structural conformation of native and PEGylated proteins. Ting Zhao et.al have recorded the CD spectra of 5 μmol/L HSA and equimolar quantity of PEGylated HSA in the far UV (190 – 260 nm). The results indicated that the secondary protein structure of HSA is not changed significantly due to PEGylation.[16,17]
Xiaoyan Suo at 2009 used intrinsic fluorescence spectroscopy that mostly is assigned to Trp and Tyr residues and CD analysis to investigate the secondary structural conformation of unmodified and PEGylated HSA using solid- phase PEGylation and liquid phase PEGylation method. The far-UV spectra results suggest that both PEGylation methods have little influence on the secondary structural conformation of HAS.[31]

Molecular Hydrodynamic radii of native and PEGylated proteins

Dynamic light scattering (DLS) measurement on a multi-angle laser photometer equipped with a linearly polarized gallium arsenide (GaAs) laser (λ=658 nm), at a scattering angle of 99°and Stokes–Einstein equation* is applied to determine the molecular Hydrodynamic radii (Rh) of PEGylated and native HSA by Ting Zhao et.al[16,17] and by X Shang et al.[19]
* Stokes–Einstein equation:
$Rh=kBT/6\pi\eta 0D$
Rh : [μm] hydrodynamic radius
kB: [-] Boltzmann's constant
T : [Kelvin] The absolute temperature
η0: [cP] The solvent viscosity
D : [1/s] The translational diffusion coefficient

Analyzing the extent of PEGylation

Birgit K. et al[37] have used standard electron multiplier (SEM) and a high mass (HM) detector combined with MALDI linear TOF-MS system for analyzing PEGylated glycoproteins in various Mw range (60 600kDa). Their study identified MALDI TOF MS as a powerful analytical tool to achieve the detailed structural information about the number of attached PEG chains to the high molecular mass (glyco) proteins, which also is useful in monitoring the production process and in final quality control of (glyco) protein PEGylation. In addition this system has advantage in using only small amounts of sample (100 fmol), and as a result of the availability of two detector systems on one instrument, it is possible to choose the optimal ion detection system can for the required molecular mass range of the PEGylated (glyco)protein. At this study as a middle molecular mass model protein PEGylated HSA by 20kDa PEG reagent was used. They concluded that the standard SEM detector provides better S/N ratios for proteins such as HSA having MW below 100 kDa, while the HM detector shows considerably improved detection of (glyco) proteins above 100 kDa. Figure 3 is a graph they have obtained for PEGylated and Native HSA.
Pedro Cabrales et al[24] also have used NMR and MALDI-TOF-MS methods to analyze the extent of PEGylation of HSA.

Conclusion

Increasing the apparent size of HSA by using different methods of PEGylation leads to increase in its half life that is important factor in treatment of some critical illnesses. Some of various factors including the molecular weight of PEG, structure and the number of PEG chains attached to the protein; the PEGylation site on the protein; PEGylation chemistry; pH, temperature and incubation condition of reaction, and protein to PEG molar ratio that are affective in the properties of PEGylated protein have studied for HSA.
The results of site specific and N-terminal PEGylation in liquid phase have indicated no significant effect on pharmacokinetic of modified protein and PEGylated HSA is found to be superior to native HSA in capillary permeability increase related illness. Multi PEGylation of HSA in liquid phase either at free generated thiol sites or at reactive primary amino groups, resulted HexaPEGylated HSA with higher colloidal oncotic pressure (COP) compared to HSA at 5% (HSA5) and almost equal to HSA at 10% (HSA10) that makes PEG-HSA conjugates a better potential candidate as a plasma volume expander.
In solid-phase PEGylation method as a result of advantage of controlling the PEGylation process, in spite of using succinimidyl carbonate (SC)-mPEG that can result multi PEGylated protein, due to the steric hindrance of the solid-phase to the PEGylation and the interaction between solid phase and the amino groups at proteins surface, homogenous monoPEGylated HSA has produced. Although the yield of PEGylation is low at this method, however this method has advantage in producing homogeneous PEGylated protein, because in purification steps, broad and overlapped peaks due to heterogeneity of the products with varied PEG number and position of attachment will prevent. Also, PEGylated HSA by this method showed little conformational changes using Circular dichroism and intrinsic fluorescence studies.
Anion exchange chromatography (AExC), size exclusion chromatography (SEC), and hydrophobic interaction membrane chromatography (HIMC) are efficient methods for separation and purification of PEGylated HAS. To characterize the extent of PEGylation and the purity of separated PEG-HSA, SDS-PAGE and Native PAGE are recomended.

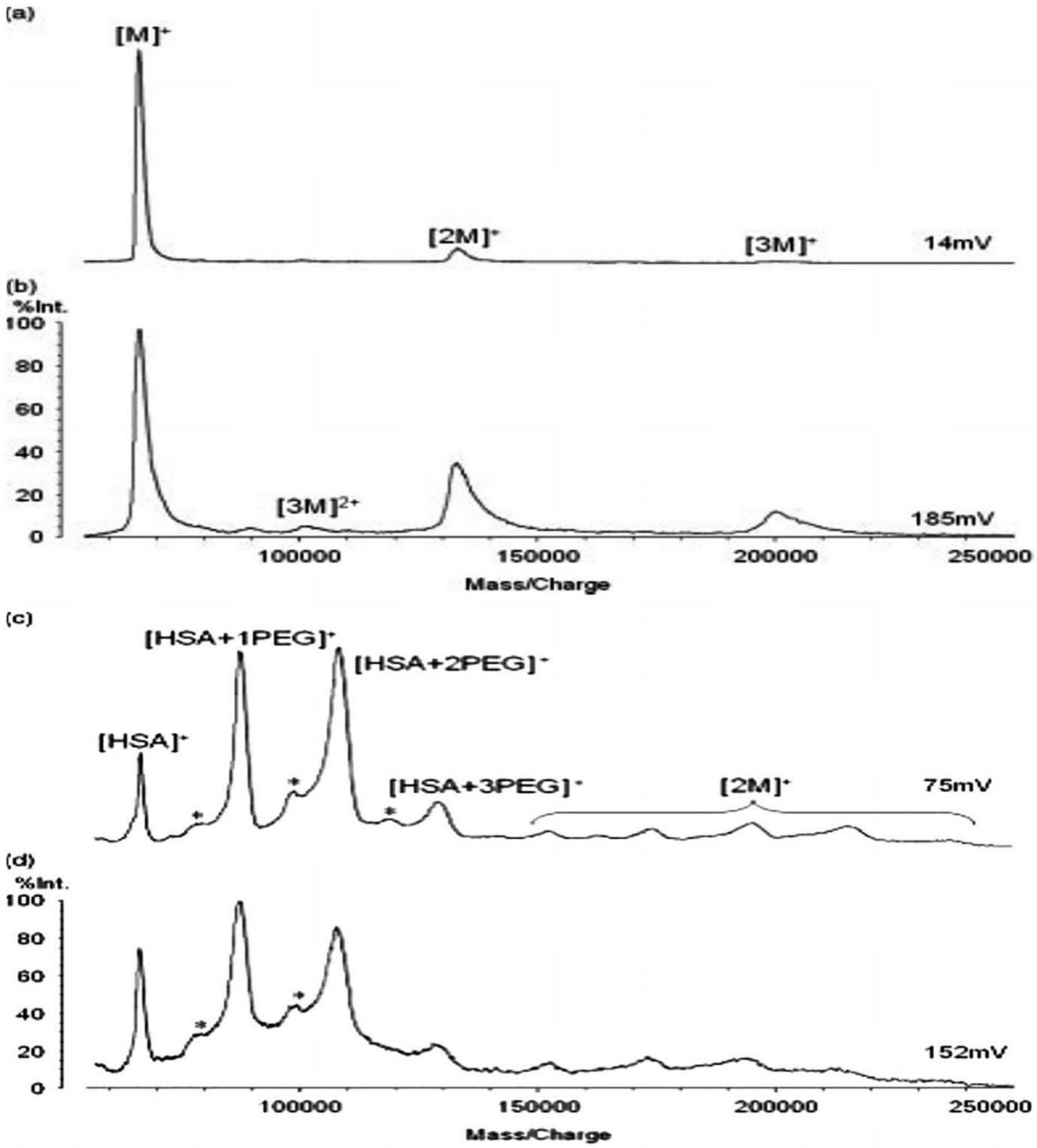

Figure 3. Positive ion MALDI linear TOF MS of HSA: (a) with the standard SEM detector and (b) with the HM detector and for PEGHSA (c) and (d), respectively. The asterisks in the PEGHSA MS indicate the 10-kDa smaller satellite peaks of the PEGylated species, which would suggest a one-armed 20-kDa PEG chain[38]

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Ethical Issues

Not applicable.

Conflict of Interest

The authors report no conflicts of interest.

Abbreviations

- Bicinchoninic acid (BCA)
- Circular dichroism (CD)
- Colloidal osmotic pressure (COP)
- Human serum albumin (HSA)
- Hydrophobic interaction chromatography (HIC)
- Hydrophobic interaction membrane chromatography (HIMC)
- Ion exchange chromatography (IEC)
- Poly ethylene glycol (PEG)
- Reversed phase chromatography (RP- HPLC)
- Size exclusion chromatography (SEC)

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