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PEGylated Hemoglobin: Role of surface configuration of PEG for the modulation of hemoglobin vasoactivity

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SUMMARY

PEGylation induced changes in the colligative properties of HbA have been implicated as potential modulators of its vasoactivity. However, our recent studies have indicated that this modulation is not a direct correlate of the colligative properties. With a view to delineate the role of surface charge and/or the pattern of surface decoration with PEG for the vasoinactivation of hemoglobin, HbA has now been modified by thiolation mediated PEGylation that does not alter its surface charge. Incubation of HbA with PEG5K maleimide in the presence of iminothiolane generated a PEGylated Hb, namely (SP-PEG5K)6-Hb, that carried an average of ~six PEG5K chains/Hb. The total PEG mass in (SP-PEG5K)6-Hb, its enhanced molecular volume, molecular radius, viscosity, oncotic pressure and O2 affinity are comparable to that of another PEGylated Hb, namely (SP-PEG20K)2-Hb, that carries two PEG20K chains/Hb, one on each of its two Cys-93(β). However, (SP-PEG5K)6-Hb exhibited significantly reduced vasoconstriction mediated response relative to that of (SP-PEG20K)2-Hb. These results demonstrate that surface decoration of Hb by conservative PEGylation with an average of six PEG5K chains neutralizes its vasoactivity. More significantly, this study demonstrates that, in addition to the colligative properties, a surface configuration accomplished by conjugation of multiple copies of small PEG chains is more effective for decreasing the vasoactivity of Hb than a corresponding increase in total PEG mass achieved through conjugation of a small number of large PEG chains. The simplicity in the generation of this new vasoinactive PEG-Hb conjugate makes it a potential candidate for a Hb based oxygen carrier.
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INTRODUCTION

Vasoactivity of acellular Hb has been a major impediment in the development of Hb-based oxygen carriers (1-6). This has been attributed, at least in part, to the NO scavenging effect of Hb (5-12). Reduction of NO binding through site-directed mutagenesis is one of the approaches being evaluated for the reduction of the vasoactivity of acellular Hb (10,13). 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 (14). However, the recent studies of Winslow and co-workers (15-17) 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 high 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 isopeptidyl linkages (18). 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 (19-21). We have recently reported the preparation of three site-specifically PEGylated Hbs, namely (SP-PEG5K)_2-Hb, (SP-PEG10K)_2-Hb, and (SP-PEG20K)_2-Hb (19). 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
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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 PEG500 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 thiolis (22, 23) 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 thiolis of Cys-93(β) and the extrinsic thiolis 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-PEG5K)6-Hb, has been generated. This new PEGylated Hb exhibits a size enhancement, colligative properties and O2 affinity comparable to that of (SP-PEG20K)2-Hb, but has a significantly reduced vasoactivity relative to the latter. These results establish that the PEGylation induced colligative 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 colligative properties of the PEGylated Hb in conjunction with the configuration of PEG on the surface of Hb.

**EXPERIMENTAL PROCEDURES**

**Materials:** HbA was purified from the human erythrocyte lysate by DE-52 chromatography (24). 2-Iminothiolane was a product of BioAffinity Systems, Rockford, IL. 4,4′-dithiopyridine (4-PDS) was purchased from Aldrich Chemical Co. Polymerized bovine Hb solution (Oxyglobin, average MW 180,000) was a product of Biopure Corp., Boston, MA. Synthesis of the mono functional maleimido-phenyl (Mal-Phe) derivatives of PEG5000, PEG10000 and PEG20000, and preparation of the site-specifically PEGylated Hbs, namely (SP-PEG5K)2-Hb, (SP-PEG10K)2-Hb, and (SP-PEG20K)2-Hb were carried out as described earlier (19).

**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 mM tetramer) in
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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 mM, but it could also be carried out at HbA concentrations of either 0.25 mM or 1 mM. In the two-step reaction, HbA (1.0 mM) in PBS pH 7.4 was first incubated with 10 mM iminothiolane overnight at 4°C. The reaction mixture was then diluted with an equal volume of 20 mM 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.

Identification of the sites of PEGylation: This was determined by tryptic peptide map analysis, as previously described (25). Briefly, the globin chains of Hb prepared by acid-acetone precipitation were dissolved in 100 mM 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 an 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 recovery of peptide α-T4 (a peptide derived by arginyl peptide bond cleavage), was used as an internal standard, and the ratio of the peak area of each peptide to the α-T4 peptide was used to elucidate differences between the HbA control and the PEGylated Hb.

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 (26-30).

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 (31). The number of PEG chains conjugated per Hb tetramer was determined by proton NMR spectroscopy, as described by Jackson et al (32). Analyses of the size enhancement of Hb as a function of the various thiolation
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mediated PEG-maleimide based PEGylation conditions, determination of the molecular radius of the
PEGylated Hbs, their O₂ equilibrium curves, and measurements of their viscosity and colloidal osmotic
pressure were carried out as previously described (19). Structural characterization of the PEGylated Hbs by
proton NMR spectroscopy was carried out as previously described (33).

RESULTS

Iminothiolane dependent thiolation mediated maleimide chemistry based PEGylation of Hb:

A. 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 mM) in PBS, pH 7.4, incubated with a 20
dfold molar excess of Mal-Phe-PEG5000 (10 mM) 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 HbA reacted with
Mal-Phe-PEG5000 in the absence of iminothiolane (curve b) corresponds to that of (SP-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) (19). On inclusion of iminothiolane in the reaction mixture of HbA and Mal-Phe-PEG5000, the
modified HbA eluted earlier than the (SP-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 mM
iminothiolane eluted at a position close to that of (SP-PEG10K)₂-Hb (Fig. 2, curve d), and that generated in the
presence of 5 mM iminothiolane eluted in between the elution positions of (SP-PEG10K)₂-Hb and (SP-
PEG20K)₂-Hb (Fig. 2, curve e).
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The retention time did not change significantly on increasing the iminothiolane concentration further to 7.5 mM and 10 mM; 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 mM) by a 10 fold molar excess of iminothiolane (5 mM), 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 tetramer. 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 HbA generated by reaction of HbA (0.5 mM) with 10 mM Mal-Phe-PEG5000 (20 fold excess over Hb) in the presence of 5 mM iminothiolane (10 fold excess over Hb) revealed the presence of about 0.5 moles of reactive thiols per tetramer. 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 HbA obtained by reaction at 4°C were quite comparable to that obtained at
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room temperature.

**B. 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 mM 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 (34,35). Hence, the one step *in situ* thiolation mediated protocol was selected for the preparation of the PEGylated HbA for all subsequent studies.

**Purification of PEGylated-Hb:** HbA (0.5 mM 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 (32, 36), and by –SH titration is presented in Table I, along with those of two site specifically PEGylated Hbs, namely (SP-PEG5K)2-Hb and (SP-PEG10K)2-Hb (i.e., Hb PEGylated at Cys-93(β)). The values determined for (SP-PEG5K)2-Hb and (SP-PEG10K)2-Hb are in agreement with the expected values of two copies each of PEG5K.
Vasoinactive PEGylated hemoglobin 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-PEG5K)\(\_\)-Hb.

The apparent molecular size of (SP-PEG5K)\(\_\)-Hb was estimated by comparison with the SEC profile of oligomeric forms of Hb (generated by inter-tetrameric crosslinking of \(\alpha\alpha\)-fumaryl Hb using Bis Mal-Phe-PEG600) (Fig. 4, inset, upper panel). As can be seen, the hydrodynamic volume of (SP-PEG5K)\(\_\)-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\(^\circ\)C without any significant autooxidation for periods of at least up to one year.

The sites of conjugation of PEG-chains in the (SP-PEG5K)\(\_\)-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 II. The data revealed complete modification of Cys-93(\(\beta\)), and predominant modification of four lysine residues, namely Lys-60(\(\alpha\)), Lys-120(\(\beta\)), Lys-11(\(\alpha\)), and Lys-8(\(\beta\)); only minor modification of the \(\alpha\)-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-PEG5K)\(\_\)-Hb are on the two Cys-93(\(\beta\))s and the remainder are distributed for the most part on a limited number of lysines and to a lesser degree on the \(\alpha\)-amino groups. The tryptic peptide map of the (SP-PEG5K)\(\_\)-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.

**Molecular, colligative and functional characterization of (SP-PEG5K)\(\_\)-Hb**

\(^1\)H-NMR spectroscopy of (SP-PEG5K)\(\_\)-Hb: A comparison of the proton NMR spectra of (SP-PEG20K)\(2\)-Hb and (SP-PEG5K)\(\_\)-Hb with that of HbA in 0.1 M phosphate buffer at pH 7.0 and 29\(^\circ\)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 [40,000 Da in (SP-PEG20K)\(2\)-Hb vs 30,000 Da in (SP-PEG5K)\(\_\)-Hb]. With the exception
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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($\beta$) with PEG-20000 or at Cys-93($\beta$) and at least four of its $\varepsilon$-amino groups with PEG-5000. Fig 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 micro-environment of the heme of the PEGylated Hb-samples. Fig 5C shows the hyperfine shifted $N_{\delta}^1$H-resonances of proximal histidine residues of the $\alpha$- and the $\beta$-chains of PEGylated Hbs in the deoxy form. The chemical shift at -75 ppm assigned to $N_{\delta}^\beta$ of the proximal histidine of the $\beta$-chain is shifted upfield by -2 to -3 ppm reflecting the perturbation of the $\beta$-heme environment in the PEGylated samples. This upfield shift is somewhat more pronounced in (SP-PEG20K)$_2$-HbA than in (SP-PEG5K)$_6$-Hb. Fig. 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 $\beta$-heme of Hb as a result of PEGylation of the molecule. The resonance at 14 ppm, assigned to an important H-bond between $\alpha$-Tyr(42) and $\beta$-Asp(99) in the $\alpha_1\beta_2$ subunit interface (37) is unchanged in the PEGylated samples. Thus, there are no significant changes in the $\alpha_1\beta_2$ subunit interface of the PEGylated Hb.

**Functional properties of (SP-PEG5K)$_6$-Hb:** The $O_2$ affinity of (SP-PEG5K)$_6$-Hb in 50 mM Bis-Tris/50 mM Tris acetate buffer, pH 7.4 and 37°C and its modulation in the presence of allosteric effectors is shown in Table III. 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 $\beta\beta$-cleft, had no significant influence on the $O_2$ affinity of (SP-PEG5K)$_6$-Hb. On the other hand, the presence 1 M sodium chloride lowered the $O_2$ affinity of the PEGylated Hb, but only slightly compared to unmodified HbA. L35, an allosteric effector that reduces the $O_2$ affinity of Hb by binding at the $\alpha\alpha$-end of the molecule, also reduced the $O_2$ affinity of (SP-PEG5K)$_6$-Hb. However, as with NaCl, this effect
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was markedly reduced compared to that observed with unmodified HbA. Thus, the PEGylation of HbA significantly reduces the propensity of HbA to respond to the presence of the allosteric effectors DPG, chloride and L35.

In addition to the measurements in the BisTris-Tris buffer, pH 7.4, the O$_2$ affinity of (SP-PEG5K)$_6$-Hb was also determined in PBS, pH 7.4. Even in PBS, (SP-PEG5K)$_6$-Hb exhibited a higher O$_2$ affinity than control HbA (a P$_{50}$ of 8.5 mmHg vs 15.3 mmHg for HbA). Furthermore, the increase in O$_2$ affinity observed in PBS was more significant than that observed in the BisTris-Tris buffer.

Molecular radius of (SP-PEG5K)$_6$-Hb: The molecular radius of (SP-PEG5K)$_6$-Hb, as determined by dynamic light scattering measurements, along with that of (SP-PEG20K)$_2$-Hb is presented in Table IV. As can be seen, the molecular radius of (SP-PEG5K)$_6$-Hb is slightly smaller than the radius of (SP-PEG20K)$_2$-Hb. It may also be seen from the data presented in Table IV, that the molecular volume of HbA is increased >10 fold when it is surface decorated either with about six copies of PEG5000 chains or with 2 copies of PEG20000.

Colligative properties of (SP-PEG5K)$_6$-Hb: A comparison of the viscosity of (SP-PEG5K)$_2$-Hb and (SP-PEG5K)$_6$-Hb as a function of Hb concentration is presented in Fig. 6A. (SP-PEG5K)$_2$-Hb showed only a small increase in viscosity with the increase in protein concentration, the viscosity being directly proportional to the concentration of the protein. On the other hand, even though the viscosity of (SP-PEG5K)$_6$-Hb is comparable to that of (SP-PEG5K)$_2$-Hb in dilute solutions, the viscosity of the former increased exponentially with the concentration of the protein.

The colloidal osmotic pressure of the PEGylated Hbs as a function of the protein concentration is shown in Fig 6B. As can be seen, the oncotic pressure of the PEGylated Hbs increased as a function the protein concentration. Like the viscosity, the increase in COP is small with (SP-PEG5K)$_2$-Hb, and appears to increase linearly with the protein concentration. On the other hand, the oncotic pressure of (SP-PEG5K)$_6$-Hb increased exponentially with the increase in protein concentration. These results suggest that the oncotic pressure of PEGylated Hb is a direct correlate of its viscosity. Similar results were observed with PEGylated Hbs carrying 2 PEG chains/tetramer (19).
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Vasoactivity of (SP-PEG5K)$_6$-Hb: (i) Comparison of the systemic and microvascular responses to PEGylated-Hb: Changes in the A2 arteriolar diameter (expressed as percent of baseline values) after various periods of 10% top load infusions of (SP-PEG5K)$_2$-Hb and (SP-PEG5K)$_6$-Hb in the hamster skin fold-window model are shown in Fig. 7. As can be seen, (SP-PEG5K)$_6$-Hb conserves the arteriolar diameter more closer to the starting value than that observed with (SP-PEG5K)$_2$-Hb. These results demonstrate that the PEGylation of acellular Hb, to a level of ~six copies of PEG5000 chains reduces its intrinsic vasoactivity significantly relative to that observed with two PEG5000 chains. This is consistent with the notion that the increased PEG mass mediated colligative properties of the PEGylated Hb play a role for the modulation of its vasoactivity.

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 tetramer for the microcirculation were also 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. 8. Saline was used as the control; the results obtained with unPEGylated Hb, (SP-PEG20K)$_2$-Hb and (SP-PEG5K)$_6$-Hb are compared. MAP increased and the heart rate decreased after infusion of (SP-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 relatively unchanged (data not shown). Interestingly, MAP and heart rate were not statistically changed after infusion with (SP-PEG5K)$_6$-Hb and remained close to the baseline values. Although there was a small decrease in FCD with (SP-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-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
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(ii) Effect of extreme hemodilution with (SP-PEG5K)_{6-Hb}: Previous studies by Tsai et al (30) have demonstrated that functional capillary density is an accurate predictor of survival during acute blood loss. To determine if the (SP-PEG5K)_{6-Hb} is effective in the treatment of hemorrhagic shock i.e., to evaluate the ability of (SP-PEG5K)_{6-Hb} to keep the capillaries open to facilitate the circulation of the O_{2}-carrier under conditions of severe blood loss, the (SP-PEG5K)_{6-Hb} solution was analyzed in an extreme hemodilution protocol. To achieve a scenario closer to actual practice, the hemodilution was accomplished in three steps. Each volume exchange was a percent of the animal's total blood volume, estimated at 7% of the bodyweight. The animal's blood was first hemodiluted in two steps with a plasma expander to a Hb concentration of 7 g/dl, a recommended red cell transfusion threshold. The third step of the hemodilution was with the test solution to achieve a final systemic hematocrit equivalent to 25% of baseline.

The results obtained with (SP-PEG5K)_{6-Hb} are presented in Fig. 9, and are compared with that obtained with the Biopure product Oxyglobin (glutaraldehyde polymerized bovine Hb), a commercially available hemoglobin based oxygen carrier. The first two steps of the hemodilution protocol were performed with a 6% solution of Dextran-70 to reduce the hematocrit to 40% of baseline (60% red blood cell exchange) and the third step was performed with the test solutions - 5 g Hb/dl of (SP-PEG5K)_{6-Hb} and 14 g Hb/dl of Oxyglobin. As can be seen, (SP-PEG5K)_{6-Hb} maintained the functional capillary density at 65.5% ± 5% of the base-line whereas the Oxyglobin maintained the functional capillary density only at 37 ± 9% of the baseline.

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-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 (20, 38-40). In view of its proximity to the protein surface, the succinimidophenyl moiety of
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the PEG-Hb conjugate is anticipated to be shielded from the macro-environment by the hydration shell of the PEG chains around the protein and hence unlikely to be immunogenic. However, if the succinimidophenyl linkage should turn out to be immunogenic, PEG reagents with alkyl linkages could be used to generate the desired material.

(SP-PEG5K)₆-Hb, has many of the attributes that have been advanced as needed for minimizing the vasoactivity of acellular Hb (15, 41): (i) Increased O₂ affinity to limit the O₂ off-loading by acellular Hb in arterioles, thus minimizing the potential for vasoconstriction through autoregulatory mechanisms, (ii) Retention of the cooperative binding to insure off-loading of O₂ 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 O₂ 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-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 (15-17) was generated by the active ester chemistry (18) which results in the loss of the net positive charge of the protein, and has been suggested to carry an average of ten PEG-5K chains/Hb. The results of the present study suggest that the conservation of the net surface charge of the parent Hb is not crucial to generate a non-hypertensive Hb and that the nonhypertensive property can be endowed to Hb with less than ten PEG5K chains. In addition, the absence of a deoxygenation step in the present protocol, unlike that used for the generation of the Enzon PEG-bovine Hb, makes the scale-up of this PEGylation process simple and cost effective. Furthermore, the results of the present study also demonstrate that under conditions of extreme hemodilution, (SP-PEG5K)₆-Hb, a size enhanced PEGylated Hb, is far better than the polymerized bovine Hb (Biopure product Oxyglobin) in maintaining the functional capillary density, a property shown to be an accurate predictor of survival during acute blood loss (30).

More strikingly, the results of the present study suggest an important role for the surface configuration
Vasoinactive PEGylated hemoglobin

of the PEG chains on the Hb molecule in neutralizing its vasoactivity. Although the molecular and colligative properties, and the O₂ affinity of (SP-PEG5K)₆-Hb and (SP-PEG20K)₂-Hb are comparable, 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 colligative properties. Thus, in conjunction with enhanced molecular size and colligative properties, and high O₂ affinity, the architecture/surface configuration of the PEG on the Hb molecule, i.e., the number and size of the PEG-chains, and possibly the site of PEGylation appears to play a key role in the modulation of the vasoactivity of the Hb molecule. A plausible explanation for the difference in the vasoactive properties of (SP-PEG5K)₆-Hb and (SP-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 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, 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, which in turn would enable the generation of well-defined, site-specifically PEGylated Hbs that are much needed for such studies.

In conclusion, it is apparent from the results of the present study that, in addition to the increase in colligative properties, a surface coverage of Hb accomplished by conjugation of multiple copies of small PEG chains vs conjugation of two long PEG chains to obtain comparable PEG mass is desirable due to its potential for reducing the vasoactivity of the molecule. 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 \textit{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 (42-45). Thus it appears that the selection of a PEGylation strategy for a particular
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protein will be dependent on the specific application under consideration.

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Vasoinactive PEGylated hemoglobin

H827-H836


LEGENDS TO FIGURES

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

Figure 2. *In situ* thiolation mediated maleimide chemistry based PEGylation of Hb: Influence of iminothiolane
concentration on the size enhancement of Hb with maleidophenyl PEG5000. HbA (0.5 mM in tetramer) in
PBS, pH 7.4 was incubated with 10 mM 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 mM Mal-Phe-PEG-5000 in the absence
of iminothiolane; Curves c, d, and e represent PEGylation in the presence of 1, 2.5 and 5 mM, respectively of
iminothiolane (2, 5 and 10 fold molar excess over Hb). The elution positions of (SP-PEG10K)2-Hb and (SP-
PEG20K)2-Hb are indicated by dashed down arrow and solid down arrow, respectively. Inset shows the
kinetics of thiolation of HbA (0.5 mM) in the presence of a 10 fold molar excess of iminothiolane (5 mM).

Figure 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 mM) in PBS, pH 7.4 was incubated with 10 mM iminothiolane overnight at
4°C, followed by dilution with an equal volume of 20 mM Mal-Phe-PEG5000 and further incubated at 4°C for
6 h. B: Thiolated HbA obtained by incubation of HbA (1 mM) in PBS, pH 7.4 with 10 mM iminothiolane
overnight at 4°C (i.e., the product of the first step of the two-step protocol).

Figure 4. Purification of (SP-PEG5K)6-Hb by size exclusion chromatography on a Superose 12 Prep grade
column (2.6 cm x 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-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
Vasoinactive PEGylated hemoglobin
tetrameric, octameric, dodecameric and hexadecameric forms of $\alpha\alpha$-fumaryl Hb.

Figure 5. NMR Spectra of (SP-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-PEG20K)$_2$-Hb and (SP-PEG5K)$_6$-Hb, respectively.

Figure 6. A: Viscosity of PEGylated Hb as function of protein concentration. Open triangles represent (SP-PEG5K)$_2$-Hb; open circles represent (SP-PEG5K)$_6$-Hb. B: Colloidal osmotic pressure of (SP-PEG5K)$_6$-Hb as a function of protein concentration. Open triangles represent (SP-PEG5K)$_2$-Hb, and open circles represent (SP-PEG5K)$_6$-Hb.

Figure 7. Changes in the $A_2$ arteriolar diameter (% of base line) in response to a 10% top load (hypervolemic) infusion of (SP-PEG5K)$_2$-Hb (solid bar) and of (SP-PEG5K)$_6$-Hb (open bar) as a function of time.

Figure 8. Changes in mean arterial pressure, heart rate and functional capillary density in response to a 10% hypervolemic infusion with (SP-PEG20K)$_2$-Hb and (SP-PEG5K)$_6$-Hb as compared to saline and unPEGylated Hb controls.

Figure 9. Functional capillary densities on extreme hemodilution with (SP-PEG5K)$_6$-Hb (5 g Hb/dL) and its comparison with the Biopure product Oxyglobin (Polymerized bovine hemoglobin, 14 g/dL).
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**Abbreviations:**

Hb, hemoglobin; PEG, poly(ethylene glycol); 4-PDS, 4,4’-dithiopyridine; SP, succinimidophenyl; 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; Oxyglobin, glutaraldehyde polymerized bovine hemoglobin, a product of Biopure Corp. Boston, MA; hypervolemic infusion, infusion of a known volume of the test solution without removal of an equal volume of blood; isovolemic hemodilution, progressive infusion of the test solution with a simultaneous withdrawal of an equal volume of blood at the same rate, to maintain the total blood volume.
Table I

Quantitation of PEGylation in PEG-Hb conjugates

<table>
<thead>
<tr>
<th>Hb Sample</th>
<th>Number of PEG chains per Hb (moles/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>By -SH titration*</td>
</tr>
<tr>
<td>HbA</td>
<td>0</td>
</tr>
<tr>
<td>(SP-PEG5K)&lt;sub&gt;2&lt;/sub&gt;-Hb</td>
<td>2.1</td>
</tr>
<tr>
<td>(SP-PEG10K)&lt;sub&gt;2&lt;/sub&gt;-Hb</td>
<td>2.1</td>
</tr>
<tr>
<td>(SP-PEG5K)&lt;sub&gt;6&lt;/sub&gt;-Hb</td>
<td>6.5</td>
</tr>
</tbody>
</table>

*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.

**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. (32).
Table II

Identification of sites of PEGylation in (SP-PEG5K)_6-Hb

<table>
<thead>
<tr>
<th>Residue modified</th>
<th>% Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys-93(β)</td>
<td>100</td>
</tr>
<tr>
<td>Lys-60(α)</td>
<td>65</td>
</tr>
<tr>
<td>Lys-120(β)</td>
<td>62</td>
</tr>
<tr>
<td>Lys-11(α)</td>
<td>36</td>
</tr>
<tr>
<td>Lys-8(β)</td>
<td>29</td>
</tr>
<tr>
<td>Val-1(β)</td>
<td>12</td>
</tr>
<tr>
<td>Val-1(α)</td>
<td>8</td>
</tr>
</tbody>
</table>

The sites of PEGylation in (SP-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 3.12 groups per αβ dimer, and hence 6.24 groups per Hb molecule, since Hb is a tetramer consisting of two αβ dimers.
Table III

Oxygen affinity of (SP-PEG5K)$_6$-Hb and its Modulation by Allosteric Effectors

<table>
<thead>
<tr>
<th>Effector</th>
<th>(P_{50}) mmHg (n)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HbA</td>
</tr>
<tr>
<td>None</td>
<td>8.0 (2.5)</td>
</tr>
<tr>
<td>DPG</td>
<td>22.5 (2.3)</td>
</tr>
<tr>
<td>NaCl</td>
<td>24.0 (2.4)</td>
</tr>
<tr>
<td>L35</td>
<td>57.0 (1.7)</td>
</tr>
</tbody>
</table>

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

*\(P_{50}\), partial pressure of \(O_2\) at half saturation; n, Hill coefficient.
Table IV

Molecular Dimensions of HbA PEGylated with Mal-Phe-PEG5000

<table>
<thead>
<tr>
<th>Sample</th>
<th>Calc. Mass Daltons</th>
<th>PEG Mass Daltons</th>
<th>Radius nm</th>
<th>Mol. Volume nm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA</td>
<td>64000</td>
<td>0</td>
<td>3.12</td>
<td>127</td>
</tr>
<tr>
<td>(SP-PEG5K)₅-Hb</td>
<td>94000</td>
<td>30000</td>
<td>6.81</td>
<td>1322</td>
</tr>
<tr>
<td>(SP-PEG20K)₂-Hb*</td>
<td>104000</td>
<td>40000</td>
<td>7.04</td>
<td>1461</td>
</tr>
</tbody>
</table>

*Data from Manjula et al (19).
Thiolated Hb (Transient Intermediate species)

\[
\text{Hb} - \text{CH}_2 - \text{CH}_2 - \text{CH}_2 - \text{NH}_2 + \text{S}^=\text{NH}_2 + \text{PEG} \text{NH} - \text{C} - \text{O} - \text{PEG}
\]

2-Iminothiolane Maleimidophenyl Carbamate of PEG 5000

Thiolated Hb (Transient Intermediate species)

\[
\text{Hb} - \text{CH}_2 - \text{CH}_2 - \text{CH}_2 - \text{NH} - \text{C} - \text{CH}_2 - \text{CH}_2 - \text{SH} + \text{2-Iminothiolane}
\]

\[
\text{Hb} - (\text{CH}_2 - \text{CH}_2 - \text{CH}_2 - \text{NH} - \text{C} - \text{CH}_2 - \text{CH}_2 - \text{SH})_n - \text{PEG}
\]

(SP-PEG5K)_n-Hb

Figure 1
Figure 2

Absorbance

<table>
<thead>
<tr>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
</tr>
<tr>
<td>40</td>
</tr>
<tr>
<td>60</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>8</td>
</tr>
</tbody>
</table>

Titratable -SH Groups

- **a**: Control HbA
- **b**: 0 mM Iminothiolane + 10 mM Mal-Phe-PEG 5000
- **c**: 1 mM Iminothiolane + 10 mM Mal-Phe-PEG 5000
- **d**: 2.5 mM Iminothiolane + 10 mM Mal-Phe-PEG 5000
- **e**: 5 mM Iminothiolane + 10 mM Mal-Phe-PEG 5000
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
PEGylated hemoglobin: Role of surface configuration of PEG for the modulation of hemoglobin vasoactivity
Belur N. Manjula, Amy G. Tsai, Marcos Intaglietta, Ching-Hsuan Tsai, Chien Ho, Ashok Malavalli, Kim D. Vandegriff, Robert M. Winslow, Paul K. Smith, Krishnaveni Perumalsamy, Nirmala Devi Kanika, Joel M. Friedman and Seetharama A. Acharya

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