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RESEARCH ARTICLE

Temperature stability of Poly-[hemoglobin-superoxide dismutase-catalase-carbonic anhydrase] in the form of a solution or in the lyophilized form during storage at -80°C , 4°C , 25°C and 37°C or pasteurization at 70°C

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ABSTRACT

Polyhemoglobin-superoxide dismutase-catalase-carbonic anhydrase (Poly-[Hb-SOD-CAT-CA]) contains all three major functions of red blood cells (RBCs) at an enhanced level. It transports oxygen, removes oxygen radicals and transports carbon dioxide. Our previous studies in a 90-min 30 mm Hg Mean Arterial Pressure (MAP) sustained hemorrhagic shock rat model shows that it is more effective than blood in the lowering of elevated intracellular pCO_2 , recovery of ST-elevation and histology of the heart and intestine. This paper is to analyze the storage and temperature stability. Allowable storage time for RBC is about 1 d at room temperature and 42 d at 4°C . Also, RBC cannot be pasteurized to remove infective agents like HIV and Ebola. PolyHb can be heat sterilized and can be stored for 1 year even at room temperature. However, Poly-[Hb-SOD-CAT-CA] contains both Hb and enzymes and enzymes are particularly sensitive to storage and heat. We thus carried out studies to analyze its storage stability at different temperatures and heat pasteurization stability. Results of storage stability show that lyophilization extends the storage time to 1 year at 4°C and 40 d at room temperature (compared to respectively, 42 d and 1 d for RBC). After the freeze-dry process, the enzyme activities of Poly-[SFHb-SOD-CAT-CA] was $100 \pm 2\%$ for CA, $100 \pm 2\%$ for SOD and $93 \pm 3.5\%$ for CAT. After heat pasteurization at 70°C for 2 h, lyophilized Poly-[Hb-SOD-CAT-CA] retained good enzyme activities of CA $97 \pm 4\%$, SOD $100 \pm 2.5\%$ and CAT $63.8 \pm 4\%$. More CAT can be added during the crosslinking process to maintain the same enzyme ratio after heat pasteurization. Heat pasteurization is possible only for the lyophilized form of Poly-[Hb-SOD-CAT-CA] and not for the solution. It can be easily reconstituted by dissolving in suitable solutions that continues to have good storage stability though less than that for the lyophilized form. According to the P50 value, Poly-[SFHb-SOD-CAT-CA] retains its oxygen carrying ability before and after long-term storage.

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Introduction

Blood substitute firstly draws the attentions of researchers after the HIV crisis. Nowadays, the blood scarcity is a worldwide problem due to the increasing demand and lack of supply as well as HIV in some regions of the world (Chang 2007, 2009, 2012, Winslow 2006). The first generation blood substitute is based on the basic principle of polyhemoglobin (PolyHb) (Chang 1964, 1971). This has been developed independently by others and has undergone extensive clinical trials (Alayash et al. 2007, Bian et al. 2013, Chang 2014, Chang 2015, Jahr et al. 2008, Kim and Greenburg 2014 and Zhao et al. 2014). It is already approved for routine clinical use in countries where HIV in donor blood is still a major problem. This includes South Africa and Russia (Moore et al. 2009, OPK Biotech LLC 2011). However, in countries where HIV is no longer an urgent problem, PolyHb is still not yet in routine clinical use. Red blood cell (RBC) has three main functions: oxygen transport, antioxidant and CO_2 transport (Geers and Gros 2000, Guyton 1991). The first generation of blood substitute like PolyHb is merely an

oxygen carrier while the second generation blood substitute, polyhemoglobin-superoxide dismutase-catalase (Poly-[Hb-SOD-CAT]) (D'Agnillo and Chang 1998), is both an oxygen carrier and an antioxidant. This way, it functions well in preventing ischemia-reperfusion injuries (Chang 2014, D'Agnillo and Chang 1998, Powanda and Chang 2002). Other research groups have supported this finding (Hoffman 2003, Mun et al. 2003). However, this cannot transport CO_2 . CO_2 transport is one of the vital functions of RBC (Ristagno et al. 2006, Sims et al. 2001, Tronstad et al. 2010). The importance of this function has been proved by recent studies. Thus, using a novel intracellular pCO_2 microelectrode, researchers show that tissue pCO_2 is not reflected by blood pCO_2 . Furthermore, tissue pCO_2 increases with severity of hemorrhagic shock and is correlated with mortality rates (Sims et al. 2001).

Poly-[SFHb-SOD-CAT-CA] is a new type of blood substitute (Bian et al. 2011). The SFHb, SOD, CAT, CA were all cross-linked together to form a nano-sized soluble blood substitute. The *in vitro* analysis showed that the enrichment of enzymes before the crosslink highly improved the activities of SOD, CAT and CA

in Poly-[SFHb-SOD-CAT-CA]. And an optimal Poly-[SFHb-SOD-CAT-CA] with the following addition of enzymes requires an Hb: SOD:CAT:CA ratio of 1 g:18,000:310,000:130,000 U. Besides, the main part of Hb and enzymes were contained in the samples larger than 100 kDa (Bian et al. 2011). The primary *in vivo* experiment showed that Poly-[SFHb-SOD-CAT-CA] could efficiently rescue the MAP and reduces the increased pCO_2 in the ischemia rat model after 90-min shock. And this new blood substitute performed better than whole blood in reducing elevated tissue pCO_2 as well as the recovery of elevated ST-elevation (Bian and Chang 2015).

Since this new blood substitute contains abundant enzymes, the long-term stability of this polymer needs to be analyzed. When it is stored in the ambulance or in places without proper storage condition, the enzyme activities in different temperature should also be tested.

Lyophilization of PolyHb can markedly increase its storage stability (Keipert and Chang 1988, Zhao et al. 2014). We, therefore, analyze the potential use of lyophilization to increase the stability of the enzyme components. The enzymes activities and other characters of the freeze-dried samples, including molecular distribution and P50 values were assessed before and after lyophilization as well as during the long-term storage.

Besides stability, we also use the *in vitro* screening test (Chang 2007). For short, the complement activation of C3 to C3a of the plasma added with blood substitute was evaluated. Using saline and zymosan as control, the effects of Poly-[SFHb-SOD-CAT-CA] on complement activation of the plasma could be easily analyzed.

Methods

Stroma-free hemolysate preparation

Fresh bovine blood with heparin (anticoagulant) was centrifuged at 4000 g for 60 min at 4 °C to remove the plasma supernatant and upper layer of the cell pellet. The RBCs were washed four times with sterile, ice-cold 0.9% NaCl and then suspended in twice the volume of potassium phosphate (12.5 mm, pH 7.4) for 30 min in order to lyse. Then, two volumes of ice-cold reagent-grade toluene were used to remove stromal lipid. The sample was centrifuged at 15,000 g for 2 h at 4 °C to remove cellular debris.

Preparation of Poly-[SFHb-SOD-CAT-CA]

The enzymes, including SOD, CAT and CA were added to SFHb before crosslink to reach a final concentration of SOD (1050 U/ml SFHb), CAT (21,000 U/ml SFHb) and CA (1070 U/ml SFHb). The crosslinking reagent, glutaraldehyde, was used to crosslink Hb, SOD, CAT and CA together to form Poly-[SFHb-SOD-CAT-CA]. 1.3 M lysine was added at a molar ratio of 7:1 lysine/Hb before the cross-linking reaction. Trehalose was added at the ratio of 0.2 g/1 g Hb to prevent the formation of methemoglobin (MetHb). The mixture was placed on a shaker at 4 °C for 1 h at 140 rpm. Five percent glutaraldehyde was added slowly at a molar ratio of 16:1 glutaraldehyde/Hb at a rate of 0.15–0.20 ml every 5–10 min. After 24 h, the addition

of 2.0 M lysine at a molar ratio of 200:1 lysine/Hb is used to stop the reaction.

Measurements of SOD, CAT and CA activities

The enzyme activity testing methods have been presented before. For short, SOD activity was determined by inhibition of nitro blue tetrazolium reduction using xanthine–xanthine oxidase as a superoxide generator.

For the CAT activities, UV 240 nm spectrophotometric method was used to measure the rate of disappearance of H_2O_2 and test samples were used as a blank to minimize hemoglobin interferences. The CA activity is measured by the pH change of 0.02 M Tris buffer. The reaction was initiated by the addition of substrate and the time (T) needed for the pH of the reaction mixture to drop from pH 8.3 to 6.3 was recorded. The measurements in seconds were converted into W-A units according to the following formula: $1 \text{ W-A unit} = [2 \times (T_0 - T)]/T$. The units were then plotted versus the Hb/CA concentration.

Oxygen-hemoglobin dissociation curve

A TCS Hemox-Analyzer Model B (Huntingdon Valley, PA) was used to analyze the oxygen affinity for Hb, PolyHb and Poly-[Hb-SOD-CAT-CA]. Samples (5 ml) contain 0.3 g/dl cross-linked Poly-[Hb-SOD-CAT] in the testing buffer (pH 7.4). This test was performed in 37 °C to obtain oxygen–hemoglobin dissociation curves.

Lyophilization procedure

The Poly-[SFHb-SOD-CAT-CA] and SFHb added trehalose at the ratio of 1 g Hb:0.8 g trehalose as the protective agent. And after frozen at –80 °C overnight, the samples were freeze-dried by the freeze-dryer machine.

Storage stability analysis of solution and freeze-dry samples

Poly-[SFHb-SOD-CAT-CA] and free solution of SFHb, SOD, CA and CAT were prepared and then stored in different temperature for enzymes activity testing at day 0, 1, 2, 4, 8, 16, 32, 64, 128. Lyophilized Poly-[SFHb-SOD-CAT-CA] and SFHb were stored at temperatures of –80 °C, 4 °C, 20 °C, 37 °C. The enzyme activities are tested before and after freeze-dry and also tested on days 10, 20, 40, 80, 160.

The molecular weight of the samples was evaluated by Sephacryl-300 HR column. This column was equilibrated with 0.1 M Tris-HCl and 0.15 M NaCl (pH 7.4) elution buffer. The molecular weight distribution was recorded at 1 mm/min using a 280-nm UV detector.

Effect of pasteurization temperature on the stability of soluble and freeze-dry samples

The samples of Poly-[SFHb-SOD-CAT-CA] solution and freeze-dried samples were kept at 70 °C for 1, 2 and 3 h and tested the SOD, CAT and CA enzymes activities separately. The molecular

distribution of the freeze-dried samples after 3 h was also analyzed.

Effects on C3a complement activity

Rat plasma is obtained from Sprague–Dawley rats and transferred into 50 ml polypropylene heparinized tubes. The plasma is separated by centrifugation at 5500 g for 20 min at 2 °C. The fresh plasma is transferred in 400 µl aliquots into 4 ml sterile polypropylene tubes. Four-hundred microliters of plasma is combined with 100 µl Ringer's lactate solution as negative control. Four hundred microliter of plasma is combined with 100 µl of zymosan (5 mg/ml) as positive control. Poly-[SFHb-SOD-CAT-CA] is the testing samples. The samples are incubated at 37 °C at 60 rpm for 1 h in the Lab-Line Orbit Environ Shaker (United States of America). The reaction is quenched by adding 0.4 ml of the samples to 1.6 ml sterile saline in 2 ml ethylenediaminetetraacetate (EDTA) sterile tubes. The complement C3a des Arg (human), EIA kit was purchased from Amersham, Canada. The testing method is the same as the instruction given in the kit except for two minor modifications. Centrifugation is carried out at 10,000 g for 20 min and the inside of the tubes are carefully blotted with Q-tips.

Results

Effects of lyophilization on enzyme activity

After the freeze-dry process, the enzyme activities of Poly-[SFHb-SOD-CAT-CA] was $93 \pm 3.5\%$ for CAT, $100 \pm 2\%$ for SOD and $100 \pm 2\%$ for CA (Table I).

Enzyme stability of freeze-dried Poly-[SFHb-SOD-CAT-CA], Poly-[SFHb-SOD-CAT-CA] solution and un-crosslinked SFHb, SOD, CAT and CA at different temperatures

The enzymes activities of the freeze-dried Poly-[SFHb-SOD-CAT-CA] were more stable compared with the soluble Poly-[SFHb-SOD-CAT-CA] and even more so when compared to the uncrosslinked solution of SFHb, SOD, CAT and CA.

Table II is a summary of the preliminary results on the storage stability of RBCs as compared to Poly-[SFHb-SOD-CAT-CA] solution and lyophilized Poly-[SFHb-SOD-CAT-CA] (from Bian and Chang (2015)). This is followed by the results of more detailed analysis:

(1) CA activity: After 320 d of storage, the freeze-dried Poly-[SFHb-SOD-CAT-CA] stored at -80°C , 4°C , 25°C and 37°C contained $86 \pm 3\%$, $85 \pm 2\%$, $73 \pm 2.5\%$ and $55 \pm 4\%$ separately of their enzyme activities (Figure 1). After 128 d storage, Poly-[SFHb-SOD-CAT-CA] solution stored at -80°C , 4°C , 25°C , 37°C contained $69.3 \pm 3\%$, $57 \pm 1.5\%$, $42 \pm 1.5\%$, $21 \pm 4.5\%$ of their enzyme activities. However, free enzymes stored at -80°C , 4°C , 25°C , 37°C dropped to $54 \pm 6\%$, $44.3 \pm 2.5\%$, $27.4 \pm 4\%$ and $8.2\% \pm 1\%$ (Figure 1).

Table I. The change in enzyme activities after freeze drying.

Enzyme type	SOD	CAT	CA
Percentage of original after freeze-dry	100 ± 2	93 ± 3.5	100 ± 2

(2) SOD activity: Freeze-dried Poly-[SFHb-SOD-CAT-CA] kept at -80°C and 4°C , contain $79 \pm 2\%$ and $76 \pm 3\%$ of their original activities after 320 d. And samples stored at 25°C and 37°C contained $67 \pm 2\%$ and $60 \pm 1\%$ separately (Figure 2). After 128 d storage, Poly-[SFHb-SOD-CAT-CA] solution stored at -80°C and 4°C contain $81 \pm 2\%$ and $67.4 \pm 3\%$ of their original activities after 128 d. And samples stored at 25°C and 37°C contained $42 \pm 1\%$ and $13.4 \pm 2\%$ separately. For the free enzymes without crosslinking, the activities dropped to $41 \pm 1\%$ and $37.5 \pm 2\%$, $30 \pm 2\%$, $10.6 \pm 1\%$ for -80°C , 4°C , 25°C and 37°C (Figure 2).

(3) CAT activity: Freeze-dried Poly-[SFHb-SOD-CAT-CA] kept at -80°C and 4°C , contained 83% of their original activities at -80°C and 74% at 4°C after 320 d (Figure 3). At 20°C , it retains about 75% of its activity after 40 d and 50% of its activity after 320 d. At 37°C , it retains 50% of its activity after 80 d.

CAT also showed good stability in the soluble Poly-[SFHb-SOD-CAT-CA] form when the samples were stored at -80°C , 4°C and 25°C (Figure 1). Thus, the Poly-[SFHb-SOD-CAT-CA] samples retained $96 \pm 3\%$, $87.5 \pm 3\%$ and $65 \pm 1\%$ of their original activities when stored at -80°C , 4°C and 25°C . The free enzyme solution retained $81 \pm 6\%$, $77 \pm 2\%$ and $58 \pm 2\%$ when stored at -80°C , 4°C and 25°C . When kept at 37°C , there was an extensive loss of CAT activity after 128 d to $20 \pm 4\%$ even though this is better than the free uncrosslinked enzymes with only $2 \pm 3\%$ (Figure 3).

Ongoing study also show that Poly-[SFHb-SOD-CAT-CA] prepared with the new enzyme extraction method (Guo et al. 2015) also has increased temperature stability.

Molecular distribution of Poly-[SFHb-SOD-CAT-CA] solution and freeze-dried samples

For the freeze-dried samples, results showed that freeze-dry could make the complex more stable. When freeze-dried samples were stored at 23°C , 4°C or -80°C , the molecular weight distribution of the samples did not change after 1 year (Table III). Even samples kept in 37°C for 1 year were also more stable than the solution form with no free Hb (lower than 100kDa) detected (Table III).

Table II. Stability at different temperatures.

STABILITY AT DIFFERENT TEMPERATURES			
	Donor Red Blood Cells		
	Maximal time of storage allowed		
	20–25°C		4°C
	1 day?		42 days
	Poly-[Hb-SOD-CAT-CA] solution		
	T1/2 of enzyme activities:		
	20–25°C		4°C
CAT	172 days		380 days
SOD	92 days		198 days
CA	51 days		231 days
	Poly-[Hb-SOD-CAT-CA] freeze dried:		
	Day 320 % enzyme activity		
	20–25°C		4°C
CAT	73% (day 320)		85% (day 320)
SOD	67% (day 320)		76% (day 320)
CA	73% (day 320)		85% (day 320)

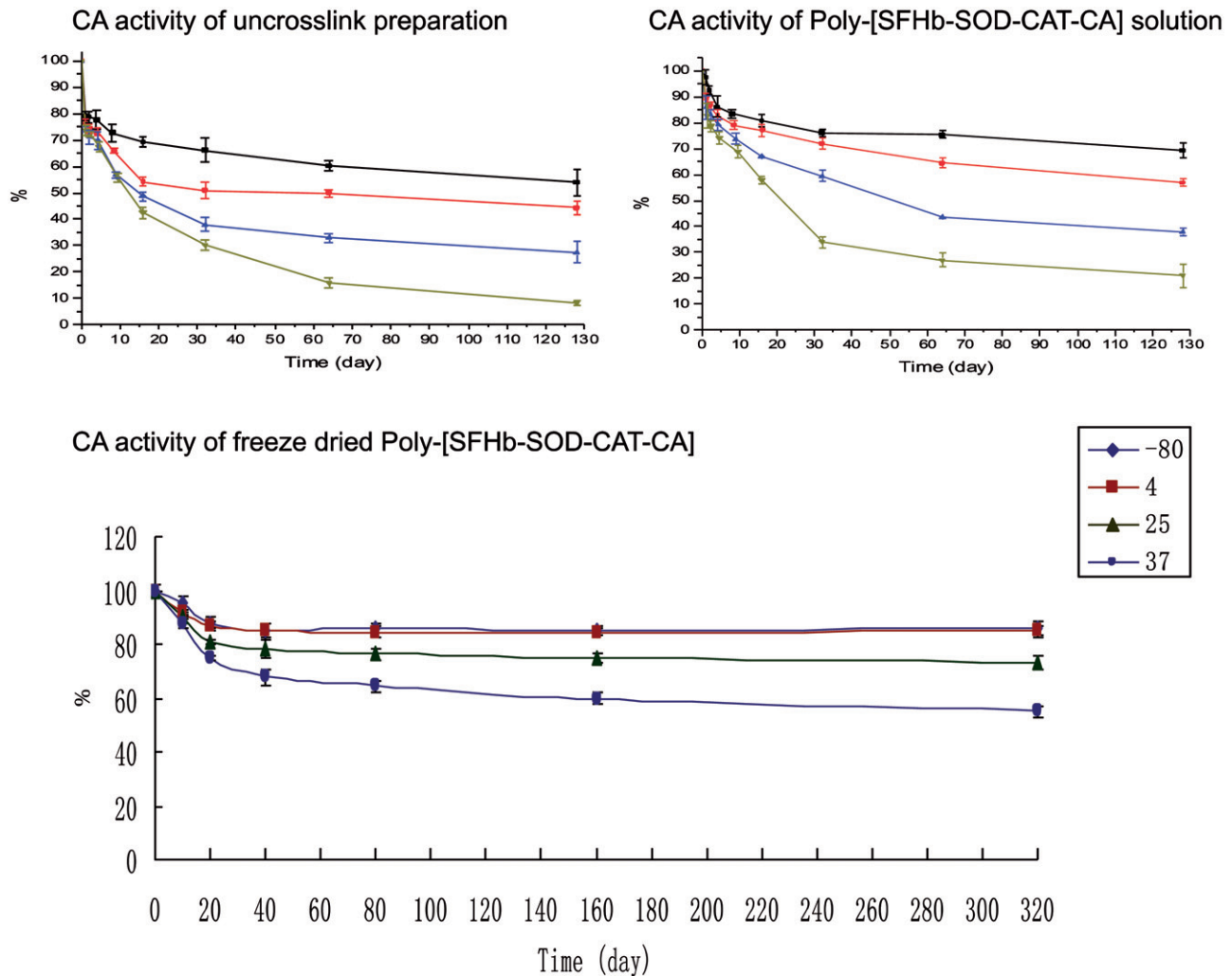


Figure 1. Storage stability of carbonic anhydrase (CA) at -80°C , 4°C , 25°C and 37°C for: (1) un-cross-linked free solution of Hb, SOD, CAT and CA; (2) polymerized solution of Poly-[SFHb-SOD-CAT-CA] and (3) freeze-dried polymerized Poly-[SFHb-SOD-CAT-CA].

For the soluble Poly-[SFHb-SOD-CAT-CA] samples, the percentage of the molecular distribution is respectively, 83% for molecular weight larger than 450 kDa; 17% for between 100 kDa and 450 kDa and not detectable for those less than 100 kDa. After 4 months storage at -80°C and 4°C , there was no significant changes in the molecular weight distribution. Storage at 25°C and 37°C for 4 months resulted in significant dissociation of molecular weight as shown in Table IV.

Stability of Poly-[SFHb-SOD-CAT-CA] solution and freeze-dried Poly-[SFHb-SOD-CAT-CA] subjected to pasteurization temperature

Lyophilized samples are much more stable at pasteurization temperature when compared to the Poly-[SFHb-SOD-CAT-CA] solution.

At the pasteurization temperature of 70°C , the enzyme activity of SOD, CAT and CA was, respectively, $100 \pm 3\%$, $67.4 \pm 1\%$ and $100 \pm 5\%$ after 1 h at 70°C , $100 \pm 2.5\%$, $63.8 \pm 4\%$ and $97 \pm 4\%$ after 2 h 70°C and $100 \pm 4\%$, $46.7 \pm 5\%$ and $94 \pm 5\%$ after 3 h 70°C (Table V). After the pasteurization, the molecular weight distribution of the freeze-dried Poly-[SFHb-SOD-CAT-CA] did not change.

For the solution, after 30-min pasteurization, the enzyme activities remained are: SOD $83.4 \pm 4\%$, CAT $28 \pm 1\%$ and CA $87.6 \pm 4\%$. After 1 h at 70°C , SOD and CA still contain $81.5 \pm 3\%$ and $77 \pm 6\%$, while the CA dropped to zero. After 2 h, the enzyme activity of SOD, CAT and CA were $64 \pm 7\%$, 0% and $62 \pm 6\%$. After 3 h, the SOD, CAT and CA activities are $57.8 \pm 10\%$, 0% and $50 \pm 6\%$, respectively.

The freeze-dry procedure improves the enzyme stability even at the pasteurization temperature of 70°C . On the other hand, when not freeze-dried the enzyme stability, especially for CAT, are not stable at the pasteurization temperature of 70°C .

Methemoglobin content

Trehalose was added to reduce the MetHb content of the Poly-[SFHb-SOD-CAT-CA] during the crosslinking process. The best ratio is 0.2 g/gHb. This resulted in the lowering of MetHb from 15% down to 8%. Trehalose is not toxic and can be removed efficiently by ultrafiltration. This protective agent is also added during the freeze-dry process to prevent the increase of MetHb. Results showed that trehalose could efficiently reduce the MetHb content when added at the ratio of 0.8 g/gHb.

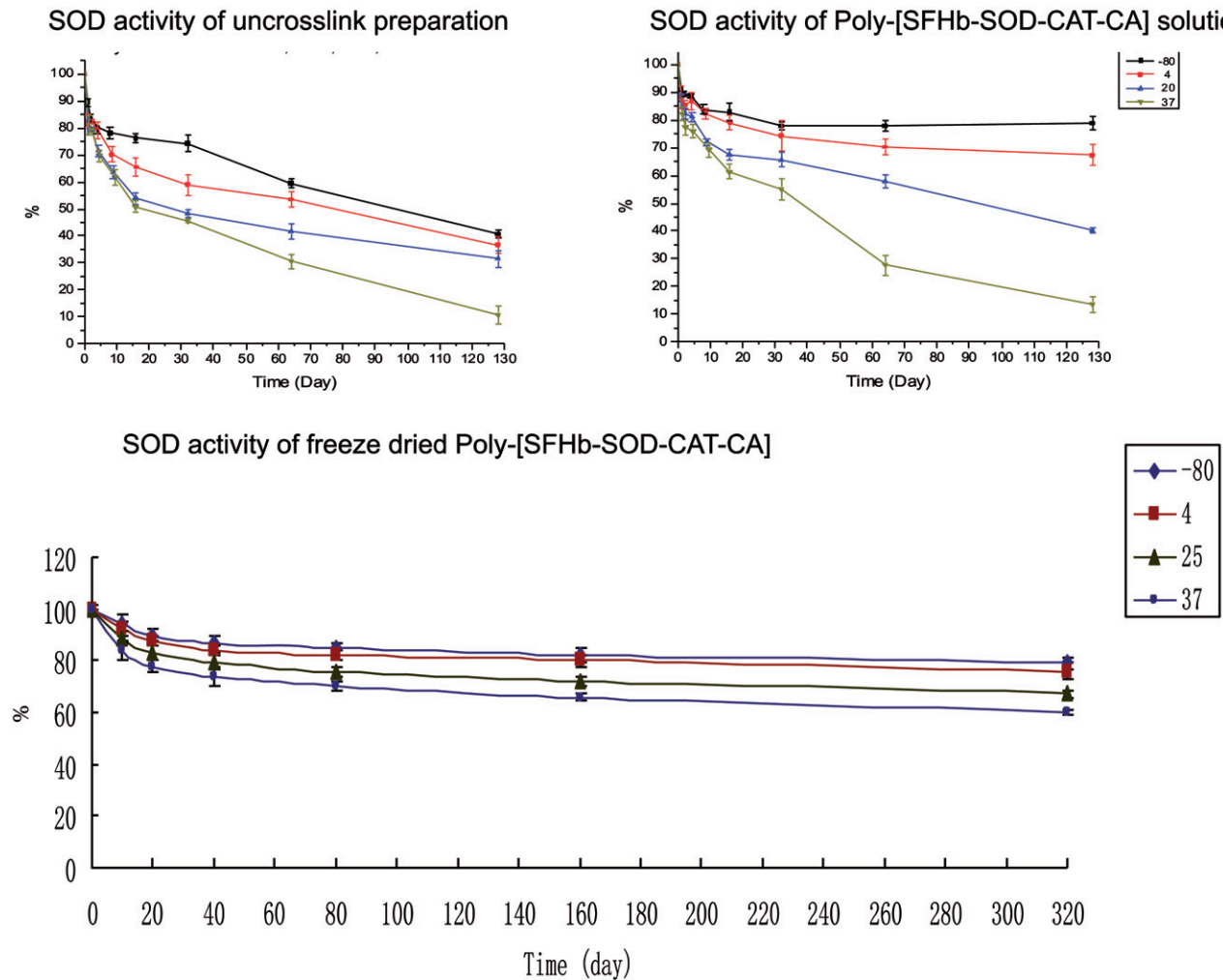


Figure 2. Storage stability of superoxide dismutase (SOD) at -80°C , 4°C , 25°C and 37°C for: (1) uncross-linked free solution of Hb, SOD, CAT and CA; (2) polymerized solution of Poly-[SFHb-SOD-CAT-CA] and (3) freeze-dried polymerized Poly-[SFHb-SOD-CAT-CA].

Oxygen carrying ability of different samples before and after long-term storage

The P50 value of Poly-[SFHb-SOD-CAT-CA] was 20.0 ± 0.74 mmHg. After storing in different temperature -80°C , 4°C , 25°C and 37°C for 3 months, the P50 value of soluble Poly-[SFHb-SOD-CAT-CA] were 21.19 ± 1.8 , 21.31 ± 1.93 , 21.88 ± 1.41 , 22.04 ± 0.55 mmHg, respectively. For the freeze-dried sample, the P50 values were 14.94 ± 0.38 , 14.22 ± 1.4 , 14.57 ± 1.1 , 13.32 ± 0.93 after 1 year storage (Table VI).

Effects on C3a complement activation

C3a des Arg levels were measured in this study as an indication of activation of the complement pathway. C3a is a multifunctional, proinflammatory mediator and has the ability to mediate hypersensitivity, anaphylactic reactions and specific antibody-antigen responses. The conversion of C3 to C3a is triggered by either classical or alternate complement pathways. C3a des Arg, can be quantified by ELISA assay. Poly-[SFHb-SOD-CAT-CA] sample were either similar to or slightly lower than levels in the negative control which is Ringer's lactate with plasma. The positive control of zymosan with plasma did exhibit higher levels of C3a. These results suggest that Poly-[SFHb-SOD-CAT-CA]

does not activate the complement pathway in rat plasma (Table VII).

Immunological studies

Ongoing immunological studies show that there is no increase in immune response to Poly-[Hb-SOD-CAT-CA] even with six folds increase in enzyme contents.

Discussion

The allowable storage time for RBC is 42 d at 4°C and around 1 d at room temperature. The above result shows that the lyophilized form of Poly-[SFHb-SOD-CAT-CA] retains most of its enzyme activity after 320 d when stored at 4°C and also in room temperature. In the soluble form, it has a T1/2 of 198 d at 4°C and 51 d in room temperature. This means that the lyophilized form can be stored for a year in room temperature and reconstituted when needed. The soluble form with a T1/2 of 51 d at room temperature is comparable to RBC stored at 4°C in the refrigerator. Without the need for refrigeration, it would be much more convenient to this to be transported even in a backpack of the medics.

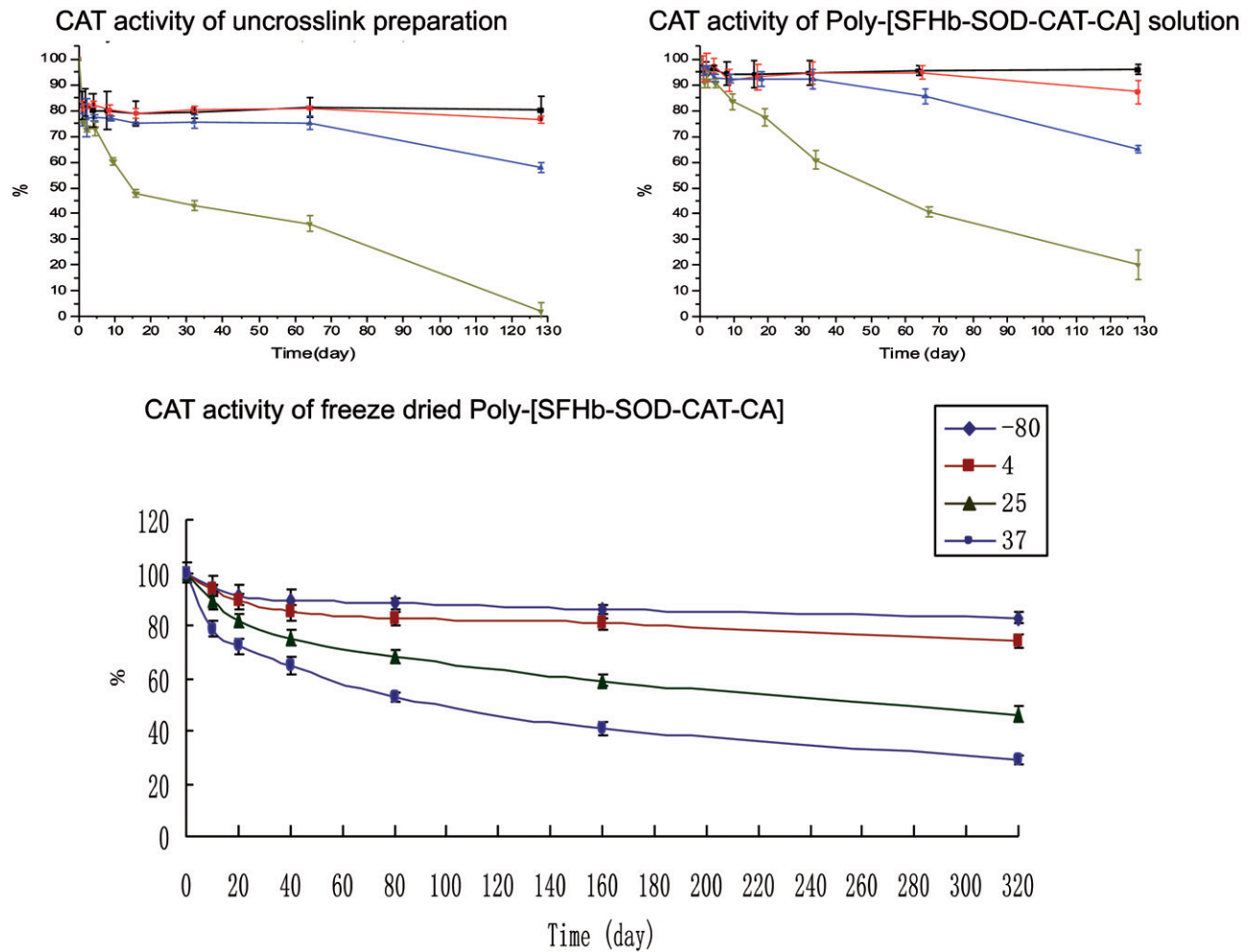


Figure 3. Storage stability of catalase (CAT) at -80°C , 4°C , 25°C and 37°C for: (1) uncross-linked free solution of Hb, SOD, CAT and CA; (2) polymerized solution of Poly-[SFHb-SOD-CAT-CA] and (3) freeze-dried polymerized Poly-[SFHb-SOD-CAT-CA].

Table III. Molecular distribution of freeze-dried samples stored for 12 months.

Freeze-dried Poly-[SFHb-SOD-CAT-CA]	Molecular weight distribution (%)		
	>450	100–450	<100
-80°C	82	18	–
4°C	78	22	–
20°C	74	26	–
37°C	63	37	–

Table IV. Molecular distribution of Poly-[SFHb-SOD-CAT-CA] solution stored for 4 months.

Solution of Poly-[SFHb-SOD-CAT-CA]	Molecular weight distribution (%)		
	>450	100–450	<100
Before storage	83	17%	
4 months stored in -80°C	83	17	–
4 months stored in 4°C	82	18	–
4 months stored in 20°C	60	21	13
4 months stored in 37°C	48%	23	31

Table V. Stability with pasteurization of the soluble and freeze-dried samples.

Enzyme activity samples		SOD	CAT	CA
Poly-[SFHb-SOD-CAT-CA] solution	0.5h (65°C)	$83.4 \pm 4\%$	$28 \pm 1\%$	$87.6 \pm 4\%$
	1 h (70°C)	$81.5 \pm 3\%$	0%	$77 \pm 6\%$
	2 h (70°C)	$64 \pm 7\%$	0%	$62 \pm 6\%$
Freeze-dried Poly-[SFHb-SOD-CAT-CA]	0.5 h (65°C)	$57.8 \pm 10\%$	0%	$50 \pm 6\%$
	1 h (70°C)	$101 \pm 4\%$	$78 \pm 5\%$	$102 \pm 4\%$
	2 h (70°C)	$100 \pm 3\%$	$67.4 \pm 1\%$	$100 \pm 5\%$
3 h (70°C)		$100 \pm 4\%$	$63.8 \pm 4\%$	$97 \pm 4\%$
		$100 \pm 4\%$	$46.7 \pm 5\%$	$94 \pm 5\%$

Table VI. The P50 value of the solution and freeze-dried sample of Poly-[SFHb-SOD-CAT-CA] stored for 4 months at the temperature shown.

Solution of Poly-[Hb-SOD-CAT-CA]	P50 value (mmHg)	Freeze-dried Poly-[Hb-SOD-CAT-CA]	P50 value (mmHg)
-80°C	19.15 ± 0.95	-80°C	14.94 ± 0.38
4°C	19.92 ± 0.74	4°C	14.22 ± 1.4
20°C	18.2 ± 1.0	20°C	14.57 ± 1.1
37°C	20.5 ± 0.28	37°C	13.32 ± 0.93

Furthermore, the lyophilized form of Poly-[SFHb-SOD-CAT-CA], unlike RBC can withstand a heat pasteurization temperature of 70°C for 2 h. This is important to allow the preparation to be free of infective agents for use in patients.

The complement activation studies show that the cross-linking and lyophilizing procedure did not result in any problems related to complement activation. Further, ongoing detailed immunological studies in animals are ongoing.

Table VII. The C3a complement activity of Poly-[SFHb-SOD-CAT-CA].

Sample	Average O.D.	Net O.D. (Av. O.D. – NSB)	Percent bound (%)	Human C3a des Arg (ng/ml)
Blank	0	–	–	–
NSB	0.102	0	–	–
Bo	0.676	0.574	100	0
Saline + plasma (negative control)	0.639	0.537	93.55	0.035 ± 0.014
Zymosan + plasma (positive control)	0.587	0.485	84.49	0.24 ± 0.04
PolyHb-SOD-CAT-CA + plasma	0.640	0.538	93.97	0.028 ± 0.07

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper. This research has been supported by the Canadian Blood Service and the Canadian Institutes of Health Research. The opinions expressed in this article are those of the authors and not necessary those of the government granting agencies or the government of Canada.

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