Influence of the chemistry of conjugation of poly(ethylene glycol) to Hb on the oxygen-binding and solution properties of the PEG-Hb conjugate

Tao HU*, Muthuchidambaram PRABHAKARAN†, Seetharama A. ACHARYA*‡ and Belur N. MANJULA*¹

*Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY 10461, U.S.A., †Department of Biomedical Engineering, Florida International University, Miami, FL 33174, U.S.A., and ‡Department of Medicine, Albert Einstein College of Medicine, Bronx, NY 10461, U.S.A.

Our recent studies on PEG–Hb [poly(ethylene glycol)–Hb] conjugates generated by thiolation-mediated maleimide-chemistry based PEGylation demonstrated that the vasoactivity of the PEG–Hb conjugates is a function of the configuration of the PEG chains on the surface of the protein and is independent of the PEG/protein-mass ratio [Manjula, A. G. Tsai, Intaglietta, H.-C. Tsai, Ho, Smith, Perumalsamy, Kanika, Friedman and Acharya (2005) *Protein J.* **24**, 133–146]. A Hb conjugated with six PEG5k chains (SP-PEG5k)₆-Hb, was vasoinactive. In an attempt to understand whether the chemistry of conjugation of PEG to Hb has any influence on the modulation of its functional and solution properties, we have now generated a new hexaPEGylated-Hb, (propyl-PEG5k)₆-Hb, by reductive alkylation chemistry. CD (circular dichroism) spectral measurements indicated that the overall secondary structure of Hb is not adversely influenced upon PEGylation. (Propyl-PEG5k)₆-Hb exhibited an increased O₂ affinity with decreased co-operativity and decreased modulation

by allosteric effectors comparable with that of (SP-PEG5k)₆-Hb, although its Cys-93(β) is not derivatized as in the latter. On a molecular mass basis, PEG linked to Hb by reductive alkylation increased its COP (colloidal osmotic pressure) more efficiently than when linked by thiolation-mediated maleimide-chemistry. These results demonstrate that the functional properties of PEG–Hb conjugates may be a direct consequence of surface decoration of Hb with PEG, but are independent of the site (pattern) and/or the chemistry of PEGylation. However the solution properties of PEGylated Hb are influenced by the site (pattern) and/or the chemistry of PEGylation and the presence or absence of an ‘extension arm’ between the conjugating site of Hb and the PEG chain.

Key words: colloidal osmotic pressure (COP), PEGylation, PEGylated Hb, reductive alkylation, thiolation-mediated maleimide-chemistry, viscosity.

INTRODUCTION

Conjugation of polymers to therapeutic peptides and proteins to generate hybrid molecules with unique and distinct molecular properties has become a popular approach to improve their therapeutic potential. Since the pioneering work of Abuchowski et al. [1] grafting of PEG [poly(ethylene glycol)] chains to proteins (PEGylation) is one of the most widely used conjugation approaches [2–6]. A variety of chemical techniques have been developed to conjugate PEGs of various sizes to proteins, mostly via either their amino groups or cysteine residues. PEGs are inert and non-toxic polymers. PEGylation effectively increases the size of proteins increasing their circulation time and decreasing kidney filtration, it also increases their stability, causes minimal loss of activity and renders proteins non-immunogenic and non-antigenic. Accordingly, there has been significant interest in developing therapeutic PEGylated proteins for clinical applications.

Development of Hb-based blood substitutes has been a field of active investigation in the past [7–10] and vasoconstrictive activity has been a major toxicity issue in the products being studied. Recent studies have shown that PEGylation of Hb can modulate its vasoactivity. This modulation has been attributed as a consequence of the PEGylation-induced changes in the oxygen-binding and solution properties of Hb [11–13]. Our recent studies using site-specific and site-selective PEGylated Hb have demonstrated that modulation of the vasoactivity of Hb is not a direct result of the solution properties of the PEGylated Hb, but is dependent on the configuration of PEG chains on the surface

of the Hb molecule [14–18]. A hexaPEGylated Hb, (SP-PEG5k)₆-Hb, generated by surface decoration of Hb with approx. 6 methoxy poly(ethylene glycol)-5000 (PEG5k) chains by a thiolation-mediated maleimide-chemistry based PEGylation protocol that does not alter the surface charge of the protein, has been found to neutralize its vasoactivity [15,18,19].

In an attempt to investigate the factors modulating the oxygen-binding and solution properties of Hb by PEGylation, we are studying alternate design strategies for the PEGylation of Hb. Acharya et al. [20] have demonstrated that reductive alkylation of Hb by glyceraldehyde in the presence of sodium cyanoborohydride is highly selective and results in the modification of its four α-amino groups [two Val-1(α) and two Val-1(β) residues]. In the present study, we have examined the feasibility of generating site-specific PEGylated Hb by reductive alkylation with PEG-aldehyde (Figure 1). Like the thiolation-mediated maleimide-chemistry based PEGylation described above, the reductive alkylation with aldehyde also has the advantage of retaining the original positive charge on Hb and hence is a conservative PEGylation. Furthermore, Cys-93(β) of Hb, modification of which has an influence on the oxygen-binding properties of the protein, is not modified in this protocol. Thus a study of the properties of the PEGylated Hb generated by both protocols could provide information about the relationship between the sites and/or the chemistry of PEGylation and their functional and solution properties.

Reductive alkylation of Hb with methoxy poly(ethylene glycol)-5000 (PEG5k)-propionaldehyde generated a PEGylated Hb

Abbreviations used: CD, circular dichroism; COP, colloidal osmotic pressure; 2,3-DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; MALDI, matrix-assisted laser-desorption ionization; p50, partial oxygen pressure at 50% oxygen saturation; 4-PDS, 4,4'-dithiopyridine; PEG, poly(ethylene glycol); RI, refractive index; RPHPLC, reverse phase HPLC; SEC, size exclusion chromatography; SP, succinimidophenyl; TFA, trifluoro acetic acid; 2,4,6-TNBS, 2,4,6-trinitrobenzenesulphonic acid.

¹ To whom the correspondence should be addressed (email manjula@aecom.yu.edu).

Figure 1 Schematic representation of reductive alkylation-mediated PEGylation of HbA

carrying an average of 6 PEG chains. Thus this new PEGylated Hb namely (propyl-PEG5k)₆-Hb is comparable to (SP-PEG5k)₆-Hb in terms of the size and number of conjugated PEG chains. Characterization of the sites of PEGylation, influence of PEGylation on the Hb conformation and determination of the effect of the site and/or the chemistry of PEGylation on the functional and solution properties of Hb i.e. oxygen affinity, viscosity and COP (colloidal osmotic pressure) are reported in the present study.

EXPERIMENTAL

Reductive alkylation of adult Hb (HbA) with PEG5k-aldehyde

Human HbA was purified from erythrocytes as previously described [21]. HbA (0.25 mM tetramer) in 50 mM sodium phosphate buffer or in 50 mM BisTris-Acetate buffer (at the desired pH) was reacted with either PEG5k-propionaldehyde or PEG2k-propionaldehyde (PEG5k-aldehyde and PEG2k-aldehyde respectively) (Shearwater Polymers, Huntsville, AL, U.S.A.) at the concentrations indicated in the text. This reaction was carried out in the presence of a 5 fold molar excess of sodium cyanoborohydride (Sigma) either at 4 °C or at room temperature. For analytical reactions, the reaction mixture was dialysed extensively against PBS, pH 7.4 and the product examined by SEC (size exclusion chromatography) and RPHPLC (reverse phase HPLC) analysis. For the preparative reactions, the reaction mixture was subjected to diafiltration through a 70 kDa membrane versus PBS (pH 7.4) using a MinimTM Tangential Flow Filtration instrument (Pall Corporation, Ann Arbor, MI, U.S.A.) to remove unreacted PEG and other excess reagents. The retentate was monitored at regular intervals by SEC for the removal of unreacted PEG. The presence of unreacted PEG in the column effluent was detected by RI (refractive index) measurements online. The final product in the retentate was concentrated and stored frozen at -80 °C.

Tryptic-peptide mapping

Tryptic-peptide mapping of PEGylated Hb was carried out by RPHPLC as previously described [15,22]. The percentage modification of the amino acid residues in the PEGylated Hb was evaluated essentially as described by Lippincott et al. [23] and Doyle et al. [24]. Briefly, the recovery of peptide βT4 was used as an internal standard and the ratio of the peak area of each peptide in the peptide map of PEGylated Hb relative to the corresponding peak in the HbA peptide map was used to elucidate the amino acid residues modified by PEGylation.

Kinetic analysis of amino-group modification by PEG5k-aldehyde

HbA (0.25 mM) was incubated with 10 mM PEG5k-aldehyde in the presence of 50 mM NaCNBH₄ in 50 mM BisTris-Acetate buffer (pH 6.5) at 4 °C. Aliquots were taken at timed intervals and

added to 10 volumes of cold acid-acetone to terminate the reaction. The globins thus precipitated were separated from the haem by centrifugation and subjected to lyophilization. The lyophilized globins were digested using Tos-Phe-CH₂Cl (tosylphenylalanyl chloromethane, 'TPCK')-trypsin and the digest was analysed by RPHPLC as described above. Finally, kinetic analysis of the modified amino groups was accomplished by a comparison with the tryptic-peptide map of unmodified HbA. The pseudo first-order rate constant was calculated from the slope of a linear plot of log (M_∞ - M_t) against time, where M_t and M_∞ were the degree of modification at any given time (t) and at infinity, respectively [25].

CD (circular dichroism) spectroscopy

CD spectra of (propyl-PEG5k)₆-Hb and HbA were recorded on a JASCO-720 spectropolarimeter (Tokyo, Japan) at 25 °C using a 0.2 cm light path cuvette (310 μl). For the 280–200 nm absorbance spectra the Hb concentration was 1.3 μM as tetramer. For the 480–250 nm absorbance spectra the Hb concentration was 26.0 μM as tetramer. All the Hb samples were in PBS, pH 7.4. The specific ellipticity [θ] is expressed in deg.cm²/dmol on a haem basis.

COP and viscosity measurements

The COP of Hb samples was determined using a Wescor 4420 Colloidal Osmometer (Wescor, Logan, UT, U.S.A.). The measurements were performed using a series of concentrations of Hb samples in PBS (pH 7.4) at room temperature. The instrument was calibrated using Osmocoll (Wescor) reference standards.

The viscosity of the Hb samples was measured using a cone and plate rheometer (Brookfield Engineering, Middleboro, MA, U.S.A.). A series of concentrations of Hb samples in PBS (pH 7.4) were measured using the cone spindle CPE-40 (Brookfield Engineering) at a shear rate of 75 s⁻¹ at 37 °C.

Analytical methods

The determination of PEGylation-induced size enhancement of Hb by SEC on Superose 12 columns (Amersham Biosciences), RPHPLC analysis of globin chains on a Vydac C4 column (4.6 × 250 mm), oxygen-affinity measurements and SDS/PAGE were all carried out as previously described [26]. The extent of PEGylation of HbA was measured by determining the free-amino groups remaining on the protein by titration with 2,4,6-TNBS (2,4,6-trinitrobenzenesulphonic) acid [27,28]. The number of reactive SH groups in PEGylated Hb was estimated by titration with 4-PDS (4,4'-dithiopyridine) as described by Ampulski et al. [29].

Molecular modelling

Molecular models of PEGylated Hb were generated as described below and the crystal structure (1HHO) was chosen as our initial model [30]. The PEG model chosen was the PEG5k molecule, which consists of 113 consecutive ethylene oxide moieties. The initial model was generated using Insight II[®] (Accelrys Software Inc) computer graphics and the model was refined in water by simulated annealing to derive a globular structure. Various starting models were used to derive the final model and simulations were carried out using the Discover program (Insight II[®], Accelrys Software Inc) and consistent valence force-field potential. These final models were linked to Hb by aliphatic linkage at the four amino termini and to the side chain of Lys-11(α). These models were constructed without any van der Waals overlap between the

Figure 2 SEC analysis of PEGylated Hb generated by reductive alkylation with PEG5k-aldehyde

HbA (0.25 mM) in 50 mM BisTris-Acetate, pH 6.5 was incubated with PEG5k-aldehyde in the presence of NaCNBH₄ (5 fold molar excess over the aldehyde) at 4°C for 16 h. The ratios of HbA/PEG5k-aldehyde/NaCNBH₄ are 1:20:100 and 1:40:200 for curves 1 and 2 respectively. The SEC was carried out at room temperature on 2 HR10/30 Superose 12 columns (Amersham Biosciences) connected in series. The column was eluted using PBS (pH 7.4) at a flow rate of 0.5 ml/min and the effluent was monitored at absorbance 540 nm. Inset shows the SDS/PAGE analysis of (propyl-PEG5k)₆-Hb. The SDS/PAGE pattern of site-specifically PEGylated Hbs modified at Cys-93(β) with PEG5k, PEG10k and PEG20k [14] is also shown in this Figure. In these PEGylated Hbs the β-globin carries one copy of either PEG5k or PEG10k or PEG20k. Lane 1, HbA; Lane 2, (propyl-PEG5k)₆-Hb; Lane 3, (SP-PEG5k)₂-Hb; Lane 4, (SP-PEG10k)₂-Hb; Lane 5, (SP-PEG20k)₂-Hb. The numbers on the left indicate the positions of the molecular weight markers.

protein and the PEG chains. Keeping the position of the protein fixed, the PEG chains were refined for maximum surface coverage of the protein. After the initial refinement, the whole complex was refined using the steepest descent to derive the final structure.

RESULTS

Preparation of PEGylated HbA by reductive alkylation

Incubation of HbA (0.25 mM) in 50 mM BisTris-Acetate, pH 6.5 with 5 mM PEG5k-aldehyde (20 fold molar excess over HbA) in the presence of 25 mM NaCNBH₄ (5 fold molar excess over aldehyde) at 4°C overnight resulted in a significant enhancement in size as revealed by SEC (Figure 2, curve 1). However, the material eluted appeared as a broad, unsymmetrical peak consisting of a major component with a minor shoulder on the descending side of the peak suggesting product heterogeneity. Alternatively, incubation of HbA (0.25 mM) with 10 mM PEG5k-aldehyde (40 fold molar excess over HbA) in the presence of 50 mM NaCNBH₄ (5 fold molar excess over the aldehyde) at 4°C overnight resulted in the further enhancement of its size and the eluted product appeared as a single, symmetrical peak indicating homogeneity in molecular size (Figure 2, curve 2). Kinetic studies revealed that the reaction is essentially complete after 12 h at 4°C and after 6 h at room temperature (results not shown).

In addition to 50 mM BisTris-Acetate buffer (pH 6.5) the reductive alkylation of HbA with PEG5k-aldehyde was also carried out in 50 mM sodium phosphate buffer, at pH 7.0, 7.5 and 8.0, and at Hb/aldehyde/sodium cyanoborohydride ratios of 1:20:100 and 1:40:200. However, at every pH tested the resulting product was smaller in molecular size and less homogeneous compared with that obtained using BisTris-Acetate buffer (pH 6.5) at a HbA/

Figure 3 Characterization of (propyl-PEG5k)₆-Hb by RPHPLC

RPHPLC analysis was carried out on a Vydac C4 column (4.6 × 250 mm), using a linear gradient of 35–50 % acetonitrile containing 0.1 % TFA (trifluoroacetic acid) in 100 min and then 50–70 % acetonitrile containing 0.1 % TFA in 30 min. The flow rate was 1 ml/min and the effluent was monitored at absorbance 210 nm. Inset shows the SDS/PAGE analysis of the globin chains of (propyl-PEG5k)₆-Hb. Lane 1, (propyl-PEG5k)₆-Hb; lanes 2–5 are peaks 1–4 from the RPHPLC analysis.

PEG5k-aldehyde ratio of 1:40. Therefore all further studies were carried out with the material generated using 50 mM BisTris-Acetate buffer (pH 6.5), 0.25 mM HbA, at a HbA/PEG5k-aldehyde/NaCNBH₄ ratio of 1:40:200 at 4°C for 16–18 h. For large-scale preparations, the PEGylated HbA was separated from the excess PEG-aldehyde and other reagents by diafiltration through a 70 kDa membrane using a Minim Tangential Flow Filtration (Pall Corporation) system. SEC analysis of the retentate, as monitored by RI measurements, ensured the complete absence of unreacted PEG in the final product.

Estimation of the decrease in the number of free-amino groups after PEGylation, by titration with 2,4,6-TNBS, revealed that approx. 6 amino groups per Hb are modified by the PEG5k-aldehyde. Therefore this PEGylated Hb will hereafter be referred to as (propyl-PEG5k)₆-Hb. SDS/PAGE analysis of (propyl-PEG5k)₆-Hb is shown in Figure 2 (inset). As shown, unmodified HbA revealed a doublet band at approx. 16 kDa, corresponding to its α and β globins. The PEGylated Hb showed an essential absence of unmodified α- and/or β-globins and the presence of three major and two minor bands. By comparison with the site-specifically PEGylated globins modified at Cys-93(β) using PEG5k, PEG 10k and PEG20k [14], the mobilities of the three major bands in the PEGylated Hb corresponded to globin chains carrying PEG-mass equivalent to 1, 2 and 5 PEG5k chains per Hb.

Globin-chain analysis of (propyl-PEG5k)₆-Hb

Globin-chain analysis of (propyl-PEG5k)₆-Hb is shown in Figure 3. As shown, the α and the β-globins are essentially completely modified in (propyl-PEG5k)₆-Hb, and five new peaks (two major and three minor) eluting later than the α- and β-globins, are present. Thus it is apparent that despite the presence of a large number of lysine residues, the reductive alkylation with PEG5k-aldehyde is highly selective and is limited to only a few lysine residues. Furthermore, the RPHPLC profile of the globin chains was highly reproducible for several batches of

Table 1 Analysis of the mass of globin chains in (propyl-PEG5K)₆-Hb

The PEGylated globin chains isolated by RPHPLC were analysed by MALDI MS.

Sample	Mass (Da)	¹ Number of PEG chains
α -globin	² 15 126	—
β -globin	² 15 865	—
Pool 1	21 008.24	1
Pool 2	26 474.60	2
Pool 3	31 717.40	3
Pool 4	37 134.18	4

¹ The number of PEG chains in the PEGylated globins was calculated from the difference between their mass and the unPEGylated globins.

² The theoretical masses of α - and β -globin are 15 126 and 15 867 respectively.

Table 2 Identification of the sites of PEGylation in (propyl-PEG5K)₆-Hb

The sites of PEGylation in (propyl-PEG5K)₆-Hb and (propyl-PEG2K)₆-Hb were determined by a comparison of the tryptic-peptide mapping of their globin chains with that of unmodified HbA, as described in the Experimental section.

Residue modified	Percentage modification	
	(propyl-PEG5K) ₆ -Hb	(propyl-PEG2K) ₆ -Hb
Val-1(α)	100	100
Val-1(β)	100	100
Lys-8(β)	24	16
Lys-11(α)	23	11
Lys-40(α)	17	8
Lys-56(α)	16	13

the preparations, indicating the reproducibility of the reductive alkylation protocol. The peaks were pooled as indicated (because of incomplete resolution, peaks eluting in the 65–70 min region were pooled together) and analysed by SDS/PAGE (Figure 3, inset). As shown, each peak exhibited homogeneity in size. Furthermore, there is a correlation between the apparent size of the PEGylated globins and their HPLC retention-time indicating that the retention-time of the PEGylated globins is a function of the number of PEG chains (PEG-mass) conjugated.

The PEGylated globins obtained by RPHPLC were also analysed by MALDI (matrix-assisted laser-desorption ionization) MS to determine the number of PEG chains conjugated to the globin and the results are presented in Table 1. As shown, the mass of pools 1, 2, 3 and 4 corresponds to the conjugation of 1, 2, 3 or 4 PEG chains per globin chain. A comparison of the SDS/PAGE pattern of these PEGylated globins with that of the β -globin monoPEGylated using PEG5k, PEG10k and PEG20k presented in Figure 2 (inset) demonstrates that globin chains carrying multiple copies of PEG5k chains exhibit a significantly higher apparent mass, by SDS/PAGE, than the globin chains carrying the same PEG-mass as a single copy of PEG chain.

Identification of PEGylation sites

The sites of PEGylation in (propyl-PEG5K)₆-Hb were determined by a comparison of the tryptic-peptide map of its globin chains with that of the unmodified HbA and the results are presented in Table 2. These results revealed that Val-1(α) and Val-1(β) are completely modified by PEGylation. In addition, partial modification was observed at four lysine residues, namely Lys-11(α), Lys-40(α), Lys-56(α), and Lys-8(β). Thus four of the PEG chains in (propyl-PEG5K)₆-Hb are on the four amino-terminal residues and the remainder are distributed on a limited number of its lysine residues. The tryptic-peptide map of the (propyl-PEG5K)₆-Hb

Figure 4 Reactivity of the SH groups of (propyl-PEG5K)₆-Hb as measured by titration with 4-PDS

Reactivity of the SH groups of (propyl-PEG5K)₆-Hb as measured by reaction with 4-PDS, according to Ampulski et al. [29]. Hb (5 μ M) in PBS, pH 7.4, was incubated with 50 μ M 4-PDS at room temperature. The SH groups were estimated by measuring the conversion of 4-PDS to 4-thiopyridone at 324 nm as a function of time.

globins was reproducible from batch-to-batch of the preparation indicating that the PEGylation of the amino groups in Hb by the present reductive alkylation chemistry based protocol is not random but exhibits a high degree of site selectivity.

Kinetic analysis of the amino-group modification of HbA

The reactivity of the amino groups for reductive alkylation with PEG5k-aldehyde was established by measuring the extent of modification of the specific amino acid residues as a function of time, as revealed by tryptic-peptide mapping. The rate of PEGylation at Val-1(α) and Val-1(β) was significantly higher than that of the lysine residues. Furthermore, the reactivity of Val-1(β) was significantly higher than that of Val-1(α), although both groups are completely modified after 15 h. The first-order rate constants for the modification of Val-1(β), Val-1(α), Lys-8(β), Lys-11(α), Lys-40(α), and Lys-56(α) were calculated as 0.396 h⁻¹, 0.23 h⁻¹, 0.012 h⁻¹, 0.01 h⁻¹, 0.018 h⁻¹ and 0.009 h⁻¹.

Reactivity of Cys-93(β) of (propyl-PEG5K)₆-Hb

The number of reactive SH groups in (propyl-PEG5K)₆-Hb was estimated by titration with 4-PDS (Figure 4). As shown, the (propyl-PEG5K)₆-Hb also carries two reactive SH groups per molecule similar to HbA. Thus the two Cys-93(β) residues are not modified in (propyl-PEG5K)₆-Hb. More strikingly the reactivity of these SH groups is significantly enhanced in (propyl-PEG5K)₆-Hb relative to HbA.

Influence of PEG-mass on the reactivities of the amino-groups in HbA to PEG-aldehyde

To determine the influence of PEG size on the site selectivity, if any, for reductive alkylation with PEG-aldehyde, HbA was subjected to reductive alkylation with PEG2k-aldehyde (a lower molecular mass homologue of PEG5k-propionaldehyde) under the same conditions as described using PEG5k-aldehyde. HbA (0.25 mM) in 50 mM BisTris-Acetate buffer (pH 6.5) was reacted with 10 mM PEG2k-aldehyde in the presence of 50 mM NaCNBH₃ at 4°C overnight. It can be seen by the SEC profile in Figure 5(A) that as in PEG5k-aldehyde, reductive alkylation of HbA with PEG2k-aldehyde results in the complete modification of HbA and generation of a homogeneous, size-enhanced species. Consistent with its smaller size the product generated using PEG-2k-aldehyde is significantly smaller than that generated using PEG5k-aldehyde.

Figure 5 (A) SEC analysis of PEGylated Hb generated by reductive alkylation with PEG2k-aldehyde

HbA (0.25 mM) in 50 mM BisTris-Acetate, pH 6.5 was incubated with PEG2k-aldehyde in the presence of NaCNBH₄ (5 fold molar excess over the aldehyde) at 4°C for 16 h. The ratios of HbA/PEG5k-aldehyde/NaCNBH₄ were 1:40:200. The SEC was carried out as described in Figure 2. The elution profiles of (propyl-PEG5k)₆-Hb and HbA are included for comparison. (B) Globin-chain analysis of PEG2k-aldehyde-reacted HbA by RPHPLC. The RPHPLC analysis was carried out as described in Figure 3. Inset shows the SDS/PAGE analysis of the globin chains obtained by RPHPLC. Lane 1, HbA; Lane 2, HbA reacted with PEG2k-aldehyde; Lanes 3–6 are pools 1–4 from the RPHPLC analysis.

Globin-chain analysis of the PEG2k-aldehyde reacted HbA, by RPHPLC, is presented in Figure 5(B). As can be seen, similar to the PEG5k-aldehyde-reacted HbA, the product is essentially free of unmodified α- and β-globins and shows the presence of three major and a few minor PEGylated globin-chains. Like the PEGylated globin-chains of the (propyl-PEG5k)₆-Hb, the first three pools of the PEG2k-aldehyde-reacted HbA exhibited homogeneity in size by SDS/PAGE analysis (Figure 5B, inset).

The site selectivity of the PEG2k-aldehyde for the amino groups, relative to that of the PEG5k-aldehyde, was determined by a comparison with the two PEGylated Hbs by tryptic-peptide mapping. As shown in Table 2, the overall pattern of modification with PEG2k-aldehyde is similar to that of PEG5k-aldehyde. As in PEG5k-aldehyde, Val-1(α) and Val-1(β) are modified completely on reductive alkylation with PEG2k-aldehyde, however, modification at other amino acid residues is lower than that observed using PEG5k-aldehyde. Based on the tryptic-peptide

Figure 6 CD spectra of (propyl-PEG5k)₆-Hb (solid line) and HbA (dotted line)

For the far-ultraviolet light region (A) the Hb concentration was 1.3 μM. For the near-ultraviolet light region (B) the Hb concentration was 26 μM. A 0.2 cm cuvette was used for CD measurements. Molar ellipticity [θ] was calculated on a haem basis.

mapping, the overall modification accounts for the conjugation of 5–6 PEG chains per Hb. Therefore this PEGylated Hb will hereafter be referred to as (propyl-PEG2k)₆-Hb. Thus it is apparent that the length of the PEG chain does not influence the site selectivity for the modification of Val-1(α) and Val-1(β), but appears to have an influence on the rate of modification of the exposed lysine residues.

Structural characteristics of (propyl-PEG5k)₆-Hb

The structural characteristics of (propyl-PEG5k)₆-Hb were determined by the measurement of its CD spectra in the near-ultraviolet (absorbance 260–480 nm) and far-ultraviolet (absorbance 200–260 nm) regions (Figure 6). The CD spectra of (propyl-PEG5k)₆-Hb and HbA in the far-ultraviolet region, which is mainly due to the globin portions of Hb, were essentially identical. This indicates that the helical content of HbA did not change and its secondary structure was not adversely influenced upon reductive alkylation-mediated PEGylation. In the near-ultraviolet region minor differences between (propyl-PEG5k)₆-Hb and HbA were observed at the Soret region, indicating that the microenvironment of the haem is slightly changed upon PEGylation of HbA. This result is consistent with the minor changes in the cooperativity (Hill coefficient) of HbA upon PEGylation, as described below.

Functional properties of (propyl-PEG5k)₆-Hb

The results of the oxygen-binding properties in (propyl-PEG5k)₆-Hb and (propyl-PEG2k)₆-Hb in PBS, pH 7.4 at 37 °C are presented in Figure 7 and Table 3. As shown, PEGylation of HbA with both the PEG-aldehydes decreased its *p*50 (partial oxygen pressure at 50% saturation) from 14 mmHg to approx. 7 mmHg (i.e. increased oxygen affinity), the difference due to PEG size being only marginal. The Hill coefficients of the PEGylated Hbs are slightly lower than that of HbA (1.9 and 2.1 respectively, indicating a decrease in cooperativity upon PEGylation).

Figure 7 Oxygen-binding curves of (propyl-PEG5k)₆-Hb (solid line), (propyl-PEG2k)₆-Hb (dotted line) and HbA (dashed line)

(A) In the absence of effectors; (B) in the presence of 2.5 mM 2,3-DPG; (C) in the presence of 1.0 M NaCl. Inset shows the Hill coefficient of (propyl-PEG5k)₆-Hb (▲), (propyl-PEG2k)₆-Hb (●) and HbA (■). The oxygen equilibrium curves were measured using a Hem-O-Scan (Aminco) at 37 °C in PBS, pH 7.4, at a Hb tetramer concentration of 0.5 mM.

Table 3 Oxygen binding properties of (propyl-PEG5K)₆-Hb

Oxygen equilibrium curves of the samples were measured using Hem-O-Scan (Aminco) at 37 °C in PBS (pH 7.4) at a haemoglobin tetramer concentration of 0.5 mM.

Effectors	HbA		(propyl-PEG5K) ₆ -Hb		(propyl-PEG2K) ₆ -Hb	
	p50	n*	p50	n	p50	n
None	14.0	2.8	7.0	1.9	7.5	2.1
2.5 mM DPG	21.5	2.6	6.5	2.0	7.2	2.1
1.0 M NaCl	27.5	2.7	6.5	1.9	9.5	2.0
10 mM IHP	82.0	2.0	10.0	1.9	9.6	1.9

* Hill coefficient

The influence of the allosteric effectors [2,3-DPG (2,3-diphosphoglycerate) and Cl⁻] on the oxygen affinity of the PEGylated Hb has also been investigated. The p50 of HbA is increased

Figure 8 COP and viscosity of (propyl-PEG5k)₆-Hb (▼), (propyl-PEG2k)₆-Hb (●) and HbA (■) as a function of protein concentration

(A) A series of concentrations of HbA samples were measured by a Wescor 4420 Colloidal Osmometer in PBS (pH 7.4) at room temperature. Osmocoll reference standards were used to calibrate the instrument before measurements of the samples. Inset shows the correspondence between the COP of the two PEGylated Hbs compared at equivalent protein concentrations. (B) A series of concentrations of HbA samples were measured using the cone spindle (CPE-40, Brookfield Engineering) at a shear rate of 75 s⁻¹ in PBS (pH 7.4) at 37 °C. Inset shows the relationship between the viscosities of the two PEGylated Hbs compared at equivalent protein concentrations.

1.5 fold and 2.0 fold in the presence of 2,3-DPG and Cl⁻ respectively. On the other hand, the oxygen affinity of (propyl-PEG5k)₆-Hb and (propyl-PEG2k)₆-Hb is almost insensitive to 2,3-DPG (Figure 7B). Similarly, the p50 of (propyl-PEG5k)₆-Hb is also insensitive to Cl⁻, but shows a small response to IHP (inositol hexaphosphate) (Figure 7C and Table 3), whereas (propyl-PEG2k)₆-Hb showed a marginal increase in p50 with both Cl⁻ and IHP. These results indicate that the decrease in the oxygen affinity of HbA modulated by the electrostatic modification of Hb in the presence of anions is desensitized upon PEGylation of the protein.

Solution properties of (propyl-PEG5k)₆-Hb

The COP of HbA and (propyl-PEG5k)₆-Hb as a function of Hb concentration is shown in Figure 8(A). As can be seen, the COP of HbA shows only a marginal increase as protein concentration increases and is linear over the range studied. In contrast, the COP

Table 4 Comparison of the molecular radius, viscosity and COP of (propyl-PEG2K)₆-Hb, (propyl-PEG5K)₆-Hb and (SP-PEG5K)₆-Hb

The molecular radius was measured at a protein concentration of 1 mg/ml in PBS (pH 7.4) and the viscosity and COP were measured at a Hb concentration of 4 g%. The results for (SP-PEG5K)₆-Hb are from Manjula et al. [15].

	Radius (nm)	Viscosity (cp)	COP (mmHg)
HbA	3.12	0.91	12.4
(propyl-PEG2K) ₆ -Hb	3.90	1.21	41.4
(propyl-PEG5K) ₆ -Hb	5.41	2.84	111.9
(SP-PEG5K) ₆ -Hb	6.50	2.50	70.4

of (propyl-PEG5k)₆-Hb demonstrated an exponential increase as protein concentration increased. The COP of HbA PEGylated with PEG2k-aldehyde is also shown in Figure 8(A). Similar to (propyl-PEG5k)₆-Hb, this PEGylated Hb also exhibited an exponential increase in COP as protein concentration increased but relatively less than that observed with (propyl-PEG5k)₆-Hb. Inset shows the relationship between the COP of the two PEGylated Hbs at equivalent protein concentrations. As shown, the increase in COP with increase in PEG-mass suggests a slightly exponential correspondence.

The viscosity of (propyl-PEG5k)₆-Hb and (propyl-PEG2k)₆-Hb as a function of protein concentration is shown in Figure 8(B). In a similar manner to the COP, the viscosity of HbA did not change significantly with increasing concentration, whereas the viscosity of the two PEGylated Hbs increased as protein concentration increased as a function of the conjugated PEG-mass. Unlike the COP, the viscosity of the two PEGylated Hbs compared at equivalent protein concentrations exhibited a linear correspondence.

It should also be noted that a mixture of HbA and methoxy-PEG5k that corresponds to the composition in hexaPEGylated Hb exhibits significantly low viscosity and COP compared with the hexaPEGylated Hb (unpublished work). Thus the high viscosity and high COP of the PEGylated Hb is a consequence of the chemical linkage between the PEG and the protein.

Molecular radius of (propyl-PEG5k)₆-Hb

The molecular radius of (propyl-PEG5k)₆-Hb and (propyl-PEG2k)₆-Hb, as determined by dynamic light scattering, along with that of (SP-PEG5k)₆-Hb [15] is presented in Table 4. As shown, the molecular radius of (propyl-PEG5k)₆-Hb is larger than that of (propyl-PEG2k)₆-Hb, consistent with the PEG-mass conjugated. However, despite comparable PEG-mass, (propyl-PEG-5k)₆-Hb exhibits a significantly lower molecular radius than (SP-PEG5k)₆-Hb.

A comparison of the viscosity and COP of these PEGylated Hbs is also presented in Table 4. Although the viscosities of (propyl-PEG5k)₆-Hb and (SP-PEG5k)₆-Hb are similar, the COP of (propyl-PEG5k)₆-Hb is significantly higher (approx. 1.6 fold) than that of (SP-PEG5k)₆-Hb. Also the viscosity and COP of (propyl-PEG2k)₆-Hb and (propyl-PEG5k)₆-Hb are consistent with the mass of conjugated PEG.

DISCUSSION

The present study describes the generation of a new hexa-PEGylated Hb, (propyl-PEG5k)₆-Hb, by reductive alkylation of HbA using PEG5k-aldehyde. This PEGylated Hb exhibited

Figure 9 Molecular model of a hexaPEGylated-Hb generated by reductive alkylation chemistry

HbA conjugated with PEG5k chains at Val-1(α), Val-1(β), and Lys-11(α) is shown. The α - and the β -globin chains are shown in blue and red respectively. PEG chains are shown in green. The models were generated as described in the Experimental section.

homogeneity in terms of its hydrodynamic volume. Of the PEG chains in (propyl-PEG5k)₆-Hb, four are on the amino termini of the protein and the remainder are distributed in the majority on four lysine residues, namely Lys-11(α), Lys-40(α), Lys-56(α) and Lys-8(β). The complete modification of Val-1(α) and Val-1(β) at pH 6.5 is consistent with a low pK_a relative to that of the ϵ -amino groups. Partial modification of the ϵ -amino groups of Lys-8(β), Lys-11(α), Lys-40(α) and Lys-56(α) suggests that the pK_a of these lysines is low relative to that of the other surface lysine residues of Hb. An examination of the molecular models of Hb clearly shows that these amino groups are exposed to the solvent, indicating their accessibility to the macromolecular PEG reagent. A molecular model of HbA conjugated to PEG5k at Val-1(α), Val-1(β) and Lys-11(α) is illustrated in Figure 9. The molecular modelling indicates that PEG chains are not passively extended on the surface of HbA, but form loosely organized domains. Consequently the PEG chains can mask reactive functional groups over large areas of the HbA surface, thus sterically hindering their accessibility to the macromolecular PEG reagent and hence interfering with further PEGylation.

HexaPEGylation of Hb by reductive alkylation with PEG5k-aldehyde appears to have limited influence on the overall conformation of Hb, as reflected by CD measurements. The increased reactivity of Cys-93(β) in (propyl-PEG5k)₆-Hb to 4-PDS appears to be consistent with its increased oxygen affinity. However, a study using seven human Hb mutants with high oxygen-affinity demonstrated that only some of these mutants exhibit increased Cys-93(β) reactivity to 4-PDS and hence there is a lack of correlation between SH reactivity and oxygen affinity using 4-PDS as a reagent, 4-PDS suggesting to be sensitive to steric and conformational factors [31]. Thus the increased reactivity of Cys-93(β) in (propyl-PEG5k)₆-Hb to 4-PDS could also be a PEGylation-mediated influence on its microenvironment resulting in an increased accessibility of this residue to the low molecular

mass 4-PDS reagent. Further studies are needed to better delineate the molecular details of this phenomenon.

(Propyl-PEG5k)₆-Hb exhibits high oxygen-affinity and reduced cooperativity and desensitization to the influence of allosteric effectors. These functional properties of (propyl-PEG5k)₆-Hb could either be a direct consequence of the reductive alkylation of the four α -amino groups of Hb, or the influence of the long-PEG-chains linked to the amino termini, or a combination of the two. The four amino-terminal valine residues of Hb are present around the $\alpha\alpha$ and the $\beta\beta$ end of the water filled central cavity [32,33]. The amino groups of Val-1(α) and Val-1(β) are involved in unique electrostatic interactions that orientate the molecule for interactions with allosteric effectors and the arrangement of the structured water molecules in the central cavity. Since reductive alkylation-mediated PEGylation can be considered as reductive alkylation by substituted aliphatic aldehydes, a comparison can be made between the consequences of reductive alkylation with glycolaldehyde [34] and with PEG-aldehyde. This could facilitate the delineation of the role of PEG chains, in particular whether the PEG chains act as passive tentacles/domains on a reductively alkylated molecule without significant influence on the functional properties of Hb. Reductive hydroxyethylation of Val-1(α) increases the oxygen affinity of Hb and slightly reduces the modulation of oxygen affinity by Cl⁻ relative to that of Hb. Similar modification at Val-1(β) reduces the oxygen affinity of Hb and also reduces the level of modulation of oxygen affinity by the allosteric effectors 2,3-DPG and Cl⁻ to a small degree. But the hexaPEGylated Hb generated by reductive alkylation with PEG5k-aldehyde, wherein both Val-1(α) and Val-1(β) are modified, exhibits an increased oxygen affinity and complete desensitization for modulation of oxygen affinity by both 2,3-DPG and Cl⁻. However, a slight but noticeable modulation of the oxygen affinity is observed in the presence of IHP. The level of desensitization using 2,3-DPG and Cl⁻-mediated modulation observed on reductive PEGylation also appears to be sensitive to the size of the PEG chain. A hexaPEGylated Hb generated using the lower molecular mass PEG2k aldehyde shows some sensitivity to chloride, as well as IHP. Thus the consequences of reductive PEGylation of the amino terminal residues in Hb on its functional properties are distinct as compared with reductive alkylation using simple aliphatic aldehydes.

It may be noted that we have shown recently that a hexa-PEGylated Hb, (SP-PEG5k)₆-Hb, generated by thiolation-mediated maleimide-chemistry based PEGylation also exhibits a high oxygen-affinity and desensitization for the modulation of its oxygen affinity by 2,3-DPG, IHP and Cl⁻ [15]. This influence on the functional properties of Hb observed with (SP-PEG5k)₆-Hb has been suggested to be a direct consequence of the PEGylation at Cys-93(β) [15], since modification of this residue is accompanied by such properties. However, Cys-93(β) is not modified in (propyl-PEG5k)₆-Hb. Thus surface decoration of Hb by reductive PEGylation appears to induce high oxygen-affinity to Hb even in the absence of PEGylation of Cys-93(β). It may also be noted that PEG in solution has been shown to bind approximately 2–3 water molecules per repeating unit. Thus it is conceivable that the PEG chains conjugated on the protein surface could act as a scaffold and create a large cloud/shell of water molecules around the protein, thus shielding it from direct interactions with the bulk water molecule. This could restrict the R to T transition of the Hb molecule, thereby favouring the oxy conformational state with a larger number of bound water molecules over the deoxy state with a lower number of bound water molecules. Given the fact that the chemistry and site selectivity (pattern) of PEGylation in Hb by reductive alkylation and the thiolation-

Figure 10 Schematic representation comparing the linkages in the PEGylated Hb generated by reductive alkylation-mediated PEGylation and thiolation-mediated maleimide-chemistry based PEGylation

mediated maleimide-chemistry PEGylation are very distinct, it appears that the PEG-shell-induced influence on the hydration state of Hb could contribute significantly to the high oxygen-affinity and desensitization to allosteric effectors of the PEG–Hb conjugate.

In (propyl-PEG5k)₆-Hb, besides the four α -amino groups, two PEG chains are distributed among the ϵ -amino groups of Lys-11(α), Lys-40(α), Lys-56(α) and Lys-8(β). Of these Lys-11(α), Lys-56(α) and Lys-8(β) are solvent exposed and are not closely involved in interactions that influence the oxygen affinity of Hb. However, the ϵ -amino group of Lys-40(α) forms an inter-chain salt-bridge with the α -carboxyl of His-146(β), and plays a key role in cooperative oxygen binding by Hb. The positive charge of the ϵ -amino group is not altered by reductive PEGylation and thus is expected to have minimal influence on the electrostatic interactions, however, the influence of the bulky PEG chains, conjugated at this site, on the oxygen affinity cannot be predicted at this stage. However, since the reductive PEGylation of this residue in (propyl-PEG5k)₆-Hb is only partial, PEGylation at this site is unlikely to contribute significantly to the observed increase in oxygen affinity.

The results of the present study further demonstrate that the site (pattern) and/or the chemistry of PEGylation have distinct influences on the molecular dimension and solution properties of the PEGylated Hbs. Despite the comparable mass of conjugated PEG, the molecular radius of (propyl-PEG5k)₆-Hb generated by the reductive PEGylation is significantly smaller than that of (SP-PEG5k)₆-Hb generated by the thiolation-mediated PEGylation and the COP is significantly higher. Thus PEG chains linked to Hb by reductive alkylation increase its COP more effectively than a similar number of chains linked by thiolation-mediated PEGylation. Typically, infusion of a large volume of protein solution intravenously will alter the solution properties of the blood, such as the COP and viscosity. In view of its high COP (111.9 mmHg versus 27 mmHg in blood) (propyl-PEG5k)₆-Hb could increase the plasma volume effectively and thus serve as a valuable material for small-volume field resuscitation applications.

A schematic representation of the linkage between the PEG-chain and Hb in the reductively PEGylated Hb and the PEG–Hb generated by thiolation-mediated PEGylation [15,18] is shown in Figure 10. As in (SP-PEG5k)₆-Hb, the original positive charges in HbA are conserved in (propyl-PEG5k)₆-Hb and hence reductive

PEGylation is also a conservative PEGylation. However, the proximity of the PEG chain to the peptide backbone is significantly different in both PEGylated Hbs. In reductively PEGylated Hb the predominant modification is on the α -amino groups. Thus the PEG chain is in close proximity to the peptide backbone. By contrast, the predominant modification of the thiolation-mediated maleimide-chemistry PEGylation is on the ϵ -amino groups. In addition thiolation of the ϵ -amino groups introduces a 4-carbon 'extension arm' between the ϵ -amino group and the newly introduced SH group. Furthermore, the linker group between the PEG-chain and the amino group derivatized in the reductively PEGylated Hb is significantly shorter compared with that in the PEGylated Hb generated by the thiolation-mediated maleimide-chemistry based PEGylation. Thus the PEG shell is significantly closer to the protein core in (propyl-PEG5k)₆-Hb compared with (SP-PEG5k)₆-Hb. The results of the present study demonstrate that the conjugation chemistry of the PEG chains, together with the site and/or the location of the PEG chains on the protein surface, could have a significant bearing on the density of the PEG shell around the protein and hence the molecular volume, as well as the COP of PEGylated Hb.

PEGylation is a widely used bioconjugation strategy for the modification of proteins and peptides for therapeutic use [2–6]. Several PEG-conjugation chemistry techniques have been used to accomplish this aim. The influence that the conjugation site and the size of PEG conjugated has on the pharmacokinetics and pharmacological properties of proteins has been demonstrated [35–39]. It is generally considered that the surface coverage of proteins/peptides by PEG-chains is responsible for the increased therapeutic efficacy of the PEG-biomolecule conjugate. The possible influence of conjugation chemistry on the properties of the conjugate has not received much attention. To our knowledge, the present study represents the first report demonstrating the impact of PEG-conjugation chemistry on the solution properties of a PEGylated protein.

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