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Research paper

Effects of PEG size on structure, function and stability of PEGylated BSA

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ABSTRACT

The effects of PEGylation on the structural, thermal and functional stability of bovine serum albumin (BSA) were investigated using BSA and 6 linear mono-PEGylated BSA compounds. The secondary and tertiary structure of BSA measured by circular dichroism (CD) was independent of PEGylation. In contrast, the thermal stability of BSA was affected by PEGylation. The apparent unfolding temperature T_{max} measured by differential scanning calorimetry (DSC) decreased with PEGylation, whereas the temperature of aggregation, T_{agg}, measured by dynamic light scattering (DLS) increased with PEGylation. The unfolding temperature and the temperature of aggregation were both independent of the molecular weight of the PEG chain. Possible functional changes of BSA after PEGylation were measured by Isothermal Titration Calorimetry (ITC), where the binding of sodium dodecyl sulphate (SDS) to BSA and PEGylated BSA was analysed. At 25 °C, two distinct classes of binding sites (high affinity and low affinity) for BSA and one class of binding site (low affinity) for PEGylated BSA were identified. The binding isotherm was modelled assuming independence and thermodynamic equivalence of the sites within each class. From the present biophysical characterisation, it is concluded that after PEGylation BSA appears to be unaffected structurally (secondary and tertiary structure), slightly destabilised thermally (unfolding temperature), stabilised kinetically (temperature of aggregation) and has an altered functionality (binding profile). These biophysical characteristics are all independent of the molecular weight of the attached polymer chain.

1. Introduction

Modification of pharmaceutical proteins with hydrophilic polymers such as poly(ethylene glycol) (PEGylation) is an established method for prolonging the circulatory half-life of proteins, reducing self-aggregation, increasing water solubility and decreasing antibody recognition, and it has been used successfully in several marketed proteins [1,2]. Most of the benefits of PEGylated proteins reflect the properties of the PEG polymer itself [3]. The PEG polymer is heavily hydrated, and consequently it has a large hydrodynamic volume which, among other things, inhibits the approach of other macromolecules, resulting in reduced immunogenicity and decreased antibody recognition [4]. Generally, the hydration of the PEG chain determines the overall hydrodynamic properties of a PEG bioconjugate [5], and the increased molecular size is one of the most important properties of a PEGylated protein, resulting for example in a prolonged circulation time due to low rates of clearance from the liver and kidney [4]. Other than increasing the hydrodynamic volume of the protein upon PEGylation, the conformation, physical properties and electrostatics of a PEG-conjugated protein may be altered compared with the unmodified protein [5]. Possible changes include altered binding properties resulting from, e.g. changes in the amino acids charges, as well as hydrophobicity and hydrophilicity of the protein. However, most studies report an unchanged secondary and tertiary structure [6–8], a retained thermodynamic stability [9] and an increased temperature of aggregation [7] of the protein upon PEGylation, whereas the protein's biological activity is not necessarily preserved [7,10,11]. Studies regarding the stability, thermal and structural, as well as the bioactivity of the protein are among the most fundamental when developing pharmaceutical proteins, and the ideal pharmaceutical protein should have a long shelf-life and a high bioavailability.

Bovine serum albumin (BSA) is a protein with a molecular weight of 66,400 Da and serves a number of functions including transporting fatty acids and drugs, maintaining the osmotic pressure as well as being enzymatically active [12]. BSA has one free sulphydryl group, cysteine 34, which does not form a disulphide bond with another cysteine in the polypeptide backbone [13]. Furthermore, BSA binds several ligands, and it is possible to monitor the function of BSA in vitro with different binding assays. This makes BSA a suitable model protein when investigating the effect

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of protein PEGylation. Binding mPEG-maleimide (mPEG-MA) to the free cysteine of BSA ensures both mono-PEGylation and site-specific PEGylation.

The relationship between biophysical characteristics and functional properties of proteins and their PEGylated analogues are widely reported but to our knowledge only a few studies have actually investigated these hypotheses systematically [7,9,14,15]. We have investigated the effect of PEGylation with six linear PEG polymers ranging from 5 kDa to 60 kDa, providing us with a unique model system for studying the effect of PEG polymer chain length on the structure, function, thermal and kinetic stability of PEGylated BSA. Various biophysical characterisation tools, including circular dichroism (CD), differential scanning calorimetry (DSC) and dynamic light scattering (DLS), were used to study possible changes in the secondary and tertiary structure as well as the thermodynamic stability of BSA upon PEGylation. Binding of sodium dodecyl sulphate (SDS) was used to study functional changes of BSA upon PEGylation.

2. Materials and methods

2.1. Materials

Bovine serum albumin (98% pure by PAGE) from bovine plasma was purchased from Gibco Invitrogen, NZ. Phosphate-buffered saline (PBS) tablets were purchased from Sigma, St. Louis, MO, dissolved in milli q water, resulting in a 10 mM PBS (0.14 M NaCl, 0,01 M PO4, 0.003 M KCl) solution. Maleimido-reactants (Monomethoxy poly (ethylene glycol) maleimido-propionamide) were of nominal molecular weights 5000, 10,000, 20,000, 30,000, 40,000 and 60,000 Da and were a kind gift from Dr. Reddy's UK (former Dow Pharma). 0.1 M HCl, sodium chloride and 0.1 N iodine solution were purchased from Sigma–Aldrich (Germany), 70% perchloric acid from Merck (Germany), LDS sample buffer and MES SDS running buffer and Simple Blue Safe Stain all from Invitrogen (Carlsbad, CA). Sodium dodecyl sulphate (SDS) was bought from Sigma, St. Louis, MO.

2.2. PEGylation reaction

Batch PEG grafting was carried out in individual beakers (six in total) to ensure that none of the individual PEGylated BSA species were crosscontaminated. All solutions were made up in 10 mM phosphate-buffered saline at pH 7.4. Bovine serum albumin was used without further purification. About 1.2 mol of solid mPEG-MA reagent was added per mole of protein to 5 mL of 20 mg/mL native bovine serum albumin, and the protein solution was mixed with the PEG reagent and left stirring in an open 10-mL beaker at room temperature overnight. Samples were acidified with one drop of 0.1 M HCl to stop the reaction.

The samples, including a non-PEGylated native BSA sample as a control, were separated individually by ion exchange chromatography (IEC) using a HiPrep 16/10 QFF Anion Exchange column (Amersham Bioscience, Uppsala, Sweden), equilibrating the column first with 5 column volumes (CV) of 10 mM PBS pH 7.4, 1 M NaCl and then 5 CV of 10 mM PBS pH 7.4. 5 mL samples were then loaded onto the IEC column, and the samples were eluted with a 15 CV linear gradient to 10 mM PBS, 1 M NaCl at 5 mL/min. The protein elution profile was monitored by UV absorbance at 280 nm and collected in 2 mL fractions. Fractions were analysed by SDS–PAGE, and fractions containing > 80% mono-PEGylated BSA monomer, detected by SDS–PAGE, were pooled. Fractions were analysed by SDS–PAGE, and fractions containing > 80% mono-PEGylated BSA monomer, detected by SDS–PAGE, were pooled. SDS–PAGE analysis was carried out using a 12% Bis-Tris gel from Invitrogen. The gels were loaded with an average of 5 μg protein per well and run at 120 mA constant current per gel. The running buffer was MES running buffer. The gel was coloured with Coomassie blue. The amount of mono-PEGylated BSA in the fractions was detected using the BioRad Experion software.

These fractions were then further purified by loading 6 mL samples onto a size exclusion chromatography (SEC) HiPrep 16/60 Sephacryl S-200 column (Amersham Bioscience, Uppsala, Sweden), with PBS running buffer at 1 mL/min. The protein elution profile was monitored by UV absorbance, and fractions containing > 90% mono-PEGylated BSA monomer, as subsequently detected by SDS–PAGE as previously described, were pooled. The purification was carried out on an AKTAexplorer 10™ liquid chromatography system with an A-900 autosampler and detected by a differential refractometer detector (model RID-10A from Shimadzu, Tokyo, Japan). The chromatography system was operated using Unicorn software, version 4.0, (Amersham Bioscience, Uppsala, Sweden). The protein samples were frozen in liquid nitrogen and lyophilised in a Labconco Freezone 2.5 Total Lab system freeze dryer (Kansas City, MO) overnight at 3.0 Pa −49 °C. The lyophilised protein samples were kept at −20 °C until use. Prior to the experiments, all native BSA and PEGylated BSA solutions were dissolved in PBS buffer and then dialysed intensively against PBS. The dialysate was used as reference. The purity of the BSA and PEGylated BSA samples after dialysis was tested by SDS–PAGE. This gel was washed in 150 mL 0.1 M perchloric acid for 15 min until 40 mL 5% barium chloride solution and 15 mL 0.1 M iodine solution were added to detect protein bands containing PEG compounds, as described in [16]. After discolouring in water, the gel was coloured with Coomassie blue. The SDS–PAGE revealed that the individual PEGylated BSA samples contained all > 90% PEGylated BSA and approximately 5% native BSA monomer and 4 % native BSA dimer, respectively (Fig. 1).

2.3. Isothermal Titration Calorimetry (ITC)

The calorimetric measurements were made with a VP-ITC instrument from MicroCal (Northhampton, MA). Before the loading the solutions into the cell and syringe, respectively, the solutions were degassed by stirring under vacuum. The protein concentration was kept in the range 18–25 μM and determined using a Nanodrop spectrophotometer (Thermo Scientific Wilmington, DE). The concentration of SDS in PBS was 2.4 mM. The protein solution was loaded into the cell and titrated 40 times with aliquots of 5 μL surfactant solution at 25 °C. Due to a small amount of available PEGylated BSA, the ITC measurements were only carried out twice. The results were very alike. The raw ITC data were analysed using the Origin software package from Microcal. The heat of dilution, defined by the plateau level when the binding is fully saturated, was subtracted from the data also using the analysis software package from Microcal. The concentration of SDS used here is slightly above the critical micellar concentration (CMC) (measured in a separate experiment, see [18]) and a small fraction of the surfactant will thus be delivered in a micellar form. The accompanying heat of demicellization was subtracted as described earlier [18].

2.4. Circular dichroism (CD) spectroscopy

Spectra of BSA in the far-UV region (180–260 nm) were recorded on a Jasco J-810 CD Spectropolarimeter (JASCO International Co. Ltd., Hachioji City, Japan). The light path of the cuvette was 0.1 mm, and a protein concentration of 0.8 mg/mL was used. The spectra were recorded at room temperature. Each spectrum is an average of 5 scans. All spectra were background-corrected,

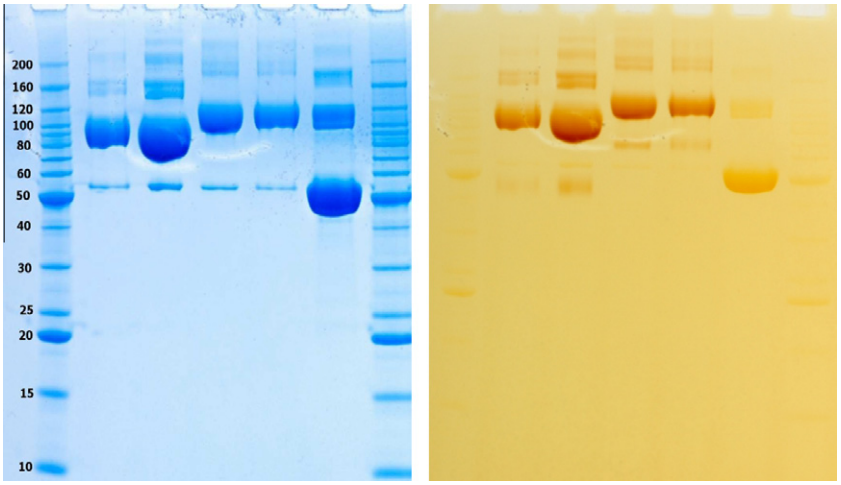

Fig. 1. The Coomassie blue stained SDS–PAGE gel (left panel). The PEG-stained SDS–PAGE gel (right panel). Lane 1 and 7: Molecular weight standard, lane 2 and 3: 20 kDa PEGylated BSA, lane 4 and 5: 30 kDa PEGylated BSA, lane 6: Native BSA. The gel is stained with iodine (right panel), discoloured in water and subsequently stained with Coomassie blue (left panel). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

smoothed and normalised to molar ellipticity (θ cm^2 dmol^{-1}). A value of 114 g/mol was used as a mean residue weight for BSA.

Spectra of BSA in the near-UV region (250–350 nm) were recorded on the same spectropolarimeter, where the light path of the cuvette was 10 mm. 0.8 mg/ml protein samples were analysed at room temperature. The recorded CD spectra resulted from the average of five scans. All spectra were background-corrected, smoothed and normalised to molar ellipticity (θ cm^2 dmol^{-1}).

2.5. Differential scanning calorimetry (DSC)

DSC experiments were carried out with a MicroCal VP-DSC (Northhampton, MA). Prior to scanning, all solutions were degassed by stirring under vacuum. A pressure of 2 atm was applied over the cells during scanning, and a scan rate of 1 °C/min was used. The concentration of BSA was 0.8 mg/mL, and buffer scans were subtracted from BSA scans. DSC data were analysed using the Origin software from MicroCal Inc., supplied with the instrument. A baseline was subtracted prior to analysis. The apparent unfolding temperature (T_{max}) values were determined as the temperature corresponding to the maximum heat capacity (C_p). The enthalpy is calculated from the area under the peak.

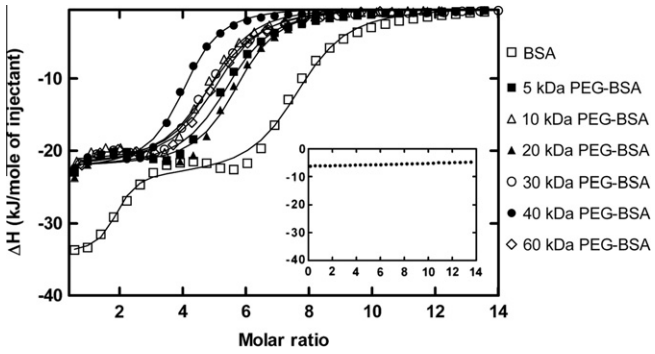

Fig. 2. Enthalpograms showing the binding of SDS to native BSA and the 6 PEGylated BSA compounds. The ordinate shows the observed enthalpy change ΔH_{obs} in kJ per mol SDS injected, and the abscissa is the concentration of SDS expressed by the surfactant: protein molar ratio.

2.6. Dynamic light scattering (DLS)

The DLS experiments were carried out with a Wyatt DynaPro Titan (Santa Barbara, CA), which employs a 829 nm laser and collects scattering intensity data at a fixed angle of 90 degrees using 1 mg/mL samples. Cuvette temperature was controlled using a thermoelectric solid-state heating module (Peltier heat pump), and a 1 °C/min scanrate was used. Solutions were examined in a quartz cuvette with 12 μL cell volume containing glass viewing windows for in situ scattering measurements. The samples were centrifuged at 10,000 rpm for 10 min prior to analysis.

3. Results

3.1. Purity

After dialysis, the purity of the protein solutions was determined by SDS–PAGE. SDS–PAGE has often been used to characterise the distribution of PEGylated proteins [19]. It is assumed that the higher molecular weight gel band ladders represented the mono- or di-PEGylated BSA compounds.

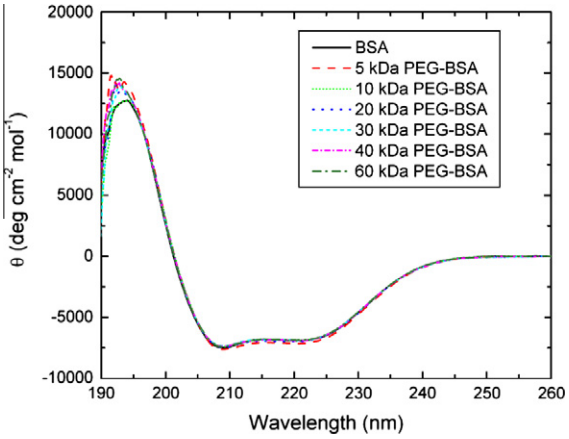

Fig. 3. Far-UV CD scans of native BSA and 6 PEGylated BSA species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

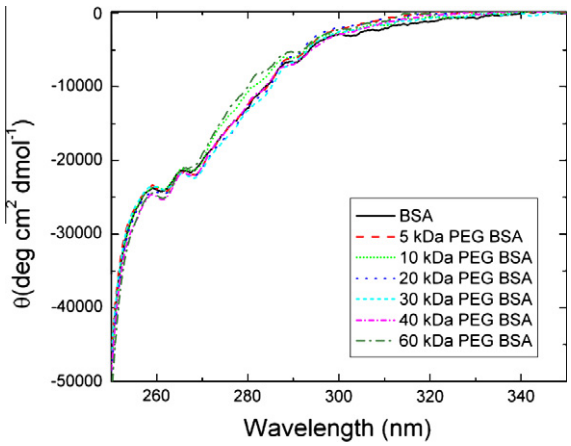

Fig. 4. Near-UV CD scans of native BSA and 6 PEGylated BSA species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

The location of the PEGylated species on the gel reveals that a 20 kDa PEGylated BSA is located at $66 + 2 \cdot 20 \sim 106$ kDa and a 30 kDa PEGylated BSA is located at $66 + 2 \cdot 30 \sim 126$ kDa. Analysis of the gel on Fig. 1 (left panel) reveals a ~5% of native BSA within the PEGylated BSA samples after purification. The purity of 5 kDa, 10 kDa, 40 kDa and 60 kDa are similar (data not shown).

3.2. Isothermal Titration Calorimetry (ITC)

ITC was used to measure the binding between BSA and the ligand SDS. ITC data for the binding to the two classes of independent sites are modelled as described in Nielsen et al. [18]. Briefly, it is assumed that the binding of SDS to native BSA molecule can be associated with two classes of independent binding sites (high affinity and low affinity), which can bind the same type of ligand. The binding sites within each class are thermodynamically similar and are characterised by a binding constant (K), an enthalpy of binding (ΔH) and a number of binding sites (N). The three values for each class of binding site are found by a non-linear, least square minimisation routine giving a maximum likelihood value (Fig. 2 and Table 1).

The enthalpogram for the binding of SDS to native BSA shows two clear inflexion points separating three plateaus (Fig. 2). This is in accordance with previously reported results [18,20] and indicates that it is a reasonable simplification to describe the multiple binding processes of SDS to BSA by modelling two classes of independent binding sites (high affinity/low affinity) for the native BSA. The enthalpograms for the binding of SDS to PEGylated BSA species show a different profile than that of native BSA, namely one clear inflection point separating two horizontal regions (Fig. 2). It seems reasonable to assume that the PEGylated BSA molecule contains only low-affinity binding sites, as the plateaus are found around the enthalpy values −20 kJ/mol and 0 kJ/mol. These values are of the same magnitude as the low-affinity sites of native BSA, supporting the conclusion that only low-affinity binding sites are found on PEGylated BSA. This class of binding site includes several sites that are thermodynamically similar [18,20] and has been modelled as a one-site model from which three parameters, namely the binding constant (K), the enthalpy of binding and number of binding sites were derived for each BSA–PEG derivative are found. ITC data for the binding to one class of independent sites are readily modelled, see Table 1. From the results of the modelling, it is suggested that the total number of binding sites is reduced upon PEGylation. The modelling parameters are based on an average of the two obtained data sets.

3.3. Circular dichroism (CD)

The secondary and tertiary structures of PEGylated BSA were investigated by far-UV and near-UV circular dichroism. Fig. 3 shows the far-UV CD scans for the native BSA and the 6 PEGylated BSA compounds. The scans show that the secondary structure of the PEGylated BSA compounds lines up with the secondary structure of native BSA. Also, there is no significant change in the secondary structure between the various PEGylated BSA species.

Fig. 4 shows the near-UV CD scans of the native BSA and the PEGylated species. The tertiary structure of BSA did not change upon PEGylation. There are small but no significant differences in the overlaid spectra around 280 nm.

3.4. Differential scanning calorimetry (DSC)

The thermal stability of BSA and PEGylated BSA was investigated by DSC. The native BSA and the 6 PEGylated BSA compounds were investigated separately, showing that the apparent unfolding temperature, T_{max}, was affected by PEGylation but not by the PEG molecular weight (Fig. 5).

The apparent T_{max} was lower for the PEGylated BSA than for the native BSA, 82.5 ± 0.7 °C for native BSA and approximately 2 °C lower for the PEGylated BSA compounds (Table 2).

A DSC scan of pure 40 kDa PEG was also performed. This showed that the thermal transition of PEG was above 100 °C (data now shown). It is hence unlikely that the PEG polymer itself does contribute to the DSC thermogram in the investigated temperature range. Even though the native BSA thermogram does not show clear signs of aggregation behaviour (exothermal signal), the native BSA solution was milk-white upon heating whereas the solutions of the PEGylated BSA compounds were transparent.

Table 1
Summary of thermodynamic and stoichiometric parameters of the binding of SDS to BSA and PEGylated BSA at T = 25 °C.

	BSA	BSA[a]	5 kDa PEG–BSA	10 kDa PEG–BSA	20 kDa PEG–BSA	30 kDa PEG–BSA	40 kDa PEG–BSA	60 kDa PEG–BSA
Low-affinity sites								
N	5.8 ± 0.1	5.8 ± 0.3	5.3 ± 0.06	4.8 ± 0.05	5.6 ± 0.06	4.7 ± 0.05	3.9 ± 0.04	4.9 ± 0.04
ΔH (kJ/mol)	−23.9 ± 0.4	−28.1 ± 1	−22.6 ± 0.3	−21.7 ± 0.3	−22.2 ± 0.3	−22.6 ± 0.3	−22.6 ± 0.3	−22.2 ± 0.2
K (M^{-1})	$3.4(\pm0.5) \times 10^4$	$1.9(\pm0.6) \times 10^5$	$3.7(\pm0.6) \times 10^5$	$4.4(\pm0.6) \times 10^5$	$4.7(\pm0.8) \times 10^5$	$3.4(\pm0.4) \times 10^5$	$4.4(\pm0.6) \times 10^5$	$4.8(\pm0.4) \times 10^5$
$T\Delta S$ (J/mol)	7.8	2.0	9.3	10.5	10.0	7.0	9.48	10.3
High-affinity sites								
N	1.7 ± 0.1	2.9 ± 0.1	–	–	–	–	–	–
ΔH (kJ/mol)	−34.3 ± 0.8	−35.5 ± 1.1	–	–	–	–	–	–
K (M^{-1})	$3.4(\pm 2.1) \times 10^7$	$(3.3\pm) \times 10^7$	–	–	–	–	–	–
$T\Delta S$ (J/mol)	8.8	7.4 ± 3	–	–	–	–	–	–

[a] Data from Nielsen et al. [18].

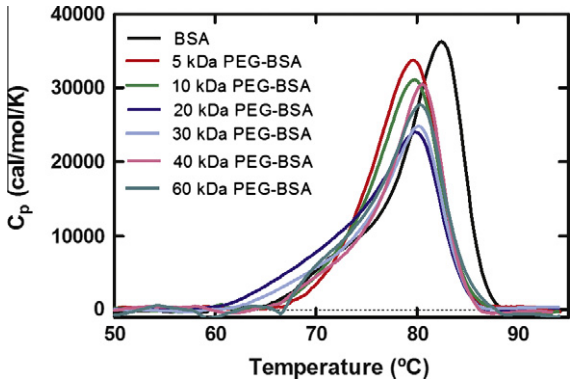

Fig. 5. DSC scan of BSA and the 6 PEGylated BSA species with various covalently attached linear PEGs. The DSC scans reveal that PEGylation slightly decreases the apparent unfolding temperature, T_{max}. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Table 2
The apparent unfolding temperature, T_{max}, and unfolding enthalpy, ΔH, measured by DSC, the aggregation temperature, T_{agg}, measured by DLS and the difference between T_{max} and T_{agg}.

	T_{max} (°C)	ΔH (kJ/mol)	T_{agg} (°C)	$T_{max} - T_{agg}$
BSA	82.5 ± 0.2	1250 ± 150	71	11.5 ± 0.2
5 kDa PEG–BSA	79.7 ± 0.2	1000 ± 100	75	4.7 ± 0.2
10 kDa PEG–BSA	79.8 ± 0.2	1060 ± 150	81	−1.2 ± 0.2
20 kDa PEG–BSA	79.8 ± 0.2	1030 ± 100	82	−2.2 ± 0.2
30 kDa PEG–BSA	80.1 ± 0.2	930 ± 130	81	−0.9 ± 0.2
40 kDa PEG–BSA	80.5 ± 0.2	950 ± 150	82	−1.5 ± 0.2
60 kDa PEG–BSA	80.4 ± 0.2	980 ± 100	77	3.4 ± 0.2

3.5. Dynamic light scattering (DLS)

The thermodynamic stabilities of BSA and PEGylated BSA were measured by DLS in seven separate experiments. The signal intensity was plotted as a function of temperature in Fig. 6. The temperature of aggregation, T_{agg}, is found by reading off the intersection of the intensity curves with three intensity threshold values 1×10^5, 2×10^5 and 3×10^5 counts/s. As the temperature of aggregation change linearly with the intensity threshold values in the investigated intensity range, we choose the temperature at the lowest intensity (1×10^6) as the temperature of aggregation. From the results, it is evident that attaching a PEG polymer to BSA increases the onset temperature for aggregation.

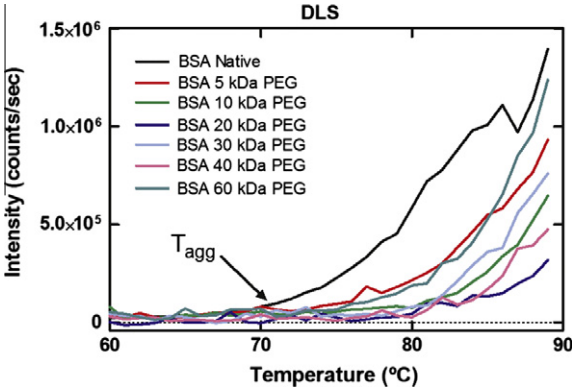

Fig. 6. DLS measurements of BSA and the 6 PEGylated BSA species with various covalently attached linear PEGs. The aggregation temperature, T_{agg}, increases with PEGylation, as expected. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

The apparent unfolding temperature, the temperature of aggregation and the difference between these two characteristic temperatures are given in Table 2.

4. Discussion

Most literature on the effect of PEGylation on the structure, stability and function of proteins has only investigated the unmodified protein and one PEGylated variant and focused on the effect of PEGylation [1,14,15]. This study expands the concepts of PEGylation by investigating the effect of varying the PEG chain length on the structure, stability and function of BSA.

The structural information obtained with far-UV CD scan show that the secondary structure of BSA is maintained upon PEGylation and similar to previously reported far-UV CD scans on native BSA [21]. In addition, the near-UV CD scans shows that the tertiary structure of BSA is maintained upon PEGylation. Both the secondary and the tertiary structure of the various PEGylated BSA species does not change with PEG chain length. The small differences in the CD scans found around 270–300 nm for 10 kDa PEGylated BSA and 60 kDa PEGylated BSA compared with native BSA and the other PEGylated BSA compounds could be due to small changes in the properties (mobility, polarisability, H-bonding) of specific aromatic amino acids, most likely tyrosine. However, the overall tertiary structure of 10 kDa PEGylated BSA and 60 kDa PEGylated BSA is still considered to be essentially identical to that of the native BSA and the other PEGylated BSA compounds.

The thermal stability of BSA and PEGylated BSA was measured by DSC, and the unfolding temperature and related unfolding enthalpy are presented in Table 2. There is a range of unfolding temperatures of BSA reported in the literature, based on studies where the ionic strength, the pH or the concentration of anionic surfactants have been changed [22–25]. Our unfolding temperature values are in the high end of the range of those reported in the literature. The enthalpies are generally of the same order of magnitude as those obtained in the literature [23,25]. The unfolding enthalpies are lower for the PEGylated BSA compounds than the native BSA, but the unfolding enthalpies are alike for all the PEGylated BSA compounds The bimodal appearance of the DSC scans is especially pronounced for native BSA (Fig. 5), where the overall DSC scan may appear to consist of two underlying unfolding events, which can be correlated with the domain structure of BSA [25]. The PEGylated BSA compounds with the smaller PEGs attached (5–30 kDa) do not seem to have this distinct bimodal appearance; however, the peaks are broadened. The broadening of DSC peaks of BSA has been reported in the literature previously [22,26,27]. The PEG molecular weight dependence on the appearance of the DSC scan may be related the fact that the molecular weight of the attached PEG has different impacts on the stability of the two domains of BSA.

From the results presented in Fig. 5, it is evident that the apparent unfolding temperature, T_{max}, for native BSA is slightly higher than that of the PEGylated BSA species. Dhalluin et al. found a slight increase in T_{max} with PEGylation using interferon-alpha (2a) [9]. How PEGylation affects the apparent unfolding temperature of a certain protein depends on several factors including the degree of PEGylation, the site of the polymer conjugation, the buffer composition and the specific local environment on the protein surface [28]. A slight decrease in T_{max} with PEGylation may be found for some proteins, whereas a slight increase may be found for other proteins. The results obtained in this study also reveal that there is no difference in the thermal stability, measured by T_{max}, between a 5 kDa PEGylated BSA and a 60 kDa PEGylated BSA. Thus, a longer PEG chain length does not contribute more to the stability of the protein than a short PEG chain. Rather, it

appears that the thermal stability is independent of the molecular weight of the attached PEG polymer. Only a few examples of similar types of systematic investigations are found in the literature. Rodrıguez-Martınez et al. [29] have investigated the effect of PEGylation on the thermodynamic stability of α-Chymotrypsin with different PEG MW and degree of PEGylation. Rodrıguez-Martınez et al. conclude that the magnitude of thermodynamic stabilisation correlates with a reduction in protein structural dynamics and was independent of the PEG MW, which is in accordance with our conclusions.

In analogy to the effect of PEGylation on the apparent unfolding temperature, the temperature of aggregation, T_{agg}, depends on PEGylation but is relatively insensitive to the size of the attached PEG polymer (Fig. 6.). The data in Table 2 show that under the investigated conditions, aggregation can be detected about 11 °C below T_{max} for the unmodified protein. In this case, T_{agg} practically coincides with the first endothermic deflection in the DSC signal. These observations suggest that the thermally unfolded form of (unmodified) BSA has a very poor colloidal stability and thus aggregates immediately upon its formation during heating. Aggregation of the PEGylated form of BSA begins at a temperature much closer to T_{max} when an appreciable fraction of the protein is denatured. This behaviour may be rationalised along the lines of the Lumry-Eyring model for irreversible protein denaturation [30]. According to the Lumry-Eyring model, irreversible protein denaturation involves at least two steps. The first step is the reversible unfolding of the native protein (N), characterised by the rate constants, k_1 and k_2. This step is followed by an irreversible change of the unfolded protein (D), into an inactivated, irreversible aggregate (A), characterised by the rate constant k_3. The unfolded state, D, is characterised by having some tertiary structure.

$$N \underset{k_1}{\overset{k_2}{\leftrightarrow}} D \overset{k_3}{\rightarrow} A \qquad K = \frac{k_1}{k_2}$$

Thus, we suggest that PEGylation of BSA slightly increases the equilibrium constant K (lowers T_{max}) but has a stronger, reducing effect on k_3, so that the aggregation of the denatured (D) is slowed down. This is illustrated by the difference between T_{max} and T_{agg} in Table 2, where there is a large difference between T_{max} and T_{agg} for the unmodified BSA but a only a small difference (positive or negative) between T_{max} and T_{agg} for the PEGylated BSA compounds.

The general suppression of protein aggregation by PEG observed here is in accord with earlier reports [7,31] and the suggestion that this is mainly due to changes in k_3 (and not K) has also been put forward before [7]. Steric hindrance of favourable intermolecular peptide fragment contacts appears to be a likely cause for the effect of PEG on k_3, but the lack of a correlation of PEG size and T_{agg} suggests that other as of yet unidentified factors may be involved in the PEG induced increase in the colloidal stability.

The ligand binding properties of BSA were investigated with ITC. Previous studies of the binding of serum albumins have reported, that the thermodynamics and stoichiometry of binding fatty acids (FA) to BSA is similar to that of binding sodium dodecyl sulphate (SDS) [18,32,33]. Because of the major experimental challenges in working with FA binding due to their insolubility, tendency to aggregate and adsorb to surfaces, we used the binding of a single sulphonic (instead of a carboxylic) acid anion to BSA. Sodium dodecyl sulphate, a sulphonic acid, forms stable micellar aggregates that are easily handled experimentally. The binding of SDS to BSA and PEGylated compounds of BSA reflects the functional properties of BSA. The type of binding sites and their thermodynamic characteristics such as the binding enthalpies, the binding constants and the number of binding sites are listed in Table 1. The values obtained by modelling the binding of SDS to PEGylated BSA are similar to the low-affinity binding site found for the native BSA. Compared with the shape of the actual enthalpogram in Fig. 2, this suggests that the PEGylated BSA molecule contains only one type of binding site. The number of binding sites, high affinity as well as low affinity in native BSA, is slightly lower than reported previously [18] but of the same order of magnitude. With the used methods, it has not been possible to investigate if any new low-affinity binding sites have been generated upon PEGylation, for example if one or two of the high-affinity sites in the native BSA molecule to appear as low-affinity binding sites in the PEGylated BSA molecule. There is surprisingly little variation within the parameters characterising the low-affinity sites of the 6 PEGylated BSA compounds. The binding properties of PEGylated BSA seem to be the same whether a small PEG polymer chain or a long PEG polymer chain is attached. Crystallographic and thermodynamic investigations of the these sites on structurally similar serum albumin have revealed that the binding sites for negatively charged ligands with large acyl chains can be roughly divided into two classes: high affinity and low affinity, and that within each class the binding parameters are similar [18,34,35]. It has been suggested that the hydrophobic regions of PEG would interact with the hydrophobic clusters on the protein surface, resulting in a structure of the PEG–protein complex, where PEG is coiled on the protein surface [15]. This may explain the change in the binding properties of BSA upon PEGylation. The PEG polymer could compete with SDS for interaction sites on the protein surface and thus weaken SDS–BSA affinities, but control ITC experiments in which an 8 kDa PEG polymer was titrated to unmodified BSA failed to show direct binding (Fig. 2).

In the protein screening process that is often performed early in the protein drug development process, one of the goals is to find out whether certain protein modifications affect the structure, stability and function of a potential pharmaceutical protein. Biophysical characterisation screening tools often include techniques such as DLS, DSC and CD, all of which complement one another in determining possible changes to the structure, kinetic and thermodynamic stability of the protein. However, this study suggests that even though the protein appears to be structurally unaffected by PEGylation, its function is significantly altered. In fact, the high-affinity sites, which are the physiologically relevant [34,36], appear to be completely inactivated by the PEGylation. Based on a comprehensive analysis of crystallographic data of human serum albumin (HSA), it has previously been reported that only one of the 35 cysteines in HSA is not a part of an S–S bridge, and this residue is not close to any binding site [20,34]. As BSA and HSA have a very similar overall structure [34], and the distance from the cleavage in HSA, where most of the high-affinity binding sites for SDS are found, to cysteine 34 is approximately 45 Å, it seems reasonable to assume that the cysteine 34 onto which the PEG is attached is not close to any of the binding site.

For this reason, the differences in the binding properties of BSA and PEGylated BSA cannot be explained by the PEG being located close to a binding site and in this way sterically hindering the binding of SDS to the high-affinity sites of BSA.

5. Conclusion

BSA was mono-PEGylated with 6 different linear PEG polymers ranging from 5 kDa to 60 kDa in molecular weight. The results from the CD scans suggested that the secondary and tertiary structure of BSA is unaltered upon PEGylation. From the DSC measurements, it was evident that the thermodynamic stability of the protein is slightly decreased by the covalently attached PEG, but independent of the PEG molecular weight. The aggregation temperature, derived from DLS measurements, increases with PEGylation, but is independent of the size of the attached PEG. The

binding of SDS to BSA and PEGylated BSA reveals that the function of BSA as a transport protein may be changed significantly when the protein is PEGylated. Of the two high-affinity binding sites and six low-affinity binding sites found on native BSA, only five of the low-affinity binding sites are retained on the PEGylated BSA species, and this change was found to be independent of the PEG chain length. Thus, PEGylation may leave a protein structurally unaffected but profoundly changed in terms of function.

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