Stability of a Novel PEGylation Site on a Putative Haemoglobin-Based Oxygen Carrier

C. E. Cooper, M. Bird, X. Sheng, M. Simons, L. Ronda, A. Mozzarelli, and B. J. Reeder

Abstract

PEGylation of protein sulfhydryl residues is a common method used to create a stable drug conjugate to enhance vascular retention times. We recently created a putative haemoglobin-based oxygen carrier using maleimide-PEG to selectively modify a single engineered cysteine residue in the α subunit (αAla19Cys). However, maleimide-PEG adducts are subject to deconjugation via retro-Michael reactions, with consequent cross-conjugation to endogenous plasma thiols such as those found on human serum albumin or glutathione. In previous studies mono-sulfone-PEG adducts have been shown to be less susceptible to deconjugation. We therefore compared the stability of our maleimide-PEG Hb adduct with one created using a mono-sulfone PEG. The corresponding mono-sulfone-PEG adduct was significantly more stable when incubated at 37 °C for 7 days in the presence of 1 mM reduced glutathione, 20 mg/mL human serum albumin, or human serum. In all cases haemoglobin treated with mono-sulfone-PEG retained >90% of its conjugation whereas maleimide-PEG showed significant deconjugation, especially in the presence of 1 mM reduced glutathione where <70% of the maleimide-PEG conjugate remained intact. Although maleimide-PEGylation of Hb seems adequate for an oxygen therapeutic intended for acute use, if longer vascular retention is required reagents such as mono-sulfone-PEG may be more appropriate.

Keywords

Vascular retention time · Recombinant human hemoglobin · Mono-sulfone-PEG adduct · Maleimide-PEG adduct

1 Introduction

In order to use a haemoglobin-based oxygen carrier (HBOC) as an oxygen therapeutic, it is necessary to increase the size of the haemoglobin (Hb) molecule to prevent renal clearance [1]. This can be achieved by PEGylation. However, whilst previously used non-specific PEGylation methods can create stable HBOC, these HBOC are heterogeneous mixtures with significantly altered protein function [1]. We therefore recently

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engineered a HBOC with homogenous PEGylation at a single site, by removing the existing reactive sulfhydryl on the β subunit (βCys93) and introducing a new surface cysteine sulfhydryl on the α subunit. The novel PEGylation site chosen (αAla19Cys) was on the surface of the protein, distant from the heme binding site and the α/β dimer/dimer interface. The resultant mutant Hb (βCys93Ala/αAla19Cys) had unaltered functional oxygen-binding properties to wild type Hb and no increase in the autoxidation or heme loss that has prevented the use of previous recombinant Hb as viable oxygen therapeutics [2]. Our PEGylation method used maleimide-PEG to conjugate to a surface sulfhydryl. However, maleimide-PEG adducts are subject to deconjugation via retro-Michael reactions and cross-conjugation to endogenous thiol species *in vivo*, such as human serum albumin (HSA) or reduced glutathione (GSH). We therefore compared our maleimide-PEG adduct with one created using a mono-sulfone-PEG less susceptible to deconjugation [3]. In the presence of high (1 mM) GSH, Hb treated with mono-sulfone-PEG retained >90% of its conjugation whereas maleimide-PEG showed significant deconjugation, with <70% of the maleimide-PEG conjugate remaining intact following 7 days incubation at 37 °C [4]. In this paper we compare the stability data in the presence of GSH with more physiological levels of competing thiols species such as HSA and IgG-depleted serum.

2 Methods

Recombinant human Hb with the βCys93Ala/αAla19Cys mutations was expressed in a pET-Duet system in an *E. coli* BL21 host and purified by ion exchange chromatography and gel filtration as described previously [2]. The purified Hb was then PEGylated at αAla19Cys with a 20 kDa maleimide-PEG or mono-sulfone-PEG [4]. Hb-PEG solutions were prepared at 2 mg/mL in CO purged PBS buffer containing 20 mg/mL HSA or 50% v/v IgG depleted human serum. Blank samples consisting of HSA, serum or HbCO in PBS were prepared as controls. Each sample was supplemented with 0.05% w/v sodium azide. Aliquots (60 µL) of the samples were then prepared. One aliquot per sample was directly frozen at −80 °C (t = 0 days). The remaining aliquots were incubated at 37 °C. At t = 1, 3 and 7 days samples were removed from storage at 37 °C and frozen at −80 °C. Once all timepoint samples had been acquired, the samples were thawed and analysed by size exclusion chromatography (SEC). Analytical SEC used TOSOH TSKgel Super SW 3000 analytical (4.6 mm × 15 cm) and guard (4.6 mm × 3.5 cm) columns. Hb-PEG and control samples were isocratically eluted with PBS. For each analysis 30 µg (15 µL) of sample was injected and elution was performed over 10 min at 30 °C with visible detection at 538 nm. The stabilities of maleimide- and mono-sulfone PEG Hb conjugate linkages were assessed by evaluating the levels of the Hb-PEG and free Hb peaks in isolation (i.e., ignoring all other species present). Chromatograms were baseline subtracted using HSA and serum blanks to account for the absorbance of interfering matrix related peaks. For normalisation and to aid comparison, the percentage of Hb-PEG was expressed as a relative percentage compared to the t = 0 days value. Each SEC sample was run in triplicate. The figures show a representative example.

3 Results

Human serum albumin (HSA) contains a free reactive cysteine with the potential to participate in cross-conjugation reactions with a Hb-PEG bond *in vivo*. As HSA runs at a similar weight to Hb-PEG on SDS-PAGE, size exclusion chromatography (SEC) was used to monitor the stability of the Hb-PEG bond by monitoring the heme absorbance at 538 nm. As shown previously [2], SEC of Hb-PEG solutions separates free unPEGylated protein from Hb-PEG (Fig. 1).

The intensity of the Hb-PEG heme peak, measured by SEC, showed that the fraction of Hb-PEG following incubation in PBS for 7 days remained at 95% of the Day 0 value, consistent with the SDS-PAGE data shown previously [2]. Figure 2 compares the stability of PEG:Hb conjugates in the presence of HSA. Compared to

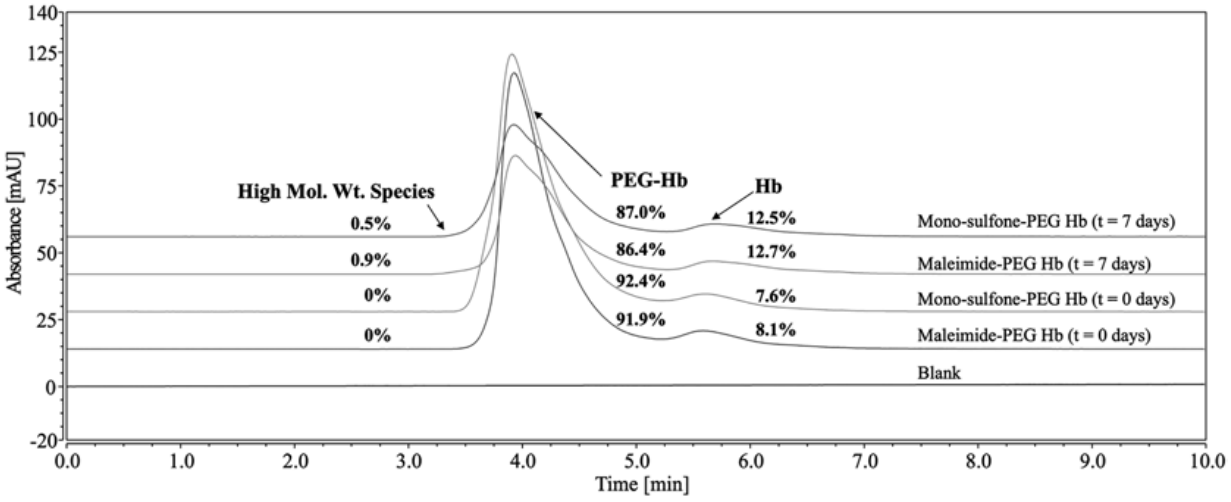

Fig. 1 SEC of Hb-PEG conjugates in PBS. Comparison with SEC standards reveals Hb peaks at 5.5 min comprise unPEGylated Hb dimer and peaks at 4 min Hb-PEG peaks. A small fraction of unidentified higher molecular weight species elutes at 3.5 min panel). Arrows identify peak of Hb species indicated. % values reflect the contribution of that species to the overall intensity

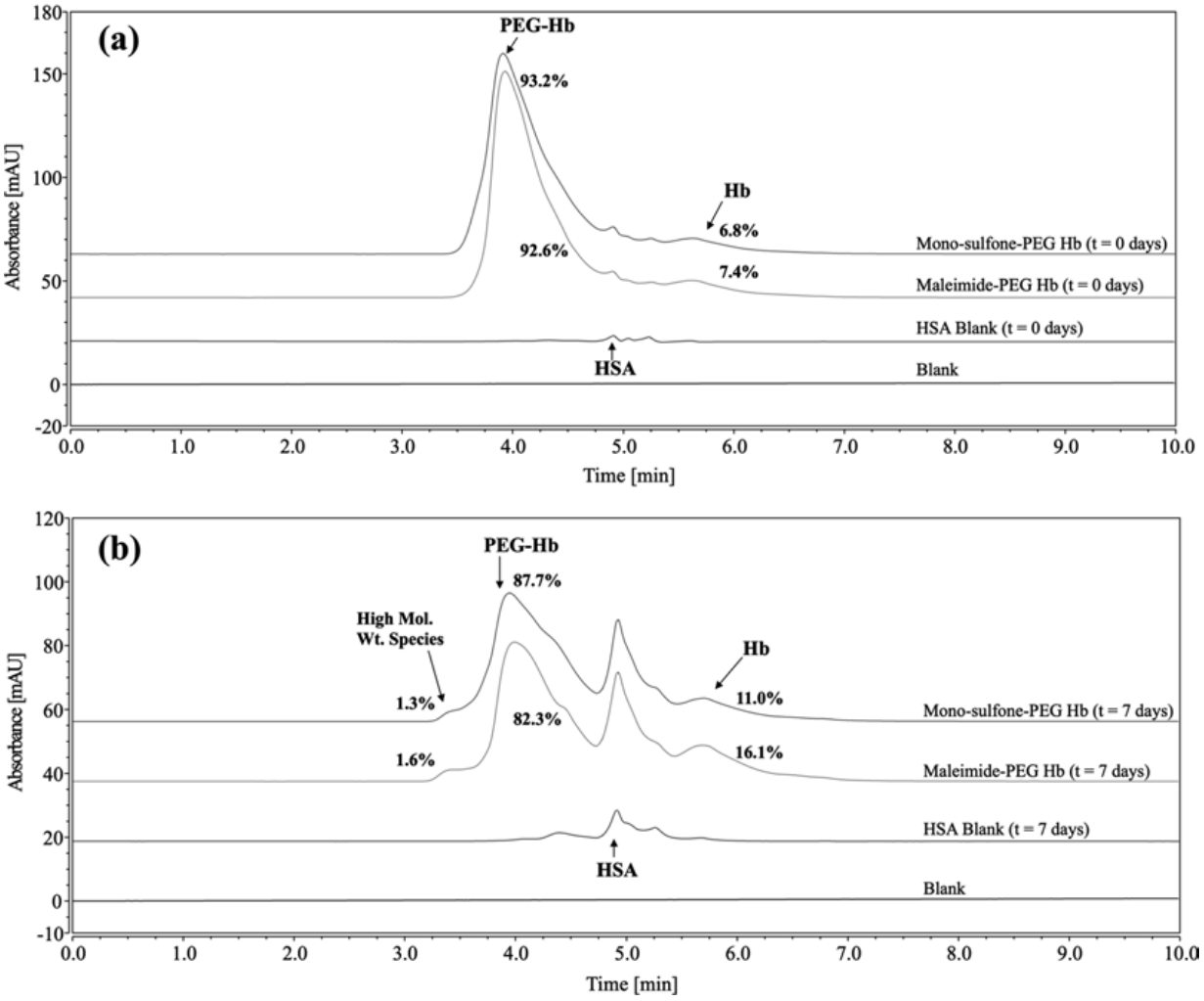

Fig. 2 SEC of Hb-PEG conjugates in HSA. SEC of Hb mutants following incubation in HSA at 37 °C immediately (**a**) and after 7 days incubation (**b**). Arrows identify peak of Hb species indicated. % values reflect contribution of that species to the overall intensity

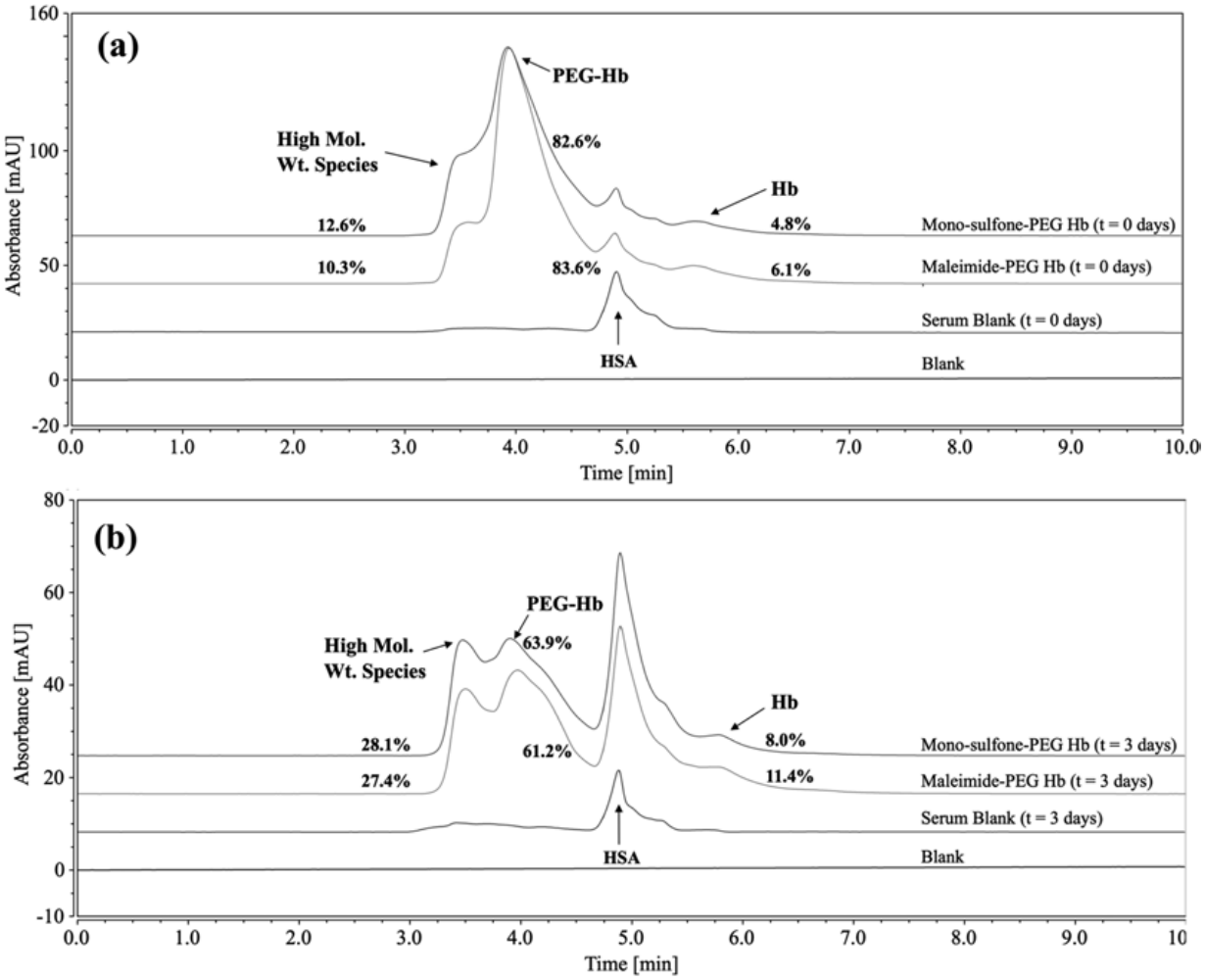

Fig. 3 SEC of Hb-PEG conjugates in human serum. SEC of Hb mutants following incubation in human serum at 37 °C immediately (**a**) and after 3 days incubation (**b**). Arrows identify peak of Hb species indicated. % values reflect the contribution of that species to the overall intensity

PBS, the extent of Hb-PEGylation following incubation in HSA is lower, suggesting cleavage of the Hb-PEG bond. Figure 3 shows the effect of a 37 °C incubation in human serum on the Hb-PEG bond. Distinct Hb-PEG and unPEGylated peaks can be seen, again allowing for quantification of the Hb-PEG/Hb ratio with time. However, unlike for HSA, it was not possible to analyse a sample on day 7 due to significant protein precipitation.

Figure 4 shows a side-by-side comparison of the stability of Hb-maleimide-PEG and Hb-mono-sulfone-PEG following incubation with PBS, GSH, HSA and serum. The mono-sulfone-PEG is stable in all conditions over a 7-day period. In contrast, in the presence of species containing competing thiols, the Hb-maleimide-PEG conjugation is labile. However, even in the most competitive conditions (1 mM GSH), 70% of the Hb-maleimide-PEG conjugation is retained after 7 days.

4 Discussion

The creation of a new cysteine residue in Hb (βCys93Ala/αAla19Cys) enables efficient PEGylation with no detectable effect on protein function or stability [2]. Although maleimide PEGylation is efficient and relatively stable, the use of alternative PEG reagents such as mono-sulfones [4] leads to a product with enhanced stability, with minimal deconjugation detectable in the presence of plasma reducing

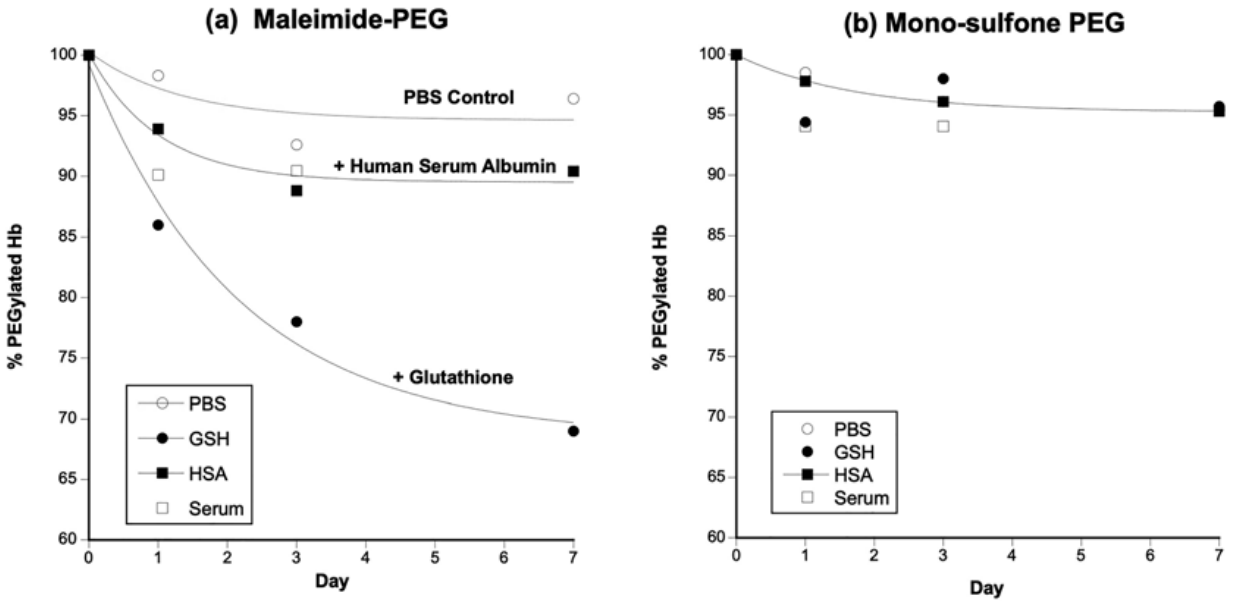

Fig. 4 Kinetics of stability of Hb-PEG conjugates. Graphs show the % drop in Hb PEGylation for maleimide-PEG Hb (**a**) and mono-sulfone-PEG Hb (**b**), assuming 100% PEGylation at t = 0 analysed by either SDS-PAGE (PBS, GSH; [4]) or SEC (HSA, serum; this paper). Curve fits are mono exponential decay; for mono-sulfone PEG, only the fit to the HSA data is shown for clarity as the fits superimpose. Note for the SEC data, the contribution of the higher molecular weight species was ignored, due to the uncertainty as to the PEGylation status of this species. This had minimal effect for the HSA calculation but did lead to greater uncertainty for the serum data

agents and free sulfhydryl residues, even after a week of incubation at 37 °C. This enhanced stability may not be relevant for current HBOC, which are targeted as acute therapeutics with circulatory half-lives of at most 2–3 days [1]. In these cases, HBOCs are likely cleared primarily by phagocytosis rather than by filtration in the kidney following loss of conjugation. However, if a longer lived HBOC with half-lives of weeks rather than days is required, this paper shows more stable PEG linkages such as mono-sulfone are likely to prove more suited than maleimide PEG.

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Disclosure Cooper and Reeder have patents relating to the use of recombinant haemoglobin as a component of an oxygen therapeutic. Bird and Sheng were employees of Abzena Ltd. at the time this work was undertaken.

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