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Synthesis and characterization of hemoglobin conjugates with antioxidant enzymes via poly(ethylene glycol) cross-linker (Hb-SOD-CAT) for protection from free radical stress

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Abstract

Hemoglobin (Hb) conjugated with the antioxidant enzymes (SOD and CAT), by employing dicarboxymethylated poly(ethylene glycol), was designed for protection of hemoglobin against free radicals. In this study, the conjugation process was confirmed by employing SDS-PAGE and SEC techniques. The average molecular weight of the conjugates was estimated to be around 1000 kDa. The enzymatic activities of the SOD and CAT in the conjugates (Hb-SOD-CAT) after conjugation were found to retain greater than 70% and 90% of the original bioactivity. Results show that antioxidant enzymes helped minimize methemoglobin (non-carrier of oxygen) formation during the conjugation process and also during storage at 4°C over a period of one month. In summary, the optimized (1:10 Hb/PEG) cross-linked conjugates with antioxidant enzymes showed protective properties from severe free radical stresses when incubated with hydrogen peroxide (0.1 and 1mM) and xanthine (1mM)/xanthine oxidase (10 and 20mUnits/mL) system.

Keywords

Crosslinked hemoglobin; Antioxidants (SOD and CAT); Poly (ethylene glycol); Free radical stress; Methemoglobin

1. Introduction

Cell-free hemoglobins intended for blood transfusions in hemorrhagic shock,[1] anemia,[2] ischemia heart conditions etc.,[3] have been widely investigated as artificial oxygen carriers. Cell-free hemoglobin is not stable and the tetramer dissociation is known to cause nephrotoxicity[4]. Another problem with the cell-free hemoglobin is vasoconstriction and is considered as one of the main reason for their failure as oxygen carriers in many clinical trials [5]. To overcome this problem, a number of different approaches have shown very promising results in stabilizing the tetrameric hemoglobin: crosslinking, [6] polymerization, [7] recombinant technology,[8] encapsulation[9,10] etc. Stabilizing hemoglobin by these

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approaches helped deliver oxygen efficiently in blood transfusions and other conditions under the normal partial oxygen pressures in the body. Most of these approaches only stabilize the alpha and beta tetramers of hemoglobin but do not offer much protection from autooxidation [11] and free radicals such as hydroxyl and superoxide anions [12]. Inside the RBCs, high levels of antioxidant enzymes such as Superoxide dismutase (SOD) and Catalase (CAT) help in the protection of hemoglobin from free radical damage [13]. To have a protective effect in these conditions, there is a need to design a hemoglobin-based oxygen carrier system with an antioxidant defense mechanism that has the ability to protect itself from both the hypoxia-induced free radicals and environmental radical stresses that primarily protect the hemoglobin's oxygen-carrying capability [14].

Previously, glutaraldehyde crosslinking was shown to stabilize hemoglobin except that the increasing the concentration, glutaraldehyde to hemoglobin molar ratio up to 40:1, was shown to increase the methemoglobin content [15]. Crosslinking by addition of antioxidant enzymes (SOD and CAT) with glutaraldehyde had reduced oxidation [16] and renal damage by eliminating free radical mediated injury after ischemic reperfusion [17]. However, release of free glutaraldehyde crosslinker by hydrolytic degradation of imine bonds in aqueous solutions [18] and thus associated toxicity was shown as a big concern [19]. Promotion of vasoconstriction by scavenging nitric oxide from the vasculature by glutaraldehyde crosslinked products is also a serious concern [20,21]. In clinical studies, hemoglobin's crosslinked with glutaraldehyde was shown to cause myocardial infarction and deaths [22]. Antioxidants were also introduced by using complementary chemistry of maleimides and sulfhydryls, however; the NO scavenging vasoactive nature of this method still may be a problem [23]. It is therefore desirable to provide a crosslinking reagent that is of low cytotoxicity and biocompatible.

On the other hand, PEG-based polymeric modifications have shown to reduce hypertensive responses and Nitric Oxide (NO) scavenging effect and thus inhibit vasoconstriction. This may be attributed to the beneficial effects associated with PEG polymer such as increase in molecular size and hydrodynamic volume [24]. Extensive literature on usefulness of PEG in pharmaceutical industry for proteins demonstrates that approaches employing this polymer is safe and may be used similarly to conjugate the hemoglobin. There is an effect of PEG molecular weight used in the conjugates. Hb modified by the lower molecular weight PEG is shown to lessen the vasoconstrictive effect than the higher molecular weight PEG [25]. PEG modified Hb has been shown to have unique advantages of low cooperativity and high oxygen affinity or low p_{50} [24,26]. Also it has been reported that the cysteine modification on hemoglobin using PEG-maleimide chemistry has shown to increase the autooxidation of Hb and further enhanced oxidation by PEG chains in the presence of hydrogen peroxide [27] and shows the need for inclusion of antioxidant enzymes.

Our previous research had shown that crosslinked hemoglobin improved the functionality of encapsulated isolated pancreatic beta cells in transplanted diabetic mice by maintaining oxygen supply to encapsulated cells [28,29]. However, the shelf-life of hemoglobin was limited because of its conversion to methemoglobin and free radical damage induced by autooxidation of hemoglobin. In this research, to overcome the above-mentioned problems and based on current information in the literature, the use of polyethylene glycol to conjugate the available amino groups (lysine's, arginine, asparagines, glutamine, histidine) on the oxygen carrier hemoglobin (Hb), and antioxidant enzymes SOD and CAT was investigated. Although polymerization of hemoglobin can stabilize hemoglobin, addition of SOD and CAT may be a better strategy provided the enzymatic actions inhibit the conversion of hemoglobin to methemoglobin. This particular conjugate design may also help the protection of hemoglobin from oxidation during chemical conjugation, autooxidation and free radical stress during storage, retention of the enzymatic activity post

conjugation, and their size suitability for co-encapsulation with isolated islets for cell transplantation.

2. Materials and Methods

2.1. Materials

Freshly pooled bovine red blood cells were purchased from Innovative Research (Novi, Michigan IC100-0410). Polyethylene glycol (Molecular weight: 2 kDa), Superoxide dismutase SOD-S7571 from bovine erythrocytes, Bovine liver catalase CAT-C40, tertiary butoxide, ethyl bromoacetate, N-Hydroxy-Succinimide (NHS), N,N'-Dicyclohexylcarbodiimide (DCC), hydrogen peroxide (H₂O₂), xanthine, xanthine oxidase (XO) were purchased from Sigma Chemical Co. (St. Louis, USA). Dialysis membranes were purchased from spectrum labs (Rancho Dominguez, CA). Amicon Ultra-15 Centrifugal Filter Units, 50 and 100 kDa were purchased from Millipore Corporation (Billerica, MA).

2.2. Isolation of Hemoglobin

Freshly withdrawn bovine red blood cells (RBCs) were used to isolate hemoglobin from the RBCs. Briefly; the RBCs were washed twice with normal saline solution by centrifuging at 2500 rpm for 20 minutes. Hemoglobin was extracted from the washed RBC by mixing with 4:1 water and dichloromethane by shaking for 5 minutes. Released hemoglobin that partitioned into the aqueous phase was collected by further centrifuging at 2500 rpm for 20 minutes. The process was repeated twice and the isolated hemoglobin was centrifuged to remove any remaining organic solvent. Isolated hemoglobin was dialyzed for 48 hours using 50 kDa dialysis membrane. This solution was then transferred to 100 kDa dialysis tube and dialyzed for 24 hours where only pure hemoglobin was diffused out, which can be further concentrated by using Amicon membranes. The purity of hemoglobin was verified by SDS-PAGE and quantified by using Drabkin's method for quantification [28].

2.3. Modification of PEG

PEG (2 kDa) was customized for conjugation (Fig.1.) by employing previously reported methods [28,30,31]. Briefly, the hydroxyl group of PEG was converted to ethyl-protected carboxymethylated PEG by using potassium tertiary butoxide and ethyl bromoacetate. Hydrolysis of the product by using sodium hydroxide resulted in mixture of carboxylic (mono and di) PEG's, which was further purified by ion-exchange chromatography. This was converted to NHS active ester by using DCC and NHS to yield PEG-NHS, which was used for the conjugation of amino groups on Hb, SOD and CAT. Before crosslinking the activated PEG diacid (into NHS) was characterized by ¹H NMR spectroscopy and thin-layer chromatography.

2.4. Conjugation of Hb (hemoglobin) with SOD (Superoxide dismutase) and CAT (Catalase)

In Hb-only conjugations, isolated bovine Hb was conjugated with PEG (Fig.1.) by varying Hb/PEG molar ratios between 1:5 and 1:30. And in Hb conjugations with antioxidant enzymes (SOD and CAT), identical Hb/PEG ratios that were used in Hb-only conjugations were used and the ratio between SOD and CAT was fixed at 30000 to 300000 enzyme units in all conjugation reactions[28,32]. All hemoglobin conjugation reactions in the PBS buffer (pH 7.4) (with and without antioxidant enzymes) were carried out by adding activated PEG and stirring for 3 hours at 4°C. Unreacted PEG and protein components were removed by using 100 kDa dialyzing membranes and by changing PBS buffer for 24 hrs. The product was further concentrated to the desired concentration by using the Amicon centrifugation filters. The product was filter sterilized by using 0.22-μm pore size syringe filter and stored at 4°C until further use.

2.5. SDS-PAGE

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of PEG-conjugated hemoglobin with and without antioxidant enzymes was performed on 4-12% Bis-Tris NuPAGE gels under reducing conditions by employing XCell SureLock Mini-Cell apparatus (Invitrogen life technologies, Carlsbad, CA). The samples were heated (denatured) at 70°C for 10 min and 1-10 µg equivalent hemoglobin protein was (20µl) applied onto the gel. Electrophoresis was carried out at a constant 150 V for about 90 min. SeeBlue Plus2® pre-stained molecular weight standard was added on all gels and the separation was achieved with NuPAGE® MES SDS running buffer as described in the manufacturer instructions (Invitrogen). For visualizing, the gels were rinsed with ultrapure water (Millipore) and stained by SimplyBlue™ safe stain (Invitrogen) followed by extensive washing in pure water for protein detection. The completion of the conjugation process was assessed by comparing with appropriate molecular weight standards.

2.6. SEC analysis of conjugates

Size exclusion chromatography (SEC) was performed by employing Agilent 1100 Liquid chromatography separation system for determining the weight-average molecular weight with an absorbance detection at 280nm (Agilent Technologies, Santa Clara, CA). The separation was performed on a BioSEP-S-4000 column (Hydrophilic bonded silica with 5µm particle size and 500 Å pore size) capable of resolving high molecular weight proteins ranging in size between 15 kDa - 2000 kDa (Phenomenex, Torrance, CA). The column was equilibrated by using the mobile phase (0.1 M potassium phosphate buffer, pH 7.4). Samples were diluted in PBS and separated through the column with a flow rate of 0.5 ml/min at room temperature. Fifty micro liters of crosslinked Hb-Hb and Hb-SOD-CAT conjugates solutions were injected and the molecular weight of the samples determined from a standard curve derived from the retention times of protein reference standards (Sigma molecular mass marker kit). Molecular standards include carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), beta-amylase (200 kDa), apoferritin (443 kDa), thyroglobulin (669 kDa) and Dextran Blue (2000 kDa). All the data were analyzed with Agilent Chemstation software. Molecular weight and their distributions by SEC are represented and described as by Hu Tao [33] and Nonier MF [34].

2.7. Analysis of absorption spectra of conjugated Hemoglobin's

The qualitative analysis of conjugated hemoglobin's was carried out by measuring the visible absorption spectrum of conjugated hemoglobin's with and without antioxidant enzymes, by employing UV-visible spectrophotometer (SpectraMax, Molecular devices; Sunnyvale, CA). 20 µM hemoglobin conjugates was placed in the UV cuvette and visible absorption spectrum was scanned between 450 and 750nm at a rate of 2 seconds.

2.8. Methemoglobin Quantification

The amount of methemoglobin content in conjugated samples were determined by using the method described by Patton et al[35]. Briefly, a sufficient amount of the conjugated protein was taken in a UV-visible cuvette by diluting the sample (such that it gave an absorbance less than one unit at 630 nm), referred to as A1. This value corresponds to the total hemoglobin content in the sample which consists of oxy-, deoxy-, and the methemoglobin content. Same sample was treated with one drop (50 µL) of potassium cyanide solution (1 part of 10% KCN and 1 part of PBS pH 7.5) and allowed to react for 5-10 minutes. A second absorbance measurement on the same sample was done at 630 nm referred to as A2. The difference between A2 and A1 was calculated and by using the extinction coefficient value for methemoglobin (3.7 cm mM)⁻¹ in the equation below gave the methemoglobin content in the samples. The total hemoglobin content in the sample was measured by taking

same amount of conjugates in other cuvette and by adding one drop (50 μL) of 20% $\text{K}_3\text{Fe}(\text{CN})_6$ with a dilution. After the conversion of oxy- and deoxy- hemoglobin in the sample to methemoglobin, another drop (volume) of 10% KCN was added and the absorbance peak at 540nm was measured (A3). Using the extinction coefficient of 11 (cm mM^{-1}) for cyanomethemoglobin, the hemoglobin content was calculated by equation below. Percentage methemoglobin was calculated from the ratio of total methemoglobin and hemoglobin contents in the sample.

$$\begin{aligned}\text{Methemoglobin} &= \frac{A1-A2}{3.7} * \text{dilution factor} \\ \text{Hemoglobin} &= \frac{A3}{11} * \text{dilution factor} \\ \text{Methemoglobin \%} &= \frac{\text{Methemoglobin}}{\text{Hemoglobin}} * 100\end{aligned}$$

2.9. Enzymatic Assay for Superoxide Dismutase (SOD)

The SOD activity was determined based on a previously reported cytochrome C reduction assay method [36-38]. Superoxide dismutase activity was measured by the rate of reduction of cytochrome C inhibition by the superoxide radical generated by using xanthine and xanthine oxidase system at pH 7.8 and room temperature. The samples contained a mixture of 50 mM potassium phosphate, 0.01 mM cytochrome C, 0.1 mM ethylenediaminetetraacetic acid, 0.05 mM xanthine, 0.005 units of xanthine oxidase and conjugated hemoglobins. The uninhibited rate of reduction was adjusted by changing the amount of xanthine oxidase enzyme used in the assay such that the reaction produced an initial change in the absorbance between 0.025 - 0.005 per minute at 550nm. A 50% rate reduction of cytochrome C in the inhibited samples corresponds to one unit of SOD. Background activity by hemoglobin component in the antioxidant conjugated samples was subtracted by assaying equivalent activity in Hb-only conjugated samples. Inhibition rate and enzyme units per mL were calculated by below equations:

$$\begin{aligned}\text{Percent inhibition} &= \frac{\text{Change in Absorbance per min at 550nm in uninhibited-inhibited sample}}{\text{Change in Absorbance per min at 550nm in uninhibited-bank sample}} * 100 \\ \frac{\text{units}}{\text{mL}} \text{ of enzyme} &= \frac{\text{percent inhibition} * \text{dilution factor}}{(50\%) * \text{volume of enzyme sample used in mL}}\end{aligned}$$

2.10. Enzymatic Assay for Catalase (CAT)

Catalase activity was measured by monitoring the decomposition rate of hydrogen peroxide at 240nm [39-41]. Catalase was assayed in the conjugates by measuring the enzyme catalyzed decomposition rate of hydrogen peroxide as described in Sigma-Aldrich test procedure for the enzymatic assay for Catalase (Sigma Aldrich, EC 1.11.1.6). A fresh 0.036% (w/w) hydrogen peroxide solution (H_2O_2) was prepared in 50 mM potassium phosphate buffer, pH 7.0 at room temperature such that its initial absorbance was 0.550 units. 2.9 ml of the above reagent was mixed with 0.1ml of conjugated proteins in a cuvette and the time taken for reduction in the absorbance between 0.45 to 0.40 absorbance units was calculated. The number of enzyme units in the sample was calculated as per the equation below.

$$\frac{\text{enzyme}}{\text{ml}} \frac{\text{Units}}{\text{ml}} = \frac{(3.45) * \text{Dilution Factor}}{\text{Time in minutes for decrease in Absorbance from 0.45 to 0.40} * (0.1\text{ml of enzyme sample})}$$

3.45 in the equation represent the decomposition of 3.45 μM of hydrogen peroxide in a 3.0 ml reaction mixture that decreases the absorbance from 0.45 to 0.40 absorbance units. The background activity of hemoglobin in the samples was removed by calculating the equivalent hemoglobin activities in non-antioxidant conjugated samples.

2.11. Peroxide and superoxide challenge

To examine the protective effect of hemoglobin in Hb-antioxidant enzyme conjugates, the 1:10 Hb-Hb and Hb-SOD-CAT conjugates were tested by challenging with superoxide anion generated by xanthine (1mM)/xanthine oxidase (10 and 20mUnits/mL) system and hydrogen peroxide (0.1mM and 1mM) which generates hydroxyl free radicals. 20 μM hemoglobin equivalents were used in every reaction and were incubated with free radicals for 30 min, 3hrs, 24 hrs and 48 hours at room temperature in the UV-visible cuvettes. The protection of hemoglobin and conversion to non-oxygen binding methemoglobin was evaluated by using UV-visible spectrophotometry.

2.12. Statistical Analysis

The statistical significance between conjugated hemoglobin's with and without antioxidant enzymes were analyzed by one-way analysis of variance followed by Holm–Sidak test to compare means of group (for differences between enzymatic activity and polymer conjugation ratios, conjugation and storage effect on methemoglobin formation) by analyzing estimates of variance. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. SDS-polyacrylamide gel electrophoresis

SDS-PAGE analyses (Fig.2) demonstrated the formation of crosslinked products at all ratios of Hb to PEG (1:5, 1:10, 1:15, 1:20 and 1:30), both with and without antioxidant enzymes. A range of different molecular weight conjugates were obtained (~ 100 kDa products and > 180 kDa) when compared to the molecular weight standards used in SDS-PAGE. The smear-like or ladder-like appearance of the SDS-PAGE indicates the differential mobility of the conjugates because of the differences in sizes/molecular weights of the polymerized conjugates. However, SDS analysis only demonstrated the formation of the polymerized products and does not specify the exact size of the conjugates with molecular weight higher than the limit of detection (180 kDa). This method was the first piece of evidence in confirming the crosslinking in Hb-only conjugates and Hb/antioxidant enzymes crosslinked conjugates.

3.2. SEC analysis of conjugates

SEC analysis confirmed the formation of large PEG crosslinked hemoglobin conjugates, with a broad elution spread, with and without antioxidants, in all Hb/PEG molar ratios (Fig. 3). When Hb-only (without antioxidant enzymes) conjugates were polymerized by employing PEG, a heterogeneous population of crosslinked hemoglobin was obtained with molecular weights ranging between ~ 150 kDa to ~ 2000 kDa. The chromatogram showed prominence towards the lower retention time ('left-sided') than the higher retention times ('right-side') of the broad peak, indicating that majority of the crosslinked Hb-only conjugates comprised of high molecular weight species near ~ 2000 kDa. Different Hb/PEG ratios yielded similar molecular weight distribution profiles with only slight differences. When antioxidant enzymes were added during crosslinking, the conjugates' chromatograms showed a molecular weight range between ~ 100 kDa to ~ 1500 kDa. However, Hb conjugates with antioxidant enzymes showed a major peak shift towards higher retention times ~ 100 kDa time ('right-sided') unlike Hb-only conjugates. Minimal differences were

seen when different Hb/PEG ratios were used even with the antioxidant enzymes. The average molecular weight for all the conjugates appeared to be around ~800 - 1000 kDa with broad peak profiles and dramatic differences in elution profile for the conjugation of Hb with and without antioxidant enzymes.

3.3. Visible Absorbance Spectra of Conjugated Hemoglobins

Oxyhemoglobin shows characteristic absorbance peaks at 540 and 575nm. Methemoglobin shows a characteristic absorbance peak at 630nm. Also visually, in the solution state, hemoglobin appears as red-colored and methemoglobin is dark brown. The results (Fig.4) showed that when hemoglobin is self-crosslinked, there is an increased amount of methemoglobin formation with increasing amounts of the crosslinking agent-PEG. The change in absorbance was found to vary between 0.01 - 0.2 units when Hb was self crosslinked for Hb/PEG molar ratio between 1:5 and 1:30. Significant change in the absorbance at 630nm is noticed in 1:15, 1:20 and at 1:30 Hb/PEG crosslinking ratios. However, the addition of antioxidant enzymes during crosslinking significantly decreased the formation of methemoglobin that is demonstrated by lower absorbance at 630nm. This decrease is clearly significant upto the ratio of 1:20 (Hb/PEG ratios with antioxidant enzymes) and only a slight increase is observed at 1:30 Hb/PEG ratio. The change in absorbance varied between 0.01 - 0.06 units which is dramatically lower when compared to self-crosslinked hemoglobins.

3.4. Methemoglobin Quantification

The results (Fig.5) indicate that the crosslinking of hemoglobin by PEG enhanced the conversion of hemoglobin to methemoglobin in absence of antioxidant enzymes. For self-crosslinked hemoglobins, methemoglobin content increased with the increasing amount of PEG-conjugating agent within a day. This increase was greater than 10% for Hb/PEG ratio 1:15 and greater than 40% for ratios 1:20 and 1:30. Such Hb-only conjugates are unlikely to be useful as an oxygen carrier (high amount of methemoglobin), unless there is a mechanism to reverse this process can be incorporated. However, on the addition of antioxidant enzymes during the crosslinking, there was a significant decrease in the amount of methemoglobin at all Hb/PEG ratios. The complete absence of methemoglobin was observed in Hb/PEG ratios 1:5, 1:10 and 1:15, and only 1.6 ± 0.6 % in 1:20 and 7.3 ± 1.3 % in 1:30 ratios were observed in Hb-conjugates with antioxidant enzymes SOD and CAT. These results indicate that the conjugating agent had significant effect in increasing the methemoglobin content and emphasizes the need for the addition of antioxidants enzymes to inhibit the conversion of hemoglobin to methemoglobin. Although methemoglobin is formed in all crosslinking's without antioxidants, statistical analysis showed that significant differences were found only among Hb/PEG ratios 1:15, 1:20 and 1:30 between Hb-only and Hb-SOD-CAT crosslinked formulations (p-value < 0.001).

The results also indicate that if self-crosslinked Hb conjugates are stored at 4°C for a month, all the formulations had methemoglobin content of more than 10%. A significant increase of methemoglobin was observed in Hb/PEG ratios 1:5 (from 2.2 ± 0.1 % to 10 ± 1.2 %), 1:10 (3.4 ± 0.6 % to 16.6 ± 1 %), and 1:15 (14.6 ± 0.5 % to 21.4 ± 1.5 %) formulations. The statistical analysis also showed that there was a statistically significant increase in the amount of methemoglobin among all Hb/PEG ratios (1:5 to 1:30) of crosslinked hemoglobins at days 1 and 30, without antioxidants (p-value < 0.001). However, for crosslinked hemoglobins with antioxidant enzymes SOD and CAT, the amount of increase in methemoglobin content was less than 10% in Hb/PEG ratios 1:5, 1:10, 1:15 and 1:20 conjugating ratio of the polymer. Only, 1:30 ratio showed an increased methemoglobin content (from 7.3 ± 1.3 % to 15.52 ± 2.1 %). This result also demonstrates the beneficial

effect of addition of SOD and CAT for the long-term protection of hemoglobin against oxidative stresses during storage.

3.5. Percent Retention of Enzymatic activity of Superoxide Dismutase (SOD)

For the effective removal of superoxide anion free radical and the protection of the hemoglobin from free radicals, the enzymatic activity of the conjugated SOD needs to be preserved after crosslinking with PEG and Hb in the final conjugate mix. The results show that the enzymatic activity of the conjugated SOD was reduced in comparison to the enzymatic activity of the control (free) SOD (Table 1). However, SOD's enzymatic activity in all the conjugates was shown to be 70-80% of the original. Although the enzymatic decreased compared to the enzymatic activity of the control SOD, the statistical differences between different PEG ratios of conjugated hemoglobins with SOD was not seen ($p = 0.154$). This indicates that increasing amount of the PEG used in conjugation reactions had no significant effect on SOD's enzymatic activity.

3.6. Percent Retention of Enzymatic activity of Catalase (CAT)

For the effective removal of peroxide free radicals and thus for the protection of the hemoglobin from free radicals, the enzymatic activity of the conjugated CAT is required to be preserved after crosslinking with the PEG in the final conjugate mix. The results (Table 2) show that the Catalase enzymatic activity is similar to the enzymatic activity of the free Catalase (control). The CAT activity in all the conjugates was estimated to be between 90-99%. No statistically significant differences ($p = 0.382$) in the enzymatic activity was obtained on increasing the amount of PEG polymers for conjugating hemoglobin with CAT.

3.7. Protection of Hemoglobin from Hydrogen Peroxide Challenge

On challenging (Hb/PEG 1:10 molar ratio, 20 μ M) Hb-only conjugates and Hb-SOD-CAT conjugates with hydrogen peroxide (0.1mM and 1mM) for 0.5, 3, 24, and 48 hours, there was a significant protective effect on crosslinked hemoglobins with antioxidant enzymes as shown in Fig.6. When Hb-only crosslinked conjugates were challenged with hydrogen peroxide (1mM), the absorption peak for methemoglobin appeared within 30 minutes of incubation and the increase in absorbance at 630nm was approximately 0.11 units at 30 minutes and 0.15 units at 48 hours. The corresponding characteristic hemoglobin absorbance peak decrease was found to be 0.33 (30 mins) and 0.46 (48 hrs) units at 540nm, and 0.37 (30 mins) and 0.50 (48 hrs) units at 575nm. The results further explain this similar trend of Hb-only conjugates showing damage/degradation at both concentrations (0.1 and 1mM) of hydrogen peroxide used in the *in vitro* experiments. Insignificant increase in the methemoglobin content peak after 24 hours in Hb-only formulations may be due to complete degradation of hemoglobin or further degradation of hemoglobin into ferryl hemoglobin or other degradation products of hemoglobin [42]. The characteristic hemoglobin absorbance peak changes at 540 and 575nm in Hb-only conjugates showed significant hemoglobin damage and near complete degradation of hemoglobin at 48hrs. The results also show that a small amount of peroxide is enough to cause hemoglobin damage and render it unsuitable as an oxygen carrier. This demonstrates that even though crosslinking may stabilize the tetramers of isolated hemoglobin, free radicals can easily damage the unprotected hemoglobin. On conjugating hemoglobin with antioxidant enzymes (SOD and CAT) the crosslinked formulation showed significant protection of hemoglobin. The results also show that the increase in absorbance of methemoglobin peaks are less than 0.01 units in all cases at 48 hours of challenge at room temperature, indicating near complete protection of hemoglobin formulation from hydrogen peroxide generated free radicals. The graphical overlay of hemoglobin without peroxide challenge was practically similar to the hydrogen peroxide-challenged hemoglobin conjugates containing the antioxidant enzymes. These

results strongly indicate that the hemoglobin may be afforded enhanced protection against the damaging effects of hydroxyl and peroxide radicals by incorporation of the antioxidants.

3.8. Protection of Hemoglobin from Superoxide challenge

Challenging (Hb/PEG 1:10 molar ratio) conjugates with xanthine (1mM) and xanthine oxidase mixture (10mU/mL and 20mU/mL) showed inhibition of hemoglobin conversion to methemoglobin (Inhibition of decreased hemoglobin absorption peaks at 540 and 575 nm) only if antioxidant enzymes were included in conjugation reaction (Fig.7). When Hb-only crosslinked conjugates were challenged, a slight increased absorbance at 630nm was observed with a significant decrease in absorption at 540 and 575nm indicating the reduction in the hemoglobin content within 30 minutes and a continual decrease over a period of 3 hours. However, hemoglobin conjugates with antioxidants enzymes SOD and CAT showed absorption profiles almost similar to the unchallenged conjugates indicating near complete prevention of degradation of hemoglobin and inhibition of free radical damage from the superoxide anion for at least 3 hours.

The absorption spectra of the conjugates without antioxidants enzymes after 24 hours of superoxide free radical challenge showed a significant decrease in the absorbance at 540nm (0.10 units with 10mU/mL xanthine oxidase and 0.16 units with 20mU/mL xanthine oxidase when compared to unchallenged hemoglobin) and at 575nm (0.16 units with 10mU/mL xanthine oxidase and 0.24 units with 20mU/mL xanthine oxidase). There was a significant increase in the methemoglobin absorbance at 630nm (0.13units with 10mU/mL xanthine oxidase and 0.15 units with 20mU/mL xanthine oxidase) when compared to unchallenged conjugated hemoglobin. A considerable increase (from 0 units to 0.07 units) in the amount of methemoglobin was also noticed even in formulations with antioxidants. However, when compared to the formulations without antioxidants the increase is lower (0.07 with and 0.15 units without antioxidant enzymes) and thus emphasizing the importance of antioxidant enzymes in the conjugates. Similar trend was seen with absorption spectra of the conjugates after 48 hours and evidently there is pattern suggesting better protection of hemoglobin's with antioxidants than the hemoglobin-only conjugates. Complete protection from the superoxide anion may be achieved by increasing and further optimizing the amount of SOD enzyme in the conjugates. However, in our studies we challenged hemoglobins with higher amounts of superoxide anions that that may be expected *in vivo*.

4. Discussion

SDS-PAGE distribution of the Hb-only polymerization clearly showed the heterogeneous distribution of conjugates in the crosslinked formulation, which did not differ from the Hb polymerized with antioxidant enzymes, as the band distributions of SOD and CAT in the gel was overlapped with Hb. This type of SDS-PAGE band pattern is often observed and expected in PEG-driven chemistries where chemical modifications are or cannot be tightly controlled[43]. This also indicates a high degree of intramolecular crosslinking within the hemoglobin molecule to stabilize the tetrameric structure and also a high degree of intermolecular crosslinking between Hb, SOD and CAT and thus result in an overall increase of molecular weight of the Hb conjugates. However, the difference in the average molecular weight for different PEG and Hb ratios were only slightly different with similar distribution profiles. For Hb-SOD-CAT conjugates, the intensity shift in the peak towards lower molecular conjugates or the preference for the formation of lower molecular conjugates may indicate the decrease in the intermolecular crosslinking. Although there is change in the elution profiles, as expected, the heterogeneous mixture of hemoglobin conjugates are suitable for functions such as encapsulation with isolated cells and provide oxygen near to isolated encapsulated cell [13].

The polymerization method involves conjugation of amine groups on amino acid residues, primarily lysine within the hemoglobin and also on the antioxidant enzymes [28]. It was shown in a recent study that the conjugation of PEG-chains to lysine residues of Hb by the thiolation mediated PEGylation induced structural changes by increasing the hydration shell, which further helped in the stabilization of relaxed high affinity conformation [44]. It was shown that methemoglobin levels needs to be maintained below 10% in PEG-conjugated hemoglobins to efficiently oxygenate tissues [45]. In addition, methemoglobin levels should also remain below ($< 10\%$) for extended periods following PEG-hemoglobin administration [45]. Human hemoglobin conjugated to maleimide-activated poly(ethylene glycol) was shown to increase the *in vitro* autooxidation at room temperature and was also prone to enhanced oxidation by reactive oxygen species. The rate of *in vitro* heme loss was five times higher once conjugates were oxidized to methemoglobin, and was attributed to the loss of heme in both alpha and beta chains[46]. PEGylation of hemoglobin at Cys-93(beta) and Val-1(beta) was shown to perturb the environment in the heme pocket and increase autooxidation. This was also shown to be the direct effect of chemistry of conjugation and not of the PEG molecules. However, the PEG chains enhanced autooxidation by promoting the nucleophilic attack of heme in the presence of hydrogen peroxide by increasing the water molecules in the hydration layer of hemoglobin [47]. Even though, the exact mechanism that causes the increased methemoglobin in our study is not known, it is clearly shown that the mechanism that causes the increased methemoglobin formation during conjugation is significantly minimized or reduced by addition of antioxidant enzymes, possibly a selective chemical or physical modification in hemoglobin that allowed efficient protection against oxidant stress, during conjugation and storage at 4°C . Recently, hemoglobin-SOD chimeric protein conjugates co-expressed by the human alpha and beta hemoglobin chain and manganese SOD gene together in *Escherichia coli* exhibited 44% lower autooxidation rate and have proven to be beneficial[48].

PEG crosslinking may have brought the conformational changes in the SOD enzyme active site particularly by decreasing the effect of moving positively charged lysine's involved in the substrate superoxide anion to the active site and thus altering the enzymatic activity of SOD [49]. The increasing amount PEG in the conjugation reaction did not further decrease the enzyme activity as all the 20 lysine's in dimeric SOD enzyme are not involved in conjugation due to steric hindrance. The loss of enzymatic activity depends on the type of conjugating agent and our results are consistent with observations made by Veronese et al[50]. It was shown that methemoglobin can be reduced in Hb-loaded nanoparticles by prereduction of raw Hb by using sodium dithionite and post-encapsulating with SOD and CAT [10]; however, the long-term stability, leaching out of the enzymes and Hb in non-crosslinked or polymerized state may become a concern.

In normal, *in vivo* conditions, hemoglobin is present in ferrous form (Fe^{+2}) and functions as an effective oxygen carrier in this state, which is protected by both enzymatic and non enzyme-rich antioxidant environment. Oxidized hemoglobin, methemoglobin, is converted back to hemoglobin by methemoglobin reductase (metHb) system and maintained in Fe^{+2} state at levels above 98%. Spontaneous autooxidation of hemoglobin causes the production of superoxide anion ($\text{O}_2^{\cdot -}$) by the removal of electron from the ferrous hemoglobin and its conversion into ferric (Fe^{+3}) methemoglobin. The superoxide anion will further cause the production of hydrogen peroxide by various indirect mechanisms and cause damage to hemoglobin and its surrounding cellular environment [51]. In normal conditions, iron released from hemoglobin or free Hb will be removed by complexation with Haptoglobin and avoid heme related toxicity [42]. However, in excessive oxidative situations, the hemoglobin will be converted to methemoglobin (Fe^{+3}), ferryl hemoglobin (Fe^{+4}) and further release of free iron and globulin. This excessive release causes free iron to react with H_2O_2 and cause the production of hydroxyl radical ($\text{OH}^{\cdot}$) by Haber-Weiss and Fenton

chemistry [51]. This event will further enhance the release of heme which was shown to cause cellular damage in both humans and animal models by different pathways [52]. Simoni et al. had also shown that particularly modified hemoglobin solutions had increased the lipid peroxidation and thus the cellular damage mediated by these free radical processes [53].

Current PEG crosslinked Hb-SOD-CAT has shown less than 2% methemoglobin for Hb/PEG ratio of 1:20 on contrary to a reported 7% value for Hb/glutaraldehyde ratio of 1:17 crosslinked product, immediately after crosslinking. The content of methemoglobin had further increased to 10% within only 5 days for Hb/glutaraldehyde ratio of 1:17 as compared to below 10% level for Hb/PEG ratio of 1:20 within 30 days when incubated at 4°C [54]. These results clearly show the advantage of PEG over glutaraldehyde and we anticipate the conjugates to perform even better in long-term studies. The other advantage of PEG based conjugate is its effectiveness in the severe hypoxic and low partial pressure conditions where the conjugate has a p50 of 7 mmHg (Unpublished result) as compared to 25 mmHg of high p50 glutaraldehyde system [55]. We chose the hemoglobin conjugates with Hb/PEG ratio of 1:10 for testing the protective effect against free radicals because these conjugations had shown lower methemoglobin content during conjugation and storage (for one month). Our results had shown that crosslinked hemoglobins with antioxidant enzymes had clearly minimized or inhibited the oxidation of hemoglobin by free radicals and thus its conversion to methemoglobin. In cases of isolated cell transplantations such as beta cells, cardiac cell storage, there is a need for hemoglobin conjugates that protect cells against both hypoxic and free radicals stresses. Enhancing the life of oxygen carrying agent with antioxidant enzymes may help in even longer protection of isolated cell transplantations by reducing the intracellular and extracellular oxidative stresses. The peroxide and superoxide radicals used in our studies are significantly higher than inflamed or hypoxic states and we expect to see superior functionality of our conjugates in *in-vivo* situations as these high amounts (concentrations tested in our *in-vitro* experiments) of localized hydrogen peroxide concentrations are not commonly observed [56,57].

5.0 Conclusion

The goal of the work was to develop large molecular weight hemoglobin conjugates crosslinked with antioxidant enzymes by using a biocompatible polymer such as PEG. Our results confirm the formation of large PEG crosslinked Hb-SOD-CAT conjugates and have the capability to protect Hb from free radicals and its conversion to methemoglobin during conjugation and storage. This will further allow the co-encapsulation of the designed conjugates with isolated pancreatic beta-cells for their long-term protection and extend *in vivo* functionality in the treatment of diabetes. The large size will also help prevent leakage of crosslinked conjugates when co-encapsulated with most of the isolated cells and provide oxygen supply in the close vicinity to the cells without hemoglobin degradation, where oxygen supply is crucial for cell viability.

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Fig.1.
Schematic representation of Hemoglobin cross-linking with antioxidant enzymes by employing dicarboxymethylated poly(ethylene glycol) (2 kDa).

Fig.2. Sodium dodecylsulfate-polyacrylamide gel electrophoresis showing formation of Hb cross-linked products with and without enzymes (Hb:PEG). Lane A, 1:5 Hb-Hb; Lane B, 1:10 Hb-Hb; Lane C, 1:15 Hb-Hb; Lane D, 1:20 Hb-Hb; Lane E, 1:30 Hb-Hb; Molecular Weight Standard; Lane F, 1:5 Hb-SOD-CAT; Lane G, 1:10 Hb-SOD-CAT; Lane H, 1:15 Hb-SOD-CAT; Lane I, 1:20 Hb-SOD-CAT; Lane J, 1:30 Hb-SOD-CAT.

Fig.3. Size Exclusion Chromatogram of cross-linked Hemoglobins with and without antioxidant enzymes, with Hb/PEG ratios 1:5 to 1:30 and SOD/CAT enzyme ratio of 30000:300000 units.

Fig.4. Absorbance spectra of cross-linked Hemoglobins with and without antioxidant enzymes, 24hrs after cross-linking, Hb/PEG ratios 1:5 to 1:30 and SOD/CAT enzyme ratio of 30000:300000 units.

Fig.5.

Effect of antioxidant enzymes on formation of methemoglobin in cross-linked Hemoglobins during storage at 4°C for one month, with Hb/PEG ratios 1:5 to 1:30 and SOD/CAT enzyme ratio of 30000:300000 units.

Fig.6.

Protective effect of the antioxidant enzymes on cross-linked Hemoglobins (Hb/PEG ratios 1:5 to 1:30 and SOD/CAT enzyme ratio of 30000:300000), when challenged with 0.1 and 1mM hydrogen peroxide for 30 minutes, 3, 24 and 48 hours. Change in absorbance values represents difference between free Hb and cross-linked products. Values in parentheses are for conjugates with antioxidant enzymes. a - represent conjugates challenged with 0.1mM hydrogen peroxide (H₂O₂) and b – represent conjugates challenged with 1mM hydrogen peroxide (H₂O₂).

Fig.7.

Protective effect of the antioxidant enzymes on cross-linked Hemoglobins (Hb/PEG ratios 1:5 to 1:30 and SOD/CAT enzyme ratio of 30000:300000), when challenged with 1mM xanthine and 10 and 20 mUnits/mL xanthine oxidase(XO) for 30 minutes, 3, 24 and 48 hours. Change in absorbance values represents difference between free Hb and cross-linked products. Values in parentheses are for conjugates with antioxidant enzymes. a - represent conjugates challenged with 1mM xanthine and 10mUnits/mL xanthine oxidase(XO) and b - conjugates challenged with 1mM xanthine and 20mUnits/mL xanthine oxidase(XO).

Table 1

Percent Retention of Enzymatic Activity of Superoxide dismutase in Hemoglobin Conjugates after Cross-linking with modified PEG.

Conjugate (Hb:PEG)	Superoxide dismutase enzyme activity ^{a)}
	Percent retained ^{b)}
Hb-SOD-CAT(1:5)	73 ± 2
Hb-SOD-CAT(1:10)	79 ± 3
Hb-SOD-CAT(1:15)	76 ± 6
Hb-SOD-CAT(1:20)	72 ± 5
Hb-SOD-CAT(1:30)	67 ± 5

^a Based on the enzymatic activity of free Superoxide dismutase added before conjugation;

^b Values are means ± SD (*n* =3).

Table 2

Percent Retention of Enzymatic Activity of Catalase in Hemoglobin Conjugates after Cross-linking with modified PEG.

Conjugate (Hb:PEG)	Catalase enzyme activity ^{a)}
	Percent retained ^{b)}
Hb-SOD-CAT(1:5)	91 ± 6
Hb-SOD-CAT(1:10)	93 ± 2
Hb-SOD-CAT(1:15)	96 ± 3
Hb-SOD-CAT(1:20)	91 ± 4
Hb-SOD-CAT(1:30)	90 ± 4

^a Based on the enzymatic activity of free Catalase added before conjugation;

^b Values are means ± SD (*n* =3).