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PEGYLATION OF $\alpha\alpha$ -Hb USING SUCCINIMIDYL PROPIONIC ACID PEG 5K:

Conjugation Chemistry and PEG Shell of PEG $\alpha\alpha$ -Hbs Dictate Respectively their Oxygen Affinity and Resuscitation Fluid Properties

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Abstract

PEGylation of intramolecularly crosslinked Hb has been studied here to overcome the limitation of dissociation of Hb tetramers. New hexa and deca PEGylated low oxygen affinity PEG- $\alpha\alpha$ -Hbs have been generated. Influence of PEG conjugation chemistry and the PEG shell structure on the functional properties as well as PEGylation induced plasma expander like properties of the protein has been delineated. The results have established that in the design of PEG-Hbs as oxygen therapeutics, the influence of conjugation chemistry and the PEG shell structure on the oxygen affinity of Hb needs to be optimized independently besides optimizing the PEG shell structure for inducing resuscitation fluid like properties.

Introduction

One of the major obstacles for the development of hemoglobin based oxygen carrier (HBOC) has been the *in vivo* hypertensive activity of acellular Hb. In recent years, PEGylation has emerged as a novel chemical platform that attenuates the *in vivo* hypertensive activity of acellular Hb (Cole and Vandegriff 2011, Acharya et al. 2005). This new concept has energized the field of blood substitutes and represents the beginning of new era in the design and development of nonhypertensive Hb based oxygen carriers (HBOCs). PEGylation induced plasma expander like properties make PEGylated Hbs a novel class of oxygen carrying resuscitation fluids. This is a unique and distinguishing property of this class of modified Hb molecules as HBOCs as compared to most of the previously designed Hb derivatives designed as potential blood substitutes. Sanguinate of Prolong Pharmaceutical, a PEGylated bovine Hb that carries eight copies of PEG 5K conjugated through urethane linkage (Nho et al. 1992, Ananthakrishnan et al. 2013) and MP4 of Sangart, a PEGylated Hb that carries six copies of PEG 5K conjugated through extension

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arm chemistry based thiosuccinimidyl linkage (Vandegriff et al. 2003 2004, 2009, Young et al. 2005), represent the two major PEG Hbs that are currently in clinical trial.

However, PEGylation of Hb still presents many challenges before we can harvest the clinical potential of PEG Hb as an oxygen therapeutic resuscitation fluid. There are two major limitations with the PEGylation of Hbs. Direct PEGylation of uncrosslinked Hb weakens the interdimeric interactions of the tetramers and hence facilitates the dissociation of PEGylated tetramers into PEGylated dimers. This increases the oxygen affinity and this reduces its propensity to deliver oxygen. The conjugation chemistry used for PEGylation of Hb also appears to dictate the extent to which a given level of PEGylation induces the weakening of the interdimeric interactions. The weakening of interdimeric interaction can also increase the rate of autooxidation as well as the subsequent heme degradation product mediated toxicity. The reductive alkylation chemistry based PEGylation using PEG aldehydes has the maximum effect on the PEGylation induced dissociation of Hb (Hu et al. 2005). On the other hand acylation chemistry based PEGylation using the succinimidyl active esters of PEG propionic acid has the least impact on the tetramer stability of the hexaPEGylated uncrosslinked Hbs generated using PEG 5K (Li et al. 2008). The extension arm (EA) chemistry synergized with maleimide chemistry based PEGylation has the unique advantage of attenuating the PEGylation induced weakening of the interdimeric interactions (Li et al. 2009, Li et al. 2006, Manjula et al. 2005). This advantage of EAF PEGylation is not an all none phenomenon; this protective role is reduced beyond the stage of hexaPEGylation (using PEG 5K), even when the EAF hexaPEGylation is carried out with reversible protection of Cys-93(β) (Li et al. 2009).

The second limitation of PEGylating Hb is due to its influence on oxygen affinity since PEGylated Hbs are invariably generated high oxygen affinity species. The increase in the oxygen affinity resulting from PEGylation can be dissected out as emerging from a site specific modification of the PEG chains and that induced by the presence of PEG shell surrounding the central Hb core. The PEG shell on Hb molecule increases the viscosity on the molecular surface of Hb (microviscosity). The viscous PEG should slow down the both the entry and escape of the oxygen to and from the heme center. The influence of the packing density of the PEG shell of PEG Hbs on the tissue oxygenation, when the overall oxygen affinity of PEG Hbs are comparable, is an area that has not been addressed so far. The studies available on the tissue oxygenation by PEG5K2 Hb, PEG10K2 Hb, PEG5K4 canine Hb, and PEG5K6 Hb, all of which have comparable oxygen affinities clearly reflect the role of packing density of PEG shell (microviscosity) on the oxygen delivery by PEGylated Hbs (Acharya et al. 2007, Manjula et al. 2003).

Winslow and his colleagues advanced the concept of a high O₂ affinity for Hb based oxygen therapeutics is an essential property to attenuate the *in vivo* hypertensive activity of acellular Hb. They argued that designing blood substitutes with an oxygen affinity comparable to that of RBCs, is flawed since this molecule will release all its oxygen on the arterial side of the circulation leading to autoregulation mediated vasoconstriction (Cole et al. 2008, Winslow 2005, Winslow 2008, Tsai et al. 2003). However, the oxygen affinity of the two PEGylated Hbs in clinical trials appears to be too high to deliver adequate levels of O₂ to tissues under physiological conditions. The oxygen affinity of Hemospan of Sangart is even higher than

that of Sanguinate of Prolong. Besides PEG Hbs are used at significantly lower concentrations than the previously designed HBOC candidates. These aspects of PEG Hb that makes them well suited for targeting tissue oxygenation of hypoxic areas in the body is establishing a new basis for the design oxygen therapeutics aimed at minimizing the risk of damaging organs that are potentially hypoxic (Vandegriff and Winslow 2009, Young et al. 2005, Vandegriff et al. 2003). Since solutions of PEG Hbs are very viscous, the clinical application of the PEG Hb as blood substitute requires the optimization of the PEGylation platform using the just amount of conjugated PEG mass needed to prevent *in vivo* hypertensive activity and the right increase in viscosity to maximize the amount of PEG Hb that may be infused.

It should be noted that one of the consequences of designing a blood substitute with a very low oxygen affinity is that the patients have to be transfused with fresh blood (Tsai et al. 2010, Tsai et al. 2007) before the oxygen saturation in the tissues of patients falls in the range of the P50 of the current versions of PEG-Hbs. The oxygen carrying capacity of Hb in RBC represents essentially most of the oxygen carrying capacity of the system even when as much as 40 to 50 % the blood volume is replaced by a 4 gm % solution of MP4 (Hemospan) or Sanguinate. Assuming the total initial circulating concentration of Hb (in RBC) is around 12 gm %, after a 50 % blood volume exchange with the present versions of PEG Hbs, the Hb in RBC will account for only 6 gm %, a value below the transfusion trigger. At this level, PEG Hb will represent only about 2 gm %, around 25 % the total Hb in circulation. Thus, as long as the oxygen affinity of PEG Hb is high and PEG Hb present in the plasma is at a lower concentrations relative to Hb in RBC, RBC is still the primary oxygen carrying material. The extent to which oxygen is delivered by high oxygen affinity PEG Hb in circulation is dictated by the oxygen tension in hypoxic regions in the tissues and the P50 of PEG Hb. Therefore, the current versions of high oxygen affinity PEGylated Hbs are primarily active plasma expanders (Sriram et al. 2012, Cabrales et al. 2008), and these semisynthetic hybrid biopolymers perform only a secondary function of targeting the oxygen delivery to hypoxic regions.

Accordingly, a nonhypertensive PEG Hb with an oxygen affinity that is intermediate to that of current versions of PEG-Hbs and RBC has been considered as better choice for designing materials that can perform the function of targeting the oxygen delivery by RBC to hypoxic regions. This is expected to satisfy to a significant degree, the requirements of the designed function of oxygen therapeutics, oxygenation of hypoxic regions in the body (Acharya et al. 2007).

One of the early chemically modified hemoglobin designed to serve as blood substitute is diaspirin crosslinked hemoglobin (Winslow 2000); this is an intramolecularly cross-linked Hb, the fumaryl crosslink being between the α chains (lysine 99 α 1-lysine 99 α 2) in the middle of the central cavity. Stabilization of the tetrameric hemoglobin against dissociation into dimers by engineering intramolecular crosslinking was successful in overcoming the nephrotoxicity. The oxygen affinity of this intramolecularly crosslinked Hb is close to that of RBC and also to that of HbA in the presence of diphosphate glycerate (DPG). The vasoactivity of this molecule was even higher than the parent molecule. PEGylation could attenuate the *in vivo* hypertensive activity of this molecule as well by inducing resuscitation

fluid like properties. The oxygen affinity of this intramolecularly crosslinked Hb molecule will be lower than that of PEGylated uncrosslinked Hb and this could result in a desirable oxygen carrying resuscitation fluid. Accordingly, PEGylation of $\alpha\alpha$ Hb has been undertaken to engineer “active plasma expander” properties to $\alpha\alpha$ Hb that still maintains an oxygen affinity lower than the current versions of PEG Hbs in clinical trials.

Direct PEGylation of $\alpha\alpha$ -Hb, i.e. without the intervention of extension arm chemistry, has been undertaken here as the PEGylation induced dissociation is no longer a concern with the intramolecularly crosslinked molecule. Accordingly, succinimidyl ester of propionic acid PEG (SPA PEG) has been chosen to conjugate the PEG chains to modify the protein through the formation of stable isopeptide linkages (Figure 1). In this acylation chemistry based PEG conjugation, the positive charge on the surface amino groups is neutralized on conjugation. HexaPEGylation and decaPEGylation of the $\alpha\alpha$ Hb with PEG 5K using this acylation based PEGylation platform will generate a more negatively charged PEGylated protein. Reaction of $\alpha\alpha$ Hb with succinimidyl propionate has also been carried out in an attempt to generate hexa and deca propionyl $\alpha\alpha$ Hb as conjugation chemistry control for the PEGylation reaction. Accordingly, an attempt is made here to delineate the influence of conjugation chemistry and of intramolecular crosslinking in Hb on the PEGylation induced enhancement in plasma expander like properties as well as the PEGylation induced increase in the oxygen affinity of the molecule. Deca and hexa propionyl $\alpha\alpha$ Hb has also been generated to segregate the contribution of the PEG shell from that of covalent modification of $\alpha\alpha$ Hb by propionylation (Figure 1). Further, the influence of the succinimidyl chemistry mediated formation of urethane linkage between Hb and PEG (Figure 1) using succinimidyl carbamate PEG5K (SC PEG5K) has also been investigated here by generating the hexaPEGylated $\alpha\alpha$ Hb to map the influence of conjugation chemistry on both the functional and solution properties.

Materials and methods

Hemoglobin solutions

HbA was purified from the human erythrocyte lysate by DE-52 chromatography (Manjula and Acharya 2003). HbA was intramolecularly cross-linked with bis-(3,5-dibromosalicyl) fumarate (DBBF) as described previously (Chatterjee et al. 1986, Nacharaju et al. 2007). Briefly, HbA (1 mM) was incubated overnight with 8 mM sodium tripolyphosphate in 50 mM Bistris acetate buffer (pH 6.5) at 4 °C to block modifications of the β chains by DBBF. Then the HbA solution was deoxygenated and incubated with 1.9 mM DBBF in 50 mM Bistris acetate buffer (pH 6.5) at 37 °C for 3 h. The reaction was stopped by adding 20 mM Gly-Gly. The sample was dialyzed against 50 mM Tris acetate buffer, pH 8.0, at 4 °C overnight and then applied to a Q-Sepharose high performance column (2.6 × 65 cm, 350 mL of column volume, Pharmacia). The column was equilibrated with 50 mM Tris acetate at pH 8.0 (buffer A) using an AKTA Purifier 10 System (Amersham Pharmacia Biotech.), and eluted with a linear gradient of 0-100% buffer B, 50 mM Tris acetate at pH 6.8, at 4 °C (Manjula and Acharya 2003). The column was eluted at a flow rate of 2 ml/min. The effluent was monitored at 540 and 630 nm. The DBBF cross-linked $\alpha\alpha$ -HbA was collected on a Frac-900 collector (Amersham Pharmacia Biotech.).

PEGylation of Hb

0.5 mM HbA or $\alpha\alpha$ Hb in PBS was incubated with 5 mM or 10 mM SPA PEG5K at 4 °C for 3 hours. The unreacted PEG reagent was removed by centrifugal filtration against PBS, using Centricon (50 KD cut off membrane, Millipore Corp.) at 5,000 rpm for 30 min four times.

Oxygen Affinity Measurements

The oxygen equilibrium curves (OECs) of Hb solutions were obtained using a Hemox analyzer (TCS Scientific, New Hope, PA). This instrument measures the oxygen tension with a Clark oxygen electrode (Model 5331 oxygen Probe; Yellow Springs Instrument, Yellow Springs, OH) and simultaneously calculates the Hb oxygen saturation via dual-wavelength spectrophotometer. The concentration of Hb samples was 20 μ M (heme). The measurement was carried out in PBS, pH 7.4 at 37 °C. The dependence of the oxygen affinities of Hb samples on pH was carried out in 100 mM phosphate buffer at a range of pH 6.5 to pH 8.5 at 37 °C (Fronticelli et al. 1984).

Analytical Methods

Size-exclusion chromatography (SEC) was performed, as described previously (Manjula and Acharya 2003), at room temperature on two HR10/30 Superose 12 columns (Amersham Biosciences) connected in series using a fast performance liquid chromatography instrument (Pharmacia). The column was eluted using PBS at pH 7.4 and a flow rate of 0.5 mL/min, and the effluent was monitored at absorbance 540 nm. The reactivity and number of thiol groups on Hb samples were estimated by reaction with 4, 4'-dithiopyridine (4-PDS) (Aldrich Chemical Co.) according to Ampulski et al. (Ampulski et al. 1969).

Identification of sites of PEGylation

PEGylation sites were established by tryptic peptide mapping as described previously (Hu et al. 2007). 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 RPHPL on a Vydac C18 column (10 $\times$ 25 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.

Solution Properties of PEGylated Hb

The molecular radius of the PEGylated HSA was determined at 25 °C at 1 mg/mL by dynamic light scattering measurements on a DynaPro NanoStar Instrument (Wyatt Technology, Goleta, CA). Each sample was measured four times and the average of the measurements is presented. The viscosity of the PEGylated Hb in PBS was measured with a cone and plate Rheometer (Brookfield, Middleboro, MA) at 37 °C. The instrument was calibrated with water prior to measurements of the viscosity of the Hb samples. The colloid osmotic pressure (COP) of the PEGylated Hb in PBS was determined using a Wescor 4420 Colloidal Osmometer (Wescor, Logan, UT, USA) with a 30 KD Mw cut off membrane at

room temperature. The instrument was calibrated with Osmocoll reference standard prior to measurements of the samples. Each sample was measured at least twice and the average of the measurements is presented.

Results

Influence of non-conservative PEGylation of $\alpha\alpha$ -fumaryl-Hb on molecular and solution properties

The amount of PEG conjugated to $\alpha\alpha$ Hb in the two PEGylated samples was estimated by $^1\text{H-NMR}$ method as discussed previously (Manjula et al. 2005) and the results are shown in Table 1. The adduct generated using a 10 fold molar excess of PEG reagent (over the protein) carries an average 6 copies of PEG-5K chains per tetramer while the adduct generated using a 20 fold molar excess of PEG reagent carries on an average 10 copies of PEG-5K chains per tetramer. Accordingly the former conjugation product is referred to as hexaPEGylated derivative, i.e. (Propionyl-PEG5K) $_6$ - $\alpha\alpha$ Hb and the latter product is referred as decaPEGylated material, i.e., (Propionyl-PEG5K) $_{10}$ - $\alpha\alpha$ Hb.

FPLC patterns of hexa and decaPEGylated $\alpha\alpha$ Hb in Superose 12 columns has been compared with those of unmodified Hb and the crosslinked Hb (Figure 2). Intramolecular crosslinking of Hb has essentially no influence on its hydrodynamic volume as reflected by FPLC pattern. HexaPEGylation results in a significant enhancement of the hydrodynamic molecular volume of $\alpha\alpha$ Hb (curve 3). The decaPEGylation increases the hydrodynamic volume further as reflected by the elution pattern (curve 4). This elution pattern is more asymmetric by comparison with the hexaPEGylated material, suggesting a higher degree of heterogeneity in terms of the hydrodynamic volume.

The molecular radius of (Propionyl-PEG5K) $_6$ - $\alpha\alpha$ Hb and (Propionyl-PEG5K) $_{10}$ - $\alpha\alpha$ Hb as determined by dynamic light scattering measurements, is presented in Table 2. The molecular radius of control $\alpha\alpha$ Hb is 3.1 nm, same as the control uncrosslinked HbA. The intramolecular crosslinking has little influence on the molecular dimension of Hb. The radius of (Propionyl-PEG5K) $_6$ - $\alpha\alpha$ Hb is 6.0 nm, the conjugation of just 30 kD PEG mass (only half of the mass of Hb) nearly doubles the molecular radius of the molecule. The conjugation of another four copies of PEG-5K chains (another 20 kD PEG mass), however increases the radius of the PEGylated protein only by another 0.7 nm. Accordingly, it is clear that as new chains are conjugated to PEG Hb, they start filling the molecular space occupied by PEG chains previously conjugated, i.e., as the total mass of PEG chains conjugated to Hb is increased by increasing the number of PEG chains (using the same molecular size PEG chains). The PEG chains in the PEG shell pack tightly against one another. As shown in Table 2, the packing density of PEG shell of (Propionyl-PEG5K) $_6$ - $\alpha\alpha$ Hb is 39.8 Dal/nm 3 and that of (Propionyl-PEG5K) $_{10}$ - $\alpha\alpha$ Hb is 46.7 Dal/nm 3 . For comparison, the packing density of the central protein core is around 513 Dal/nm 3 . Thus the PEG shell engineered around Hb can be considered as a loosely packed molecular envelop around a central hard sphere. The packing density of this molecular envelope increases as the number of PEG chains in the PEG shell increases.

The intramolecular crosslinking of Hb has no influence on the viscosity and COP of a 4 gm % solution. The PEGylation increases the viscosity and COP of Hb; and the solution properties of $\alpha\alpha$ -Hb are a function of extent of PEGylation (Table 2). On decaPEGylation, i.e., when the number of conjugated PEG chains are increased from 6 to 10, the COP and viscosity of the adduct increase to 100.6 mmHg and 3.1 cP, respectively. The increase in the COP appears to be more pronounced for the addition of four more copies of PEG 5K chain than that of the increase for viscosity.

Influence of conjugation chemistry on the resuscitation fluid like properties of PEG Hb

PEGylation induced plasma expander like properties of these products are compared in Table 3. The influence of the presence of intramolecular crosslinking on the molecular radius of PEGylated products confirms the PEGylation induced dissociation of PEGylated tetrameric Hb suggested by the FPLC studies of the PEGylated Hbs. The COP of the hexaPEGylated Hb sample is also impacted by the presence of intramolecular crosslinking in Hb. COP is a colligative property, reflecting the number of particles in solution. The dissociation of Hb tetramers into dimers increases the number of particles. Being a colligative property, the increased COP reflects the presence of a significant amount of dissociated PEGylated Hb dimers in the preparation of hexaPEGylated uncrosslinked Hb. The intramolecular crosslinking in Hb appears to prevent the dissociation of Hb into dimers after PEGylation, hence the COP of this hexaPEGylated intramolecularly crosslinked Hb is lower.

The role of PEG conjugation chemistry and $\alpha\alpha$ -fumaryl crosslink in modulating oxygen affinity of hexaPEGylated Hb

The oxygen affinity of Hb is reduced significantly by the presence of the intramolecular crosslinking. Direct hexaPEGylation of uncrosslinked Hb with an isopeptide linkage or a urethane linkage between PEG chains and Hb results in an increase in oxygen affinity (Table 4). However, there is some degree of sensitivity to the PEG conjugation chemistry, the PEG conjugation through an isopeptide linkage does reduce the oxygen affinity of both PEGylated Hbs, as the conjugation through urethane linkage. The presence of intramolecular crosslinking reduces the oxygen affinity significantly. The increase in the oxygen affinity of the intramolecularly crosslinked Hb on hexaPEGylation is essentially independent of the chemistry of conjugation, a result that is very distinct seen on the hexaPEGylation of uncrosslinked Hb.

The urethane linkage and isopetidyl linkage increase the molecular radius of Hb on PEGylation whether the Hb has an intramolecular crosslinking it or not. Thus the overall influence of active ester chemistry mediated PEGylation is nearly identical whether the linkage generated between Hb and PEG is an isopeptide or a urethane, i.e. the overall influence of PEGylation on the interdimeric interactions of Hb is independent of the linkage and is essentially dictated by the active ester chemistry that presumably determines the site selectivity of PEGylation.

Influence of the propionylation vs propionylation based PEGylation on the modulation of the oxygen affinity of intramolecularly crosslinked Hb

The increase in the oxygen affinity of $\alpha\alpha$ fumaryl Hb has also been examined as a function of extent of PEGylation using isopeptide chemistry PEGylation platform (Table 5). Upon hexaPEGylation of $\alpha\alpha$ Hb, the P_{50} is lowered from 32 mmHg to 22.8 mmHg. This represents a significant (30 %) increase in the oxygen affinity of the molecule. Concomitant with this increase in oxygen affinity, the cooperativity (Hill coefficient) is decreased significantly, namely to 1.7 from 2.8. When the level of PEGylation is increased to decaPEGylation, the oxygen affinity increased further. The P_{50} of (Propionyl-PEG5K)₁₀- $\alpha\alpha$ Hb is 17.6 mmHg (a 45% increase in the oxygen affinity). The influence of the increased PEGylation on the Hill coefficient is marginal, which drops from 1.7 to 1.6.

The increase in the oxygen affinity of $\alpha\alpha$ fumaryl Hb on PEGylation may be coming either from the chemical modification of the side chains of Hb by propionic acid, i.e. propionylation or the presence of PEG chains at the distal end of the propionyl chains on Hb. Both structural aspects may contribute to the increase, and these aspects may be additive or synergistic. Accordingly we have prepared hexapropionyl and decapropionyl $\alpha\alpha$ fumaryl Hb and have established their influence on the oxygen affinity of the $\alpha\alpha$ fumaryl Hb. Surprisingly, the oxygen affinity of both hexa and decapropionyl derivatives of $\alpha\alpha$ fumaryl Hb are higher than that of the corresponding PEGylated derivatives. Thus the PEG-shell, irrespective of whether it is made up of six copies of PEG 5K chains or of ten copies of PEG chains, attenuates the high oxygen affinity inducing propensity of propionylation of $\alpha\alpha$ fumaryl Hb.

Intramolecularly crosslinking of Hb impacts the sensitivity of Hb to H^+ (Bohr effects) significantly (Figure 4). The Bohr effect dropped from 1.76 of native molecule Hb to 0.73 of $\alpha\alpha$ fumaryl Hb (expressed for the tetramer). The hexaPEGylation of the crosslinked Hb has only a marginal influence on the Bohr effect, reduces the cooperativity noticeably. Further increase in the level of PEG conjugation to Hb decaPEGylation reduces the cooperativity, with a further slight reduction in the Bohr effects beyond that of hexaPEGylation.

The influence of propionylation of $\alpha\alpha$ Hb on its Bohr effect has been investigated. Again, the PEG shell of both hexaPEGylated and decaPEGylated $\alpha\alpha$ fumaryl Hb attenuates the influence of the respective level of the propionyl PEGylation of the protein.

Influence of the PEG-shell on the reactivity of thiol of Cys-93(β) of oxy $\alpha\alpha$ -fumaryl Hb

The numbers of reactive SH groups in oxy samples of HbA, $\alpha\alpha$ -Hb, (Propionyl-PEG5K)₆- $\alpha\alpha$ -Hb, and (Propionyl-PEG5K)₁₀- $\alpha\alpha$ -Hb are 2.08, 2.01, 1.90, 1.90, respectively (Figure 5). The pseudo first order plots are nearly linear for every sample (plots not shown). The apparent first-order rate constants were obtained from the initial slope of each reaction are summarized in Table 6. The reactivity of SH groups of Hb (0.165 min^{-1}) is reduced slightly on $\alpha\alpha$ fumaryl crosslinking (0.151 min^{-1}), in spite of the fact that the oxygen affinity of the crosslinked Hb is considerably lower than that of HbA. On hexa and deca PEGylation of $\alpha\alpha$ fumaryl Hb, the reactivity of Cys-93 (β) to form the mixed disulfide with thiopyridine is

decreased further, being 0.112 min^{-1} and 0.098 min^{-1} , respectively. The oxygen affinity of the PEGylated crosslinked Hb is higher than the uncrosslinked Hb. However, the higher oxygen affinity, stabilization of oxy conformation by PEGylation, did not induce a higher chemical reactivity to the thiol of Cys-93(β) of the corresponding PEGylated samples.

The influence of the PEG shell on the chemical reactivity of Cys-93(β) has also been mapped by preparing hexapropionylated and decapropionylated $\alpha\alpha$ -Hb and establishing the thiol reactivities of these derivatives (Table 6). The reactivities of SH of Cys-93(β) of Propionyl6- $\alpha\alpha$ Hb and Propionyl10- $\alpha\alpha$ Hb are 0.118 and 0.103, which are essentially comparable to the respective PEGylated derivatives, i.e., (Propionyl-PEG5K)₆- $\alpha\alpha$ -Hb and (Propionyl-PEG5K)₁₀- $\alpha\alpha$ -Hb, respectively. The influence PEGylation on the microenvironment of Cys-93(β) is apparently a direct consequence of the chemistry of the conjugation, i.e. propylation, and the PEG shell by itself has little influence on the micro-circumstance of Cys-93(β).

Identification of the sites of PEGylation on $\alpha\alpha$ Hb

The site selectivity of PEGylation of $\alpha\alpha$ -Hb using SPA PEG has been established by tryptic peptide mapping of the globin chains from the PEGylated proteins. The tryptic peptides in the maps of PEGylated products have been quantitated and compared with the unmodified Hb, and the results are summarized in Table 7. PEGylation is distributed over a number of the Lys residues, more than the number of PEG conjugated to the molecule, reflecting the chemical heterogeneity of the sample with respect to the sites, in spite of a reasonable symmetrical elution pattern of the materials in SEC on Superose 12 columns (Figure 2).

The extent of PEGylation at any given site is not quantitative in the hexaPEGylated sample, i.e. it is significantly less than complete modification of that residue. The PEGylation is distributed over a number of amino groups. For a homogeneous hexaPEGylated Hb sample only three residues per $\alpha\beta$ dimer should be modified. The tryptic maps indicate that the ϵ -amino groups of ten lysine residues Hb are involved in PEGylation. There are 24 amino groups per $\alpha\beta$ dimer. The total number of individual sites of PEGylation on Hb do not vary significantly on increasing the extent of PEGylation from hexaPEGylation to decaPEGylation, only the extent to which each of these sites are PEGylated increases. The differences in the reactivity of multiple amino groups of Hb for acylation reaction (and hence for PEGylation) is not significant enough under these reaction conditions to exhibit high site selectivity. Accordingly hexa and the decaPEGylated doubly modified Hbs generated are not chemically homogeneous even though they exhibit molecular size homogeneity on FPLC analysis.

Among the 10 sites of HbA modified by SPA PEG, the ϵ -amino group of Lys-90(α) is the most reactive site in HbA with nearly 75 % and 95 % modification in hexa and decaPEGylated materials, respectively. Lys-120(β) is PEGylated to about 52 and 55 % respectively in the hexa and deca PEGylated Hbs respectively. Lys-82(β) is modified to an extent of 42 and 44 % respectively in the two products. The rest of the 7 lysine residues, Lys-11(α), Lys-16(α), Lys-40(α), Lys-56(α), Lys-139(α), Lys-8(β), and Lys-17(β), are only lightly modified (12-25%). Except for Lys-139(α) that shows a significant increase in the extent of PEGylation on increasing the extent of PEGylation from hexa to decaPEGylation,

the modification on the remaining residues in the decaPEGylate material (relative to the hexaPEGylated material), is only marginal.

The PEGylation extent of the protein has also been quantitated from the tryptic peptide mapping and compared with the quantitation determined by the NMR method (Table 1). The quantitation of PEGylation by the NMR method correlates well with the value calculated for the hexaPEGylated $\alpha\alpha$ -Hb. However, the correlation is not good with the decaPEGylated material. The quantitation of the tryptic peptide maps for products with higher level of PEGylation, apparently, seems to be complicated by the incomplete digestion of the heavily PEGylated chains leading to a lower level of estimation of the extent of PEGylation.

DISCUSSION

The early paradigms for the design of HBOCs has been that (i) oxygen affinity of HBOC should be comparable to that of Hb inside erythrocytes and (ii) the Hb should be stabilized by intramolecular crosslinking to prevent its dissociation into dimers *in vivo* and to attenuate the kidney filtration to avoid the nephrotoxicity. The recognition of the principles of autoregulation of circulation and that is activated by the oxygen content on the arterial side of circulation has raised concerns on the potential activation of the autoregulatory principles by the low oxygen affinity of Hb placed in plasma. This can induce a level of vasoconstriction, and in particular the higher potency of intramolecular crosslinked Hb. Winslow and his colleagues have accordingly advanced the concept that the oxygen affinity of HBOC's should be high and noncooperative to attenuate the premature release of oxygen by blood substitutes on the arterial side and the activation of the auto regulatory mechanisms to induce vasoconstriction.

Another new paradigm advanced for the design of blood substitute is to develop it as oxygen carrying plasma expanders (Intaglietta et al. 2006). This change in the design strategy came from the observation that DecaPEGylated bovine Hb of Enzon is unique and distinct from other derivatives of Hb designed so far as HBOC in that it is a conjugated protein and exhibits plasma expander like properties (Shorr et al. 1999, Song et al. 1995). A new PEG conjugated Hb, EAF P5K6 Hb, was designed and developed at Einstein using the new Extension Arm Chemistry based PEGylation platform. This is a high oxygen affinity PEG conjugated Hb, and is non-hypertensive even though it has only six copies of PEG-5K conjugated to it (Acharya et al. 2005, Li et al. 2006, Manjula et al. 2005). A prototype hexaPEGylated human Hb has been developed by Sangart based on the Einstein PEGylation platform. The choice of the hexaPEGylated Hb, MP4, for commercialization as blood substitute represents the marriage of two major conceptual aspects of the new paradigm for the design of blood substitutes, the high the oxygen affinity concept and the inducing plasma expander like properties to Hb in order to overcome the hypertensive activity of acellular Hb *in vivo*.

The plasma expander like properties of MP4 and reduced level of autoxidation mediated *in vivo* toxicity (Vandegriff et al. 2006, Hu et al. 2008) makes this PEG conjugated Hb excellent candidate for targeting delivery of oxygen to hypoxic regions of the body given its high oxygen affinity. Accordingly, the class of high oxygen affinity PEG conjugated Hb are

not ideally optimized molecules for delivering oxygen to all regions of the body. For oxygen delivery by high oxygen affinity Hbs, the oxygen tension in surrounding tissues should drop to the region corresponding to the P50 of these high oxygen affinity PEG conjugated Hbs. The oxygen affinity for MP4 is around 5 mmHg and around 8 mm Hg for EAF P5K6 Hb. Accordingly, these molecules should essentially function as an effective plasma volume expander at the systemic level, and as oxygen delivering plasma expanders if there are regions of ischemia and hypoxia. Besides, the high oxygen affinity PEG conjugated Hbs under clinical trial are used only in small amounts. Accordingly, they represent only a small fraction of the total Hb in circulation (Hb present in RBC plus PEG conjugated Hb transfused and present in the plasma). Given the oxygen carrying capacity of Hb in RBCs in these situations is not limiting, the high oxygen affinity PEG Hb present in plasma is unlikely to deliver much oxygen to normoxic regions. An oxygen affinity for conjugated Hb that is in the range of 15 mmHg is considered as an optimum oxygen tension to deliver quantifiable amounts of oxygen when it presents as an oxygen carrier in the plasma in the presence of red blood cells and the amount of Hb in RBC becomes limiting in terms of oxygen carrying capacity, close to “transfusion trigger”.

The $\alpha\alpha$ -fumaryl Hb, a low oxygen affinity intramolecular crosslinked Hb, is considered as the choice Hb molecule for PEGylation to generate hexaPEGylated Hbs with an oxygen affinity intermediate to that of RBC and the current versions of PEG conjugated Hbs that are in clinical trial. Reversible protection of the thiol of Cys-93(β) of $\alpha\alpha$ -fumaryl Hb is a necessary to generate PEG conjugated Hb with an oxygen affinity in this range if EAF PEGylation is the chosen PEGylation platform (Li et al. 2009). The chemical manipulation of reversible protection of Cys-93(β) is needed to generate a EAF hexaPEGylated Hb with a lower oxygen affinity molecule, i.e., it is necessary to target the EAF PEGylation exclusively to the ϵ -amino residues of Lys residues. The reversible protection protocol also attenuated the increase in the PEGylation induced autoxidation (Li et al. 2009). Accordingly, other PEGylation platforms that do not PEGylate at Cys-93(β) and can generate hexaPEGylated products should be as good as the product generated using EAF PEGylation (with reversible protection of Cys-93(β)). These may be preferred simpler platforms to design second generation low oxygen affinity Hbs using $\alpha\alpha$ -Hb, especially since PEGylation induced dissociation of tetramer will not be a problem with $\alpha\alpha$ -fumaryl Hb.

The direct PEGylation of Hb by the active ester chemistry platform, does not modify the Cys-93(β). Accordingly, the PEGylation of Hb can be carried out without the reversible protection of Cys-93(β). The presence of the $\alpha\alpha$ -fumaryl intramolecular crosslinking overcomes limitation of the PEGylation induced dissociation of Hb into PEGylated $\alpha\beta$ dimers, when this direct PEGylation platforms is used to PEGylate this crosslinked Hb. Accordingly direct hexaPEGylation and decaPEGylation of $\alpha\alpha$ -fumaryl Hb has been undertaken here to generate molecules with an oxygen affinity lower than the two PEG Hbs under clinical trials, i.e., hexaPEGylated molecule (MP4) generated by EAF PEGylation platform using maleimidopropyl PEG-5K and decaPEGylated molecule (Sanguinate) generated using succinimidyl carbamate of PEG-5K.

A very significant observation here is that the increase in molecular radius of hexaPEGylated $\alpha\alpha$ Hb seen on increasing the number of PEG 5K chains in the PEG shell

from 6 to 10 copies is very small. Consequently, the packing density of PEG within the PEG-shell encasing the central protein core, $\alpha\alpha$ Hb is increased considerably as the level of PEGylation is increased. This observation is consistent with the results on the EAF PEGylation of Albumin (36). The biochemical, biophysical and the microcirculatory consequences of the increased rigidity of the PEG-shell as the extent of PEGylation is increased have not been explored as of now. The PEGylated Hb with multiple copies of PEG chains consists of structures with distinct molecular regions of different packing densities. The protein core of high packing density is covered with a soft (spongy) cover of PEG, which gives these semisynthetic hybrid biopolymers a degree of compressibility (Li et al. 2006) or ability to change their molecular shapes when subjected to stress situations (for example shear stress). These properties of PEGylated proteins are expected to be of considerable merit in dictating the vasodilatory and microcirculatory responses of PEGylated Hb and PEGylated albumin (Sriram et al. 2012).

The increase in the number of PEG chains in the PEG shell is also accompanied by an increase in COP. The COP of PEGylated Hb is more than doubled as the numbers of PEG chains increased to ten from six. The increase in the COP of the PEGylated proteins is an exponential correlate of the number of the PEG chains in the PEG shell (Ananda et al. 2012). The viscosity of the PEGylated Hb and PEGylated Albumin at 4 gm % is not a direct correlate of the number of PEG chains, however it is an exponential functional of number of PEG chains just as the increase of COP.

One of the significant microvascular properties of P5K6 Albumin is its ability to mimic the physiological consequence of dextran-500, a high viscosity plasma expander. This mimicry involves the dextran 500 mediated enhanced endothelial NO production and supra perfusion (Sriram et al. 2012). How does the low viscosity plasma expander like P5K6 $\alpha\alpha$ Hb or EAF P5K6 albumin mimic the *in vivo* physiological reactions of a high viscosity plasma expander? The viscosity of dextran 500 (a 6 gm % solution) as well as the molecular radius is nearly three times of that of hexa-PEGylated $\alpha\alpha$ Hb (or hexaPEGylated albumin, viscosity measured at 4 gm % solution). But the COP of the hexaPEGylated albumin (of hexaPEGylated $\alpha\alpha$ Hb) is significantly higher than that of dextran 500. Accordingly, we speculate that the uniqueness of the structure of hexaPEGylated albumin and of hexaPEGylated Hb in terms of having regions in the molecules with different packing densities endows these PEGylated molecules with novel resuscitation fluid properties.

It may be noted that the molecular radius of the decaPEGylated $\alpha\alpha$ -fumaryl Hb generated here is considerably smaller than the molecular radius reported for Enzon decaPEGylated bovine Hb by Vandergtiff et al. (Vandegriff et al. 1997) as well as MP4. The molecular radius has been determined here for a decaPEGylated intramolecularly crosslinked Hb by light scattering. The Enzon decaPEGylated Hb does not have an intermolecular crosslinking, and approach for the determination of molecular radius of decaPEGylated bovine Hb and of MP4 was based on the COP of the solution, a colligative property. This calculation of the molecular radius from COP of the protein solution makes an intrinsic assumption that PEGylated bovine Hb does not dissociate into PEGylated dimers. Accordingly, further studies of decaPEGylated intramolecularly crosslinked bovine Hb will be needed to

understand this difference in the molecular radius of decaPEGylated bovine Hb vs. decaPEGylated $\alpha\alpha$ -fumaryl Hb.

Direct PEGylation of $\alpha\alpha$ -fumaryl Hb using succinimidyl ester of PEG-5K propionic acid also increases the oxygen affinity of the molecule just as EAF PEGylation (Li et al. 2008). The oxygen affinity increase seen with hexaPEGylation is significantly lower than that seen with EAF hexaPEGylation of $\alpha\alpha$ -fumaryl Hb carried out without reversible protection of thiol of Cys-93(β). But when the EAF hexaPEGylation of $\alpha\alpha$ -fumaryl Hb is carried out targeting exclusively to the ϵ -amino groups with a reversible protection of Cys-93(β), the increase in oxygen affinity is comparable or only slightly higher than the direct nonconservative hexaPEGylation that involves the formation of isopeptide linkages between PEG and protein. Thus the overall oxygen affinity increasing influence of the PEG shell, appears to be independent of the chemistry of PEGylation, conservative vs. nonconservative. The very high oxygen affinity of EAF PEGylated $\alpha\alpha$ -fumaryl Hb when carried out without reversible protection of Cys-93(β), is an additive (or synergistic) effect of EAF hexaPEGylation targeted to the ϵ -amino groups and of the maleimide modification of Cys-93(β) (Li et al. 2009).

The increase in the oxygen affinity of $\alpha\alpha$ Hb on the nonconservative direct PEGylation appears to be a function of the level of PEGylation. The oxygen affinity of the decaPEGylated intramolecularly crosslinked Hb is higher as compared to the hexaPEGylated sample. When PEGylation is increased to a higher level, an increase in the packing density of PEG shell also takes place. However, the sites modified by the PEGylation did not change as a function of increase in the extent of PEGylation, the extent to which each site is functionalized is only increased. Besides, the increase in the oxygen affinity of the crosslinked Hb by direct hexaPEGylation does not appear to be a function of the chemistry of the linkage between Hb and PEG chain. PEG conjugation by urethane linkage vs. isopeptide linkage has comparable influence on oxygen affinity of crosslinked Hb when the PEGylation levels are comparable.

The increase in oxygen affinity of Hb on PEGylation has been suggested to be a consequence of PEGylation resulting in a preferential stabilization of the oxy conformation of Hb vs. deoxy conformation (Khan et al. 2001). This observation suggests that the only way to overcome this effect of PEGylated Hb and generate lower oxygen affinity PEGylated Hbs is to use a lower oxygen affinity Hb as the starting material for PEGylation. In the present study, the structure of Hb has been reengineered by introducing deoxy conformation stabilizing $\alpha\alpha$ -fumaryl intramolecular cross bridge to lower the oxygen affinity of Hb (relative to unmodified Hb). However, when the Hb molecule has been reengineered to lower its P50, it may be necessary to consider this as a new molecule since the thermodynamics of the reversible equilibrium of oxy to deoxy conformational transition is likely to be distinct as compared to the parent molecule.

The oxygen carrying capacity of blood is dictated by the amount of total Hb present. This capacity is unlikely to be influenced by chemical modification introduced to lower the oxygen affinity of Hb to be placed in the plasma. The saturation of the molecule with oxygen takes place in the presence of abundant oxygen in the lungs. Accordingly, it can be

assumed that if there are any differences in the tissue oxygenation pattern among PEG Hbs with comparable oxygen affinities, such a difference should emerge from the influence of PEGylation pattern on propensity of the molecules to deliver its oxygen to the tissue. Crosslinking of Hb, besides reducing oxygen affinity, slightly lowers cooperativity and lowers significantly the Bohr effect, and PEGylation enhances this molecular effect. Each of these will have more influence in terms of oxygen delivery than on oxygenation of the molecule, as the tissue oxygenation is a controlled environment of oxygen tension of the tissues, very different than the environment in the lungs.

The lowering of oxygen affinity of Hb by the $\alpha\alpha$ -fumaryl crossbridge at neutral pH is accompanied by a reduction in the Bohr effect. The change in the Bohr effect is essentially the dependence of affinity of oxygen to the heme of Hb as a function of pH, does not have any influence on the oxygen carrying capacity of the molecule. The significance of the molecular properties of the designed blood substitutes have not been fully appreciated so far. In fact it has been argued by Winslow that the only molecular property relevant to be a blood substitute is high oxygen affinity (Cole et al. 2008, Winslow 2005, 2008). Many enzymes have been shown to change their pH optima for the catalysis on the chemical modification of their functional groups. This is generally associated with the change in the pK_a of ionization behavior of functional groups that has a pK_a around neutral pH, generally His residue in the microenvironment of active site of enzymes. In the case of Hb, these represent the acid Bohr groups, the surface His residues, carboxyl groups and the alpha amino groups. It may be noted that the isoelectric focusing pattern of $\alpha\alpha$ -fumaryl Hb is indistinguishable as compared to that of unmodified Hb, in spite of the fact the introduction of $\alpha\alpha$ -fumaryl Hb results in the loss of two positive charges, suggesting the change in the pK_a of some other amino acid at neutral pH. However we have no information at this stage on the mapping of Bohr groups of $\alpha\alpha$ Hb, but this has been well mapped for uncrosslinked Hb by NMR by Chien Ho and his colleagues (Busch and Ho 1990). The oxygen tension of the tissues, couples with the cooperativity and the pH of the blood (Bohr effect) dictates how much of the oxy Hb within the RBC will remain oxygenated and how much of the modified Hb placed in plasma will remain as oxy Hb pH remains as oxy Hb, i.e. how much of the oxygen will be unloaded. As long as the oxygen tension in the tissue is higher than the P50 of modified Hb molecule, modified Hbs in the plasma are inert oxygen storing plasma expanders, and the oxygen delivering task is fully executed by Hb inside the red blood cells. The level of tissue oxygenation will function as a trigger to activate the oxygen delivering capacity of the oxygen carrying plasma expanders.

The changes in the molecular properties of Hb resulting as consequences of PEGylation can be dissected out as originating from the modification of the protein by the conjugation chemistry and coupled with that from the PEG-shell. The studies presented here have established that hexa and deca propionylation has essentially no influence on the molecular radius, viscosity and COP of Hb, i.e., the plasma expander like properties are essentially endowed to the molecule by the PEG-shell engineered on the molecular surface. On the other hand hexa and deca propionylation of $\alpha\alpha$ -fumaryl Hb increases the oxygen affinity. Interestingly, the increase in the oxygen affinity is higher by comparison to the increase seen on the respective PEGylation. The PEG shell attenuates the increase induced by the conjugation chemistry.

This dissection of the contribution of the conjugate chemistry vs. the PEG-shell (Fig 6) on the oxygen affinity of $\alpha\alpha$ Hb suggests that the earlier conclusion that PEGylation stabilizes the oxy conformation of Hb (Khan et al. 2001) only represents an integrated picture of the conjugation chemistry and PEG shell on Hb. The influence predominantly comes from the conjugation chemistry. Therefore, it follows that the intrinsic effect of the PEG shell engineered around Hb is to reduce the oxygen affinity of Hb, or favor the release of oxygen from heme. Thus the conjugation chemistry and PEG shell appears to achieve very different and distinct influences on the molecular property of Hb, i.e. ability to reversibly bind oxygen. The same result is seen in terms of Bohr effect. PEG-shell attenuates the impact of conjugation chemistry induced influence on the Bohr effect of crosslinked Hb.

The earlier conclusions that PEGylation stabilizes the oxy conformational state of Hb were based on the EAF PEGylated uncrosslinked Hb (Khan et al. 2001). In a more recent study (Li et al. 2006), we have carried out a similar chemical dissection of the functional consequence of EAF PEGylation of Hb and have established that the Extension Arm chemistry increase in the oxygen affinity of Hb. When these extension arms are decorated with PEG shell, some attenuation of the EA chemistry induced the increase in oxygen affinity of Hb is accomplished. Accordingly, we conclude, that the influence of PEGylation of Hb on the molecular properties are direct correlate of the conjugate chemistry, and PEG-shell by itself has only marginal influence. On the other hand all the plasma expand like properties of PEG Hb are a direct consequence of the PEG shell. This gives us a better handle to modulate the functional properties of Hb, once we have optimized the PEGylation platform to achieve the active plasma expansion function of Hb.

The results presented here along with our earlier studies establish that every one of the PEGylated Hbs designed should be considered as very distinct molecular species in terms of the functional properties. On the other hand in terms of the PEGylation induced solution properties, as a first approximation, we can still continue to with the working hypothesis, as when the pattern of PEGylation is conserved (number and size of PEG chains conjugated) their solution properties should be comparable. Accordingly, detailed correlation of microcirculatory properties of PEGylated Hbs with PEGylation platform and the functional properties with the conjugation chemistry is necessary in designing the best oxygen delivering plasma expanders.

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References

- Acharya SA, Friedman JM, Manjula BN, Intaglietta M, Tsai AG, Winslow RM, Malavalli A, Vandegriff K, Smith PK. Enhanced molecular volume of conservatively PEGylated Hb: (SP-PEG5K)6-HbA is non-hypertensive. *Artif Cells Blood Substit Immobil Biotechnol.* 2005; 33:239–255. [PubMed: 16152690]
- Acharya SA, Acharya VN, Kanika ND, Tsai AG, Intaglietta M, Manjula BN. Non-hypertensive tetraPEGylated canine haemoglobin: correlation between PEGylation, O₂ affinity and tissue oxygenation. *Biochem J.* 2007; 405:503–511. [PubMed: 17425516]

- Acharya SA, Intaglietta M, Tsai AG. Modulation of the polyethylene glycol-hemoglobin structure to increase the efficiency of plasma expansion and O₂ carrying capacity. *Transfus Altern Transfus Med*. 2007; 9:254–264.
- Ampulski RS, Ayers VE, Morell SA. Determination of the reactive sulfhydryl groups in heme proteins with 4, 4'-dipyridinedisulfide. *Anal Biochem*. 1969; 32:163–169. [PubMed: 5407964]
- Ananda K, Manjula BN, Meng F, Acharya VN, Intaglietta M, Acharya SA. Packing density of the PEG-shell in PEG-albumins: PEGylation induced viscosity and COP are inverse correlate of packing density. *Artif Cells Blood Substit Immobil Biotechnol*. 2012; 40:14–27. [PubMed: 21623695]
- Ananthakrishnan R, Li Q, O'Shea KM, Quadri N, Wang L, Abuchowski A, Schmidt AM, Ramasamy R. Carbon monoxide form of PEGylated hemoglobin protects myocardium against ischemia/reperfusion injury in diabetic and normal mice. *Artif Cells Nanomed Biotechnol*. 2013 [Epub ahead of print].
- Busch MR, Ho CE. Effects of anions on the molecular basis of the Bohr effect of hemoglobin. *Biophys Chem*. 1990; 37:313–322. [PubMed: 2285794]
- Cabralas P, Tsai AG, Ananda K, Acharya SA, Intaglietta M. Volume resuscitation from hemorrhagic shock with albumin and hexaPEGylated human serum albumin. *Resuscitation*. 2008; 79:139–146. [PubMed: 18621463]
- Chatterjee R, Welty EV, Walder RY, Pruitt SL, Rogers PH, Arnone A, Walder JA. Isolation and Characterization of a New Hemoglobin Derivative Cross-linked between the α Chains (Lysine 99 α_1 →Lysine 99 α_2). *Biol Chem*. 1986; 261:9929–9937.
- Cole R, Vandegriff K, Szeri A, Savas O, Winslow R. Targeted O₂ delivery by blood substitutes: in vitro arteriolar simulations of first- and second-generation products. *Microvasc Res*. 2008; 76:169–179. [PubMed: 18671987]
- Cole RH, Vandegriff KD. MP4, a vasodilatory PEGylated hemoglobin. *Adv Exp Med Biol*. 2011; 701:85–90. [PubMed: 21445773]
- Fronticelli C, Bucci E, Orth C. Solvent regulation of oxygen affinity in hemoglobin. *J Biol Chem*. 1984; 259:10841–10844. [PubMed: 6469985]
- Hu T, Prabhakaran M, Acharya SA, Manjula BN. Influence of the chemistry of conjugation of poly(ethylene glycol) to Hb on the oxygen-binding and solution properties of the PEG-Hb conjugate. *Biochem J*. 2005; 392:555–564. [PubMed: 16111474]
- Hu T, Manjula B, Brenowitz M, Acharya SA. Influence of intramolecular cross-links on the molecular, structural and functional properties of PEGylated haemoglobin. *Biochem J*. 2007; 402:143–151. [PubMed: 17049048]
- Hu T, Li D, Manjula BN, Acharya SA. Autoxidation of the site-specifically PEGylated hemoglobins: role of the PEG chains and the sites of PEGylation in the autoxidation. *Biochem*. 2008; 47:10981–10990. [PubMed: 18808150]
- Hu T, Li D, Manjula BN, Brenowitz M, Prabhakaran M, Acharya SA. PEGylation of Val-1(α) destabilizes the tetrameric structure of hemoglobin. *Biochem*. 2009; 48:608–616. [PubMed: 19119852]
- Intaglietta M, Cabralas P, Tsai AG. Microvascular perspective of oxygen-carrying and -noncarrying blood substitutes. *Ann Rev Biomed Eng*. 2006; 8:289–321. [PubMed: 16834558]
- Khan I, Dantsker D, Samuni U, Friedman AJ, Bonaventura C, Manjula B, Acharya SA, Friedman JM. Beta 93 modified hemoglobin: kinetic and conformational consequences. *Biochem*. 2001; 40:7581–7592. [PubMed: 11412112]
- Li D, Manjula BN, Acharya AS. Extension arm facilitated PEGylation of hemoglobin: correlation of the properties with the extent of PEGylation. *Protein J*. 2006; 25:263–274. [PubMed: 16718519]
- Li D, Hu T, Manjula BN, Acharya SA. Non-conservative surface decoration of hemoglobin: influence of neutralization of positive charges at PEGylation sites on molecular and functional properties of PEGylated hemoglobin. *Biochim Biophys Acta*. 2008; 1784:1395–1401. [PubMed: 18452721]
- Li D, Hu T, Manjula BN, Acharya SA. Extension arm facilitated pegylation of alphaalpha-hemoglobin with modifications targeted exclusively to amino groups: functional and structural advantages of free Cys-93(beta) in the PEG-Hb adduct. *Bioconjug Chem*. 2009; 20:2062–2070. [PubMed: 19842622]

- Manjula BN, Acharya AS. Purification and molecular analysis of hemoglobin by high-performance liquid chromatography. *Methods in Molecular Medicine: Hemoglobin Disorders*. 2003; 82:31–47.
- Manjula BN, Tsai A, Upadhyaya R, Perumalsamy K, Smith PK, Malavalli A, Vandegriff K, Winslow RM, Intaglietta M, Prabhakaran M, Friedman JM, Acharya AS. Site-specific PEGylation of hemoglobin at Cys-93(beta): correlation between the colligative properties of the PEGylated protein and the length of the conjugated PEG chain. *Bioconjug Chem*. 2003; 14:464–472. [PubMed: 12643758]
- Manjula BN, Tsai AG, Intaglietta M, Tsai CH, Ho C, Smith PK, Perumalsamy K, Kanika ND, Friedman JM, Acharya SA. Conjugation of multiple copies of polyethylene glycol to hemoglobin facilitated through thiolation: influence on hemoglobin structure and function. *Protein J*. 2005; 24:133–146. [PubMed: 16096719]
- Meng F, Manjula BN, Tsai AG, Cabrales P, Intaglietta M, Smith PK, Prabhakaran M, Acharya SA. Hexa-thiocarbamoyl phenyl PEG5K Hb: vasoactivity and structure: influence of rigidity of the conjugation linkage on the PEGylation induced plasma expander-like solution properties of PEG-Hb adducts. *Protein J*. 2009; 28:199–212. [PubMed: 19653083]
- Nacharaju P, Friedman JM, Prabhakaran M, Acharya SA, Manjula BN. Combining the influence of two low O₂ affinity-inducing chemical modifications of the central cavity of hemoglobin. *Biochem*. 2007; 46:4554–4564. [PubMed: 17381072]
- Shorr RG, Kwong S, Gilbert C, Benesch RE. Changes in the functional properties of bovine hemoglobin induced by covalent modification with polyethylene glycol. *Artif Cells Blood Substit Immobil Biotechnol*. 1999; 27:185–202. [PubMed: 10226683]
- Song D, Olano M, Wilson DF, Pastuszko A, Tammela O, Nho K, Shorr RG. Comparison of the efficacy of blood and polyethylene glycol-hemoglobin in recovery of newborn piglets from hemorrhagic hypotension: effect on blood pressure, cortical oxygen, and extracellular dopamine in the brain. *Transfusion*. 1995; 35:552–558. [PubMed: 7631386]
- Sriram K, Tsai AG, Cabrales P, Meng F, Acharya SA, Tartakovsky DM, Intaglietta M. PEG-albumin supraplasma expansion is due to increased vessel wall shear stress induced by blood viscosity shear thinning. *Am J Physiol Heart Circ Physiol*. 2012; 302:H2489–2497. [PubMed: 22505638]
- Tsai AG, Vandegriff KD, Intaglietta M, Winslow RM. Targeted O₂ delivery by low-P50 hemoglobin: a new basis for O₂ therapeutics. *Am J Physiol Heart Circ Physiol*. 2003; 285:H1411–1419. [PubMed: 12805024]
- Tsai AG, Johnson PC, Intaglietta M. Is the distribution of tissue pO₂ homogeneous? *Antioxid Redox Signal*. 2007; 9:979–984. [PubMed: 17508919]
- Tsai AG, Cabrales P, Intaglietta M. The physics of oxygen delivery: facts and controversies. *Antioxid Redox Signal*. 2010; 12:683–691. [PubMed: 19757988]
- Vandegriff KD, McCarthy M, Rohlfes RJ, Winslow RM. Colloid osmotic properties of modified hemoglobins: chemically cross-linked versus polyethylene glycol surface-conjugated. *Biophys Chem*. 1997; 69:23–30. [PubMed: 9440206]
- Vandegriff KD, Malavalli A, Wooldridge J, Lohman J, Winslow RM. MP4, a new nonvasoactive PEG-Hb conjugate. *Transfusion*. 2003; 43:509–516. [PubMed: 12662285]
- Vandegriff KD, Malavalli A, Minn C, Jiang E, Lohman J, Young MA, Samaja M, Winslow RM. Oxidation and haem loss kinetics of poly(ethylene glycol)-conjugated haemoglobin (MP4): dissociation between in vitro and in vivo oxidation rates. *Biochem J*. 2006; 399:463–471. [PubMed: 16813564]
- Vandegriff KD, Winslow RM. Hemospan: design principles for a new class of oxygen therapeutic. *Artif Organs*. 2009; 33:133–138. [PubMed: 19178457]
- Nho K, Glower D, Bredehoeft S, Shankar H, Shorr R, Abuchowski A. PEG-bovine hemoglobin: safety in a canine dehydrated hypovolemic-hemorrhagic shock model. *Biomater Artif Cells Immobilization Biotechnol*. 1992; 20(2-4):511–524. [PubMed: 1391472]
- Winslow RM. alphaalpha-crosslinked hemoglobin: was failure predicted by preclinical testing? *Vox Sang*. 2000; 79:1–20. [PubMed: 10971209]
- Winslow RM. Targeted O₂ delivery by low-p50 hemoglobin: a new basis for hemoglobin-based oxygen carriers. *Artif Cells Blood Substit Immobil Biotechnol*. 2005; 33:1–12. [PubMed: 15768561]

- Winslow RM. Cell-free oxygen carriers: scientific foundations, clinical development, and new directions. *Biochim Biophys Acta*. 2008; 1784:1382–1386. [PubMed: 18555029]
- Young MA, Riddez L, Kjellström BT, Bursell J, Winslow F, Lohman J, Winslow RM. MalPEG-hemoglobin (MP4) improves hemodynamics, acid-base status, and survival after uncontrolled hemorrhage in anesthetized swine. *Crit Care Med*. 2005; 33:1794–1804. [PubMed: 16096458]

Abbreviations

$\alpha\alpha$ Hb	hemoglobin intramolecularly crosslinked at Lys-99(α)
COP	colloidal osmotic pressure
Hb	hemoglobin
HBOC	hemoglobin based oxygen carrier
HexaPEGylation	conjugation with 6 copies of PEG chains
P50	oxygen pressure at half saturation
4-PDS	4, 4'-dithiopyridine
PEG	poly(ethylene glycol)
PEG5K2 Hb	hemoglobin conjugated with 2 copies of PEG5K
PEG10K2 Hb	hemoglobin conjugated with 2 copies of PEG10K
PEG5K6 Hb	hemoglobin conjugated with 6 copies of PEG5K
PEG5K4 canine Hb	canine hemoglobin conjugated with 4 copies of PEG5K
(Propionyl)₆- $\alpha\alpha$Hb	$\alpha\alpha$ Hb conjugated with 6 propionyl groups
(Propionyl)₆- Hb	Hb conjugated with 6 propionyl groups
(Propionyl-PEG5K)₆-$\alpha\alpha$Hb	propionylated PEG- $\alpha\alpha$ Hb conjugate with 6 copies of PEG5K chains
(Propionyl-PEG5K)₆-Hb	propionylated PEG-Hb conjugate with 6 copies of PEG5K chains
SC PEG5K	succinimidyl carbamate PEG5K
SEC	Size-exclusion chromatography
SPA PEG5K	succinimidyl ester of propionic acid PEG5K
(Urethane-PEG5K)₆-$\alpha\alpha$Hb	urethane PEG- $\alpha\alpha$ Hb conjugate with 6 copies of PEG5K chains
(Urethane-PEG5K)₆-Hb	urethane PEG-Hb conjugate with 6 copies of PEG5K chains

Figure 1.

Schematic representation of the conjugation chemistry used for PEGylation of $\alpha\alpha$ -fumaric Hb. Succinimidyl esters are used to generate either isopeptide and of urethane linkage between Hb and PEG: (A) Succinimidyl ester of propionic acid PEG (SPA PEG), (B) N-Succinimidyl propionate, and (C) succinimidyl carbamate PEG (SC PEG).

Figure 2. SEC analysis of modified Hbs. 1, HbA control; 2, $\alpha\alpha\text{Hb}$; 3, $(\text{Propionyl-PEG5K})_6\text{-}\alpha\alpha\text{Hb}$; 4, $(\text{Propionyl-PEG5K})_{10}\text{-}\alpha\alpha\text{Hb}$.

Figure 3. SEC analysis of modified Hbs: 1, HbA; 2, $\alpha\alpha$ Hb; 3, (Propionyl-PEG5K)₆- $\alpha\alpha$ Hb; 4, (Propionyl-PEG5K)₁₀- $\alpha\alpha$ Hb; 5, (Urethane-PEG5K)₆- $\alpha\alpha$ Hb; 6, (Urethane-PEG5K)₁₀- $\alpha\alpha$ Hb.

Figure 4.

The oxygen affinity of HbA control, $\alpha\alpha$ Hb, (Propionyl-PEG5K)₆- $\alpha\alpha$ Hb, and (Propionyl-PEG5K)₁₀- $\alpha\alpha$ Hb at different pH of 100 mM phosphate buffer.

Figure 5. Kinetics of the reactions of SH groups of HbA, $\alpha\alpha$ -Hb, Propionyl₆- $\alpha\alpha$ Hb, Propionyl₁₀- $\alpha\alpha$ Hb, (Propionyl-PEG5K)₆- $\alpha\alpha$ Hb, and (Propionyl-PEG5K)₁₀- $\alpha\alpha$ Hb with 4,4-dipyridine disulfide (4-PDS).

Figure 6.
Schematic representation of the PEGylated $\alpha\alpha\text{Hb}$ molecule.

Table 1Estimation of PEGylation $\alpha\alpha$ Hb.

	Number of Copies of PEG5K/$\alpha\alpha$-Hb molecule^a	
	¹HNMR	Tryptic peptide mapping
(Propionyl-PEG5K)6 $\alpha\alpha$ -Hb	6	6
(Propionyl-PEG5K)10- $\alpha\alpha$ -Hb	10	8

^a Approximated to the nearest integer.

Table 2Molecular and Solution properties of PEGylated $\alpha\alpha$ Hb.

Properties	HbA	(Propionyl-PEG5K) ₆ - $\alpha\alpha$ Hb	(Propionyl-PEG5K) ₁₀ - $\alpha\alpha$ Hb
Molecular radius(nm)	3.1	6.0	6.7
Molecular volume(nm ³)	125	904	1259
PEG shell Volume(nm ³)	0	779	1134
PEG mass ^a (Dal)	0	31000	53000
PEG Density (Dal/nm ³)	0	39.8	46.7
COP ^b (mm Hg)	7.4	41.4	100.6
Viscosity ^b (cP)	0.8	2.0	3.1

^aThe PEG mass are calculated from the extent of PEGylation as determined by by ¹H-NMR method and as described in context.

^bHb concentration is 4%. The COP is tested at room temperature. The viscosity is tested at 37 °C.

Table 3

Influence of Conjugation Chemistry on the Plasma Expander Like Solution Properties of PEG-Hb conjugates.

	Hydrodynamic radius (nm)	Viscosity ^a (cP)	COP ^a (mmHg)
HbA	3.1	0.9	10.3
ααHb	3.1	--	--
(Propionyl-PEG5K) ₆ -Hb	5.8	2.1	55.4
(Propionyl-PEG5K) ₆ -ααHb	6.2	2.0	41.4
(Urethane-PEG5K) ₆ -Hb	5.8	2.6	63.8
(Urethane-PEG5K) ₆ -ααHb	6.4	2.3	49.5

^a Hb concentration is 4%. The COP is tested at room temperature. The viscosity is tested at 37 °C.

Table 4Influence of Conjugation Chemistry on O₂ affinities of PEG-Hb conjugates.

	P ₅₀ /mmHg	Hill number
HbA	14.0	2.8
ααHbA	32.0	2.8
(Propionyl-PEG5K) ₆ -Hb	10.0	1.8
(Propionyl-PEG5K) ₆ -ααHb	22.8	1.7
(Urethane-PEG5K) ₆ -Hb	8.0	2.0
(Urethane-PEG5K) ₆ -ααHA	23.3	2.1

Table 5Influence of PEGylation of the Function properties of $\alpha\alpha$ -fumaryl Hb

Sample	Oxygen affinity ^a		Bohr effect ^b	
	P ₅₀ (n) mmHg	Increase fold in P ₅₀	- logP ₅₀ / pH	Reduction%
HbA	13.7 (3.0)	--	1.76	--
$\alpha\alpha$ Hb	32.0 (2.8)	2.3	0.73	59
(Propionyl-PEG5K) ₆ - $\alpha\alpha$ Hb	22.8 (1.8)	1.7	0.62	65
(Propionyl-PEG5K) ₁₀ - $\alpha\alpha$ Hb	17.6 (1.6)	1.2	0.44	75
(Propionyl) ₆ - $\alpha\alpha$ Hb	17.5 (1.7)	1.2	0.42	75
(Propionyl) ₁₀ - $\alpha\alpha$ Hb	13.3 (1.6)	1.0	0.36	80

^aThe P₅₀ and *n* were determined in PBS, pH 7.4.

^bThe oxygen affinity were determined in 100 mM phosphate buffer, pH 6.6, 7.0, 7.4, 8.0, and 8.5.

Table 6Influence of Conjugation Chemistry and of PEG shell on the Reactivity of Cys-93(β).

Pseudo-first-order rate (min^{-1})	
HbA	0.165
$\alpha\alpha$ -HbA	0.151
Propionyl ₆ - $\alpha\alpha$ -Hb	0.118
Propionyl ₁₀ - $\alpha\alpha$ -Hb	0.103
(Propionyl-PEG5K) ₆ - $\alpha\alpha$ -Hb	0.112
(Propionyl-PEG5K) ₁₀ - $\alpha\alpha$ -Hb	0.098

The rates were calculated from initial slop of the time courses in Fig 4

Table 7Site of PEGylation of $\alpha\alpha$ Hb.

Residue	(Propionyl-PEG5K) ₆ - $\alpha\alpha$ -Hb modification %	(Propionyl-PEG5K) ₁₀ - $\alpha\alpha$ -Hb modification %
Lys-11(α)	13	23
Lys-16(α)	17	31
Lys-40(α)	12	14
Lys-56(α)	16	20
Lys-90(α)	75	93
Lys-139(α)	25	44
Lys-8(β)	17	21
Lys-17(β)	23	29
Lys-82(β)	42	44
Lys-120(β)	52	56