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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YZ Bian, C Guo & TMS Chang
Artificial Cells & Organs Research Centre,
Departments of Physiology, Medicine & Biomedical Engineering
Faculty of Medicine, McGill University, Montreal, Quebec, Canada H3G 1YH
www.medicine.mcgill.ca/artcell artcell.med@mcgill.ca

Corresponding author:
Professor TMS Chang, Director, Artificial Cells and Organs Research Centre
Faculty of Medicine, McGill University. Artcell.med@mcgill.ca

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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 30mm Hg 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 red blood cell (rbc) is about 1 day at room temperature and 42 days at 4C. Also, rbc cannot be pasteurized to remove infective agents like H.I.V. 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 temperature and heat pasteurization stability. Results of storage stability show that lyophillization extends the storage time to 1 year at 4C and 40 days at room temperature (compared to respectively 42 days and 1 day for rbc). After the freeze-dry process, the enzyme activities of Poly-[SFHb-SOD-CAT-CA] was 100±2% for CA , 100±2% for SOD and 93±3.5 % for CAT. After heat pasteurization at 70°C for 2 hours, lyophilized Poly-[Hb-SOD-CAT- CA] retained good enzyme activities of CA 97±4%, SOD 100±2.5% and CAT 63.8±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.

Introduction

Blood substitute firstly draw 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 **(refs).** It is already approved for routine clinical use in countries where HIV in donor blood is still a major problem. This includes South African and Russia. (Moore et al., 2009; OPK Biotech LLC. 2011). However, in countries where HIV is no longer a urgent problem, PolyHb is still not yet in routine clinical use. Red blood cell (RBC) has three main functions: oxygen transport, anti-oxidant and CO_2 transport (Guyton, 1991; Geers, C. & Gros, G., 2000). The first generation of blood substitute like PolyHb is merely an oxygen carrier while the second generation blood substitute, Poly-[Hb-SOD-CAT] (D'Agnillo & Chang, 1998), is both an oxygen carrier and an anti-oxidant. This way it functions well in preventing ischemia reperfusion injuries (Chang et al., 2004; D'Agnillo & Chang, 1998; Powanda & Chang, 2002). Other research groups have supported this finding (Mun, K.C. 2003; Hoffman, A, 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 C, 2010). The importance of this function has been proved by recent studies. Thus using a novel intracellular pCO2 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 & Chang, 2011). The SFHb, SOD, CAT, CA were all crosslinked together to form a nano-size 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 1g: 18,000:310,000:130,000U. Besides, main part of Hb and enzymes were contained in the samples larger than 100kDa (Bian & Chang, 2011). The primary in vivo experiment showed that Poly-[SFHb-SOD-CAT-CA] could efficiently rescue the MAP and reduces the increased PCO2 in the ischemia rat model after 90 min shock. And this new blood substitute performed better than whole-blood in reducing elevated tissue PCO2 as well as the recovery of elevated ST-elevation. (Bian & 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 store condition, the enzyme activities in different temperature should also be tested.

Lyophilization of PolyHb can markedly increase its storage stability (Keipert P & Chang, 1998; Zhao L & Yang, 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 ~~the~~ 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.

Method:

Stroma-free Hemolysate Preparation

Fresh bovine blood with heparin (anticoagulant) was centrifuged at 4000g for 60 minutes at 4 °C to remove the plasma supernatant and upper layer of cell pellet. The red blood cells 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 2 volumes of ice-cold reagent-grade toluene were used to remove stromal lipid. The sample was centrifuged at 15000g for 2 hours 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 crosslink reagent, glutaraldehyde, was used to crosslink Hb, SOD, CAT and CA together to form Poly-[SFHb-SOD-CAT-CA]. 1.3M lysine was added at a molar ratio of 7:1 lysine/Hb before the cross-linking reaction. Trehalose was added at the ration of 0.2 g/ 1g Hb to prevent the formation of methemoglobin. The mixture was placed on a shaker at 4°C for 1 hour at 140 rpm. 5% 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 minutes. After 24 hours, the addition of 2.0M 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 nitroblue tetrazolium (NBT) reduction using xanthine-xanthine oxidase as a superoxide generator.
For the CAT activities, UV 240nm spectrophotometeric 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. 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 W-A unit = [2 x (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 37C to obtain oxygen-hemoglobin dissociation curves.

Lyophilization procedure: The Poly-[SFHb-SOD-CAT-CA] and SFHb added trehalose at the ratio of 1gHb:0.8g 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 0.1M Tris-HCl and 0.15M NaCl (pH 7.4) elution buffer. The molecular weight distribution was recorded at 1mm/min using a 280nm 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 hour, 2 hours and 3 hours and tested the SOD, CAT and CA enzymes activities separately. The molecular distribution of the freeze-dried samples after 3 hours was also analyzed.

Effects on C3a Compliment Activity

Rat plasma is obtained from Sprague-Dawley rats and transferred into 50mL polypropylene heparinized tubes. The plasma is separated by centrifugation at 5500g for 20 minutes at 2 °C. The fresh plasma is transferred in 400uL aliquots into 4mL sterile polypropylene tubes. 400uL of plasma is combined with 100uL Ringer's lactate solution as negative control. 400uL of plasma is combined with 100uL of zymosan (5mg/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 hour in the Lab-Line Orbit Environ Shaker. The reaction is quenched by adding 0.4mL of the samples to 1.6mL sterile saline in 2mL 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 10,000g 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±3.5 % for CAT, 100±2% for SOD and 100±2% for CA. (Table 3).

Table 1. The change in enzyme activities after freeze drying.

Enzyme type	SOD	CAT	CA
Percentage of original after free-dry (%)	100±2	93±3.5	100±2

Enzymes 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 temperature

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 2 is a summary of the preliminary results on the storage stability of red blood cells as compared to Poly-[SFHb-SOD-CAT-CA] solution and Lyophilized Poly-[SFHb-SOD-CAT-CA] (from Bian & Chang 2015). This is followed by the results of more detailed analysis

Table 2

STABILITY AT DIFFERENT TEMPERATURES

Donor Red Blood Cells
Maximal time of storage allowed

20-25C	4C
1 day?	42 days

Poly-[Hb-SOD-CAT-CA] solution
T1/2 of enzyme activities:

	20-25C	4C
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

	20-25C	4C
CAT	73%(day 320)	85%(day 320)
SOD	67%(day 320)	76% (day 320)
CA	73% (day 320)	85% (day320)

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

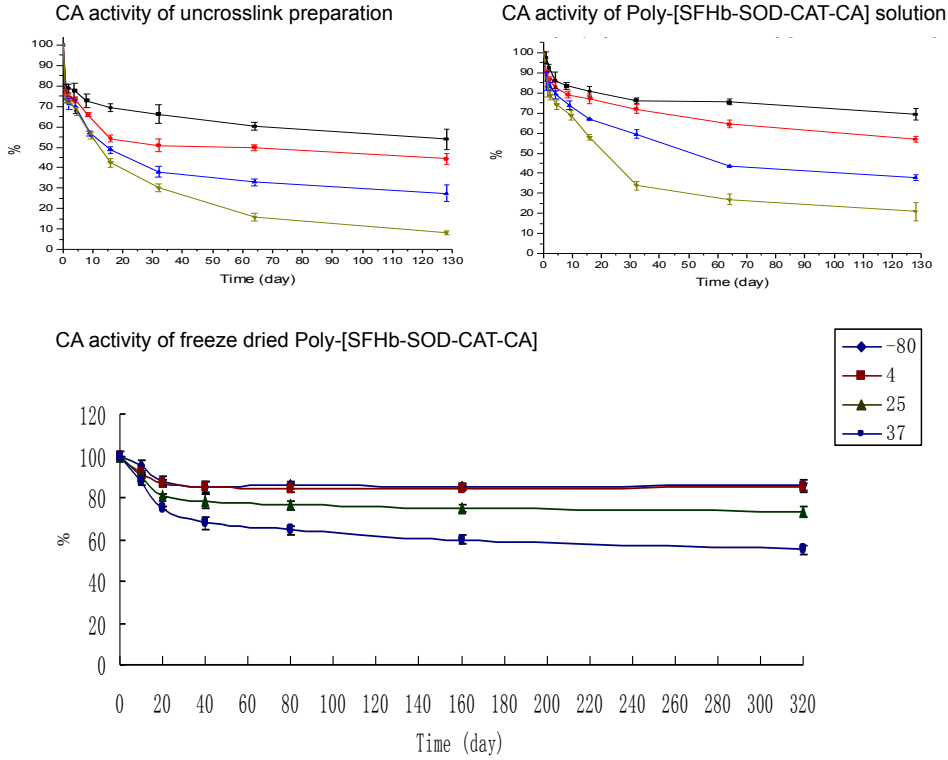

Figure 1. Storage stability of carbonic anhydrase (CA) at -80°C, 4°C, 25°C and 37°C for (1) un-crosslinked free solution of Hb, SOD, CAT, CA; (2) polymerized solution of Poly-[SFHb-SOD-CAT-CA]. and (3) freeze-dried polymerized Poly-[SFHb-SOD-CAT-CA].

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

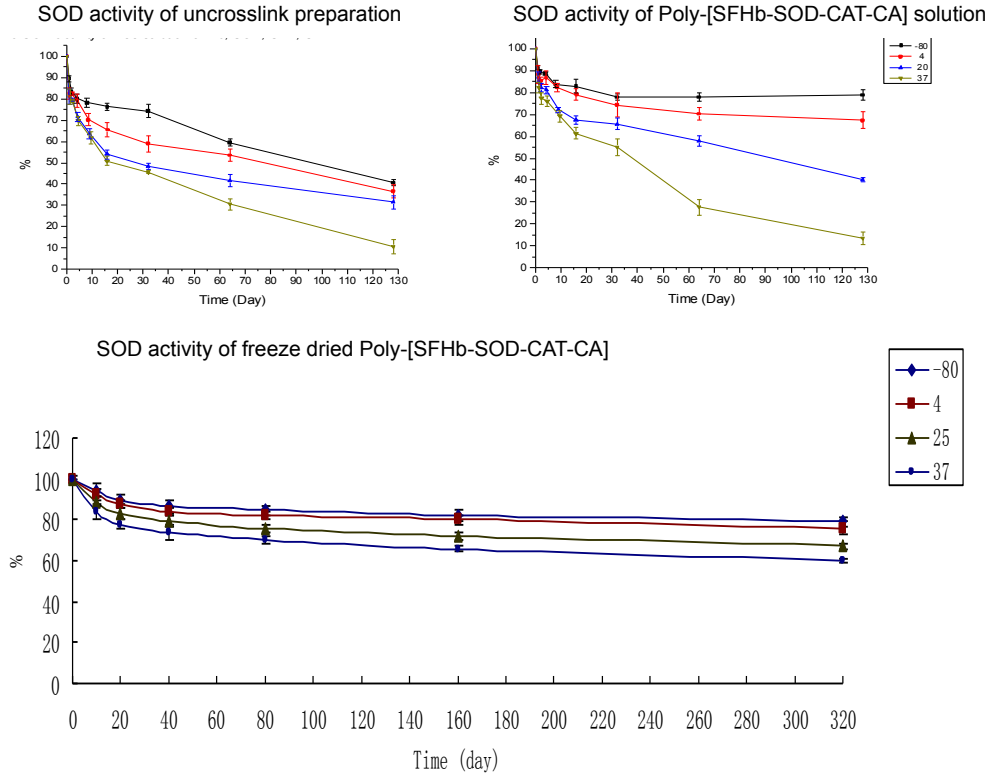

Figure 2. Storage stability of superoxide dismutase (SOD) at -80ºC, 4ºC, 25ºC and 37ºC for (1) un-crosslinked free solution of Hb, SOD, CAT, CA; (2) polymerized solution of Poly-[SFHb-SOD-CAT-CA]. and (3) freeze-dried polymerized Poly-[SFHb-SOD-CAT-CA].

(3) CAT activity: Freeze-dried polySFHb-SOD-CAT-CA kept at -80 ºC and 4 ºC, contained 83% of their original activities at -80 ºC and 74% at 4 ºC after 320 days. (Fig.3). At 20 ºC it retains about 75% of its activity after 40 days and 50% of its activity after 320 days. At 37 ºC it retains 50% of its activity after 80 days.

CAT also showed good stability in the soluble poly-[SFHb-SOD-CAT-CA] form when the samples were stored at -80 ºC, 4 ºC and 25 ºC (Fig. 1). Thus, the poly-[SFHb-SOD-CAT-CA] samples retained 96%±3%, 87.5%± 3% and 65%±1% of their original activities when stored at -80 ºC, 4 ºC and 25 ºC. The **free enzyme** solution retained 81%±6%, 77%±2%, and 58%±2% when stored at -80 ºC, 4 ºC and 25 ºC. When kept at 37ºC, there was extensive loss of CAT activity after 128 days to 20%±4 even though this is better than the free uncross-linked enzymes with only 2%±3%. (Fig.3),

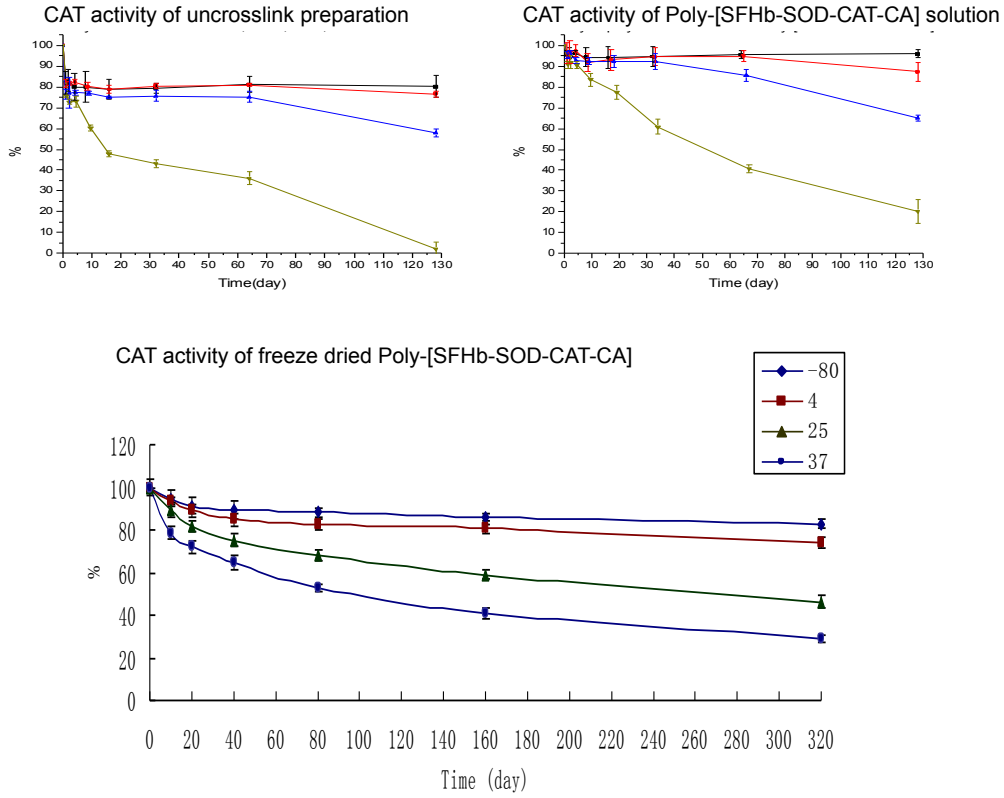

Figure 3. Storage stability of catalase (CAT) at -80°C, 4°C, 25°C and 37°C for (1) un-crosslinked free solution of Hb, SOD, CAT, CA; (2) polymerized solution of Poly-[SFHb-SOD-CAT-CA]. and (3) freeze-dried polymerized Poly-[SFHb-SOD-CAT-CA

On going study also show that poly-[SFHb-SOD-CAT-CA] prepared with the new enzymes extraction method (Guo, Gynn, Chang 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 23C, 4C or -80C the molecular weight distribution of the samples did not change after 1 year. (Table 4). Even samples kept in 37C for 1 year were also more stable than the solution form with no free Hb (lower than 100kDa) detected (Table 4).

Table 3. Molecular distribution of freeze dried sample store for 12 months

Freeze-dried PolySFHb-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%	

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

Table 4. Molecular distribution of PolySFHb-SOD-CAT-CA solution stored for 4 months

Solution of PolySFHb-SOD-CAT-CA	Molecular weight distribution		
	>450	100-450	＜100
Before storage	83%	17%	
4 months store in -80°C	83%	17%	
4 months store in 4°C	82%	18%	
4 months store in 20°C	60%	21%	13%
4 months store in 37°C	48%	23%	31%

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.

Table 5. Stability with pasteurization of the soluble and freeze dry samples.

Enzyme activity Sample		SOD	CAT	CA
Poly-[SFHb-SOD-CAT-CA] solution	0.5h (65ºC)	83.4 ± 4%	28 ± 1%	87.6 ± 4%
	1h (70ºC)	81.5 ± 3%	0	77±6%
	2h (70ºC)	64 ± 7%	0	62±6%
	3h (70ºC)	57.8±10%	0	50±6%
Freeze-dried Poly-[SFHb-SOD-CAT-CA]	0.5h (65ºC)	101 ± 4%	78 ± 5%	102 ± 4%
	1h (70ºC)	100±3%	67.4±1%	100±5%
	2h (70ºC)	100±2.5%	63.8±4%	97±4%

	3h (70°C)	100±4%	46.7±5%	94±5%

At the pasteurization temperature of 70°C the enzyme activity of respectively SOD. CAT and CA was respectively 100±3%, 67.4±1% and 100±5% after 1 hour at 70°C ; 100±2.5%, 63.8±4% and 97±4% after 2 hours 70°C; and 100±4%, 46.7±5% and 94±5% after 3 hours 70°C (Tabl5) After the pasteurization, the molecular weight distribution of the freeze-dried Poly-[SFHb-SOD-CAT-CA] did not change.
For the solution, after 30mins pasteurization, the enzyme activities remained are: SOD 83.4±4%; CAT 28 ±1% and CA 87.6±4%. After 1 hour at 70°C, SOD and CA still contain 81.5±3% and 77±6%, while the CA dropped to zero. After 2 hours, the enzymes activity of SOD, CAT and CA were 64±7% 0% and 62±6%. After 3 hours, the SOD, CAT and CA activities are ~~as~~ 57.8 ±10%, 0% and 50±6% respectively.
The freeze-dry procedure improves the enzyme stability even at the at the pasteurization temperature of 70°C. On the other hand, when not freeze-dried the enzymes 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 0.2g/gHb. This resulted in the lowering of met-Hb 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 0.8g/gHb.

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

Table 6. The P50 Value of solution and freeze-dried sample of Poly-[SFHb-SOD-CAT-CA] stored for 4 months at temperature shown.

Solution of PolyHb-SOD-CAT-CA	P50 value (mmHg)	Freeze-dried PolyHb-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

The P50 value of PolySFHb-SOD-CAT-CA was 20.0±0.74 mmHg. After stored in different temperature-80 °C, 4 °C, 25 °C, and 37 °C for 3 month, the P50 value of soluble PolySFHb-SOD-CAT-CA were 21.19±1.8, 21.31±1.93, 21.88±1.41, 22.04±0.55mmHg respectively. For the freeze dry 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 6)

Effects on C3a Compliment Activition

C3a des Arg levels were measured in this study as an indication of activation of the complement pathway. C3a is a multifunctional, pro-inflammatory 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 7)

Table 7. The C3a compliment 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

Immunological studies

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

Discussion

The allowable storage time for rbc is 42 days at 4 ^{o}C and around 1 day at room temperature. The above result shows that the lyophilized form of PolySFHb-SOD-CAT-CA retains most of its enzyme activity after 320 days when stored at 4 ^{o}C and also in room temperature. In the soluble form it has a T1/2 of 198 days at 4 ^{o}C and 51 days 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 days at room temperature is comparable to rbc stored at 4^{o}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.

Furthermore, the lyophilized form of PolySFHb-SOD-CAT-CA, unlike rbc can withstand a heat pasteurization temperature of 70 ^{o}C for 2 hours. This is important to allow the preparation to be free of infective agents for use in patients.
The complement activation studies shows that the crosslinking and lyophilizing procedure did not result in any problems related to complement activation. Further ongoing detailed immunological studies in animal are ongoing.

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