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Derivatization of haemoglobin with periodate-generated reticulation agents: evaluation of oxidative reactivity for potential blood substitutes

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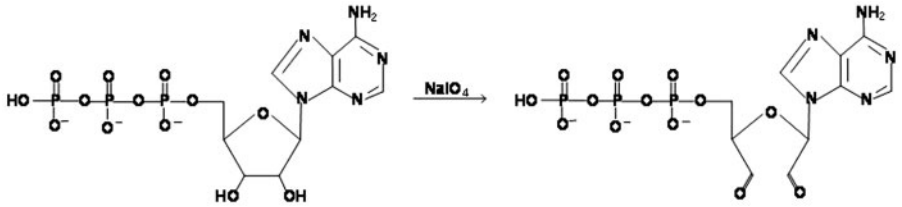
Periodate modification of the sugar moiety in sugars, including adenosine triphosphate (ATP), has previously been employed in order to prepare dialdehyde-type reagents, which were then utilized in crosslinking reactions on haemoglobin, yielding polymerized material with useful dioxygen-binding properties and hence proposed as possible artificial oxygen carriers ('blood substitutes'). Here, the periodate protocol is shown to be applicable to a wider range of oxygen-containing compounds, illustrated by starch and polyethylene glycol. Derivatization protocols are described for haemoglobin with such periodate-treated crosslinking agents, and the dioxygen-binding properties and redox reactivities are investigated for the derivatized haemoglobins, with emphasis on pro-oxidative properties. There is a general tendency of the derivatization to result in higher autooxidation rates. The peroxide reactivity of the met (ferric) form is also affected by derivatization, as witnessed, among others, by varying yields of ferryl [Fe (IV)-oxo] and free radical generated. In cell, culture tests (human umbilical vein epithelial cells, HUVEC), the derivatization protocols show no toxic effect.

Keywords: Blood substitute/free radical/haemoglobin/high-valent iron/oxidative stress.

Abbreviations: ATP, adenosine triphosphate; EPR, electron paramagnetic resonance; Hb, hemoglobin; HBOC, Hemoglobin-based oxygen carriers; HUVEC, human umbilical vein epithelial cells; MTT, 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide; oATP, oxidized adenosine triphosphate; oPEG, oxidized polyethylene glycol; PEG, polyethylene glycol; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

Haemoglobin-based oxygen carriers (HBOC) are known to manifest their toxicity at molecular level along several coordinates *(1, 2)*. On one hand, chemically derivatized globins may feature oxygen affinities drastically different when compared with those exhibited by erythrocyte-encapsulated native haemoglobin. One working hypothesis is that even more dangerous are the two other coordinates of haemoglobin (Hb) toxicity, namely, reactivity towards oxidative/nitrosative stress agents (such as hydrogen peroxide or nitric oxide) and, connected to this indirectly, extravasation which allows native haemoglobin and its derivatives to access cells outside blood where they engage in unwanted redox reactions *(1, 2)*. Haemoglobin's haem centre is, among others, reactive towards hydrogen peroxide (and peroxides in general), especially under conditions where due to natural autooxidation processes the haemoglobin reaches the ferric state *(1, 2)*. The product of the reaction between ferric haemoglobin and peroxides is, as with most haemoproteins (e.g. catalases, peroxidases, cytochromes P450), a species formally described as Fe (IV) with an oxo ligand (ferryl) *(3–5)*. Ferryl haemoglobin is constantly produced in human blood, and this process is enhanced under conditions of stress (be it physical effort or disease-related); pathological implications of this process have been discussed, including the concept according to which much of the problems arising in clinical trials of HBOC's are correlated with un-tamed peroxide reactivity of the HBOC *(1, 5–7)*.

For the reasons outlined above, one may expect that purified haemoglobin can only be a reasonable blood substitute candidate once its redox reactivities can be controlled/reduced. Such reduction has been achieved so far via four routes. A first approach has involved inter-protein crosslinking of haemoglobin, thereby generating particles of large molecular weight/volume, which have increased stability in the bloodstream and avoid homogenous close contact between haemoglobin and endothelium, thereby reducing the haemoglobin-NO reactivity *(1, 2)*. A second approach towards reducing haemoglobin's pro-oxidant activity has involved derivatization of the protein surface with polymeric systems, with results much similar to those achieved by the protein–protein crosslinking approach described above. Polyethylene glycols have formed the focus of such strategies, although oligo and polysaccharides have also been employed *(1, 2)*. A third approach has been to genetically modify human haemoglobin; distinct site-directed mutations have been identified that drastically reduce reactivity



Scheme 1 Oxidation of adenosine triphosphate by periodate.

towards NO by modifying amino acids gating access of small molecules towards the haem site; possible examples of similarly rational design of haemoglobin to remove pro-oxidant activity have more recently been reported *(6, 8)*. A fourth approach has been to leave haemoglobin intact and to engineer its environment either by adding antioxidant systems (enzymes and/or small molecules) or by constructing nanomaterials mimicking cells which would encapsulate the haemoglobin thereby helping to isolate it from stress agents found in blood upon transfusion *(2, 9)*.

Others have extensively characterized the process whereby periodate treatment of the sugar moiety within adenosine triphosphate (ATP) can be oxidized to a di-aldehyde, cf. Scheme 1, after which the dialdehyde was used to crosslink haemoglobin *(10–12)*. Since ATP itself acts as an effector for haemoglobin, it was speculated that an ATP-derivatized HBOC may exhibit interesting dioxygen-binding behaviour, of use in HBOC applications; such modifications were indeed demonstrated *in vitro* and initial positive results were obtained *in vivo* as well *(12)*. Palmer and co-workers have shown that the procedure can be extended to other mono- di- or tri-saccharides, or even to dextran *(13)*. Here, we show that one can employ a large number of hydroxyl-containing compounds, exemplified in the form of starch and polyethylene glycol, for crosslinking haemoglobin; in examining these products, we focus on the oxidative/pro-oxidant reactivity, which indeed is shown to have affected. Nevertheless, in cell culture tests [human umbilical vein epithelial cells (HUVEC)], the derivatization protocols show no toxic effect.

Materials and Methods

Bovine Hb was purified following a general protocol of Antonini and Brunori *(14)*, and manipulated in phosphate buffer saline (PBS) unless otherwise mentioned. Haemoglobin concentrations in text are given per haem rather than per tetramer. Adenosine triphosphate (ATP, Merck, Germany), hydrogen peroxide (30%, Sigma-Aldrich, Germany), polyethylene glycol (PEG-4000) and starch (Merck, Germany) were used as received without any further purification.

UV–vis spectra were recorded on Agilent 8453 (Agilent Inc.) and Cary 50 (Varian Inc.) instruments. For EPR spectra, a Bruker EMX Micro spectrometer with a liquid nitrogen cooling system was employed for EPR spectra. Instrument conditions were: microwave frequency 9.43 GHz, microwave power 15.89 mW, modulation frequency 100 kHz, modulation amplitude 5 G, sweep rate 22.6 G/s; time constant 81.92 ms, average of three sweeps for each spectrum, temperature 100 K.

For ATP oxidation, the protocol followed indications given by Palmer and co-workers *(13)*: 50 mM solutions of ATP were prepared in 18.1 MΩ deionized water. The solution was oxidized with sodium periodate ($NaIO_4$) for 1 h at room temperature in order to ring-open the 1,2-diols to yield dialdehydes (Scheme 1). The product of ATP oxidation via this protocol is designated as oATP. Starch and polyethylene glycol oxidation protocols were performed similarly to ATP, with differences as follows: starch solutions were 4 g/l, and PEG solutions were 2 mM. The respective products are referred to as oPEG (oxidized PEG) and oST (oxidized starch).

For polymerization of Hb with oATP, 1 mM Hb was reacted with oATP at concentrations of 0.5, 2, 7, 10, 15 and 20 mM. The reaction was performed under stirring at 4°C; a 2-fold excess of $NaBH_4$ was then added to each reaction vessel for 30 min to quench the reaction. The product was dialysed in 50 mM Tris buffer with 150 mM NaCl (pH 7.4) to remove excess $NaBH_4$ and unreacted cross-linker. The protein was then chromatographed on a Sephacryl S-300 size exclusion column (GE Healthcare, Sweden) with a mobile phase of 150 mM NaCl, 20 mM Tris buffer at pH 7.4.

The derivatized forms of Hb were, where required, separated using a HiTrap SP HP anion exchange column (GE Healthcare) controlled by a FPLC system. Using 20 mM Tris pH 7.4 as mobile phase, the derivatized Hb was bound to the stationary phase of the column, whereas the unmodified Hb was not. The modified Hb was then eluted with Tris buffer pH 7.4 containing 200–1,000 mM NaCl. The separated protein fractions were further analysed by 15% SDS–PAGE and gel filtration size exclusion chromatography on a Superdex 200 5/150 GL column (GE Healthcare), 0.25 ml/min flow rate; molecular weights were determined based on a calibration curve employing a molecular weight standard kit (Sigma-Aldrich) containing carbonic anhydrase (29 kDa), bovine serum albumin, (BSA, 66 kDa), alcohol dehydrogenase (150 kDa), amylase (200 kDa), apoferritin (443 kDa), thyroglobulin (669 kDa) and blue dextran (void volume marker). Dioxygen affinity and autooxidation measurements were at room temperature (~23°C) and 37°C respectively.

The biological system used for *in vitro* testing was the HUVEC. This cell line is a generous gift from Assoc. Prof. Marina Nechifor from University of Bucharest, Faculty of Biology. Cells were defrosted carefully, cultivated in special culture flasks (Nunclon) with RPMI-1640 (Sigma) culture medium, supplemented with foetal calf serum (FCS, Sigma), Penicillin–Streptomycin (Sigma) and glutamine in humidified Heto Holten Cellhouse 154 incubator at 37°C and 5% CO_2 level. Cells were adherent, and several cell passages were performed using enzymatic procedures. Experiments were made when confluence of the cells achieved 80% on the flask surface. Cells were then plated on 96-well flat-bottomed microtiter plates (Nunclon), and were kept 24 h in the incubator. To obtain a suitable density of cells on each well on the day of the measurement, and considering the HUVEC cells proliferation rate, we used different cell densities pro well: 1×10^5 per well cell for the 24 h experiment, 7.5×10^3 cells for 48 h incubation, and 6×10^3 cells for the 72 h long experiment. Wells were then treated with compounds to be tested (10 µl in each well), with untreated cells left as reference; blank cell-free wells contained cell culture media; as coloration reference, cell-free wells were treated with culture media and compound. Each compound was tested in triplicate, and three different experiments were completed. To assess cytotoxicity we performed the widely used quantitative colorimetric MTT assay *(15, 16)* for determination of cell viability changes. The method's advantages are its sensitivity, simplicity and relatively short duration. The assay is based on the ability of viable cells mitochondria to reduce the 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) to a purple formazan product. The formazan product is analysed with a scanning multiple spectrophotometer and its quantity as measured by the amount of 492 nm absorbance values is

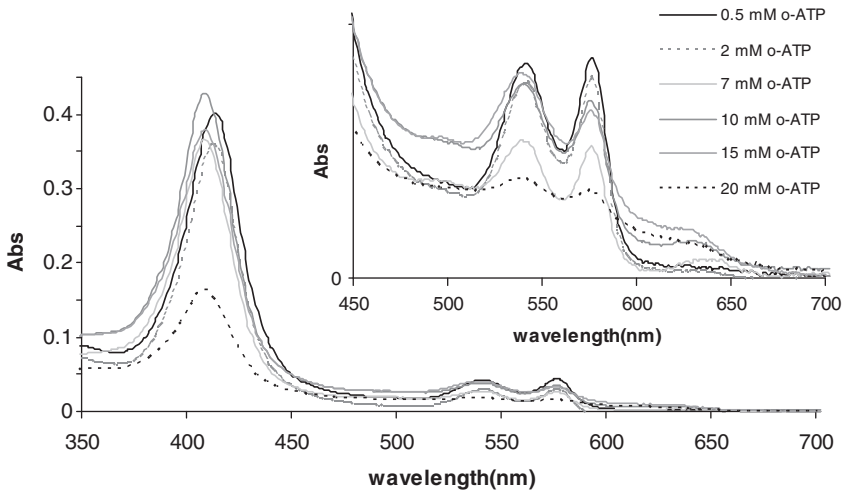


Fig. 1 The UV–vis spectra of the o-ATP-derivatized Hb; oATP concentrations employed in derivatization reactions are indicated.

directly proportional to the number of living cells in cell culture. After 24, 48 or 72 h incubation with the Hb molecules, MTT solution was added to each well at a final concentration of 1 mg/ml per well and the plates were incubated at 37°C for another hour. Dimethyl sulphoxide (DMSO) was added to each well to dissolve the formazan, and spectrophotometric absorbance measurements were made using a BioTek Synergy 2 multimodal fluorescence microplate plate reader. Statistical analysis employed the GraphPad Prism 5 biostatistics software. We established the survival curves for compounds **1**, **2** and **3.** One-way analysis of variance (ANOVA) and Dunnett Multiple Comparison test ($P < 0.05$, $r^2 = 0.94$–0.86) were completed for all individually inhibitory effects.

Results and Discussion

Derivatization with ATP

Figure 1 shows UV–vis spectra of Hb derivatized with oxidized ATP (oATP). The 630 nm features indicate that with oATP concentrations higher than 10 mM the derivatized Hb features significant percentages of ferric form (~50% and higher), resulted from oxidation of the oxy-Hb during the derivatization process. Control experiments (not shown) where the derivatization was performed anaerobically on the deoxy form of Hb, did not avoid the oxidation side reaction, suggesting that it involves direct oxidation by periodate or its congeners, rather than favuorization of autooxidation because of protein derivatization.

Figure 2 illustrates size-exclusion chromatograms of oATP-derivatized Hb, demonstrating increases in molecular weights directly dependent on the dose of oATP used for derivatization. With low oATP concentrations (0.2–7 mM) the apparent size of the protein appears to suffer negligible modifications, suggesting that the derivatization has occurred on the surface of the Hb tetramer without leading to the polymerization expected of a dialdehyde reagent such as oATP. This surface derivatization is verified by the fact that such oATP non-polymeric fractions do bind to anion-exchange columns, whereas native Hb does not (chromatograms not shown). For Hb samples treated with higher concentrations, up to 20 mM oATP, Fig. 2 shows an increase in molecular weight to ~350 kDa in the 15-mM oATP sample. However, Fig. 1 shows

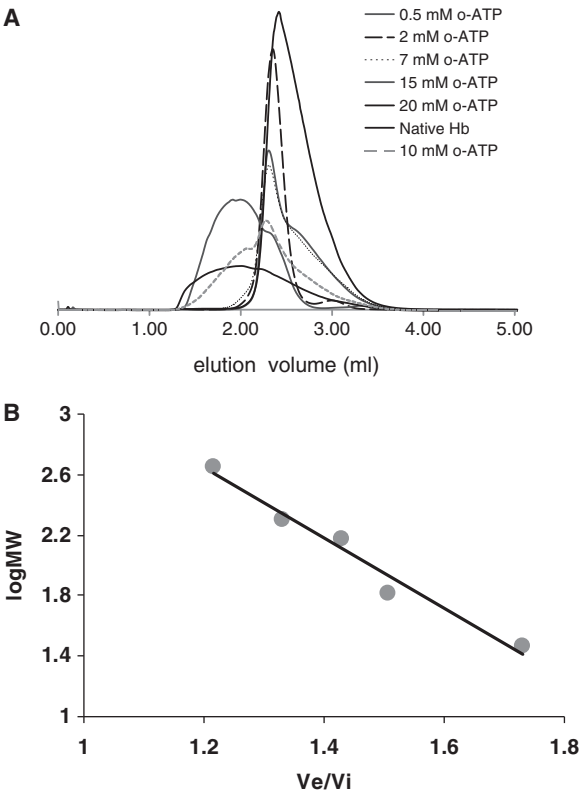


Fig. 2 Gel-filtration data demonstrating polymerization of Hb by oATP. (A) Size exclusion chromatograms of oATP-derivatized Hb. Estimated average molecular weights (kDa) are: native Hb—64; 0.5 mM oATP—100; 2 mM oATP—80; 7 mM—100; 15 mM—350; 20 mM—290. (B) The corresponding calibration curve (c.f. 'Materials and Methods' section).

that the 20-mM oATP treatment has led to a significant decrease in protein concentration, and even the 15-mM oATP sample shows signs of a perturbed haem environment, with the 630 nm band no longer clearly defined. As such, the 15 and 20 mM oATP samples were not analysed any further as potentially useful for blood substitute material.

Overall, Figs 1 and 2 indicate that low oATP concentrations may be used to selectively derivatize the surface of the Hb tetramer without inducing polymerization; polymerization requires higher oATP concentrations but has the disadvantage of inducing Hb oxidation. This latter aspect may either be a result of an increased tendency in autooxidation in the Hb polymer, or simply a reversible side-reaction under conditions where the Hb autooxidation rate is not affected by oATP. Table I shows that the former explanation is most likely valid: the autooxidation rates of oATP-Hb are somewhat higher than those of native Hb (increases by 120–190%).

The presence of the oATP adenine ring attached to the Hb surface may be expected to provide a sink for free radicals and may thus offer an extra line of defence for the haem against oxidative stress agents. This hypothesis was tested as shown in Table II and Fig. 3, by examining the direct reaction between Hb and peroxide as well as the catalytic activity as ascorbate peroxidase, both of which are representatives of oxidative stress reactions expected to take place during a transfusion experiment *(17, 18)*. Thus, the reaction of ferric Hb with hydrogen peroxide, monitored by the absorbance at 425 nm characteristic of the strongly oxidizing ferryl [Fe (IV)] form, proceeds to yield distinctly lower amounts of high-valent iron in the oATP-treated haemoglobins. These decreases range between ~20 and 40%, depending on the ATP : Hb ratio. The lower ferryl yields do not appear to be mirrored by predictable changes in ascorbate peroxidase reactivity: changes in this respect are, as shown in Table II, relatively small and most likely the result of a balance between two factors different in nature and possibly in sign: the ability of the adenine rings to quench free radicals and thus protect ascorbate from oxidation, and the protein structural changes brought about by derivatization (the latter are also witnessed by Table I data). One notable feature, looking at Fig. 3 for all derivatized haemoglobins, is the clearly decreased stability of the ferryl species, which accumulates at constant concentration in native Hb throughout the duration of the experiment, but decays to a large extent within the same timeframe in derivatized haemoglobins.

Table I. Increases in autooxidation rates of Hb brought about by derivatization with oATP.

Hb	Increase (%)
+0.5 mM oATP	193
+2 mM oATP	171
+7 mM oATP	121
+10 mM oATP	150

Table II. Increases in ascorbate peroxidase activity of Hb and in ferryl yields brought about by derivatization with oATP.

Hb	Activity (increase %)	Ferryl (percent decrease relative to native Hb)
+0.5 mM oATP	+18	−33
+2 mM oATP	+26	−18
+7 mM oATP	−35	−33
+10 mM oATP	−15	−37

Conditions: for direct reaction with peroxide—room temperature, PBS, 10 μM Hb, 100 μM H_2O_2; for enzymatic activity—room temperature, 50 mM pH 5 acetate, 10 μM Hb, 400 μM ascorbate. 800 μM H_2O_2.

Derivatization with starch

Treatment of starch with periodate was expected to yield a poly-aldehyde-type reagent which would efficiently react with exposed lysine residues on the surface of Hb, leading to polymerization in a manner analogous to what was shown for glutaraldehyde-based polymerization *(19–22)*. Figure 4 illustrates electronic absorption UV–vis spectra of Hb derivatized with periodate-oxidized starch under various conditions, the amount of periodate used decidedly controlled the degree to which Hb retained its oxy state. Figure 5 then shows size-exclusion chromatograms demonstrating that indeed polymerized fractions have been produced in these reactions.

Similarly to oATP-derivatization, starch derivatization also resulted in an increase in autooxidation rate

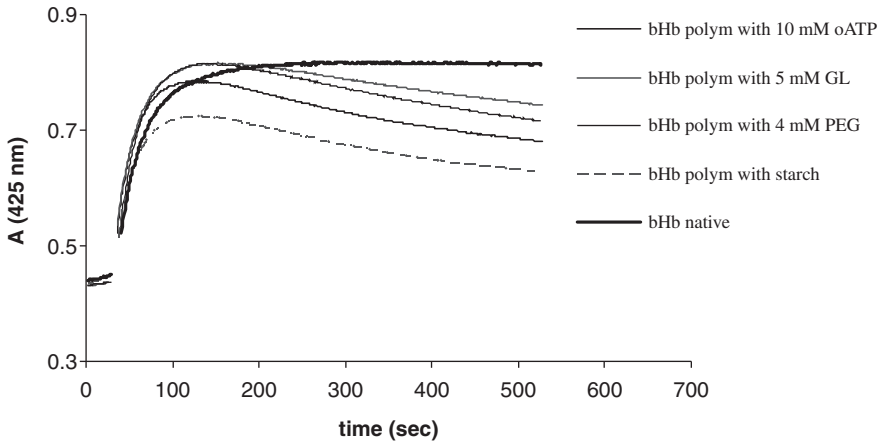


Fig. 3 The reaction of derivatized haemoglobins with H_2O_2. Conditions: room temperature, 10 μM protein, 80 μM H_2O_2, PBS 7.4.

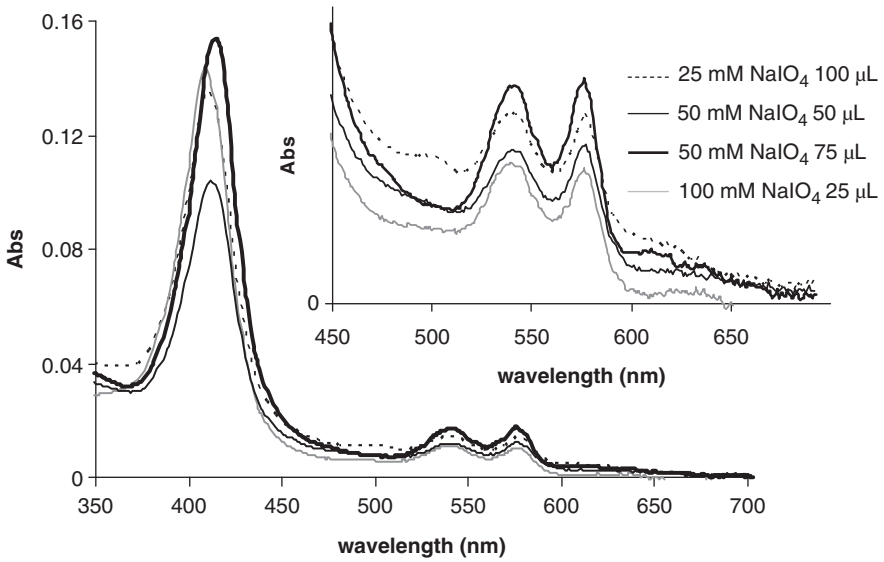


Fig. 4 The UV–vis spectra of the starch polymerized Hb. Conditions: PBS, room temperature.

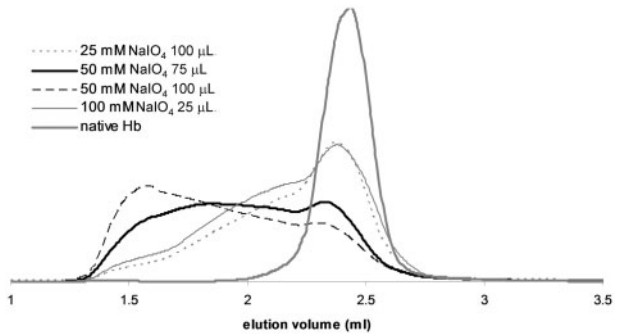


Fig. 5 Size exclusion chromatograms of starch-derivatized Hb. Average molecular weights were: +25 mM $NaIO_4$, 100 µl: 80 kDa; +50 mM $NaIO_4$, 75 µl: 90–470 kDa; +50 mM $NaIO_4$, 100 µl: 100–1000 kDa; +100 mM $NaIO_4$, 25 µl: 80–130 kDa.

(by 320% for the samples polymerized with 0.6 g/l starch and 40 mM $NaIO_4$). Figure 3 illustrates how this is accompanied by a decrease in the yield of high-valent ferryl iron (to 70% compared to native Hb) as well as by a decrease in its stability. On the other hand, the enzymatic peroxidase-type reaction, which relies of the very formation of the ferryl species, is 32% smaller than in native Hb.

Derivatization with PEG

Since polyethylene glycol features multiple hydroxyl groups similarly to a sugar moiety, it may be expected that oxidation with periodate may convert polyethylene glycol to a poly-aldehyde capable of crosslinking proteins—Hb included. Figure 6 shows SDS–PAGE data illustrating that this is indeed the case: aggregates of various molecular weights can indeed be obtained from Hb with periodate-oxidized polyethylene glycol (oPEG). Also shown in Fig. 6 is a size-exclusion chromatogram illustrating how even in the sample obtained with 4 mM PEG, where according to the SDS–PAGE only dimeric Hb units appear to have resulted from

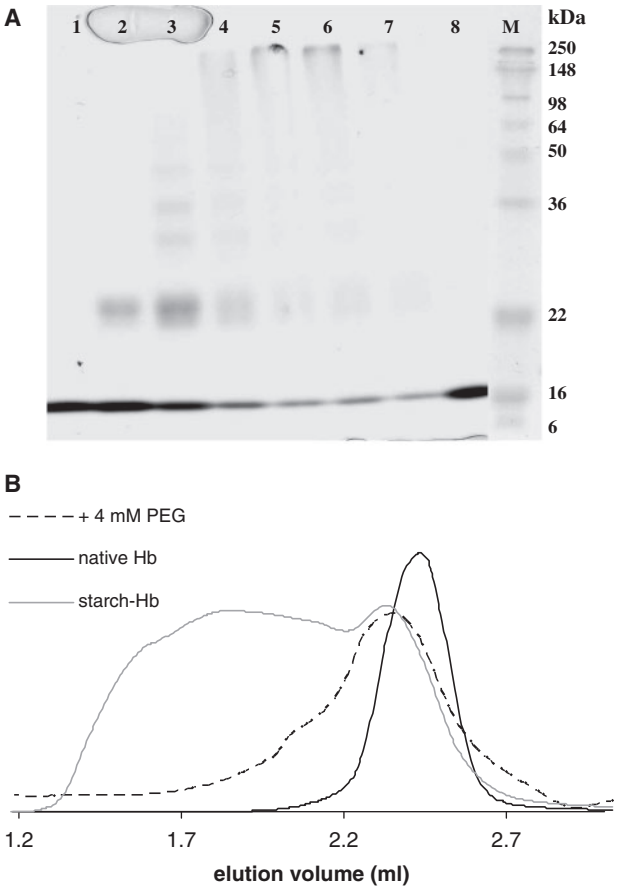


Fig. 6 Chromatographic evidence for derivatization of Hb with oxidized starch and polyethylene glycol. (A) SDS–PAGE illustrating the influence of PEG concentration on polymerization degree. SDS–PAGE 15%; 1- 1 mM PEG; 2- 3 mM PEG; 3- 5 mM PEG; 4- 10 mM PEG; 5- 15 mM PEG; 6- 17 mM PEG; 7- 20 mM PEG; 8- native Hb. (B) Size-exclusion chromatogram for Hb derivatized with 3 mM periodate-reacted PEG.

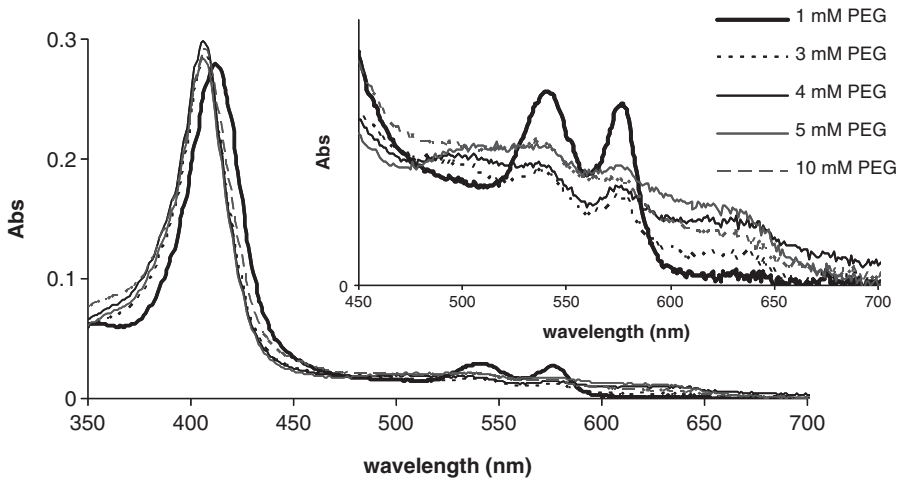


Fig. 7 The UV–vis spectra of the oPEG-polymerized Hb after dialysis to remove crosslinking/oxidizing agents. Conditions: room temperature, PBS.

derivatization, these newly-formed bonds are inter-tetrameric in nature as they do lead to an apparent increase in molecular weight to 80 kDa.

Figure 7 shows UV–vis spectra of oPEG-derivatized Hb. A marked tendency towards oxidation upon crosslinking is noted: of the several conditions tested in Fig. 7, only the one employing 1 mM PEG is seen to tolerate Hb in its oxy form—all the others showing essentially fully oxidized Hb as witnessed by the decrease in absorbance at 540–580 nm and by the diagnostic absorption maximum at 630 nm. The 1 mM PEG sample, whose UV–vis spectrum appears to be unaffected by autooxidation, shows essentially no polymerization (as witnessed by the Fig. 6 SDS–PAGE).

The yield of ferryl formed upon reaction of 3 mM-PEG-Hb with peroxide is, as illustrated in Fig. 3, essentially identical to that of native Hb, while its stability is, as for all other derivatized Hb's, smaller. On the other hand, the enzymatic peroxidase-type reaction is 33% smaller than in native Hb.

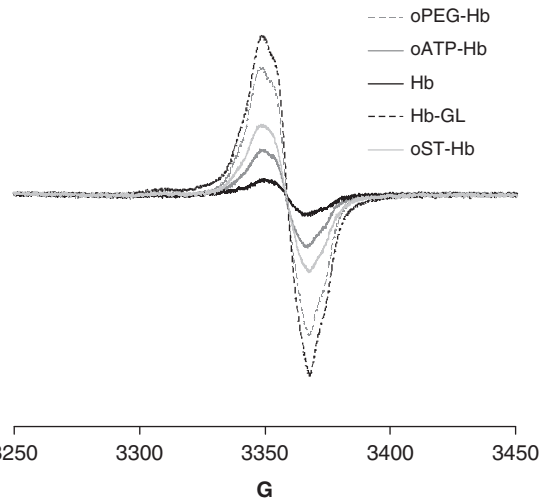


Fig. 8 EPR spectra of 200 µM globins treated with 400 µM H_2O_2 in PBS and frozen at 30 s after mixing: Hb, native Hb; Hb-GL, glutaraldehyde-polymerized Hb; oPEG-Hb, Hb derivatized with oxidized PEG; oATP-Hb, Hb derivatized with periodate-oxidized ATP; oST-Hb, Hb derivatized with periodate-oxidized starch.

EPR-detected free radicals

Treatment of Hb with hydrogen peroxide leads to relatively low levels of protein-located free radicals—the nature of which was previously described in much detail *(23)*. Figure 8 shows these free radicals in the derivatized haemoglobins examined in the current study, alongside native Hb and glutaraldehyde-reticulated Hb *(22)* [the latter taken as a prototypical derivatized Hb, as it forms the basis for a blood substitute already approved for limited human use *(1)*]. It can be noted that all derivatization methods lead up to 10 times more free radical—the maximum yield being reached in glutaraldehyde-derivatized Hb, followed closely by oPEG-Hb. On the other hand, scaled superposition of the signals (not shown) confirms that the shape of the free radical signal is not different in the five proteins examined here—which, among others, suggests that the adenine ring in oATP-Hb is not a stable site for free radicals [at least not by comparison with tyrosine residues within Hb, which dominate our EPR spectra *(23)*].

HUVEC tests

The effect of Hb-based potential blood substitutes on human cell cultures, including the HUVEC line, has previously been studied and shown to allow for identification of possible toxic effects of cell-free Hb (derivatized or not) *(1, 9, 24–26)*. Figure 9 shows the effect of Hb derivatization with protocols described here, on the HUVEC toxicity of Hb. As expected, pure native Hb shows slight toxicity on these cells. oATP and starch-derivatized Hb act in very similar manner, suggesting that derivatization protocols described here offer some promise for further design of non-toxic polymeric aggregates of proteins—for blood substitutes or elsewhere.

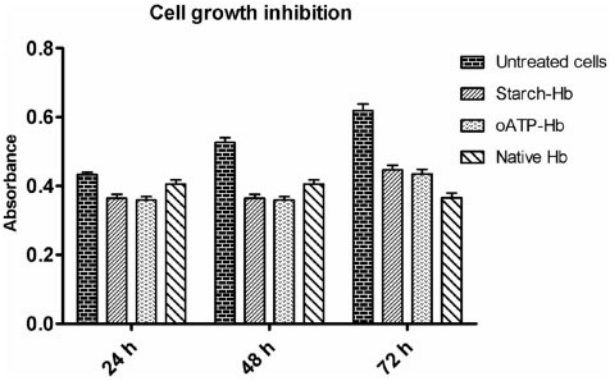


Fig. 9 Survival of HUVEC cells after exposure to oATP-Hb (1), oST-Hb (2) and native Hb (3).

To conclude, derivatization protocols were shown here for Hb with periodate-oxidized sugar, nucleotide and polyethylene glycol, and the products were characterized in terms of molecular weight distribution, dioxygen binding, and pro-oxidant reactivity. These derivatizations entailed essentially negligible effects on the toxicity of Hb towards human HUVEC cells.

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Conflict of interest

None declared.

References

1. Alayash, A.I. (2004) Oxygen therapeutics: can we tame haemoglobin? *Nat. Rev. Drug Discov.* **3**, 152–159
2. Chang, T.M.S. (2009) Nanobiotechnology for hemoglobin based blood substitutes. *Crit. Care Clinics* **25**, 373–382
3. Sono, M., Roach, M.P., Coulter, E.D., and Dawson, J.H. (1996) Heme-containing oxygenases. *Chem. Rev.* **96**, 2841–2888
4. Shaik, S., Kumar, D., de Visser, S.P., Altun, A., and Thiel, W. (2005) Theoretical perspective on the structure and mechanism of cytochrome P450 enzymes. *Chem. Rev.* **105**, 2279–2328
5. Silaghi-Dumitrescu, R., Reeder, B.J., Nicholls, P., Cooper, C.E., and Wilson, M.T. (2007) Ferryl haem protonation gates peroxidatic reactivity in globins. *Biochem. J.* **403**, 391–395
6. Reeder, B.J., Grey, M., Silaghi-Dumitrescu, R.L., Svistunenko, D.A., Bulow, L., Cooper, C.E., and Wilson, M.T. (2008) Tyrosine residues as redox cofactors in human hemoglobin: implications for engineering nontoxic blood substitutes. *J. Biol. Chem.* **283**, 30780–30787
7. Reeder, B.J., Svistunenko, D.A., Cooper, C.E., and Wilson, M.T. (2004) The radical and redox chemistry of myoglobin and hemoglobin: from *in vitro* studies to human pathology. *Antioxidant Redox Signaling* **6**, 954–966
8. Olson, J.S., Foley, E.W., Rogge, C., Tsai, A.L., Doyle, M.P., and Lemon, D.D. (2004) NO scavenging and the hypertensive effect of hemoglobin-based blood substitutes. *Free Radic. Biol. Med.* **36**, 685–697
9. Chen, J.Y., Scerbo, M., and Kramer, G. (2009) A review of blood substitutes: examining the history, clinical trial results, and ethics of hemoglobin-based oxygen carriers. *Clinics* **64**, 803–813
10. Harris, D.R. and Palmer, A.F. (2008) Modern cross-linking strategies for synthesizing acellular hemoglobin-based oxygen carriers. *Biotechnol. Prog.* **24**, 1215–1225
11. Simoni, J., Simoni, G., Newman, G., and Feola, M. (1996) An improved blood substitute. *In vivo* evaluation of its hemodynamic effects. *Asaio J.* **42**, M773–M782
12. Riess, J.G. (2001) Oxygen carriers (''Blood Substitutes'') sRaison d'Etre, chemistry, and some physiology. *Chem. Rev.* **101**, 2797–2919
13. Eike, J.H. and Palmer, A.F. (2004) Oxidized mono-, di-, tri-, and polysaccharides as potential hemoglobin cross-linking reagents for the synthesis of high oxygen affinity artificial blood substitutes. *Biotechnol. Prog.* **20**, 953–962
14. Antonini, E. and Brunori, M. (1971) *Hemoglobin and Myoglobin in their Reaction with Ligands*, North-Holland, Amsterdam, Holland
15. Denizot, F. and Lang, R. (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* **89**, 271–277
16. Furukawa, T., Kubota, T., Suto, A., Takahara, T., Yamaguchi, H., Takeuchi, T., Kase, S., Kodaira, S., Ishibiki, K., and Kitajima, M. (1991) Clinical usefulness of chemosensitivity testing using the MTT assay. *J. Surg. Oncol.* **48**, 188–193
17. Cooper, C.E., Silaghi-Dumitrescu, R., Rukengwa, M., Alayash, A.I., and Buehler, P.W. (2008) Peroxidase activity of hemoglobin towards ascorbate and urate: a synergistic protective strategy against toxicity of hemoglobin-based oxygen carriers (HBOC). *Biochim. Biophys. Acta* **1784**, 1415–1420
18. Deac, F.V., Todea, A., Bolfa, A.M., Podea, P., Petrar, P., and Silaghi-Dumitrescu, R. (2009) Ascorbate binding to globins. *Rom. J. Biochem.* **46**, 115–121
19. Eike, J.H. and Palmer, A.F. (2004) Effect of NaBH4 concentration and reaction time on physical properties of glutaraldehyde-polymerized hemoglobin. *Biotechnol. Prog.* **20**, 946–952
20. Eike, J.H. and Palmer, A.F. (2004) Effect of Cl^- and H^+ on the oxygen binding properties of glutaraldehyde-polymerized bovine hemoglobin-based blood substitutes. *Biotechnol. Prog.* **20**, 1543–1549
21. Eike, J.H. and Palmer, A.F. (2004) Effect of glutaraldehyde concentration on the physical properties of polymerized hemoglobin-based oxygen carriers. *Biotechnol. Prog.* **20**, 1225–1232
22. Deac, F., Todea, A., and Silaghi-Dumitrescu, R. (2009) *Glutaraldehyde derivatization of hemoglobin: a potential blood substitute*. (Silaghi-Dumrescu, R. and Garban, G., eds.), pp. 165–173, Cluj University Press, Cluj-Napoca, Romania
23. Svistunenko, D.A. (2005) Reaction of haem containing proteins and enzymes with hydroperoxides: the radical view. *Biochim. Biophys. Acta* **1707**, 127–155
24. Buehler, P.W., Vallelian, F., Mikolajczyk, M.G., Schoedon, G., Schweizer, T., Alayash, A.I., and Schaer, D.J. (2008) Structural stabilization in tetrameric or polymeric hemoglobin determines its interaction with endogenous antioxidant scavenger pathways. *Antioxid. Redox Signal.* **10**, 1449–1462

25. Ortegon, D.P., Davis, M.R., Dixon, P.S., Smith, D.L., Josephs, J.D., Mueller, D.L., Jenkins, D.H., and Kerby, J.D. (2002) The polymerized bovine hemoglobin-based oxygen-carrying solution (HBOC-201) is not toxic to neural cells in culture. *J. Trauma* **53**, 1068–1072

26. Sullivan, J.P. and Palmer, A.F. (2006) Targeted oxygen delivery within hepatic hollow fiber bioreactors via supplementation of hemoglobin-based oxygen carriers. *Biotechnol. Prog.* **22**, 1374–1387