Non-hypertensive tetraPEGylated canine haemoglobin: correlation between PEGylation, O₂ affinity and tissue oxygenation

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TetraPEGylated canine Hb, [SP (succinimidophenyl)-PEG5K]₄-canine-Hb, with PEGylation at its four reactive cysteine residues (α_{111} and β_{93}) has been prepared and characterized. The hydrodynamic volume and the molecular radius of (SP-PEG5K)₄-canine-Hb are intermediate to those of di- and hexaPEGylated human Hb as expected. However, the COP (colloidal osmotic pressure) of tetraPEGylated canine Hb is closer to that of hexaPEGylated human Hb than to that of diPEGylated human Hb. The O₂ affinity of tetraPEGylated canine Hb is higher than that of canine Hb and comparable with that of hexaPEGylated Hb. The O₂ affinity of tetraPEGylated canine Hb is not responsive to the presence of DPG (diphosphoglycerate) or chloride, but it retains almost full response to L-35, an allosteric effector that interacts at the α -end of the central cavity. The tetraPEGylated canine Hb is vasoinactive in hamster in 10 % top load infusion studies. It is also essentially non-hypertensive in an extreme

exchange haemodilution protocol in hamster just as di- and hexaPEGylated human Hb. The O₂ delivery by tetraPEGylated canine Hb is comparable with that of hexaPEGylated Hb but not as efficient as diPEGylated Hb. These results demonstrate that PEGylation-induced solution properties of PEG [poly(ethylene glycol)]-Hb conjugates are dictated by the level and chemistry of PEGylation and the interplay of these plays a critical role in tissue oxygenation. The studies imply the need to establish the right level (and/or pattern) of PEGylation and O₂ affinity of Hb-PEG adducts in designing O₂-carrying plasma volume expanders, and this remains the primary challenge in the design of PEGylated Hb as blood substitutes.

Key words: blood, non-hypertensive tetraPEGylated canine haemoglobin, O₂ affinity, poly(ethylene glycol) (PEG), PEGylation, tissue oxygenation.

INTRODUCTION

Studies of Rohlf et al. [1] and Winslow et al. [2] showing that Enzon PEGylated bovine Hb is non-hypertensive have opened a new approach to neutralize or mask the 'pressor effect' of acellular Hb. The solution properties of Enzon PEGylated bovine Hb are uniquely different from other modified Hbs in clinical trials as blood substitutes [3–10] and comparable with those of plasma volume expanders. PEGylation of Hb endows the molecule with excellent plasma volume expansion properties [11–17]. Thus designing Hb with solution properties of plasma volume expanders appears to be a new strategy to neutralize the vasoactivity of Hb [18].

To evaluate and develop this concept further, we developed a simple protocol for PEGylation of proteins [19,20] termed 'EAF-PEGylation (extension arm-facilitated maleimide chemistry-based PEGylation)'. This thiolation and maleimide chemistry based approach [19–21] has been optimized to generate a hexaPEGylated Hb, [SP (succinimidophenyl)-PEG5K]₆-Hb. This product exhibits an unusually high hydrodynamic volume for its mass (molecular mass is only ~94 kDa, but its molecular volume is comparable with that of a globular protein with a molecular mass of 256 kDa), comparatively high viscosity and COP (colloidal osmotic pressure) and is essentially vasoinactive and non-hypertensive [20–22]. The non-hypertensive Enzon PEGylated Hb has ten PEG5K chains conjugated per tetramer of bovine Hb [23], while the EAF-PEGylation neutralizes the

'pressor effect' with only six copies of PEG5K chains conjugated to human Hb.

The non-hypertensive hexaPEGylated Hb exhibits a high degree of molecular size homogeneity, but it lacks chemical homogeneity in terms of the site selectivity of the PEGylation. There are approximately seven ϵ - and/or α -amino groups/ $\alpha\beta$ -dimer that are the reactive sites for thiolation-mediated PEGylation and the extent of PEGylation at individual reactive sites varied from 12 to 100 % [21]. Accordingly, the molecular size homogeneity of the hexaPEGylated Hb suggests that the product is probably a mixture of multiple PEGylated species of Hb each with six to eight copies of PEG [poly(ethylene glycol)] chains per tetramer. Therefore it is likely that more than one pattern of hexaPEGylation of Hb with maleimide PEG5K can result in the neutralization of its vasoactivity.

Cabral et al. [24] studied two PEGylated Hbs, human Hb PEGylated at its two Cys⁹³ (β) either with maleimide PEG5K or maleimide PEG10K [25]. In an extreme haemodilution study in hamsters, both samples were vasoinactive and non-hypertensive. Interestingly, diPEGylated Hb caused tissue *p*O₂ (partial pressure of O₂) to be higher than that by hexaPEGylated Hb.

This has prompted us to develop homogeneous tetra- and hexa-PEGylated Hbs to correlate the PEGylation with tissue oxygenation. Engineering a desired number of cysteine residues on the surface of Hb (without perturbing its structure/conformation) by site-directed mutagenesis as targeted sites for conjugating maleimide PEG chains is a simple alternative strategy

Abbreviations used: COP, colloidal osmotic pressure; DPG, diphosphoglycerate; EAF-PEGylation, extension arm-facilitated maleimide chemistry-based PEGylation; FCD, functional capillary density; Hct, haematocrit; IEF, isoelectric focusing; MAP, mean arterial blood pressure; PEG, poly(ethylene glycol); *p*O₂, partial pressure of O₂; PQM, phosphorescence quenching microscopy; RBC, red blood cell; RP-HPLC, reverse-phase HPLC; SEC, size-exclusion chromatography; SP, succinimidophenyl.

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to EAF PEGylation to generate non-hypertensive homogeneous PEGylated Hb. Before entering into the phase of developing recombinant Hbs with the desired number of surface cysteine residues for such studies, we have initiated PEGylation of animal Hbs that carry additional reactive cysteine residues as compared with human Hb as a prelude to advance the site-directed mutagenic strategy. In the present study, the reaction conditions have been optimized to generate a tetraPEGylated canine Hb by PEGylating it at Cys¹¹¹(α) and Cys⁹³(β). In this paper, we show that tetraPEGylation neutralizes the 'pressor effect' and achieves a tissue pO_2 that appears to be slightly better than that with hexaPEGylated Hb, even though the O_2 affinity is comparable. Studies have also been carried out with other animal Hbs, namely feline Hb, murine Hb and chicken Hb, to gain some insight into the reactivity of the new surface cysteine residues of these Hbs towards maleimide PEG. These studies have identified the sites α_{13} , α_{111} , α_{130} and β_{13} as the potential positions in human Hb for engineering maleimide PEG-reactive cysteine residues as the recombinant DNA approach to generate site specifically PEGylated (tetra- and/or hexa-PEGylated Hbs) homogeneous products with the desired number of PEG chains. The molecular, structural and functional characterization of tetraPEGylated canine Hb and the study of its microvascular responses and tissue pO_2 attained after exchange transfusion in the hamster are presented here.

MATERIALS AND METHODS

Preparation of Hb

Human, canine, feline, chicken, and minor components of murine Hbs were prepared from the lysates of the respective RBCs (red blood cells) by DE-52 cellulose chromatographic procedures.

PEGylation of canine Hb and analysis of the PEGylated samples

Hb samples (0.25 mM) in PBS (pH 7.4) at 4°C were reacted with a 20-fold molar excess of maleimide phenyl PEG5K over the reactive thiol groups of Hb overnight. The reaction mixture was dialysed against PBS buffer (three changes). Dialysis tubing with a 12–15 kDa molecular-mass cutoff was used for dialysis. The dialysed samples were concentrated by ultrafiltration to 32 mg/ml (~0.5 mM) and analysed by analytical SEC (size-exclusion chromatography) as described previously [21]. Effluent from the column was monitored at 540 nm (Hb and its PEGylated product), and by refractive index (PEG as the reagent or as PEG–Hb conjugate).

The relative reactivity of the surface cysteine residues of other animal Hbs was compared by reacting respective animal Hbs (0.5 mM) at pH 7.4 with 12.5 mM maleimide phenyl PEG5K for 18 h in a cold room (at 4°C) and then analysing the material by SEC using FPLC [25].

Purification of tetraPEGylated canine Hb

Chromatographic purification of the PEGylated sample was carried out by ion-exchange chromatography on a Q-Sepharose HP column using an AKTA Purifier 10 System. Canine Hb reacted with the maleimido-phenyl PEG5K was first dialysed extensively against PBS (pH 7.4) using Minim (a tangential flow filtration system). The removal of excess maleimide PEG was monitored by FPLC analysis. The effluent was monitored at 540 nm (for Hb) and by refractive index for PEG. Once all the free PEG had been dialysed out, PEGylated canine Hb was subjected to dialysis against 50 mM Tris acetate

(pH 8.5). This buffer was also the starting buffer for the ion-exchange chromatography. The protein was eluted from the column using a linear gradient generated by 50 mM Tris acetate buffer (pH 8.5) and 50 mM Tris acetate buffer (pH 7.0). The column was eluted at a flow rate of 1.5 ml/min. The elution of the protein was continuously monitored at 280, 540 and 600 nm. The major peak was pooled (as marked in Figure 2) and subjected to further structural and functional studies.

O_2 affinity measurements

The oxygen equilibrium curves of PEGylated Hbs were generated at an Hb concentration of 0.5 mM, at 37°C in PBS (pH 7.4), using Hem-O-Scan (Aminco). The measurements were made in the absence and presence of allosteric effectors. The oxygenation curves for each sample were measured in duplicate or triplicate as needed. The average of satisfactory duplicates/triplicates (differing from each other by no more than 0.5 mmHg) is provided.

Dynamic light scattering

Dynamic light scattering for molecular radius measurement was performed using a DynaPro instrument (Protein Solutions, Lakewood, NJ, U.S.A.). Samples at the protein concentration of 1 mg/ml were centrifuged at 15 700 *g* for 4 min prior to analysis. Normally three measurements are taken for each sample, and these measurements are reproduced within the range of 0.1–0.2 nm when the molecular radius of the PEGylated sample is in the region of 3–6 nm.

Other analytical methods

SEC analysis of PEGylated proteins on Superose 12 columns (1 cm × 30 cm), RP (reverse-phase)-HPLC analysis of globin chains on a Vydac C4 column (4.6 mm × 250 mm) and SDS/PAGE analysis were carried out as previously described [21–27]. IEF (isoelectric focusing) electrophoresis was operated using precast resolve gels from Isolab and a blend of pH 6–8 resolve ampholytes. Gels were electrofocused for 3 h to resolve the components in the sample completely. The COP and viscosity of PEGylated proteins were measured as described by Hu et al. [27]. Colloidal osmotic measurements were made in triplicate and these were reproduced within a difference of 1–2 mmHg. The average of these determinations were used. Viscosity measurements were also made in triplicate and these readings were also very reproducible, and an average of three runs was used for presentation of the data.

Systemic and microvascular studies

Investigations were performed in male golden Syrian hamsters (55–65 g body weight) fitted with a dorsal skinfold chamber window [28]. This model has been extensively used for investigations of the intact microvasculature of adipose and subcutaneous tissues and skeletal muscle in conscious animals for extended periods. Pentobarbital sodium (50 mg/kg intraperitoneally) was used for window implantation and for carotid artery and jugular vein catheterization. The microvasculature was examined 4–5 days after the initial surgery, and only animals passing established systemic and microcirculatory inclusion criteria, which included having tissue void of low perfusion, inflammation and oedema [29], were entered into the study. Animal handling and care followed the NIH (National Institutes of Health) Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the local Animal Care Committee.

Hypervolaemic infusion

Animals were infused with a bolus of tetraPEGylated canine Hb equivalent to 10 % of their blood volume via the jugular vein at a rate of 0.2 ml/min (top load). MAP (mean arterial blood pressure), arteriolar diameters and FCD (functional capillary density) were studied 30 min after the start of infusion.

Acute isovolaemic exchange transfusion (haemodilution) protocol

Progressive haemodilution to a final systemic Hct (haematocrit) level of 11 % was accomplished with three isovolaemic exchange steps. This protocol is described in detail in our previous reports [30]. Briefly, the volume of each exchange–transfusion step was calculated as a percentage of the blood volume, estimated as 7 % of body weight. An acute anaemic state was induced by lowering the systemic Hct by 60 % with two steps of progressive isovolaemic haemodilution using 6 % Dextran 70 (molecular mass, 70 kDa), exchange levels 1 and 2. Level 1 exchange was 40 % of blood volume and level 2 exchange was 35 % of blood volume. Level 2 exchange was followed by level 3, exchanging 35 % of blood volume using a non-oxygen carrier and the test materials. Because mixed blood and dilution material is withdrawn during the exchanges, a 110 % blood volume exchange was needed to reduce the Hct to 11 %. The transfusion solution was filtered (in-line, 0.2 μ m filter), at a rate of 100 μ l/min (CMA100 Microinjection Pump; CMA) and infused into the jugular vein catheter. Blood was simultaneously withdrawn by hand at the same rate from the carotid artery catheter according to a previously established protocol [24]. Data were taken after a 5 min stabilization period.

MAP

MAP was measured using a pressure transducer connected to the arterial catheter implanted in the carotid artery (Biopak Lab Systems, Santa Barbara, CA, U.S.A.).

FCD

FCD (cm^{-1}) is the total length of RBC-perfused capillaries divided by the area of the microscopic field of view [30]. Capillary segments are considered functional if RBCs are observed to transit over a 30 s period. FCD was tabulated from the capillary lengths with RBC flow in an area comprising ten successive microscopic fields ($420 \times 320 \mu\text{m}^2$). Detailed mappings were made of the chamber vasculature to study the same microvessels throughout the experiment.

Microvascular flow

Arteriolar and venular blood flow velocities were measured online using the photodiode cross-correlation technique (Fiber-Optic Photo Diode and Velocity Tracker model 102B; Vista Electronics, San Diego, CA, U.S.A.) [31]. The centreline velocity (V) was corrected according to vessel size to obtain the mean RBC velocity. The video image shearing technique was used to measure vessel diameter (D) online [32]. Blood flow (Q) was calculated from the measured parameters as $Q = V \times \pi(D/2)^2$.

Tissue $p\text{O}_2$ distribution

High-resolution non-invasive tissue $p\text{O}_2$ measurements were made using PQM (phosphorescence quenching microscopy) [33]. PQM is based on the oxygen-dependent quenching of phosphorescence emitted by the albumin-bound metalloporphyrin after pulsed light excitation. Tissue $p\text{O}_2$ was measured in tissue regions in between functional capillaries. The reported values

Figure 1 SEC analysis of canine Hb PEGylated with maleimide PEG5K as a function of incubation time

The PEGylation of Hb was monitored as reflected by the enhancement in the molecular volume (hydrodynamic volume) of Hb by SEC of the reaction mixture. The canine Hb incubated with maleimido-phenyl PEG5K for different periods of time has been analysed by SEC. FPLC patterns of canine Hb PEGylated with maleimido-phenyl PEG5K at 4 °C for 1.5 h (curve b), 6 h (curve c) and 22 h overnight (curve d) are shown. The pattern of unmodified canine Hb (curve a) is also given for comparison.

are the average of several determinations in several animals. The system set-up and procedure for delivering the dye have been described previously [33].

Statistical analysis for animal studies

Results presented are means $\pm$ S.D. unless otherwise indicated. One-way ANOVA was used to detect statistically significant differences between the study groups. If significance was obtained ($P < 0.05$), *post hoc* analysis was performed with Bonferroni multiple comparison tests between groups. Results are presented either as absolute values or percentage change from baseline to focus on the change in the parameters rather than the variability. For example, a -10% and 10% change from baseline would mean a 10% reduction and 10% increase in the parameter from its value at baseline respectively.

RESULTS

TetraPEGylation of canine Hb using maleimide phenyl PEG-5000

PEGylation of canine Hb with a 25-fold molar excess of maleimide phenyl PEG5K on the hydrodynamic volume of the protein is shown in Figure 1. Very little unmodified Hb remains in the reaction mixture after incubation of canine Hb for 90 min; almost all protein elutes at an earlier position on the FPLC analysis. A shoulder is seen on the descending side of the peak, apparently reflecting the presence of small amounts of diPEGylated canine Hb. As the incubation time is increased, this shoulder disappears, with a slight shift in the peak position of the major peak, and reflects the complete tetraPEGylation of canine Hb. The shoulder disappears completely after overnight incubation. Given the similar hydrodynamic volume of the PEGylated canine Hb and diPEGylated human Hb with two copies of PEG10K conjugated [25], the PEGylated canine Hb is considered to be tetraPEGylated Hb, with four copies of PEG5K.

Chromatographic purification of tetraPEGylated Hb

The ion-exchange chromatographic pattern of tetraPEGylated canine Hb on a Q-Sepharose column is shown in Figure 2. The chromatographic profile of PEGylated canine Hb shows one major component and two minor components eluting slightly after the

Figure 2 Ion-exchange chromatographic purification of the tetraPEGylated canine Hb on a Q-Sepharose high performance column

Purification was carried out as described previously for purification of human Hb diPEGylated at Cys⁹³(β) with maleimide PEG [25]. The major peak corresponds to tetraPEGylated canine, and the region of the chromatogram pooled to isolate this material is indicated.

major component. The major component of the PEGylated canine Hb was pooled and concentrated to a 6 % (by weight) solution.

Characterization of tetraPEGylated canine Hb

The apparent hydrodynamic volume of tetraPEGylated canine Hb, as reflected by FPLC analysis, is smaller than that of hexaPEGylated Hb, generated by the EAF-PEGylation protocol (Figure 3A).

The HPLC pattern of Q-Sepharose-purified PEGylated canine Hb (Figure 3B) has very few unmodified globin chains, suggesting the nearly complete modification of the globin chains. The PEGylated canine Hb carries three components: the major one accounting for nearly 90 % of the sample and a minor one that eluted slightly later than the major fraction. Trace amounts of a third component eluted after the minor component. The mass data established that the major component corresponds to globin chains with one copy of the PEG5K chain, and the second component carries two copies of PEG5K chains per globin chain.

Figure 3 Structural analysis of PEGylated canine Hb

(A) Comparison of SEC patterns of tetraPEGylated canine Hb (the major component eluting from the Q-Sepharose HP column) with that of HbA, and hexaPEGylated Hb generated using thiolation-mediated maleimide chemistry-based PEGylation maleimide PEG5K [21]. (B) RP-HPLC pattern of canine Hb. (Upper curve) PEGylated canine Hb before chromatographic purification. (Lower curve) PEGylated canine Hb after chromatographic purification on Q-Sepharose HP. (C) SDS gel patterns of tetraPEGylated canine Hb before and after Q-Sepharose chromatography and (SP-PEG5K)₂-Hb. The tetraPEGylated canine Hb carries one major band. The mobility of this band corresponds to that of human β -globin PEGylated with PEG5K at its Cys⁹³(β), present in the sample of diPEGylated Hb. Lane 1, protein markers; lane 2, canine Hb; lane 3, tetraPEGylated canine Hb before Q-Sepharose purification; lane 4, tetraPEGylated Hb after purification on the Q-Sepharose column; and lane 5, diPEGylated human Hb. (D) IEF pattern of PEGylated canine Hb. Lane 1, canine Hb; lane 2, tetraPEGylated canine Hb after purification on a Q-Sepharose column; lane 3, diPEGylated human Hb; lane 4, hexaPEGylated Hb generated by EAF PEGylation; lane 5, control human Hb.

Thus the globin chains of PEGylated canine Hb are primarily monoPEGylated forms of PEG5K. However, PEG maleimide also appears to react at other sites besides the cysteine residues at α_{111} and β_{93} of canine Hb.

Figure 3(C) compares the SDS/PAGE pattern of PEGylated canine Hb before and after the Q-Sepharose purification. The pattern of diPEGylated Hb, (SP-PEG5K)₂, Hb PEGylated at Cys⁹³(β) with maleimide phenyl PEG is also shown for comparison. The globin chains of PEGylated canine Hb moved at a position corresponding to that of the PEGylated β -globin chain of (SP-PEG5K)₂-Hb, a globin chain with one copy of PEG5K. Thus the globin chains of PEGylated canine Hb are, predominantly, monoPEGylated species. It may also be seen that very small but noticeable amounts of protein are seen at the position of the unmodified globin chains and also another band moving slower than the monoPEGylated globin chains. The position of this higher molecular mass band corresponds to that of β -globin chain modified at Cys⁹³(β) with maleimide PEG10K present in (SP-PEG10K)₂-Hb [25]. Accordingly, this band represents the small amounts of diPEGylated globin chains present in this sample. Q-Sepharose purification of the PEGylated canine Hb reduces the amount of unmodified globin chains present in the sample. These studies confirm that the predominant product of PEGylation of canine Hb is tetraPEGylated canine Hb.

IEF gels (Figure 3D) established that the tetraPEGylated canine Hb does not contain unmodified canine Hb. However, the PEGylated sample electrofocused as a diffused band, while diPEGylated Hb [which has the PEGylation only on Cys⁹³(β)] focused as a sharp band [25] and hexaPEGylated Hb generated by the thiolation-mediated maleimide chemistry-based PEGylation also electrofocused as a diffused band. Thus the diffused IEF pattern of the tetraPEGylated canine Hb appears to be a consequence of PEGylation of Hb with multiple copies of PEG chains.

The molecular radius of canine Hb is 3.1 nm and is comparable with that of HbA (3.0 nm). TetraPEGylation of canine Hb exhibits a molecular radius of 5.32 nm. This value is very close to that of (SP-PEG10K)₂-Hb (5.54 nm), but noticeably smaller than that of the hexaPEGylated Hb [(SP-PEG5K)₆-Hb], which is approx. 6.5 nm [21,25].

Viscosity and COP of tetraPEGylated Hbs

The COP of tetraPEGylated canine Hb is presented in Figure 4(A). As the concentration of tetraPEGylated canine Hb and hexaPEGylated Hb is increased, the COP increased in an exponential fashion. The COP of tetraPEGylated canine Hb is not intermediate to that of di- and hexaPEGylated Hbs; it is closer to that of hexaPEGylated Hb, (SP-PEG5K)₆-Hb, in the protein concentration ranges studied. The conjugation of two copies of PEG5K to Cys⁹³(β) and four copies of PEG5K to the amino groups of human Hb using 'extension arm' appears to have nearly the same influence as the conjugation of four copies of PEG5K to Cys¹¹¹(α) and Cys⁹³(β) of canine Hb. For generating the tetraPEGylated canine Hb the 'extension arms' engineered on to the ϵ -amino groups of surface lysine residues of Hb in generating hexaPEGylated Hb are not needed for PEGylation. Since the maleimide PEG reacts directly with the thiol groups of internal cysteine residues in tetraPEGylation of canine Hb, the PEG chains are closer to the peptide backbone in the tetraPEGylated canine Hb than in hexaPEGylated human Hb. Conjugating the PEG chains to the thiol groups of cysteine residues appears to be a more efficient method of setting COP to Hb than EAF-PEGylation.

The viscosity of the tetraPEGylated canine Hb is shown in Figure 4(B) as a function of protein concentration and is also compared with that of (SP-PEG5K)₆-Hb and control canine Hb.

Figure 4 COP and viscosity of tetraPEGylated canine Hb as a function of protein concentration

(A) COP and (B) viscosity data for (SP-PEG5K)₂-Hb compared with (SP-PEG5K)₆-Hb. Open squares, canine Hb; filled circles, (SP-PEG5K)₂-Hb; open triangles, tetraPEGylated canine Hb; filled triangles, (SP-PEG5K)₆-HbA. The COP and viscosity of each sample were measured in triplicate and values presented are the averages.

The viscosity of the tetraPEGylated canine Hb increases in a slightly exponential fashion when studied as a function of the protein concentration. Its viscosity is intermediate to that of (SP-PEG5K)₆-Hb and of diPEGylated Hb.

O₂ affinity of tetraPEGylated canine Hb

The O₂ affinity of tetraPEGylated canine Hb has been compared with that of hexaPEGylated Hb in Table 1. The O₂ affinity of canine Hb and human Hb are comparable. On tetraPEGylation of canine Hb the O₂ affinity is increased. Just as the hexaPEGylated human Hb, the tetraPEGylated canine Hb does not respond to the presence of allosteric effectors, DPG (diphosphoglycerate) and 1.0 M sodium chloride. Interestingly, the tetraPEGylated canine Hb retains almost full sensitivity to the allosteric effector L-35. The effector L-35 is known to modulate the O₂ affinity of Hb by interacting at the $\alpha\alpha$ -end of the central cavity. On the other hand, the O₂ affinity of hexaPEGylated Hb does not show much sensitivity to the presence of L-35. Thus, although tetraPEGylated canine Hb and hexaPEGylated human Hb exhibit nearly comparable O₂ affinities, the structure/conformation of these two PEGylated proteins at the $\alpha\alpha$ -end of the central cavity appears to be very different. It may be noted that human Hb diPEGylated at Cys⁹³(β) also exhibits an O₂ affinity comparable with that of tetraPEGylated and hexaPEGylated Hbs, but it shows some sensitivity to the presence of DPG that binds at the $\beta\beta$ -cleft and the presence of sodium chloride, besides conserving the full sensitivity to L-35 [25]. As the level of PEGylation of

Table 1 Oxygen affinity of tetraPEGylated canine Hb

p_{50} , partial pressure of oxygen at 50 % saturation of Hb samples; n , Hill coefficient. Oxygenation curves are generated by using a Hemoscan at 37 °C and pH 7.4 (PBS). A protein concentration of 0.5 mM was used for these determinations. Oxygenation curves are generated in duplicate for each sample; the maximum variation between the two oxygenation curves of a sample is 0.2 mmHg when the p_{50} value is in the region of 10–25 mmHg. When the p_{50} values are in the range of 70–80 mmHg, the variability is approx. 1 mmHg. With very low oxygen affinity samples, the saturation at 178 mmHg is assumed to correspond to 100 % saturation. These values accordingly provide a relative comparison between the effector-modulated oxygen affinity of the control and the modified samples of Hb.

Effector	(SP-PEG5K) ₄ -canine-Hb		Canine Hb		HbA		(SP-PEG5K) ₆ HbA	
	p_{50}	(n)	p_{50}	(n)	p_{50}	(n)	p_{50}	(n)
None	10.0	2.2	13.0	2.1	15.0	2.5	8.5	2.2
DPG	10.5	2.1	22.0	2.0	24.0	2.3	8.5	2.0
NaCl	11.0	1.4	20.0	2.1	32.0	2.4	8.2	1.9
L-35	74.0	2.1	77.0	1.6	80.0	1.7	12.0	1.5

Hb increases, the molecules are desensitized first to allosteric effectors that bind at the $\beta\beta$ -cleft and then to those that bind at the $\alpha\alpha$ -end of the central cavity.

Systemic and microvascular responses of tetraPEGylated canine Hb to hypervolaemic infusion

The systemic and microvascular responses to a 10 % hyper-volaemic infusion (top load) of tetraPEGylated canine Hb were compared with the responses to unmodified canine Hb, diPEGylated human Hb and hexaPEGylated human Hb (Figure 5). Canine Hb behaved as human Hb in these assays increasing MAP immediately upon infusion by comparison with di- and hexaPEGylated Hb (Figure 5A). TetraPEGylated canine Hb also caused hypertension; however, this was related to a significant increase in arteriolar diameter that was statistically significant ($P < 0.05$) when compared with the arteriolar diameter changes of the other Hbs (Figure 5B). TetraPEGylated canine Hb caused no change in FCD from baseline, an effect that was not statistically different from that found using di- and hexaPEGylated Hb; however, FCD was significantly reduced ($P < 0.05$) relative to baseline using canine Hb (Figure 5C).

Extreme haemodilution studies with tetraPEGylated canine Hb

Microvascular responses and the effect on MAP of tetraPEGylated canine Hb used in the extreme haemodilution protocol were compared with the earlier studies using diPEGylated human Hb and hexaPEGylated human Hb [21,24] (Figure 6). MAP and FCD following infusion with the PEG-Hbs were significantly lowered relative to baseline (Figures 6A and 6C), but not different from each other. Microvascular flow in animals transfused with tetraPEGylated canine Hb and hexaPEGylated Hb are similarly reduced and statistically lower than baseline, while diPEGylated human Hb yields a flow that is not statistically different from baseline (Figure 6B). These results suggest that flow tends to be higher as the level of PEGylation is lowered.

Tissue pO_2 attained with tetraPEGylated canine Hb is the same as that in animals infused with hexaPEGylated Hb (Figure 6D), diPEGylated Hb providing the highest level of tissue oxygenation, and the difference being statistically significant.

These results indicate that in extreme haemodilution PEG-Hbs tend to provide improved microvascular function as the level of PEGylation is lowered. Figure 6(D) shows that the combined effect of lower blood pressure, better FCD and improved flow, where as with the exception of microvascular flow none of the differences between parameters are statistically significant between groups, has a cumulative effect resulting in a statistically significantly greater tissue pO_2 for diPEGylated human Hb.

Figure 5 Systemic and microcirculatory responses to a 10% top load of canine Hb (Dog-Ao), tetraPEGylated canine Hb, di (SP-PEG5K)₂-Hb and hexaPEGylated [SP-PEG5K)₆-Hb] human Hb

As the PEGylation increased from di- to tetra- and hexa-, the molecular volume, the viscosity and COP are all increased. (A) Changes in the MAP, (B) arteriolar diameter and (C) FCD. Studies performed in this current investigation with native canine Ao and tetraPEGylated canine Hb were both performed in four and five animals in which a total of 32 arterioles (average diameter at baseline of $58 \pm 12 \mu\text{m}$) were followed in both groups. $P < 0.05$: *, relative to baseline; †, Dog-P5K4 compared with Dog-Ao; §, P5K4 compared with P5K2, P5K6. Light grey bar, native canine Ao (the present study); open bar, di-, P5K2-Hb [24]; dark grey bar, tetra-, P5K4-canine Hb (the present study); black bar, hexa-, P5K6-Hb [20].

Comparison of the reactivity of cysteine residues of animal Hbs for PEGylation with maleimide phenyl PEG5K

Many other animal Hbs that carry additional surface cysteine residues are, in principle, potential candidates for generating PEGylated Hb with multiple copies of PEG chains using maleimide phenyl PEG-5000 (Table 2). The reactivity of the additional cysteine residues of these animal Hbs for PEGylation with maleimide phenyl PEG5K will determine their ability to generate homogeneous PEGylated Hbs with a defined number of PEG chains (Figure 7). The reactivity of the additional cysteine

Figure 6 Microcirculatory and tissue oxygenation studies of tetraPEGylated Hb in extreme haemodilution

Hamster window chamber model haemodiluted to Hct 11% with 70 kDa dextran. TetraPEGylated canine Hb (Tetra) is compared with hexaPEGylated Hb, (SP-PEG5K)₆Hb (Hex) [27], and diPEGylated Hb, (SP-PEG5K)₂Hb (Di) [24]. (A) MAP; (B) microvascular flow in arterioles (A, $n = 54$ vessels with diameter $66 \pm 19 \mu\text{m}$) and venules (V, $n = 45$ vessels with diameter $83 \pm 29 \mu\text{m}$); (C) FCD; (D) tissue $p\text{O}_2$. $P < 0.05$: *, relative to baseline; †, Di compared with Tetra and Hex.

Table 2 Reactive cysteine residues of animal Hbs that are potential candidates for generation of homogeneous PEGylated Hbs using maleimide PEG5K

Hb	Number of reactive cysteine residues in oxy conformation	Position in sequence	Expected PEGylated Hb
Human Hb	2	Cys ⁹³ (β)	(SP-PEG5K) ₂ -Hb
Canine Hb	4	Cys ¹¹¹ (α) Cys ⁹³ (β)	(SP-PEG5K) ₄ -Hb
Chicken Hb	4	Cys ¹³⁰ (α) Cys ⁹³ (β)	(SP-PEG5K) ₄ -Hb
Sheep Hb	4	Cys ¹¹¹ (α) Cys ⁹³ (β)	(SP-PEG5K) ₄ -Hb
Murine Hb	4	Cys ¹³ (β) Cys ⁹³ (β)	(SP-PEG5K) ₄ -Hb
Feline Hb	6	Cys ¹³ (α) Cys ¹¹¹ (α) Cys ⁹³ (β)	(SP-PEG5K) ₆ -Hb

residues has been analysed by PEGylation of these animal Hbs using 12.5 mM PEG maleimide and a Hb concentration of 0.5 mM. With human Hb, this represents a 12.5-fold molar excess of PEG reagent over the reactive cysteine residues. The presence of a new surface cysteine residue in the α -chains of canine and chicken Hbs (as compared with the absence of these in human Hb) and in the β -chain of murine Hb causes the PEG reagent used in these experiments to have a 6.25-fold molar excess over the reactive cysteine residues in these animal Hbs (two equivalents per $\alpha\beta$ -dimer). In the case of feline Hb that carries two reactive cysteine residues in its α -chain besides the reactive Cys⁹³(β), the amount of the PEG maleimide used in the present study represents (only 3.125-fold molar excess over reactive cysteine residues) a limited excess of the reagents. Accordingly, to minimize the contribution of the differential reactivity of the reactive cysteine residues in the maximum modification, we have used an overnight PEGylation reaction.

Under these PEGylation conditions, the major component in the samples of PEGylated human Hb is diPEGylated Hb,

Figure 7 Comparison of the chemical reactivity of cysteine residues of animal Hbs with maleimide phenyl PEG5K

The Hbs at a concentration of 0.5 mM were reacted with 12.5 mM maleimide PEG5K at pH 7.4 (PBS) at 4 °C for 18 h. Curve a, control human Hb; curves b–e, animal Hbs PEGylated with maleimide PEG5K; curve b, human Hb; curve c, feline Hb; curve d, chicken Hb; curve e, canine Hb.

generated by the PEGylation of the two Cys⁹³(β) of human Hb. The shoulder on the ascending side of the diPEGylated Hb peak presumably represents a species of tetraPEGylated human Hb, which is generated by the PEGylation of Cys⁹³(β) and two of the four α -amino groups of Hb. Some reaction of maleimides with the α -amino groups of Hb has been reported earlier, and accordingly some reaction at the α -amino groups of Hb with maleimide is not a surprise, given the excess of maleimide used and long incubation periods. The presence of additional reactive cysteine residues [besides Cys⁹³(β)] in canine, chicken and feline Hbs has facilitated the formation of tetraPEGylated species of Hb. The presence of a second set of reactive cysteine (per dimer) in canine and murine Hbs results in a near-quantitative modification of these Hbs as tetraPEGylated species of the respective Hbs. With feline Hb, a hexaPEGylated Hb is expected; however, only a tetraPEGylated product seems to have been generated. This probably reflects a lower reactivity of Cys¹³(α) or Cys¹¹¹(α) of feline Hb relative to that of canine Hb, and further studies of PEGylation of this Hb as a function of maleimide PEG are needed to determine the conditions needed to generate homogeneous hexaPEGylated feline Hb.

DISCUSSION

Our results show that site-specifically tetraPEGylated canine Hb causes elevation of blood pressure upon its delivery as a 10% top load, a test that is generally agreed upon to characterize an infusion material as vasoactive. Notably this effect is accompanied by the statistically significant increase in arteriolar diameter, which lowers peripheral vascular resistance due to the 4th power dependence of this parameter on the diameter. Tsai et al. [34] recently reported that the increase in blood pressure in identical top load experiments using hexaPEGylated Hb was not the consequence of vasoactivity, since PEG–Hbs maintain cardiac index at normal values, while unmodified Hb and other small Hb molecules such as $\alpha\alpha$ -cross-linked Hb [34], and polymerized bovine Hb (Oxyglobin; Biopure, Boston, MA, U.S.A.) lower cardiac output and significantly increase blood pressure. Notably the same non-PEG-modified molecules cause significant vasoconstriction, an effect also seen in the present study with unmodified canine Hb, but not with tetraPEGylated canine Hb. In fact the latter material causes a significant ($P < 0.05$) vasodilator effect. The vasoactivity of the PEG Hbs is further

evidenced by the lack of changes in FCD induced by the 10 % top load (Figure 5C).

In extreme haemodilution exchange transfusion studies, diPEGylation of human Hb [24,25], tetraPEGylation of canine Hb, as well as hexaPEGylation of human Hb result in the near complete neutralization of the elevation of blood pressure effect of acellular Hbs. However, the microvascular responses of the three PEGylated Hbs are not identical (Figure 6).

Of these three PEGylated Hbs, the hexaPEGylated Hb, (SP-PEG5K)₆-Hb, represents a chemically heterogeneous population. However, it exhibits a high degree of molecular size homogeneity [21] reflecting the presence of a number of isomeric forms of hexaPEGylated species of human Hb. The present results that the homogeneous tetraPEGylated canine Hb is vasoinactive suggests that the pattern of PEGylation (surface decoration) may not be crucial in neutralizing the vasoactivity of Hb. The PEGylation-induced molecular properties, i.e. molecular volume, viscosity and COP, apparently play a major role in neutralizing the vasoconstrictive activity of acellular Hb. This result along with the PEGylation studies of other animal Hbs that carry multiple reactive cysteine residues has now opened up the opportunity to design and develop a multiple homogeneous non-hypertensive oxygen-carrying plasma volume expander that carries a total PEG-mass lower than that in the hexaPEGylated Hb, for example, MP4 [22,35] that is currently in Phase III clinical trial.

In tetraPEGylated canine Hb, the PEG chains are conjugated to the thiol groups of intrinsic cysteine residues of Hb. Accordingly this tetraPEGylated Hb does not carry extension arms, between the protein side chain and the PEG chain conjugated. The PEGylation reaction is site-selective and thus its chemical structure is well defined unlike that of the hexaPEGylated human Hb [21].

An interesting aspect of the present study is that although viscosity of the tetraPEGylated canine Hb is lower than that of the hexaPEGylated Hb, the COP of the two PEGylated proteins are comparable. Accordingly, it may be suggested that the longer side chains of lysine residues and/or the 'extension arms' on the ϵ -amino groups present in hexaPEGylated Hb [20,21] reduce the intrinsic propensity of the conjugated PEG chains to endow COP to Hb, i.e. chemistry of conjugation of PEG chains to Hb plays a major role in dictating the molecular properties endowed to the PEGylated protein. It may also be noted that (propyl-PEG5K)₆-Hb that carries six copies of PEG5K chains [26] conjugated to the surface amino groups of Hb by alkylamine linkages, without the intervention of 'extension arms', exhibits a higher COP than hexaPEGylated Hb. These results are also consistent with the above conclusion that the chemistry of the conjugation of PEG chains to Hb modulates the COP of PEGylated Hbs. The differentiation of pressor responses due to vasoactivity compared with that due to maintenance of cardiac output and volume effects is further supported by the comparatively high COP of this material, which led to volume expansion as previously reported for PEG Hbs [34].

The tetraPEGylation of canine Hb has essentially neutralized the 'pressor effect'. The molecular models of (SP-PEG5K)₂-Hb, (SP-PEG5K)₄-Hb and (SP-PEG5K)₆-Hb have demonstrated that even hexaPEGylation with PEG5K does not provide the complete coverage of the molecular surface of Hb [37], suggesting the neutralization of pressor effect is not a direct correlate of the coverage of the molecular surface.

PEGylation studies of other animal Hbs with multiple reactive cysteine residues have suggested that Cys¹³(β), or Cys¹³(α), or Cys¹¹¹(α) and/or Cys¹³⁰(α) if engineered on to human Hb are the potential PEGylation sites. This will facilitate the design of new homogeneous preparations of tetraPEGylated and/or hexaPEGylated Hbs respectively. Design of such cysteine mutants

of Hb by site-directed mutagenesis as substrates for maleimide chemistry-based PEGylation also provides us with an opportunity to avoid PEGylation at position 93(β) and also to engineer additional mutations into Hb to modulate the other properties of Hb, i.e. reduced rate of autoxidation, increased stability and lower oxygen affinity.

We studied the tissue oxygenation by three different PEGylated Hbs, that differ from one another in terms of the extent of PEGylation (number of copies of PEG chains per tetramer) but still with comparable oxygen affinity. This study was an extreme haemodilution protocol, where the oxygen-carrying capacity of the normal RBCs was reduced to 25 % of normal with a conventional plasma expander leading to a RBC-related Hb concentration of approx. 3.5 g/dl. This concentration is augmented by exchange transfusion with the test materials to approx. 4.5 g/dl total Hb, thus providing a sensitive test of the tissue oxygenation properties of the added material. As shown in Figure 6, the MAP, and the FCD of these preparations are comparable although the increased PEGylation from di to tetra and hexa increased the molecular volume, viscosity and COP. However, significant differences are seen in terms of mean flow upon infusion of these materials, the diPEGylated material giving the best flow rates, which corresponded to the highest tissue pO_2 . Thus tissue oxygenation is not a direct correlate of the O_2 affinity of the PEGylated protein; other properties such as the flow properties induced by the PEGylated protein could influence tissue oxygenation. It is also important to consider the possible contribution of facilitated diffusion of O_2 when Hb is used in plasma as the oxygen carrier [36]. Given the influence of the molecular dimensions of the protein on facilitated diffusion, and the fact that this aspect will vary significantly as a function of the level of PEGylation, we need to consider this structural aspect of PEGylated Hbs as well.

In summary, vasoinactive PEGylated Hb can be generated by decorating Hb with only four copies of PEG5K chains, one each on four globin chains of tetramer. Furthermore, tetraPEGylated canine Hb achieves a tissue oxygenation comparable with that seen with hexaPEGylated Hb. Accordingly, establishing the right amount of PEGylation on Hb remains a challenge in designing Hbs as plasma volume expanders, followed by that of engineering the optimal O_2 affinity that maximizes O_2 delivery to the tissues. The chemistry of PEGylation used here to generate tetraPEGylated canine Hb is different from the one used for generating hexaPEGylated Hb (EAF-PEGylation). Thus the present study paves the way for the use of mutant human Hbs designed by site-directed mutagenesis as the substrate for generation of homogeneous PEGylated Hbs to design new PEGylated Hbs with the desired level of O_2 affinity and of PEGylation to identify the best PEGylated Hbs that could be used as blood substitutes.

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