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Conjugation of Multiple Copies of Polyethylene Glycol to Hemoglobin Facilitated Through Thiolation: Influence on Hemoglobin Structure and Function

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PEGylation induced changes in molecular volume and solution properties of HbA have been implicated as potential modulators of its vasoconstrictive activity. However, our recent studies with PEGylated Hbs carrying two PEG chains/Hb, have demonstrated that the modulation of the vasoconstrictive activity of Hb is not a direct correlate of the molecular volume and solution properties of the PEGylated Hb and implicated a role for the surface charge and/or the pattern of surface decoration of Hb with PEG. HbA has now been modified by thiolation mediated maleimide chemistry based PEGylation that does not alter its surface charge and conjugates multiple copies of PEG5K chains. This protocol has been optimized to generate a PEGylated Hb, $(SP\text{-}PEG5K)_6$-Hb, that carries ~six PEG5K chains/Hb – HexaPEGylated Hb. PEGylation increased the O_2 affinity of Hb and desensitized the molecule for the influence of ionic strength, pH, and allosteric effectors, presumably a consequence of the hydrated PEG-shell generated around the protein. The total PEG mass in $(SP\text{-}PEG5K)_6$-Hb, its molecular volume, O_2 affinity and solution properties are similar to that of another PEGylated Hb, $(SP\text{-}PEG20K)_2$-Hb, that carries two PEG20K chains/Hb. However, $(SP\text{-}PEG5K)_6$-Hb exhibited significantly reduced vasoconstriction mediated response than $(SP\text{-}PEG20K)_2$-Hb. These results demonstrate that the enhanced molecular size and solution properties achieved through the conjugation of multiple copies of small PEG chains to Hb is more effective in decreasing its vasoconstrictive activity than that achieved through the conjugation of a comparable PEG mass using a small number of large PEG chains.

KEY WORDS: Colligative property; hydrodynamic volume; PEG shell; PEGylated hemoglobin; PEGylation; vasoactivity.

1. INTRODUCTION

Vasoactivity of acellular Hb has been a major impediment in the development of Hb-based oxygen carriers (Hess *et al.*, 1993; Saxena *et al.*, 1999; Sloane *et al.*, 1999; Thomson *et al.* 1994). This has been attributed, at least in part, to the NO scavenging effect of Hb (Doherty *et al.*, 1998; Dou *et al.*, 2002; Hess *et al.*, 1993; Kilbourn *et al.*, 1994; Motterlini *et al.*, 1996; Muldoon *et al.*, 1996; Thomson *et al.*

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[6]Abbreviations: Hb, hemoglobin; PEG, poly(ethylene glycol); PEGylation, conjugation with PEG; PEGylated, conjugated with PEG, 4-PDS, 4,4-dithiopyridine; SP, succinimidophenyl; SE, succinimidoethyl; PBS, phosphate buffered saline; Tris, tris(hydroxymethyl) amino methane; SEC, size exclusion chromatography; COP, colloidal osmotic pressure; FCD, functional capillary density; MAP, mean arterial pressure; hypervolemic infusion, infusion of a known volume of the test solution without removal of an equal volume of blood.

1994). Reduction of NO binding through site-directed mutagenesis is one of the approaches being evaluated for the reduction of the vasoactivity of acellular Hb (Eich *et al*., 1996; Doherty *et al*., 1998; Dou *et al*., 2002). The other approach involves enhancement of the molecular size of Hb to reduce or possibly prevent the extravasation of Hb, based on the concept that the vasoactivity of acellular Hb is due to its extravasation into the interstitial spaces and trapping of NO (Macdonald and Motterlini, 1994). However, the recent studies of Winslow and co-workers (Vandegriff *et al*., 1997; Rohlfs *et al*., 1998; Winslow *et al*., 1998) utilizing Hb preparations varying in molecular size namely, oligomerized Hb, polymerized Hb and PEGylated Hb have suggested a lack of correlation between their NO binding activity and pressor effect. These studies also suggested that PEGylation modulates the Hb induced hypertension and thus could be an efficient approach to reduce the vasoactivity of Hb. The higher viscosity and oncotic pressure of the PEGylated Hb were implicated as the potential modulators of the vasoactivity of Hb.

In the PEGylated Hbs described in the above study, the PEG chains are conjugated to the surface amino groups of the protein through urethane linkages (Nho *et al*., 1994). Thus, this PEGylation is accompanied by a loss of the net positive charge of the protein (non-conservative PEGylation). In an attempt to delineate the role of PEGylation induced changes in solution properties, surface charge and the pattern of surface decoration of PEG on Hb for the modulation of its vasoactivity, we have been exploring new chemical approaches for the PEGylation of Hb without altering its surface charge i.e., conservative PEGylation (Acharya *et al*., 1996; Manjula *et al*., 2000; Manjula *et al*., 2003). We have recently reported the preparation of three site-specifically PEGylated Hbs, namely $(SP\text{-}PEG5K)_2$-Hb, $(SP\text{-}PEG10K)_2$-Hb, and $(SP\text{-}PEG20K)_2$-Hb (Manjula *et al*., 2003). Each of these PEGylated Hbs carry two copies of PEG chains/Hb, conjugated at its two Cys-93(β) residues, but differ in the size of the PEG chains i.e., PEG5000, PEG10000 and PEG20000, respectively. The hydrodynamic volume, molecular radius, viscosity and oncotic pressure of Hb increased with PEGylation and exhibited a correlation with the mass of the PEG conjugated (i.e., chain length). However, the vasoactivity of the preparations was not a direct correlate of the PEG mass. Thus, the neutralization of the vasoactivity was not a direct correlate of the colligative properties of the PEGylated Hb. There appeared to be a threshold for the PEG chain length beyond which the ability to modulate the vasoactivity was decreased, despite an increase in the colligative properties. Furthermore, the surface coverage of Hb with the PEG chain was also not directly proportional to the length of the PEG chain and suggested a potential relation between the surface coverage by the conjugated PEG and the vasoactivity.

In the present study, the maleimide chemistry based PEGylation protocol has been used to conjugate multiple copies of PEG5000 chains to Hb to accomplish a more uniform surface coverage of the protein without altering its surface charge. Since there are only two reactive -SH groups in oxy Hb (the two Cys-93(β) residues), we used the propensity of iminothiolane to react with protein amino groups to generate new thiols (Jue *et al*., 1978; Traut *et al*., 1973) to introduce additional PEG-maleimide reactive sites on Hb (Fig. 1). Thus, PEGylation of oxy Hb in the presence of iminothiolane is targeted to the two intrinsic thiols of Cys-93(β) and the extrinsic thiols generated on the ε-amino groups by the iminothiolane. Employing this protocol and using Mal-Phe-PEG5000 as the PEGylating reagent, a new PEGylated Hb carrying an average of ~six PEG5000 chains on the Hb, namely $(SP\text{-}PEG5K)_6$-Hb, has been generated. This new PEGylated Hb exhibits a size enhancement, solution properties and O_2 affinity comparable to that of $(SP\text{-}PEG20K)_2$-Hb, but has a significantly reduced vasoactivity relative to the latter. These results establish that the PEGylation induced solution properties, without an accompanying loss of surface positive charge, can neutralize the vasoactivity of Hb and demonstrate that the PEGylation mediated modulation of the vasoactivity of Hb is a function of the solution properties of the PEGylated Hb in conjunction with the configuration of PEG on the surface of Hb.

2. MATERIALS AND METHODS

2.1. Materials

HbA was purified from the human erythrocyte lysate by DE-52 chromatography (Manjula and Acharya, 2003). 2-Iminothiolane was a product of

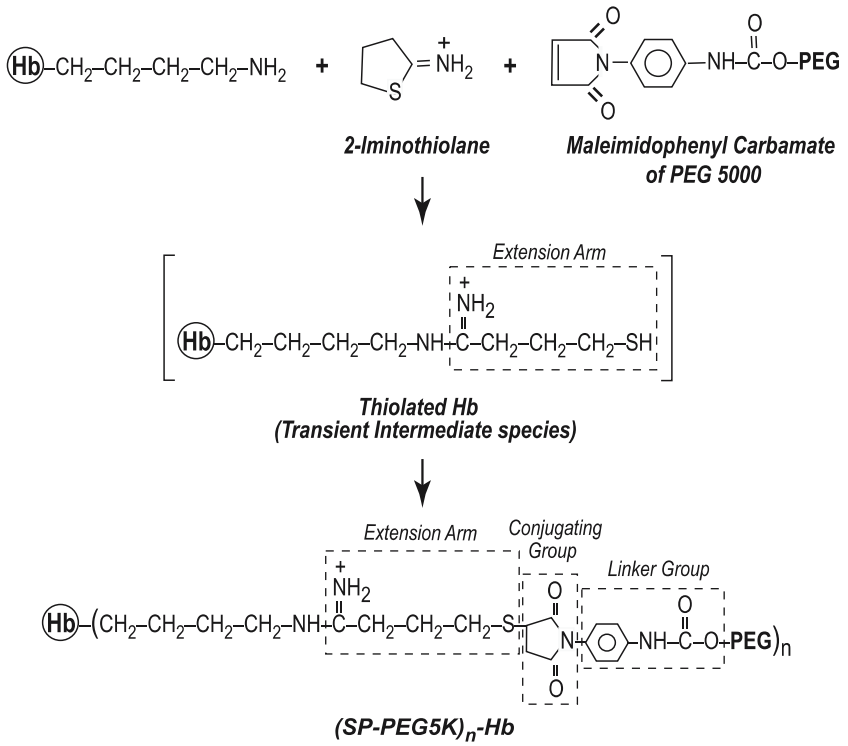

Fig. 1. Schematic representation of the iminothiolane dependent thiolation mediated maleimide chemistry based PEGylation of Hb.

BioAffinity Systems, Rockford, IL. 4,4′-dithiopyridine (4-PDS) was purchased from Aldrich Chemical Co. Synthesis of the mono functional maleimidophenyl (Mal-Phe) derivatives of PEG5000, PEG10000 and PEG20000, and preparation of the site-specifically PEGylated Hbs, namely $(SP\text{-}PEG5K)_2$-Hb, $(SP\text{-}PEG10K)_2$-Hb, and $(SP\text{-}PEG20K)_2$-Hb were carried out as described earlier (Manjula *et al*., 2003).

2.2. Iminothiolane Dependent Thiolation Mediated Maleimide Chemistry Based PEGylation of HbA

This was carried out either as a one-step or as a two-step reaction. In the one step reaction, HbA (0.5 m*M*) in PBS, pH 7.4 was incubated with Mal-Phe-PEG5000 in the presence of iminothiolane, at the concentrations indicated in the text, either at 4°C or at room temperature. Generally, 4 to 6 h incubation was needed for completion of the reaction at room temperature. Reactions at 4°C were routinely carried out for ~16 h. The reaction was generally carried out at a HbA concentration of 0.5 m*M*, but it could also be carried out at HbA concentrations of either 0.25 m*M* or 1 m*M*. In the two-step reaction, HbA (1.0 m*M*) in PBS pH 7.4 was first incubated with 10 m*M* iminothiolane overnight at 4°C. The reaction mixture was then diluted with an equal volume of 20 m*M* Mal-Phe-PEG5000 in PBS, pH 7.4, and the PEGylation was carried out for 6 h of 4°C. In both the one-step and the two-step reactions, after the desired incubation period, the reaction mixtures were dialyzed extensively against PBS, pH 7.4 prior to analysis.

2.3. Identification of the Sites of PEGylation

This was determined by tryptic peptide map analysis, as previously described (Rao *et al*., 1994). Briefly, the globin chains of Hb prepared by acid-acetone precipitation were dissolved in 100 m*M* ammonium bicarbonate to a final concentration of 1 mg/ml, and digested for 3 h at 37°C with TPCK-trypsin (Sigma Chemical Co., St. Louis, MO) at a enzyme to substrate ratio of 1:100 (w/w). The resulting tryptic peptides were analyzed by RPHPLC on a Vydac C18 column (10 × 250 mm) using a linear gradient of 5–50% acetonitrile containing 0.1% TFA in 160 min, followed by a linear gradient of 50–70% acetonitrile containing 0.1%

TFA in 20 min at a flow rate of 2 ml/min. The elution of the peptides was monitored at 210 nm. 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.

2.4. Vasoactivity of PEGylated Hbs

Analysis of the vasoactivity and microvascular hemodynamics of the PEGylated Hb were carried out in a hamster skin fold window microcirculation model, essentially according to the procedures previously described (Mirhashemi *et al*. 1988; Tsai *et al*. 1995; Tsai *et al*., 1996; Tsai *et al*. 1998; Kerger *et al*. 1996).

2.5. Analytical Methods

The thiolation of Hb by 2-iminothiolane was followed by estimating the number of thiol groups formed as a function of time using 4,4′-dithiopyridine (4-PDS), as described by Ampulski *et al* (1969). The number of PEG chains conjugated per Hb tetramer was determined by proton NMR spectroscopy, as described by Jackson *et al*. (1987). Analyses of the size enhancement of Hb as a function of the various thiolation mediated PEG-maleimide based PEGylation conditions, determination of the molecular radius of the PEGylated Hbs, their O_2 equilibrium curves, and measurements of their viscosity and colloidal osmotic pressure were carried out as previously described (Manjula *et al*. 2003). Structural characterization of the PEGylated Hbs by proton NMR spectroscopy was carried out as previously described (Plateau and Gueron, 1982).

3. RESULTS

3.1. Iminothiolane Dependent Thiolation Mediated Maleimide Chemistry Based PEGylation of Hb

(i) One step protocol: *In situ* thiolation mediated PEGylation of Hb: In this protocol, thiolation of HbA with iminothiolane is carried out in the presence of the PEG-maleimide. 2-Iminothiolane, by itself, does not carry any free thiol group and the thiol group is generated only after it reacts with the ε-amino groups of the protein. Thus, HbA can be incubated with iminothiolane in the presence of the PEG-maleimide without the concern of the thiolating reagent itself consuming the PEGylating reagent. Thus, as new thiol groups are generated *in situ* they are trapped by the PEG maleimide. Hence, HbA with multiple thiol groups is not accumulated as an intermediate in the reaction mixture.

The size exclusion chromatographic pattern of HbA (0.5 m*M*) in PBS, pH 7.4, incubated with a 20 fold molar excess of Mal-Phe-PEG5000 (10 m*M*) for 4.5 h at room temperature, in the absence and in the presence of varying concentrations of iminothiolane is shown in Fig. 2. The retention time of

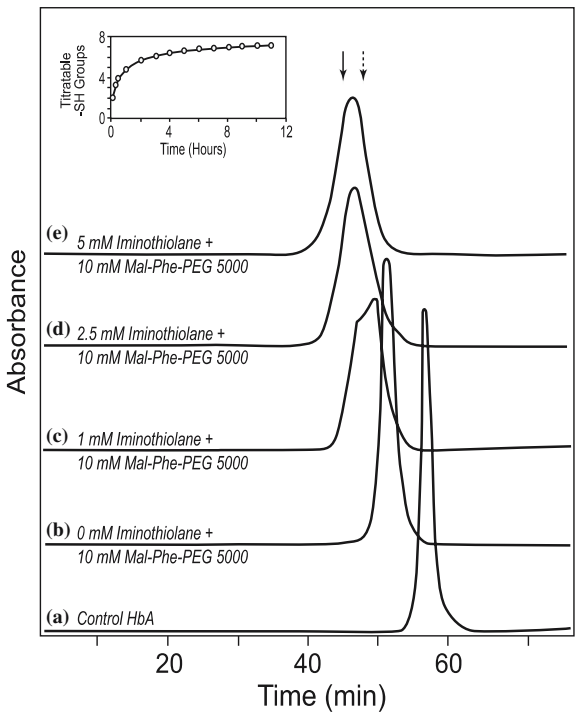

Fig. 2. *In situ* thiolation mediated maleimide chemistry based PEGylation of Hb: Influence of iminothiolane concentration on the size enhancement of Hb with Mal-Phe-PEG5000. HbA (0.5 m*M* in tetramer) in PBS, pH 7.4 was incubated with 10 m*M* Mal-Phe-PEG5000 for 4.5 h at room temperature either in the absence or in the presence of a known concentration of iminothiolane. The products were analyzed by size exclusion chromatography using two analytical Superose 12 columns (HR 10/30, Amersham Biosciences) connected in series. The column was eluted with PBS, pH 7.4, at a flow rate of 0.5 ml/min, and the effluent was monitored at 540 nm. Curve a, Control HbA; curve b, HbA incubated with 10 m*M* Mal-Phe-PEG5000 in the absence of iminothiolane; Curves c, d, and e represent PEGylation in the presence of 1, 2.5 and 5 m*M*, respectively of iminothiolane (2, 5 and 10 fold molar excess over Hb). The elution positions of $(SP\text{-}PEG10K)_2$-Hb and $(SP\text{-}PEG20K)_2$-Hb are indicated by dashed down arrow and solid down arrow, respectively. Inset shows the kinetics of thiolation of HbA (0.5 m*M*) in the presence of a 10 fold molar excess of iminothiolane (5 m*M*).

HbA reacted with Mal-Phe-PEG5000 in the absence of iminothiolane (curve b) corresponds to that of $(SP\text{-}PEG5K)_2$-Hb, a product that has been previously identified as HbA conjugated with two PEG5000 chains, one each at its two Cys-93(β) (Manjula *et al*. 2003). On inclusion of iminothiolane in the reaction mixture of HbA and Mal-Phe-PEG5000, the modified HbA eluted earlier than the $(SP\text{-}PEG5K)_2$-Hb from the size exclusion chromatographic column (Fig. 2, curves c, d and e). Furthermore, the retention time of the PEGylated Hb exhibited an inverse relation with the iminothiolane concentration, suggesting that an increased level of thiolation of HbA is responsible for the increased apparent molecular size of Hb. The PEGylated Hb generated in the presence of 2.5 m*M* iminothiolane eluted at a position close to that of $(SP\text{-}PEG10K)_2$-Hb (Fig. 2, curve d), and that generated in the presence of 5 m*M* iminothiolane eluted in between the elution positions of $(SP\text{-}PEG10K)_2$-Hb and $(SP\text{-}PEG20K)_2$-Hb (Fig. 2, curve e).

The retention time did not change significantly on increasing the iminothiolane concentration further to 7.5 and 10 m*M*; however, the elution pattern of the PEGylated Hb peak became slightly broader, and revealed a small shoulder on the ascending side of the peak (data not shown). These results suggested that under the conditions described above, optimal PEGylation of HbA is achieved in the presence of a 10 fold molar excess of iminothiolane.

The number of –SH groups introduced on to HbA (0.5 m*M*) by a 10 fold molar excess of iminothiolane (5 m*M*), was determined independently as a function of time by titration with 4-PDS, and the results are shown in Fig. 2, inset. The two thiol groups at zero time represent the two reactive thiols of Cys-93(β). As can be seen from the figure, the thiolation of Hb by iminothiolane exhibits an initial fast phase wherein about 4 new thiols are introduced in the first two hours and a subsequent slow phase wherein only approximately one additional thiol group is introduced. After 11 h of incubation, the thiolated HbA carried a total of ~7 reactive-SH groups per Hb. Thus, a quantitative PEGylation of such a thiolated HbA will generate a molecule carrying an average of seven PEG5000 chains. A 4-PDS titration of the PEGylated Hb generated by reaction of HbA (0.5 m*M*) with 10 m*M* Mal-Phe-PEG5000 (20 fold excess over Hb) in the presence of 5 m*M* iminothiolane (10 fold excess over Hb) revealed the presence of about 0.5 moles of reactive thiols per molecule. In conjunction with the results of the kinetics of thiolation, this result suggests that an average of ~6.5 copies of PEG5000 chains are introduced on to HbA in generating this PEGylated Hb.

Titration with 4-PDS also revealed that increasing the concentration of iminothiolane from 10 fold molar excess to 30 fold molar excess nearly doubles the total number of thiols on the HbA. However, the size enhancement of Hb on PEGylation in the presence of this iminothiolane concentration was only marginal (data not shown). These results are suggestive of a crowding effect induced by the ~six PEG5000 chains incorporated on the molecular surface of Hb, and hence resistance to further PEGylation.

The rate of thiolation of HbA was not significantly influenced when the temperature was lowered from room temperature to 4°C. However, the rate of PEGylation appeared to slow down. A 9 h incubation ensured the completion of the reaction; however, routinely the reaction was carried out overnight. The elution characteristics of the PEGylated Hb obtained by reaction at 4°C were quite comparable to that obtained at room temperature.

(ii) Two-step Reaction: Thiolation of Hb followed by PEGylation: In the two-step protocol, HbA was first reacted with iminothiolane to achieve the desired level of thiolation, and the resulting thiolated HbA was subjected to PEGylation. Thus, unlike the one-step protocol described above, thiolated HbA is generated as a product of the first step in this protocol. Size exclusion chromatographic profile of the PEGylated Hb prepared by the two-step protocol, as described under 'Methods', is shown in Fig. 3a. As can be seen, this PEGylated Hb elutes as a slightly broader peak compared to the product generated by the *in situ* thiolation mediated PEGylation protocol. Besides, analysis of the thiolated HbA intermediate (i.e., the product of the first step, obtained prior to the addition of the PEG maleimide), indicated that about 10 to 15% of the protein eluted at the position corresponding to the octameric and dodecameric forms of HbA, suggesting air oxidation of the new thiols introduced on to Hb (Fig. 3b). However, the generation of the oligomerized products was completely inhibited when the thiolation was carried out in the presence of 20 m*M* N-ethylmaleimide. The susceptibility of the thiols generated by reaction with iminothiolane for side reactions has also been observed in other studies on the generation of bioconjugates (McCall *et al*.,

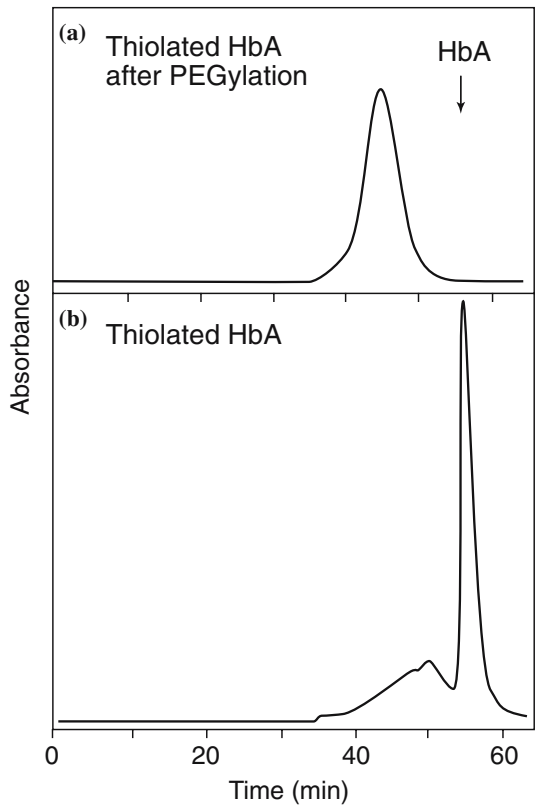

Fig. 3. Size exclusion chromatographic analysis of PEGylated Hb generated by the two-step thiolation mediated PEGylation protocol. The chromatographic conditions are the same as in Fig. 2. (a) HbA thiolated first and then PEGylated. HbA (1 m*M*) in PBS, pH 7.4 was incubated with 10 m*M* iminothiolane overnight at 4°C, followed by dilution with an equal volume of 20 m*M* Mal-Phe-PEG5000 and further incubated at 4°C for 6 h. (b) Thiolated HbA obtained by incubation of HbA (1 m*M*) in PBS, pH 7.4 with 10 m*M* iminothiolane overnight at 4°C (i.e., the product of the first step of the two-step protocol).

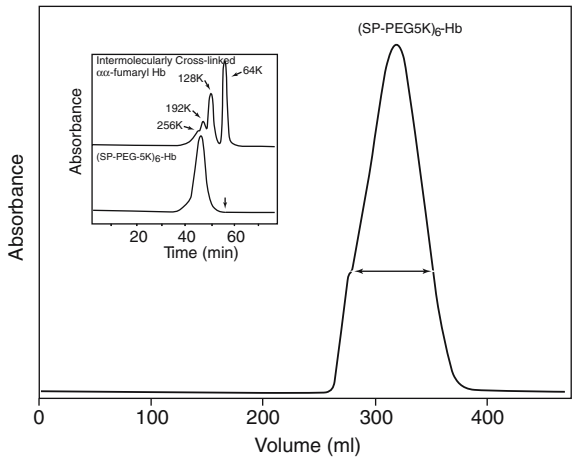

Fig. 4. Purification of $(SP\text{-}PEG5K)_6$-Hb by size exclusion chromatography on a Superose 12 Prep grade column (2.6 cm × 130 cm) using an AKTA Explorer 10 Protein Purification System (Amersham Biosciences). Protein load: 180 mg. The column was eluted with PBS, pH 7.4 at a flow rate of 1 ml/min, and the effluent was monitored at 540 nm. The inset compares the molecular size of the purified $(SP\text{-}PEG5K)_6$-Hb with that of oligomeric αα-fumaryl Hb (i.e., intra-molecularly crosslinked Hb oligomerized by inter tetrameric crosslinking using Bis Mal-Phe-PEG600). The SEC profile of oligomeric αα-fumaryl Hb helps to mark the position of tetrameric, octameric, dodecameric and hexadecameric forms of αα-fumaryl Hb.

1990; Singh *et al*., 1996). Hence, the one step *in situ* thiolation mediated protocol was selected for the preparation of the PEGylated Hb for all subsequent studies.

3.2. Purification of PEGylated-Hb

HbA (0.5 m*M* in PBS, pH 7.4) PEGylated using a 10 fold molar excess of iminothiolane in the presence of a 20 fold molar excess of Mal-Phe-PEG5000 (2 fold molar excess over iminothiolane) overnight at 4°C was subjected to size exclusion chromatography on a Prep grade Superose 12 column. A typical chromatographic profile of a preparation of PEGylated Hb is shown in Fig. 4. As can be seen, but for a small shoulder on the ascending side, the PEGylated Hb eluted as a single, fairly symmetrical peak. The PEGylated Hb peak was pooled as indicated and the protein concentrated to about 6 g/dl. The purity of the PEGylated-Hb thus isolated was further confirmed by analytical SEC analysis (Fig. 4, inset, lower panel). The PEG content of the purified PEGylated-Hb as determined by NMR analysis (Jackson *et al*., 1987; Chamow *et al*., 1994), and by –SH titration is presented in Table 1, along with those of two site specifically PEGylated Hbs, namely $(SP\text{-}PEG5K)_2$-Hb and $(SP\text{-}PEG10K)_2$-Hb (i.e., Hb PEGylated at Cys-93(β). The values determined for $(SP\text{-}PEG5K)_2$-Hb and $(SP\text{-}PEG10K)_2$-Hb are in agreement with the expected values of two copies each of PEG5K and PEG10K, respectively. The purified PEGylated-Hb was found to carry an average of 6.7 copies of PEG-5000 chains per tetramer by NMR analysis. This product will hereafter be referred to as $(SP\text{-}PEG5K)_6$-Hb.

The apparent molecular size of $(SP\text{-}PEG5K)_6$-Hb was estimated by comparison with the SEC profile of oligomeric forms of Hb (generated by inter-tetrameric crosslinking of αα-fumaryl Hb using Bis Mal-Phe-PEG600) (Fig. 4, inset, upper panel). As

Table 1. Quantitation of PEGylation in PEG-Hb conjugates

Hb Sample	Number of PEG chains per Hb (moles/mole)	
	By –SH titration[a]	By NMR[b]
HbA	0	0
$(SP\text{-}PEG5K)_2$-Hb	2.1	1.93
$(SP\text{-}PEG10K)_2$-Hb	2.1	2.22
$(SP\text{-}PEG5K)_6$-Hb	6.5	6.69

[a]Number of PEG groups estimated by indirect method. The number of PEG groups attached was estimated by titration of the thiol groups before and after PEGylation.
[b]Number of PEG groups estimated by direct method. The mass of PEG in a given PEGylated Hb sample was estimated by NMR analysis as described by Jackson *et al.* (1987).

can be seen, the hydrodynamic volume of $(SP\text{-}PEG5K)_6$-Hb corresponds to that of a Hb oligomer of a molecular mass of about 256,000 daltons (i.e., a tetrameric form of Hb). No detectable autooxidation of the PEGylated Hb to generate met-Hb type of products was observed either during the thiolation mediated maleimide chemistry based PEGylation reaction or during the subsequent purification steps. The PEGylated-Hb thus isolated could be stored at −80°C without any significant autooxidation for periods of at least up to one year.

The sites of conjugation of PEG-chains in the $(SP\text{-}PEG5K)_6$-Hb were determined by a comparison of the tryptic peptide map of its globin chains with that of the unmodified HbA. The results are presented in Table 2. The data revealed complete modification of Cys-93(β), and partial modification of five lysine residues, namely Lys-40(α), Lys-120(β), Lys-61(α), Lys-7(α), and Lys-8(β); only minor modification of the α-amino groups was observed. Together, this accounted for an average of 6.2 residues modified per Hb, a value that is close to the number of PEG chains per Hb estimated by NMR analysis and by thiol titration. Thus, two of the PEG chains in $(SP\text{-}PEG5K)_6$-Hb are on the two Cys-93(β)s and the remainder are distributed for the most part on a limited number of lysines and to a lesser degree on the α-amino groups. The tryptic peptide map of the $(SP\text{-}PEG5K)_6$-Hb globins was reproducible from batch to batch of the preparation, indicating that the PEGylation of the amino groups of Hb by the present thiolation mediated maleimide chemistry based protocol is not random, but exhibits a high degree of site selectivity.

Table 2. Identification of sites of PEGylation in $(SP\text{-}PEG5K)_6$-Hb

Residue modified	% Modification
Cys-93(β)	100
Lys-40(α)	61
Lys-120(β)	60
Lys-61(α)	26
Lys-7(α)	24
Lys-8(β)	24
Val-1(β)	13

The sites of PEGylation in $(SP\text{-}PEG5K)_6$-Hb were identified by a comparison of the tryptic peptide map of its globin chains with that of unmodified HbA, as described under 'Methods'. The number of groups PEGylated is calculated to be 3.08 groups per αβ dimer, and hence 6.16 groups per Hb molecule, since Hb is a tetramer consisting of two αβ dimers.

3.3. Subunit Interface Interactions of $(SP\text{-}PEG5K)_6$-Hb

A comparison of the proton NMR spectra of $(SP\text{-}PEG5K)_6$-Hb and $(SP\text{-}PEG20K)_2$-Hb with that of HbA in 0.1 *M* phosphate buffer at pH 7.0 and 29°C in both the carbonmonoxy and deoxy forms is presented in Fig. 5a. These two samples carry a comparable amount of total PEG-mass/Hb [(30,000 Da in $(SP\text{-}PEG5K)_6$-Hb vs 40,000 Da in $SP\text{-}PEG20K)_2$-Hb]. With the exception of broader resonances observed with the PEGylated samples due to an increase in the molecular size as a result of PEGylation, there is no significant difference in the chemical shift over the spectral region of 10 to 14 ppm indicating no alterations in the $\alpha_1\beta_1$ interface of Hb as a result of PEGylation either only at Cys-93(β) with PEG-20000 or at Cys-93(β) and at least four of its ε-amino groups with PEG-5000. Figure 5b compares the ring-current-shifted proton resonances of the two PEGylated Hbs with that of HbA in the carbonmonoxy form. There are some alterations in the ring-current shifted proton resonances reflecting some perturbation in the microenvironment of the heme of the PEGylated Hb-samples. Figure 5c shows the hyperfine shifted $N_\delta H$ ^{1}H-resonances of proximal histidine residues of the α- and the β-chains of PEGylated Hbs in the deoxy form. The chemical shift at −75 ppm assigned to $N_\delta H$ of the proximal histidine of the β-chain is shifted upfield by −2 to −3 ppm reflecting the perturbation of the β-heme environment in the PEGylated samples. This upfield shift is somewhat more

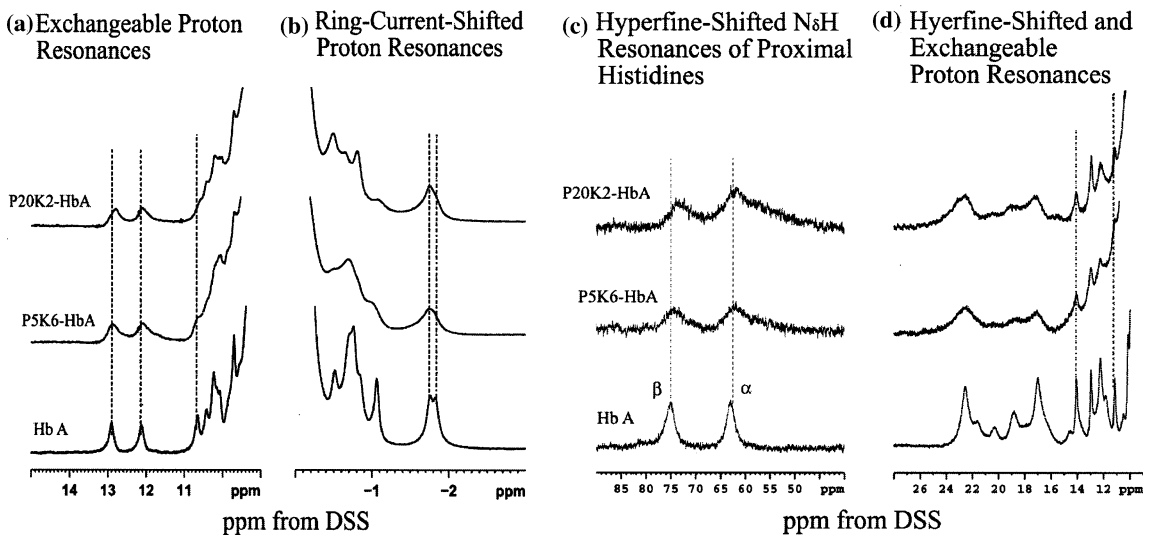

Fig. 5. NMR Spectra of $(SP\text{-}PEG5K)_6$-Hb. 300 MHz ^{1}H-NMR spectra of 5 g% solutions of PEGylated Hbs. Panel a shows the exchangeable proton resonances of CO-forms of the PEGylated Hbs and Panel b shows ring current shifted proton resonances of the same. Panel c shows the hyperfine shifted $N_\delta H$ resonances of the proximal histidine in the deoxy state whereas Panel d shows the hyperfine shifted and exchangeable proton resonances in the deoxy state. P20K2-Hb and P5K6Hb refer to $(SP\text{-}PEG20K)_2$-Hb and $(SP\text{-}PEG5K)_6$-Hb, respectively.

pronounced in $(SP\text{-}PEG20K)_2$-HbA than in $(SP\text{-}PEG5K)_6$-Hb. Figure 5d compares the hyperfine shifted and exchangeable proton resonances of the two PEGylated samples of HbA with that of HbA in the deoxy form. The hyperfine-shifted resonances are broader than that of HbA. Besides, there are some changes in the resonances in the spectral region from 16 to 24 ppm, reflecting changes in the microenvironment of the β-heme of Hb as a result of PEGylation of the molecule. The resonance at 14 ppm, assigned to an important H-bond between α-Tyr(42) and β-Asp(99) in the $\alpha_1\beta_2$ subunit interface (Fung and Ho, 1975) is unchanged in the PEGylated samples. Thus, there are no significant changes in the $\alpha_1\beta_2$ subunit interface of the PEGylated Hb.

3.4. Functional Properties of $(SP\text{-}PEG5K)_6$-Hb

The O_2 affinity of $(SP\text{-}PEG5K)_6$-Hb in 50 m*M* Bis–Tris/50 m*M* Tris acetate buffer, pH 7.4 and 37°C and its modulation in the presence of allosteric effectors is shown in Table 3. The P_{50} of Hb is lowered (i.e., the O_2 affinity is increased) on PEGylation, from the control value of 8.0 mmHg to 6.5 mmHg. The presence of a five fold molar excess of DPG, an effector that lowers the O_2 affinity of HbA by binding at the ββ-cleft, had only a marginal influence on the O_2 affinity of $(SP\text{-}PEG5K)_6$-Hb. Similarly, the presence 1 *M* sodium chloride lowered the O_2 affinity of the PEGylated Hb only slightly compared to unmodified HbA. L35, an allosteric effector that reduces the O_2 affinity of Hb by binding at the αα-end of the molecule, also had only a limited influence on the O_2 affinity of $(SP\text{-}PEG5K)_6$-Hb. These results indicate that the surface decoration with multiple copies of PEG5K chains has desensitized the propensity of HbA to respond to the presence of the allosteric effectors DPG, chloride and L35.

Table 3. Oxygen affinity of $(SP\text{-}PEG5K)_6$-Hb and its Modulation by Allosteric Effectors

	P_{50}·mmHg (*n*)[a]	
Effector	HbA	$(SP\text{-}PEG5K)_6$-Hb
None	8.0 (2.5)	6.5 (2.2)
DPG (3 m*M*)	22.5 (2.3)	8.5 (2.0)
NaCl (1 *M*)	24.0 (2.4)	8.2 (1.9)
L35 (10 m*M*)	57.0 (1.7)	12.0 (1.5)

The O_2 affinity measurements were carried out in 50 m*M* Bis–tris/50 m*M* tris acetate, pH 7.4 at 37°C using Hem-O-Scan (Aminco). The protein concentration was 0.6 m*M*. The samples analyzed contained less than 2% met Hb.

[a]P_{50}, partial pressure of O_2 at half saturation; *n*, Hill coefficient.

The O_2 affinity of $(SP\text{-}PEG5K)_6$-Hb in PBS (10 m*M* phosphate buffer, containing 150 m*M* NaCl), pH 7.4 and its comparison with that reported previously for the site-specifically PEGylated Hbs (Manjula *et al.* 2003) is presented in Table 4. As can be seen, the difference in O_2 affinity between Hb and $(SP\text{-}PEG5K)_6$-Hb is enhanced in PBS relative to that observed in the Bis–Tris/Tris-Ac buffer, and is comparable to that of the site-specifically PEGylated Hbs. The amplification of the O_2 affinity increasing influence of PEGylation in PBS, pH 7.4 is reminiscent of the desensitization of the O_2 affinity of PEGylated Hb to allosteric effectors. A comparison of the O_2 affinity of $(SP\text{-}PEG5K)_6$-Hb with that of $(SP\text{-}PEG5K)_2$-Hb suggests that the surface decoration of $(SP\text{-}PEG5K)_2$-Hb with an additional four PEG5K chains by thiolation mediated PEGylation has little influence on its O_2 affinity.

The influence of PEGylation on the O_2 affinity of Hb is enhanced further when the ionic strength of the phosphate buffer is increased to 100 m*M* (Table 5). These O_2 affinity measurements were made in dilute protein solutions (Cheng *et al.* 2002). As can be seen, the O_2 affinity increasing influence of PEGylation is enhanced in the 100 m*M* phosphate buffer. The data presented in this Table V also show that PEGylation reduces the intrinsic propensity of HbA to lower its O_2 affinity as the pH is lowered. Thus, the encaging of the Hb inside the PEG shell generated by six copies of PEG5K has desensitized the molecule to respond to the changes of the ionic strength and organic allosteric effectors, and hydrogen ions. The desensitization of the Hb molecule to changes in pH is reflected by the decrease in the Bohr effect (Table 6). It may be noted that N-ethylmaleimide modification of Hb reduces the Bohr effect by about 73% (Cheng *et al.* 2002), whereas PEGylation with ~six copies of PEG5K chains has reduced the Bohr effect by about 43%. Thus, part of the Bohr effect reducing influence of Cys-93(β) modification by NEM is neutralized by the surface decoration of Hb with multiple copies of PEG5K.

Table 4. O_2 affinity of $(SP\text{-}PEG5K)_6$-Hb in PBS, pH 7.4

Protein	P_{50}, mmHg (n)[a]
HbA	15.3 (2.3)
$(SP\text{-}PEG5K)_6$-Hb	8.5 (1.9)
$(SP\text{-}PEG5K)_2$-Hb[b]	11.8 (2.3)
$(SP\text{-}PEG10K)_2$-Hb[b]	11.5 (2.2)
$(SP\text{-}PEG20K)_2$-Hb[b]	10.5 (2.1)

The O_2 affinity measurements were carried out in PBS, pH 7.4 at 37°C using a Hem-O-Scan (Aminco). The protein concentration was 1 m*M*.
[a]P_{50}, partial pressure of O_2 at half saturation; n, Hill coefficient.
[b]Data from Manjula *et al.* (2003).

Table 5. O_2 affinity of $(SP\text{-}PEG5K)_6$-Hb in 0.1 *M* Phosphate buffer as a function of pH

	P_{50}, mmHg (n)[a] at		
Protein	pH 6.5	pH 7.4	pH 7.8
HbA	22.3 (3.0)	9.3 (3.1)	5.9 (3.1)
$(SP\text{-}PEG5K)_6$-Hb	6.1 (2.0)	3.7 (1.8)	2.9 (1.8)
$(SP\text{-}PEG20K)_2$-Hb	5.0 (2.0)	3.6 (1.8)	3.2 (1.6)

The O_2 affinity measurements were carried out in 100 m*M* phosphate buffer at 29°C using a Hemox Analyzer (TCS Scientific, PA). The protein concentration was 0.025 m*M* in Hb.
[a]P_{50}, partial pressure of O_2 at half saturation; n, Hill coefficient.

3.5. Viscosity and Colloidal Osmotic Pressure of $(SP\text{-}PEG5K)_6$-Hb

A comparison of the viscosity of HbA and $(SP\text{-}PEG5K)_6$-Hb as a function of Hb concentration is presented in Fig. 6a. HbA exhibited negligible changes in viscosity with the increase in protein concentration. On the other hand, although the viscosity of $(SP\text{-}PEG5K)_6$-Hb is comparable to that of HbA in dilute solutions, it increased exponentially with the increase in protein concentration.

A comparison of the colloidal osmotic pressure of HbA and $(SP\text{-}PEG5K)_6$-Hb as a function of the protein concentration is shown in Fig. 6b. As can be seen, the colloidal osmotic pressure of both HbA and $(SP\text{-}PEG5K)_6$-Hb increased as a function the protein concentration. However, the increase in colloidal osmotic pressure is small with HbA, and is linear with the protein concentration. On the other

Table 6. Influence of PEGylation on the Bohr effect of Hb

Protein	$-\Delta P_{50}/\Delta$pH	% Reduction
HbA	0.44	–
$(SP\text{-}PEG5K)_6$-Hb	0.25	43
$(SP\text{-}PEG20K)_2$-Hb	0.14	68
$(SE)_2$-Hb[a]	0.12	73

The O_2 affinity was measured in 100 m*M* phosphate buffer at 29°C using a Hemox Analyzer.
[a]$(SE)_2$-Hb is Hb modified at its Cys-93(β) with NEM; data from Cheng *et al.* (2002).

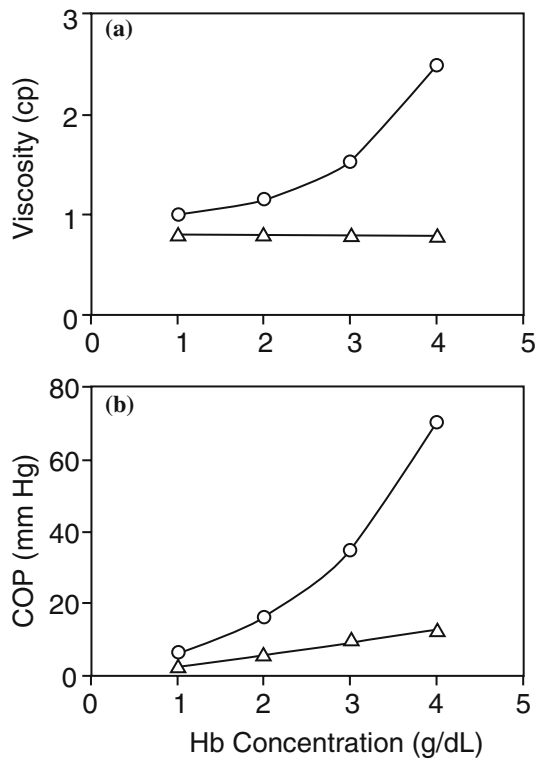

Fig. 6. (a) Viscosity of PEGylated Hb as a function of protein concentration. Open triangles represent HbA; open circles represent $(SP\text{-}PEG5K)_6$-Hb. (b) Colloidal osmotic pressure (COP) of $(SP\text{-}PEG5K)_6$-Hb as a function of protein concentration. Open triangles represent HbA, and open circles represent $(SP\text{-}PEG5K)_6$-Hb.

hand, like the viscosity, the colloidal osmotic pressure of $(SP\text{-}PEG5K)_6$-Hb increased exponentially with the increase in protein concentration. Thus, the difference in both the viscosity and colloidal osmotic pressure between HbA and $(SP\text{-}PEG5K)_6$-Hb is enhanced exponentially. These results, therefore, suggest that the colloidal osmotic pressure of $(SP\text{-}PEG5K)_6$-Hb is a correlate of its viscosity. A correlation between colloidal osmotic pressure and viscosity as a function of PEG mass was observed earlier with PEGylated Hbs carrying 2 PEG chains/Hb (Manjula *et al.* 2003).

3.6. Molecular Radius of $(SP\text{-}PEG5K)_6$-Hb

The molecular radius of $(SP\text{-}PEG5K)_6$-Hb, as determined by dynamic light scattering measurements, along with that of $(SP\text{-}PEG20K)_2$-Hb is presented in Table 7. As can be seen, the molecular radius of $(SP\text{-}PEG5K)_6$-Hb is slightly smaller than the radius of $(SP\text{-}PEG20K)_2$-Hb. It may also be seen from the data presented in Table 7, that the molecular volume of HbA is increased 9 and 11.5 fold, respectively, when it is surface decorated with about six copies of PEG5000 chains vs with 2 copies of PEG20000. A comparison of the viscosity and COP of $(SP\text{-}PEG5K)_6$-Hb with that of $(SP\text{-}PEG20K)_2$-Hb is also included in Table VII. As can be seen, both the viscosity and COP of $(SP\text{-}PEG20K)_2$-Hb are higher than that of $(SP\text{-}PEG5K)_6$-Hb and thus exhibit a correlation with the PEG mass conjugated to Hb.

3.7. Vasoactivity of $(SP\text{-}PEG5K)_6$-Hb

The relative merits of increasing the PEG mass to an equivalent level by conjugation with ~six copies of PEG5000 chains vs two copies of PEG20000 chains per Hb molecule for the microcirculation were evaluated in the hamster skin fold window model by measuring the acute systemic and microvascular response to a 10% hypervolemic infusion of the two PEGylated Hbs. The results are presented in Fig. 7. Saline was used as the control; the results obtained with unPEGylated Hb, $(SP\text{-}PEG20K)_2$-Hb and $(SP\text{-}PEG5K)_6$-Hb are compared. MAP increased and the heart rate decreased after infusion of $(SP\text{-}PEG20K)_2$-Hb. The increase in MAP was more close to unmodified Hb but the decrease in heart rate was significantly lower than that observed with unPEGylated Hb. The FCD was also decreased, much more than observed with saline, and remained closer to that of unPEGylated Hb. In this case, large arterioles tended to vasoconstrict, whereas the venules were

Table 7. Comparison of the solution properties of $(SP\text{-}PEG5K)_6$-Hb and $(SP\text{-}PEG20K)_2$-Hb

Sample	Calc. Mass (Daltons)	PEG Mass (Daltons)	Radius (nm)	Mol. Volume (nm^3)	Viscosity (cp)	COP (mmHg)
HbA	64000	0	3.12	127	0.91	5.7
$(SP\text{-}PEG5K)_6$-Hb	94000	30000	6.50	1150	2.50	15.4
$(SP\text{-}PEG20K)_2$-Hb[a]	104000	40000	7.04	1461	4.76	22.2

The viscosity and COP were measured at Hb concentrations of 4 and 2 g/dl, respectively.
[a]Data from Manjula *et al.* (2003).

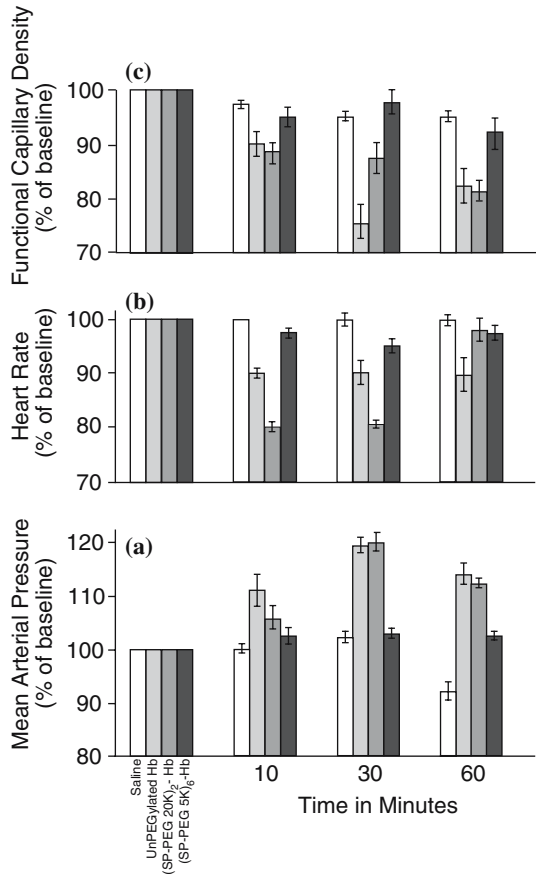

Fig. 7. Changes in mean arterial pressure, heart rate and functional capillary density in response to a 10% hypervolemic infusion with $(SP\text{-}PEG20K)_2$-Hb and $(SP\text{-}PEG5K)_6$-Hb as compared to saline and unPEGylated Hb controls.

relatively unchanged (data not shown). Interestingly, MAP and heart rate were not statistically changed after infusion with $(SP\text{-}PEG5K)_6$-Hb and remained close to the baseline values. Although there was a small decrease in FCD with $(SP\text{-}PEG5K)_6$-Hb, the values are much closer to that of the saline control than to that of unPEGylated Hb. Some arteriolar and venular constrictions were observed, but significantly less than that observed with $(SP\text{-}PEG20K)_2$-Hb. These results demonstrate that the configuration of surface decoration of Hb with PEG has a significant influence on the pressor effect and the vasoconstrictive activity of acellular Hb. Surface decoration of HbA with six copies of PEG5000 significantly reduces its acute systemic response, whereas decoration with two copies of PEG20000 (conjugation of comparable PEG mass) is not effective for achieving the same.

4. DISCUSSION

A simple and versatile procedure for enhancing the hydrodynamic volume of HbA by conjugation of multiple PEG chains without altering the surface charge of the protein (i.e., conservative PEGylation), and generation of a nonhypertensive PEGylated Hb, namely $(SP\text{-}PEG5K)_6$-Hb, by this protocol is described in the present study. Although the present studies were carried out using maleimide PEG with an aryl linker between the PEG chain and the maleimide moiety, other maleimide PEG reagents that carry an alkyl or an alkylamide linker between the PEG and the maleimide are equally efficient in the maleimide chemistry based PEGylation reactions of Hb (Acharya *et al.* 1996; Juszczak *et al.* 2002; Khan *et al.* 2001; Vandegriff *et al.* 2003).

$(SP\text{-}PEG5K)_6$-Hb exhibits an increased O_2 affinity relative to parent Hb, the extent of increase being a function of the ionic strength and the pH of the medium, and the presence or absence of allosteric effectors. These observations suggest that the structure of the 'PEG shell' architectured around the Hb molecule through multiple copies of PEG5K chains insulates the Hb molecule from experiencing the changes in the macroenvironment.

The studies of Shorr *et al.* (1999) suggested that the PEGylation of bovine Hb induces an unusual sensitivity to the oxygen affinity of Hb when measured as a function of temperature and/or protein concentration. The oxygen affinity of the deca-PEG5K-Hb increased as the temperature was lowered as well as when the concentration of the protein was lowered. The higher oxygen affinity of $(SP\text{-}PEGG5K)_6$-Hb at 29°C when measured at a protein concentration significantly lower than that at 37°C appears to be reminiscent of the observation by Shorr *et al.* (1999) with the PEGylated bovine Hb.

As noted in the Results section, the PEGylation of Hb by the present protocol is not random, and the PEG chains are distributed over a limited number of surface amino groups. In a PEGylated molecule that carries multiple PEG chains (~six in the present case), the structure of the PEG shell formed around the protein is likely to be influenced by the sites of PEGylation (i.e., the site selectivity of the amidination reaction) and the chemical nature of the linkage (with and without a change in the charge of the surface group that is reacted). Further studies altering the structure of the PEG shell in terms of sites of PEGylation, and charge at the site

after PEGylation will help in understanding the mechanism of desensitization of the molecule for changes in the macroenvironment.

$(SP\text{-}PEG5K)_6$-Hb, has many of the attributes that have been advanced as needed for minimizing the vasoactivity of acellular Hb (Rohlfs *et al.* 1998; Winslow, 1999): (i) Increased O_2 affinity to limit the O_2 off-loading by acellular Hb in arterioles, thus minimizing the potential for vasoconstriction through autoregulatory mechanisms, (ii) Retention of the cooperative binding to ensure off-loading of O_2 in the capillary beds, (iii) An enhanced molecular size (hydrodynamic volume) to reduce extravasation, (iv) an increase in the viscosity of Hb solution both to create appropriate shear stress on the arteriolar walls to maintain vascular tone and to lower the diffusion constants for oxy Hb to limit the O_2 off-loading to vessel walls, (v) A colloidal osmotic pressure greater than that of the conventionally modified Hbs to increase the effectiveness of the blood substitute as a plasma expander.

Studies with $(SP\text{-}PEG5K)_6$-Hb in hamsters, at 10% top load infusion, suggest that conjugation of an average of six PEG5000 chains on to HbA, without alteration of its surface charge, significantly reduces the Hb induced vasoactivity. The Enzon PEG5K-bovine Hb that was observed previously to be nonhypertensive (Rohlfs *et al.* 1998; Winslow *et al.* 1998) was generated by the active ester chemistry (Nho *et al.* 1994) which results in the loss of the net positive charge of the protein (nonconservative PEGylation), and has been suggested to carry an average of ten PEG-5K chains/Hb. The results of the present study demonstrate that conservation of the net surface charge of the parent Hb is not crucial to generate a non-hypertensive Hb and that with conservative PEGylation, the nonhypertensive property can be endowed to Hb with less than ten PEG5K chains.

Another difference between $(SP\text{-}PEG5K)_6$-Hb and the Enzon PEG5K-bovine Hb is the nature of the PEG chain attachment. The chemistry at the PEG anchoring site in the two PEGylated Hbs is depicted in Fig. 8. As can be seen, $(SP\text{-}PEG5K)_6$-Hb has an extension arm (a δ-mercaptobutyrimidyl moiety), a conjugating group (succinimidyl moiety), and a phenyl carbamate linker between the PEG chain and the conjugating arm. On the other hand, the Enzon PEG-Hb has neither an extension arm nor a linker group. Thus, in the Enzon PEG5K-bovine Hb, the PEG chains are attached directly on the ε-amino groups via urethane linkages, whereas in $(SP\text{-}PEG5K)_6$-Hb, they are attached through a flexible 'extension arm'. Thus, the Enzon strategy places the PEG chain very close to the protein surface (within 2 to 3 Å from the original positive charge of the ε-amino groups) whereas the thiolation mediated PEG maleimide chemistry based PEGylation places the PEG chain at a distance from the protein surface (about 15 to 21 Å away from the original positive charge of the ε-amino groups). The role of the 'extension arm' in generating a nonhypertensive Hb with only six PEG5K chains is not clear at present. However, it may be noted that the viscosity and COP of $(SP\text{-}PEG5K)_6$-Hb, a hexaPEGylated human Hb prepared by the conservative PEGylation, is comparable to that reported for the Enzon PEG-Hb, a decaPEGylated bovine Hb prepared by a nonconservative PEGylation (Vandegriff *et al.*, 1997). Thus, it appears that

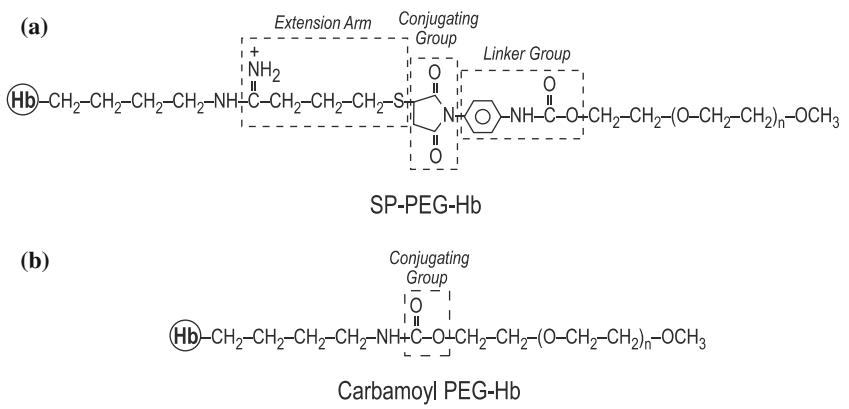

Fig. 8. Comparison of the chemistry of the extension arm, the conjugation arm and the linker group of (a) $(SP\text{-}PEG5K)_6$-Hb and (b) Enzon PEG-Hb.

the chemistry of conjugation of the PEG chains plays a significant role in determining the solution properties of the PEGylated Hbs. More detailed studies are needed to expose the correlation between the PEGylation induced solution properties of the PEGylated Hb and the conjugation chemistry, and to facilitate the rational design of new nonhypertensive Hbs.

More strikingly, the results of the present study suggest an important role for the surface configuration of the PEG chains on the Hb molecule in neutralizing its vasoactivity. Although the molecular and colligative properties, and the O_2 affinity of $(SP\text{-}PEG5K)_6$-Hb and $(SP\text{-}PEG20K)_2$-Hb are similar, the two PEG-Hb conjugates differ significantly in their vasoactive properties. Apparently, modulation of the vasoactivity of Hb is not simply a direct translation of its PEGylation induced enhancement of molecular volume and solution properties, but is governed by the pattern of surface decoration of the Hb molecule by the PEG chains (i.e., the number and size of the PEG-chains). A plausible explanation for the difference in the vasoactive properties of $(SP\text{-}PEG5K)_6$-Hb and $(SP\text{-}PEG20K)_2$-Hb could be that a better shielding of the molecular surface of Hb is afforded by multiple copies of PEG5000 chains on Hb relative to that afforded by two copies of PEG20000, thus camouflaging the acellular Hb from interactions with the vasculature. This concept of molecular surface shielding raises an important question as to whether the location of the PEG-5000 chains on the molecular surface of Hb, along with its number and size (sites of PEGylation vs surface coverage), plays any role in achieving the shielding of the molecular surface of Hb. Studies with site-specifically PEGylated Hbs with well defined number of copies of PEG chains of varying size are needed to gain further insights into this molecular aspect of the PEGylation mediated modulation of Hb induced vasoactivity. The lysines identified as the PEGylated sites in the present study could serve as potential target sites to engineer cysteine residues by site directed mutagenesis to incorporate the desired number of PEG chains on to Hb through maleimide chemistry. This strategy would enable the generation of well-defined, site-specifically PEGylated Hbs that are much needed for such studies.

In conclusion, the results of the present study demonstrate that the increase in molecular volume and solution properties of Hb accomplished by conjugation of multiple copies of small PEG chains is preferred over conjugation of two long PEG chains to obtain comparable PEG mass to endow the molecule with low vasoconstrictive activity. In contrast, in the PEGylation of other therapeutic proteins, an increase in the PEG chain length is preferred over an increase in PEG mass by increasing the number of small PEG chains to achieve longer *in vivo* half life, decreased clearance and also to retain potency without the possible loss of the bioactivity of the molecule, for example - masking of receptor binding activity due to substitution at multiple sites (Bailon and Berthold, 1998; Bailon *et al*. 2001; Lee *et al*. 1999; Satake-Ishikawa *et al*. 1992). Thus it appears that the selection of a PEGylation strategy for a particular protein (i.e., the number and/or the size of the PEG chains to be attached) will be dependent on the specific application under consideration.

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