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"BABEŞ-BOLYAI" UNIVERSITY
Faculty of Chemistry and Chemical Engineering

Blood substitutes and their interaction with oxidative and nitrosative stress agents

-PhD thesis summary-

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Keywords: hemoglobin, ascorbate, peroxide, blood substitute, oxidative stress, free radical, glutaraldehyde, BSA, bovine serum albumin, periodate-generate reticulation agents, polyethylene glycol, disuccinimyl suberate

Derivatization reaction

In most cases, derivatized hemoglobin was obtained by following the steps outlined in the diagram below. However, the reagent used is the one who dictates the necessary steps; in the diagram below, colored in blue are steps discretionary in the sense that they are missing or not in response depending on the reagent used. Thus, the oxidation reaction with sodium periodate ($NaIO_4$) is only required for some reagents - those with hydroxyl groups - such as ATP, polyethylene glycol, starch, alginate; also, introducing molecules with antioxidant character is an optional step, with the intention to reduce the autooxidation rate; reduction reaction of imine bonds by treatment with $NaBH_4$ is necessary in most reaction but may lack if we use agents such as N-hydroxysuccinimide esters like (methyl-PEG12)3-PEG4-N-hydroxysuccinimide ester) or disuccinimidyl suberate because they allow the formation of a stable amide linkages between reactive and primary amines (N-terminal α-amine or ε-amine of lysine) of protein. Except for these steps, derivatization reaction can be described as follows: Hb solution is mixed with derivatization reagent and allowed to react for a given time; the reaction was stopped by addition of sodium borohydride, which reduce imine bonds to stabile amines and also quenches excess carbonyl groups. After quenching, the reaction mixture was dialyzed in Tris buffer to remove excess $NaBH_4$ and site-products.

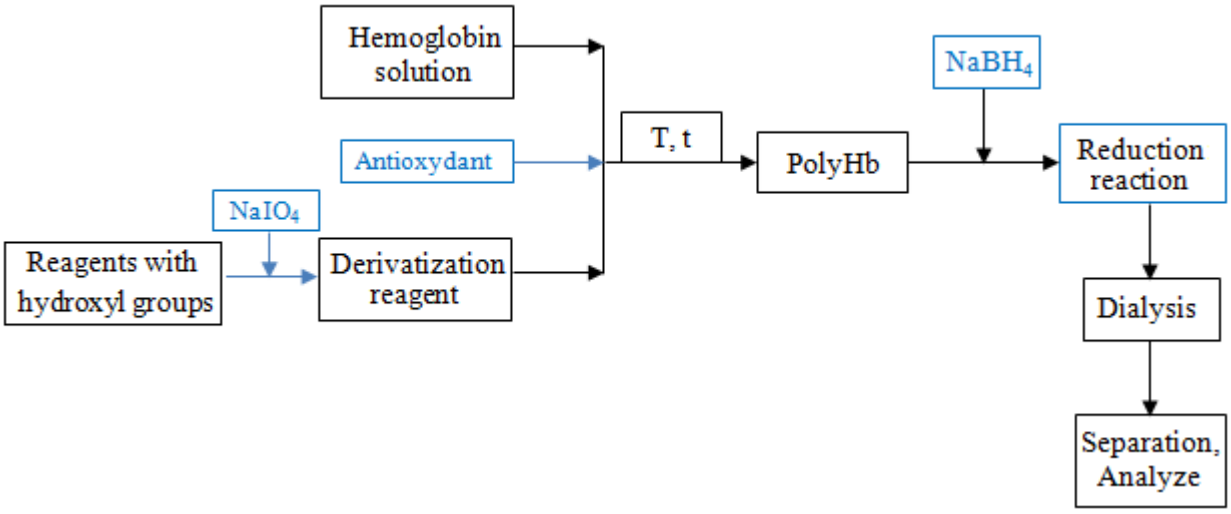
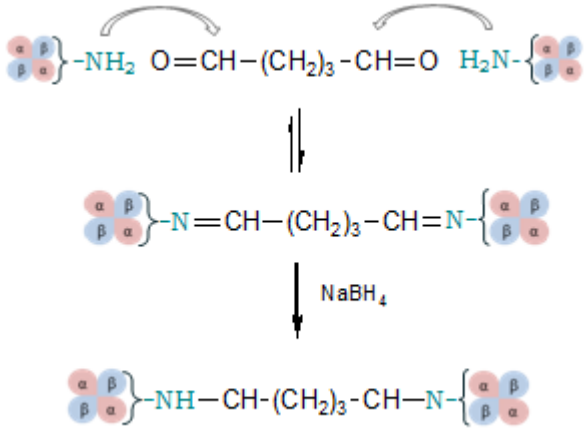


Figure 2.6. Steps used in hemoglobin derivatization

Hemoglobin derivatization with dialdehyde reagents

Hemoglobin derivatization with glutaraldehyde

Gluteraldehyde was chosen because it is a well-known crosslinking agent, and it has been used for this purpose so far, with satisfactory results. Hemoglobin derivatization with glutaraldehyde allows intermolecular bonding which is realized between the amino group of lysines and valines on Hb and carbonyl group of glutaraldehyde. This reaction yields chemically unstable imines because they are easily hydrolyzed in aqueous solution yielding the starting glutaraldehyde cross-linker and free hemoglobin. Therefore to avoid regeneration amino and carbonyl functions, the reducing agent $NaBH_4$ was used to reduce the imine bonds into stabile amine bonds (Scheme 2.1.).



Scheme 2.1. Reaction of hemoglobin with glutaraldehyde

The polymerized samples were prepared with different concentrations of reactants and then were analyzed by 15% SDS-PAGE and size exclusion chromatography. Optimal yields are observed at 1mM Hb and 5mM GL in reaction mixture. The peroxide and ascorbate affinity for the polymerized product was measured and compared with that of native Hb; The calculated Michaelis-Menten constants showed that polymerized product has low affinity for hydrogen peroxide but high for ascorbic acid.

Hemoglobin toxicity both native and derivatized with glutaraldehyde was tested on cultures of human cells and human lymphocytes. Two of the polymerized samples contained an antioxidant mixture: catalase (1000U), ascorbic acid (100μM), uric acid (100μM), cysteine (10μM), and BSA (100μM) or catalase (1000U), ascorbic acid (100μM).

Cell culture tests were performed on the endothelial cell line HUVEC (Human Umbilical Vein endothelial Cells) at the Institute of Oncology "I. Chiricuţă ". HUVEC cell line was chosen because they are belong to the blood vessels and would be the first affected by the substances studied, when they enter the bloodstream *in vivo*.

To assess the cytotoxicity we performed the widely used quantitative colorimetric MTT assay for determination of cell viability changes. The assay is based on the ability of viable cells mitochondria to reduce 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) to a purple formazan product. The formazan product was analysed with a scanning multiple spectrophotometer and its quantity as measured by the amount of 492nm absorbance values was directly proportional to the number of living cells in cell culture. After 24, 48 or 72 h incubations 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 sulfoxide (DMSO) was added to each well to dissolve the formazan.

Test results obtained from HUVEC cells indicates that native hemoglobin as well as poly-Hb display a slight inhibitory effect on HUVEC cultures. It shoul be noted that addition of antioxidants to poly-Hb does not appear to improve the performance to any significant extent.

Test in human lymphocytes shows that after 24 h, Hb compounds did not inhibit significantly the lymphocyte viability. Hemoglobin derivatives show a slightly inhibitory effect after 48 h, but not statistically significant. At 72 h a slight difference also begin to develop between native and polymerized Hb, in favour of the polymerized protein.

Hemoglobin copolymerization with glutaraldehyde

Because of the high rate of autooxidation for polymerized hemoglobin samples, we attempted derivatization in presence of an antioxidant - bovine serum albumine - which was added to hemoglobin before the polymerizing agents. Copolymerization of hemoglobin with serum albumin alleviates this problem completely, to the extent where the copolymer even has a slightly lower autooxidation rate compared to polyHb (in some cases even lower than native Hb) and lower prooxidant reactivity. Also, copolymerization with BSA lowers the amount of free radicals generated upon reaction of hemoglobin with hydrogen peroxide: the intensity of the well-known hemoglobin radical signal at ~ 3350 G is clearly lowered, in a dose-dependent manner, by copolymerization with BSA. Based on these results, serum albumin should be considered as ingredient in hemoglobin-based blood substitute. Oxygen affinity is not affected by the introduction

of albumin in the reaction mixture. The samples derivatized have higher oxygen affinity and lower cooperativity than native Hb.

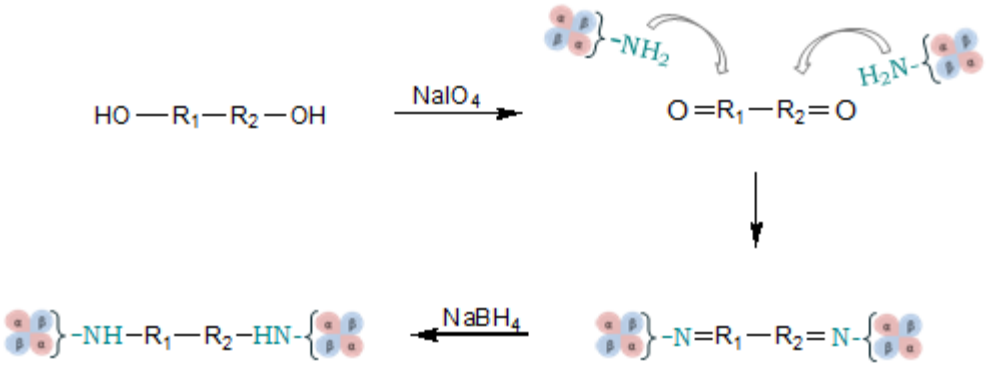
Another copolymer, prepared by our collaborators is based on rubrerythrin, a non-heme iron protein with peroxidase function in anaerobes. Unlike heme-based peroxidases or catalases, the mechanism whereby Rbr reduces hydrogen peroxide to water does not involve high-valent iron (Fe(IV) or Fe(V)) or free radicals (porphyrin- or protein-based). Moreover, the K_m for hydrogen peroxide is two orders of magnitude lower for Rbr compared to those of peroxidases and catalases. On this basis, Rbr would appear to be a distinctly safer enzyme than heme-based peroxidases or catalases for scavenging hydrogen peroxide *in vivo*.

Thus, experiments performed by our collaborators demonstrated that hemoglobin can be copolymerized with Rbr in presence of glutaraldehyde, resulting higher-molecular weight aggregates containing Rbr covalently-bound to Hb; also, the product, increased capability for reductively scavenging hydrogen peroxide, offering promise as less toxic artificial oxygen carriers/blood substitutes. The Hb-Rbr copolymer appears to fare slightly better than the corresponding native proteins at 24 hours; however, at longer times (48 and 72 hours, respectively, this advantage of the copolymers disappears.

Hemoglobin derivatization with periodate-generate reticulation agents

Periodate modification of the sugar moiety in sugars has previously been employed in order to prepare dialdehyde-type reagents, which were then utilized in crosslinking reactions on hemoglobin, yielding polymerized material with useful dioxygen-binding properties and hence proposed as possible artificial oxygen carriers. The periodate protocol is shown to be applicable to a wider range of oxygen-containing compounds, illustrated by starch, polyethylene glycol and alginate.

Derivatization reaction steps with such reagent whose carbonyl groups were generated by oxidation with $NaIO_4$ is illustrated in the diagram below. For simplicity reagent was noted OH-R_1-R_2-OH.



Scheme 2.2. Step derivatization of hemoglobin with periodate-generate reticulation agents

The dioxygen-binding properties and redox reactivities are investigated for the derivatized hemoglobins, with emphasis on pro-oxidative properties. There is a general tendency of the derivatization to result in higher autooxidation rates, especially for those obtained with polyethylene glycol. 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. Another disadvantage of the Hb-PEG sample is their enzymatic activity: 33% lower than the native hemoglobin. Treatment of Hb with hydrogen peroxide leads to relatively low levels of protein-located free radicals. It can be noted that all derivatization methods lead to up to 10 times more free radical – the maximum yield being reached in glutaraldehyde-derivatized Hb (which is used as a model, as it forms the basis for a blood substitute already approved for limited human use), 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.

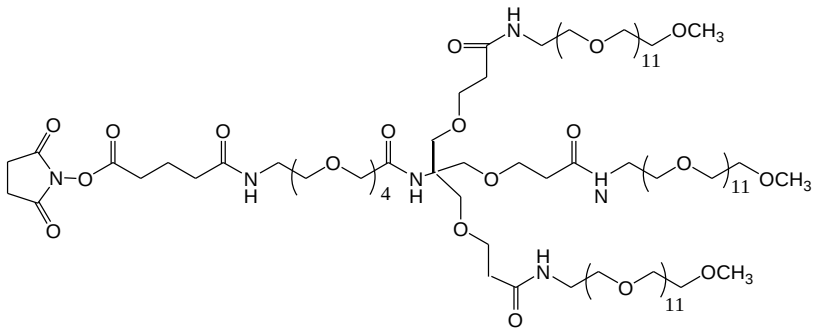
In order to reduce the autooxidation rate and prooxidant reactivity we introduced in the reaction mixture various antioxidants such as ascorbic acid, bovine serum albumin, thiosulfate, glucose. Thus, the sample derivatized without antioxidant presented a higher rate of autooxidation, while the copolymer with serum albumin alleviates this problem completely, to the extent where the copolymer even has a slightly lower autooxidation rate compared to native hemoglobin. Furthermore, the dioxygen affinity and Hill coefficient are maintained at levels close to that of native hemoglobin. These features are particularly useful since chemical derivatization of hemoglobin, especially in the form of crosslinking, can lead to autooxidation, increased affinity, and

decreased cooperativity. Also, electron paramagnetic spectra (EPR) illustrating that copolymerization with BSA lowers the amount of free radicals generated upon reaction of hemoglobin with hydrogen peroxide: the intensity of the hemoglobin radical signal is clearly lowered, in a dose-dependent manner, by copolymerization with BSA. This may be expected to be positive in a blood substitute.

Single-step hemoglobin derivatization

A new protocol is described for derivatization of hemoglobin with two N-hydroxysuccinimide esters (NHS-esters) (methyl-PEG12)3-PEG4-N-hydroxysuccinimide ester) (TMS) and disuccinimidyl suberate (DSS). They allow protein crosslinking without toxic side-products and forming peptide bond with lysine residue in one single step.

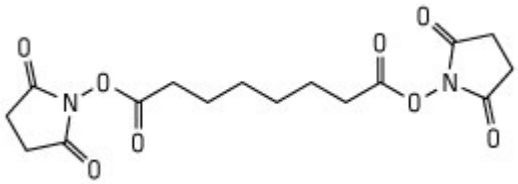
The first of the two agents, TMS, it is a branched amine-reactive PEGylation reagent (methyl-PEG12)3-PEG4-N-hydroxysuccinimide ester). Each methyl-terminated PEG (mPEG) branch contains 12 ethylene glycol units. The three branches are attached to a 4-unit PEG stem that contains an amine-reactive *N*-hydroxysuccinimide (NHS) ester at the distal end (Scheme 2.3.)



Scheme 2.3. Chemical structure of the amine-reactive PEGylation reagent used (methyl-$PEG_{12})_3$-PEG_4-N-hydroxysuccinimide ester)

Good results were obtained in case of PEGylated hemoglobin: no autooxidation occurred, but more important is that the dioxygen affinity of this derivatized Hb is very close to that of native bovine Hb with the particular observation that, unlike with a few other derivatization protocols, the dioxygen affinity is slightly lower than that of native Hb. In cell culture tests (human umbilical vein epithelial cells, HUVEC), the derivatization protocol induces no toxic effect. These results show promise towards applicability for production of hemoglobin-based blood substitutes.

DSS is a non-cleavable membrane permeable crosslinker that contains an amine-reactive N-hydroxysuccinimide ester at each end of an 8-carbon spacer who reacts with lysine residue to form peptide bond in one single step.



Scheme 2.4. Chemical strucutre of DSS

DSS may be employed for obtaining Hb polymers, and that the increase in autooxidation rate incurred by this polymerization is completely reversed when BSA is co-polymerized with Hb. The copolymers shows very little change in autooxidation and oxygen affinity compared to native Hb and low intensity of free radicals – less than in native Hb).

Alternative to bovine hemoglobin

Several types of hemoglobin were purified in order to identify cases where the rate of autooxidation, the turnover rates or affinities for substrates would be different from those of human or bovine hemoglobin, thereby possibly providing an additional advantage for preparation of a blood substitute.

Similarly to the case of bovine Hb, a low K_m for ascorbate is found, at 20-30μM. Also in agreement with data known for human and bovine Hb, the K_m values for peroxide are between 400 and 4000μM, in line with parameters of similar magnitude reported for bona fide peroxidases. Bovine and rat Hb appear to have the smallest K_m for peroxide, ~5 times smaller than that of the dog Hb. Thus, one may therefore expect that the bovine protein, by virtue of its higher affinity for peroxide, be a less useful material for blood substitutes compared to dog hemoglobin. In terms of k_{cat} values, ovine and rat hemoglobin appear the least reactive towards peroxide. These data may be taken to suggest that dog and sheep hemoglobins would have an advantage over bovine hemoglobin insofar, as they would display less pro-oxidant reactivity.

Poly bovine, canine and rat Hb were noted as the most resistant to attack of hydrogen peroxide, with highest rate constant of oxygen whereas the copolymers of bovine and goat hemoglobin were noted as having the lowest autooxidation rate. BSA copolymers of these proteins,

especially of the sheep and rat, could be candidates for blood substitutes with superior resistance to oxidative stress.

Interaction of hemoglobin-based oxygen carriers with nitric oxide (NO) and nitrite (NO_2^-)

The reaction of oxyHb (native and polymerized) with nitric oxide and nitrite were investigated by optical absorption spectroscopy, both of them form metHb and nitrate as end products.

In the first case, the rate constants were studied with the mention that the smaller values are better. Constants obtained but not significantly different between Hb native and derivatized its various forms in reaction with NO. However, no difference was observed between the values obtained.

In the second case, although the end product is known, the reaction mechanism is not yet fully elucidated. According to the latest approaches, the mechanism of this reaction can be divided into two stages: initiation and propagation of the autocatalytic stage.

The process of nitrite-induced oxidation in oxyhemoglobin is affected by blood-substitute-type derivatizations on hemoglobin; however, contrary to oxidative stress reactions, any form of polymerization seems to slow down this process. Also, we studied the influence of antioxidants like uric acid, ascorbic acid, caffeic acid, N-acetyl L-cysteine and bovine serum albumin on this reaction in order to reduce Hb oxidation rate in presence of sodium nitrite. Although the presence of albumin within the copolymer has little effect on the outcome of the nitrite-induced oxidation of oxyhemoglobin, addition of free albumin, as well as of free small-molecule antioxidants, to the reaction mixture does indeed block the process. This data supports a mechanism wherein free radicals accumulate during the lag phase of the oxy + nitrite reaction; it is proposed that the added antioxidants quench these radicals. The reason why polymerized hemoglobins also slow down the oxidation, is proposed to involve a reduced accessibility of the iron to exogenous anions and radicals.

Stability of hemoglobin-based oxygen carriers against denaturing agents

We studied the stability of hemoglobin-based blood substitutes on two factors: heat which affects hydrogen bonds and denaturing agents such as guanidine which destroys hydrophobic interactions.

Distortion, forced here by using a higher temperature than 37ºC and used in previous chapters for measuring the autooxidation rate was conducted at 60ºC and can provide clues about protein stability. To compare the behavior of derivatized Hb we studied the time of autooxidation at this temperature; also, we have looked at evolution in absorbances at wavelengths region where transition between oxy and met forms is characterized by isosbestic points. Any change in this region indicates a protein denaturing process, or presence of a third state in the autooxidation - none of the options are desirable. Thus the greatest stability at this temperature has Hb-BSA copolymer.

A parallel approach can be made used guanidine as a denaturing agent, comparing the glutaraldehyde polymerizing Hb with the native one. There is a high stability observed in the case of derivatized Hb - while the tests at 60ºC showed the opposite trend; these later results may be considered to be more physiologically relevant.

Hemerythrin-based blood substitutes

Hemerythrin-based blood substitutes has active ingredient a protein responsible for oxygen transport in the marine invertebrates that employs a non-heme diiron active site (Fe(II)-Fe(II)). Hemerythrin (Hr) was shown to avoid reactivity towards hydrogen peroxide, nitric oxide and nitrite. Another advantage is the higher molecular weight than Hb (108kDa vs 64kDa) wich should lead to lower levels of extravasation and elimination through the kidney. Until now, in our research group was successfully obtained glutaraldehyde-polymerized hemerythrin and polyethylene glycol-derivatized hemerythrin. The effects of these chemical modification on molecular weight, autooxidation rate and oxygen affinity appear to be favorable for blood substitute application.

Toxicity of hemerythrin and their chemical derivates was tested on cultures of human cells and human lymphocytes, comparing their performance with that of representative competitor, glutaraldehyde-polymerized bovine hemoglobin. Hemerythrin (native or derivatized) exhibits a proliferative effect on HUVEC cultures, as opposed to a slight inhibitory effect of Hb. A similar positive effect is displayed on human lymphocytes by glutaraldehyde-polymerized hemerythrin, but not by native or polyethylene glycol-derivatized hemerythrin. We conclude that hemerythrin and its chemical derivatives were found to be less toxic than native Hb and glutaraldehyde-polymerized Hb.

Hemoglobin and hemerythrin toxicity both native and derivatized with glutaraldehyde was tested on mice. They were divided into three equal parts which were injected with methemerythrin and oxyhemoglobin solutions in equal quantities and concentrations oxyhemoglobin and the third part,

untreated, was the reference. After 10 minutes, everyone got the same amount of blood and EPR spectra were measured. The sample with free hemoglobin shows the most intense free radical signal. In this context hemerythrin seems to exert a lower level of oxidative stress than hemoglobin - which is a promising element for hemerythrin polymers to be tested under similar conditions. The only obstacle in this line of research is possible antigenicity of hemerythrin, this issue is addressed by derivatization with glutaraldehyde, which blocks the lysine residues on the surface Hr besides inducing its polymerization, and polyethylene glycol, which is also attached to the surface protein.

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4. Oana Zolog, Augustin Mot, Florina Deac, Alina Roman, Eva Fischer-Fodor, Radu Silaghi-Dumitrescu, „A new polyethyleneglycol-derivatized hemoglobin derivative with decreased oxygen affinity and limited toxicity", *The Protein Journal*, **2011**, 30, 27-31

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2. Florina Scurtu, Oana Zolog, Bianca Iacob, Radu Silaghi-Dumitrescu, "Hemoglobin-albumin crosslinking with disuccinimidyl suberate (DSS) and/or glutaraldehyde for blood substitutes", *Artificial Cells, Blood Substitutes, and Immobilization Biotechnology*, **2012**

Completed, to be submitted:

1. Radu Silaghi-Dumitrescu, Dimitri A. Svistunenko, Daniela Cioloboc, Florina Scurtu, Chris E. Cooper, "Nitrite binding to globins: linkage isomerism, EPR silence and reductive chemistry". *Redox Biology*, **2012**