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PERIODATE-OXIDIZED ALGINATE AS POLYCONDENSATION REAGENT FOR HEMOGLOBIN

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ABSTRACT. We have previously demonstrated that derivatization of hemoglobin with periodate-modified sugar derivatives incurs increases in the pro-oxidant reactivity and, also, that serum bovine serum albumin can resolve this problem entirely. Here, we described a new polymer based on hemoglobin and another oxygen-containing compound, alginate. The rate of autooxidation increases after derivatization, but serum albumin alleviates this problem. The peroxide reactivity and oxygen affinity were also tested but no significant differences were observed between derivatized and native hemoglobin.

Keywords: *blood substitute, hemoglobin, alginate, oxidative stress*

INTRODUCTION

It was demonstrated that acellular hemoglobin, even when carefully purified, does not represent by itself a solution for hemoglobin-based oxygen carriers (HBOC) because of negative side-effects:[1-3] the first key problem with free hemoglobin is it slow molecular volume, leading to extravasation and indirectly other to problems(high oxygen affinity, vasoactive properties, renal toxicity).[4,5] The challenge in creating a reasonable blood substitute is to increase their molecular weight by chemical and/or genetic modification.[6-8] On the other hand, modification of the protein structure can affect other properties, including autooxidation rates, oxygen affinity, cooperativity, affinity for nitrosative and/or oxidative stress agents, and other functions of hemoglobin such as NO and CO_2 transport.[9]

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Intermolecularly-crosslinked hemoglobins produced using polyaldehydes have been used extensively to modify the negative properties of native Hb. The most common reagents in this respect are glutaraldehyde, formaldehyde, acetaldehyde;[10,11] however, a variety of dialdehydes can also be obtained via oxidation by sodium periodate of diverse water-soluble sugar derivatives (dextran, ATP, starch), or even compounds like polyethylene glycol.[12,13] Here, for the first time, we report that sodium alginate can also be used for crosslinking hemoglobin. Parameters like molecular size, autooxidation rate and oxygen affinity appears to be affected after the polymerization process. The process may in principle be applicable to crosslinking of other materials as well.

RESULTS ANS DISCUSSION

Oxidation of alginate with sodium periodate was performed in order to ring-open the 1,2-diols to yield dialdehydes (Figure 1) which can then be employed for polycondensation of proteins, namely hemoglobin and albumin. Figure 2 shows an SDS-PAGE gel and gel-filtration chromatograms illustrating that oxidized alginate indeed induces an increase in molecular weight. Introduction of BSA in the reaction mixture leads to lower molecular weights for copolymers comparative to the polymers as illustrated in Table 1.

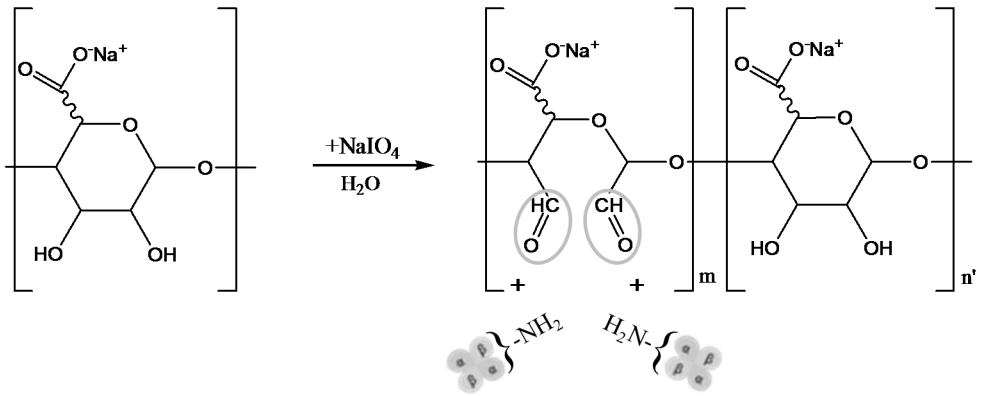

Figure 1. Oxidation of sodium alginate by periodate

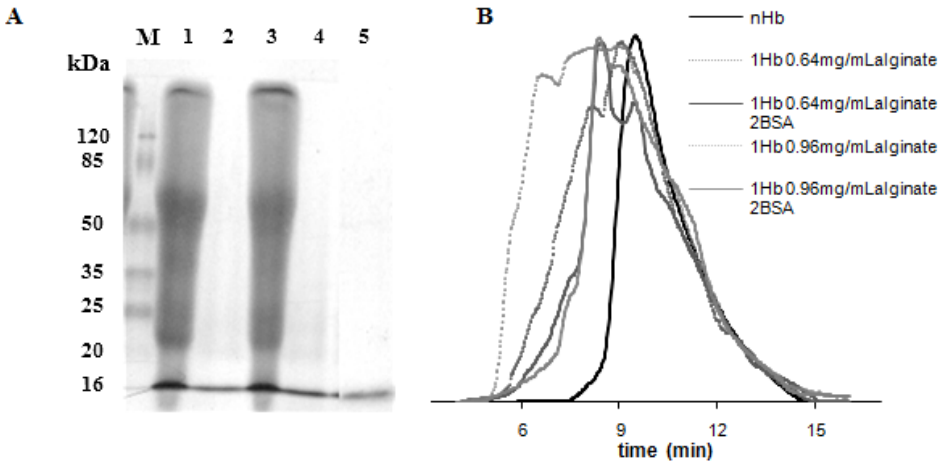

Figure2.A) SDS-PAGE illustrating the influence of alginate concentration upon polymerization degree. **1**- 1 mM Hb + 0.96 mg/mL alginate + 2 mM BSA, **2**- 1mM Hb + 0.96 mg/mL alginate, **3**- 1 mM Hb + 0.64 mg/mL alginate + 2 mM BSA, **4**- 1 mM Hb + 0.64 mg/mL alginate, **5**- native Hb. **B)**Size-exclusion chromatograms for Hb derivatized with different concentration of alginate. Conditions: 20mM Tris pH 7.4, 150 mM NaCl, room temperature

Table 1 also shows the autooxidation rate values. While oxidized alginate induces drastic autooxidation, bovine serum albumin can alleviate this problem. Thus, the amount of metHb formed is ~two times lower if in the reaction mixture is added BSA.

Table 1. Molecular weight and autooxidation rates (express in percentage of metHb formed) values.

	Molecular weight [kDa]	**Autooxidation rate(%)**
native Hb	64	14.29
1Hb + 0.64 mg/mL alginate	210 – 77	30.81
1Hb + 0.64 mg/mL alginate + 2BSA	230 – 64	18.22
1Hb + 0.96 mg/mL alginate	>500(850) – 80	44.82
1Hb + 0.96 mg/mL alginate + 2BSA	170 – 64	22.03

Figure 3 illustrates the time course at 425 nm during the reaction of hydrogen peroxide with poly- and copolymerized Hb. This wavelength is characteristic of ferryl form (FeIV – so called Compound II). [7,14,15] There is no significant difference between the derivatized Hb and the native: both the yield of ferryl form and its stability is similar with to that of native Hb.

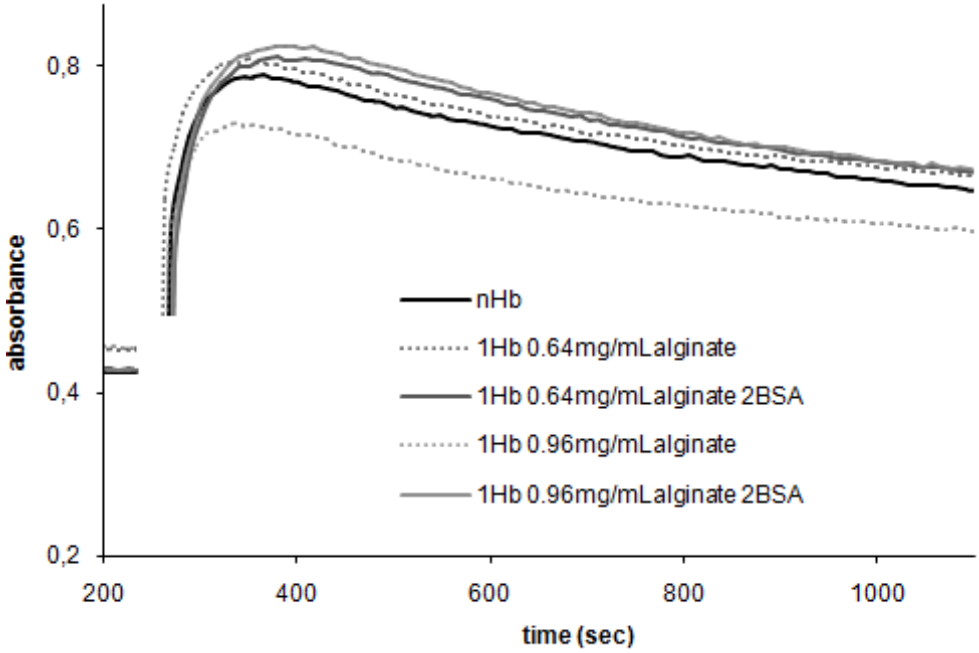

Figure 3. Time course for the reaction of ferric native Hb, poly- and copolymerized Hb with hydrogen peroxide. Conditions: 10 μM protein, 80 μM peroxide, PBS, room temperature

Figure 4 illustrates oxygen binding curves for copolymerized and native hemoglobin. It may be seen that the cooperativity effect disappears in derivatized Hb while affinity towards oxygen increases compared with native hemoglobin, in line with observations previously noted for most other polycondensates based on hemoglobin or other related proteins.[10,16-22]

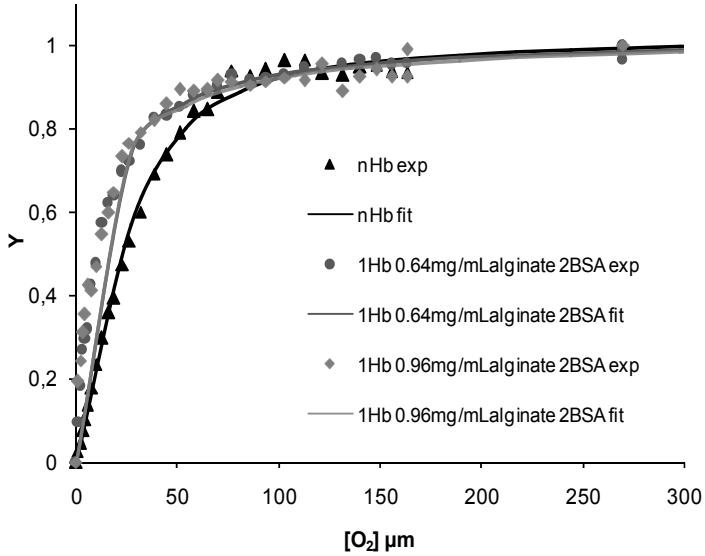

Figure 4. Oxygen saturation curves of native Hb and polymerized Hb. Conditions: PBS, room temperature

Table 2. K_d values and Hill coefficients (n) for poly- and copolymers.

	K_d	n
native Hb	22.99	1.55
1Hb + 0.64 mg/mL alginate +2BSA	10.46	1.04
1Hb + 0.96 mg/mL alginate + 2BSA	9.11	0.92

CONCLUSIONS

The derivatization procedure described here show that polymerization of hemoglobin with oxidized alginate lead to an increase in autooxidation rate, but addition of bovine serum albumin alleviates this problem. Also, molecular size and oxygen affinity was higher in polyHb than in nativeHb. By analogy with other periodate-derived hemoglobin polymers,[13] one should expect this lack of reactivity to be paralleled by improved performance on cellular cultures if using such polycondensates as (semi)-artificial carriers for molecular oxygen. On the other hand, the protocol described here can be viewed as generally applicable for protein derivatization/conjugation.

EXPERIMENTAL SECTION

Bovine hemoglobin was purified from bovine blood following a general protocol of Antonini and Brunori.[23] The blood, freshly drawn on citrate, was centrifugated 15 minutes at 5000 rpm to separate the red blood cells, which were then washed three times with 5 mM phosphate pH 7.4 + 150 mM NaCl. Hemoglobin was manipulated in phosphate buffer saline (PBS) unless otherwise mentioned and concentration in text are given per heme rather than per tetramer. The met form of the hemoglobin were prepared by ferricyanide treatment as previously described.[24-26] Bovine serum albumin (BSA, fraction V, from Sigma-Aldrich, Germany) was used as provided without further purification.

For alginate oxidation 0.01 g/mL solution were prepared in 18.1 MΩ deionized water. The solution was oxidized with sodium periodate ($NaIO_4$, Merck) (0.1 g/mL) for 1 hour at room temperature in order to ring-open the 1,2-diols to yield dialdehydes (Figure 1). For polymerization of Hb with alginate, 1 mM Hb was reacted with 0.64 mg/mL or 0.96 mg/mL alginate oxidized. The reaction was performed under stirring at 4ºC. The reaction was stopped by addition of $NaBH_4$, which reduces imine bonds to stable amines and also quenches excess carbonyl groups. The product was dialyzed in 50 mM Tris buffer with 150 mMNaCl, 7.4 to remove excess $NaBH_4$ and side-products. The resulting protein solutions were subsequently analyzed by 15% SDS-PAGE and by analytical size exclusion chromatography on a Superdex 200 5/150 GL column (GE Healthcare, Sweden), 0.25 mL/min flow rate with a mobile phase of 20 mM Tris pH 7.4 buffer with 150 mM NaCl. The absorbance was monitored at 280 nm. 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).

Autooxidation experiments were performed by incubation of oxy-Hb (native or polymerized) at 37ºC in an incubator and measuring UV-vis spectra of the Hb before and after incubation times of up to 4 hours. The change in absorbance at 630 nm was used to determine the rate of autooxidation. UV-vis spectra were recorded on Agilent 8453 (Agilent, Inc.) and Cary 50 (Varian, Inc) instruments Cary 50 (Varian, Inc) instruments.

Dioxygen affinity and autooxidation measurements were in PBS 7.4 at room temperature.

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