Published in final edited form as:

Biotechnol Prog. 2018 March ; 34(2): 521–528. doi:10.1002/btpr.2593.

Glutaraldehyde Cross-Linking Increases the Stability of *Lumbricus terrestris* Erythrocrucorin

Athul Rajesh^a, Devon Zimmerman^a, Kyle Spivack^a, Osheiza Abdulmalik^b, and Jacob Elmer^a

^aDepartment of Chemical Engineering, Villanova University, 800 East Lancaster Avenue, Villanova, PA, USA 19085

^bDivision of Hematology, Abramson Building, The Children's Hospital of Philadelphia, 34th St. & Civic Center Blvd, Philadelphia, PA, USA 19104

Abstract

Since donated red blood cells must be constantly refrigerated, they are not available in remote areas and battlefields. We have previously shown that the hemoglobin of the earthworm *Lumbricus terrestris* (LtEc) is an effective and safe substitute for donated blood that is stable enough to be stored for long periods at the relatively high temperatures that may be encountered in remote areas. The goal of this study was to increase the thermal stability of LtEc by covalently cross-linking LtEc with glutaraldehyde (gLtEc). Our results show that the melting temperatures of the gLtEc samples steadily increase as the molar ratio of glutaraldehyde to heme increases (from $T_m = 57^\circ\text{C}$ for native LtEc up to $T_m = 68^\circ\text{C}$ at a ratio of 128:1). In addition, while native LtEc is susceptible to subunit dissociation at alkaline pH (8–10), cross-linking with glutaraldehyde completely prevents dissociation of gLtEc at pH 10. Increasing the molar ratio of glutaraldehyde:heme also significantly increased the oxygen affinity of gLtEc, but this effect was decreased by cross-linking gLtEc in the deoxygenated T state. Finally, gLtEc samples cross-linked at low G:H ratios (e.g. 2:1) exhibited slight increases in oxidation rate, no significant difference in oxidation rate was observed between native LtEc and the gLtEc samples in Ringer's Solution, which contains antioxidants. Overall, cross-linking LtEc with glutaraldehyde significantly increases its thermal and structural stability without any loss of function, making gLtEc an attractive blood substitute for deployment in remote areas and battlefields.

Keywords

Erythrocrucorin; Blood Substitute; Glutaraldehyde; Hemoglobin-Based Oxygen Carrier; HBOC; *Lumbricus terrestris*

Introduction

Although transfusions of donated blood save millions of lives in hospitals each year, hemorrhagic shock is the leading cause of potentially survivable battlefield casualties.¹ Those casualties could be prevented by transfusions of donated blood, but blood is

unfortunately not available in remote areas because it must be constantly refrigerated.² In addition, shortages of both rare and common blood types also occur frequently and limit blood supplies in military and domestic hospitals. Donated red blood cells also undergo a myriad of physiochemical changes during storage that limit their shelf life to only 42 days.² For example, the iron within the heme subunits of hemoglobin is prone to oxidation (Fe^{2+} to Fe^{3+}).³ The oxidized hemoglobin is unable to transport oxygen and generates harmful reactive oxygen species (e.g. H_2O_2 and O_2^-) and degraded byproduct that may damage the red blood cells and the vasculature.⁴ The need to match donor and patient blood types can also impose logistical problems for performing blood transfusions in remote areas, since the patient's blood type may be unknown or particularly rare (A^- , B^- , $\text{AB}^{+/-}$).

These limitations have motivated decades of research into developing blood substitutes that can mimic the most important function of blood (O_2 delivery), but do not require refrigeration. The majority of blood substitutes have been hemoglobin-based oxygen carriers (HBOCs) that utilize human or bovine hemoglobin (HbA and bHb, respectively). Most of these HBOCs are polymerized, since native HbA and bHb tetramers quickly dissociate in the bloodstream into iron-carrying dimers that accumulate to toxic levels in the kidneys.⁵ Several different polymerized hemoglobins (PolyHbs) have been developed using a variety of different cross-linkers, including diaspirin⁶, glutaraldehyde⁷⁻⁹, and others.¹⁰ In addition, carboxylic acids on the surface of Hbs can also be activated with carbodiimide, thereby allowing them to directly react with amines on other subunits to form a “zero link” PolyHb.^{11,12} Unfortunately, clinical trials with most of these PolyHbs have revealed multiple severe adverse reactions, including hypertension, stroke, and heart attack.¹³ These side effects are a consequence of the PolyHbs' tendency to scavenge nitric oxide (NO), a gaseous hormone that regulates vascular tension.^{14,15} In addition, PolyHbs may also oxidize quickly *in vivo* and generate reactive oxygen species that increase oxidative stress.³

All of the major side effects observed with HbA and PolyHbs can be attributed to removing HbA and bHb from the protective environment of the red blood cell (RBC). Indeed, the RBC contains antioxidant enzymes that limit oxidation and provides a protective membrane that segregates Hb from NO and prevents Hb dimers from extravasating. Therefore, a better starting material for blood substitutes may be extracellular hemoglobins (also known as erythrocruorins, Ecs) that have evolved to function without the protection of RBCs. For example, the Ec of the common earthworm *Lumbricus terrestris* (LtEc) is a giant 3,600 kDa assembly of 144 globins and 36 linker subunits.¹⁶ Each of the globins (A, B, C, and D1/D2/D3) has an intramolecular disulfide, while the A, B, and C subunits also have intermolecular disulfides that allow them to form a covalently linked ABC trimer.^{17,18} The globins form dodecamers that are bound by linker subunits (L1, L2, L3, L4), which have their own networks of disulfide bonds and coiled coil domains that allow them to assemble into a hexagonal bilayer (HBL) that is ~30 nm in diameter ($D_{\text{HbA}} = 5-6$ nm).¹⁶ LtEc also has several calcium binding sites (1 for every 4 globins) that stabilize the protein.¹⁹ In addition to its high structural stability, LtEc also has a positive redox potential ($E_o = +112$ mV) compared to the negative redox potential of HbA ($E_o = -50$ mV), indicating that LtEc is less prone to oxidation than HbA.²⁰ LtEc also has a much smaller heme pocket in comparison to intracellular Hbs, which provides a physical barrier that prevents NO from easily reacting with bound O_2 .¹⁶ The O_2 affinity of LtEc (28 mm Hg) is quite similar to

human whole blood (28 mm Hg), although its colloid osmotic pressure (14 ± 3.4 mm Hg at 10 g/dL) is lower than whole blood (19–24.5 mm Hg). Nonetheless, transfusions of LtEc into mice and rats have also shown that it can effectively deliver O₂ without inducing vasoconstriction or hypertension.^{4,21,22} Altogether, these results suggest that LtEc may be a highly stable, effective, and safe blood substitute.

Despite these favorable properties, Ecs may still be vulnerable to dissociation or denaturation during prolonged storage at high temperatures or upon exposure to alkaline pH. For example, the Ec of *Arenicola marina* dissociates at physiological pH (7.4),²³ while LtEc dissociates under more alkaline conditions (pH 8.9).^{24,25} Studies of *Glossoscolex paulistus* Ec (GpEc) have also shown that oxidation can promote dissociation of the D subunits at pH 8.^{26,27} Conversely, subunit dissociation in GpEc has also been shown to increase the rate of subunit oxidation.²⁸ In this work, we show that the dissociation of LtEc under a wide range of conditions (e.g. pH 8–10) can be prevented by cross-linking it with glutaraldehyde. In addition, cross-linking also significantly increases the melting temperature of LtEc.

Materials and Methods

LtEc Purification

LtEc was extracted from batches of 500–1,000 *Lumbricus terrestris* specimens that were purchased from Wholesale Bait (Cincinnati, OH). After rinsing the worms to remove soil, they were quickly homogenized in a blender for 10 seconds and the homogenate was centrifuged at 3,500g for 30 minutes at 4°C to remove solid debris. The resulting red supernatant was then centrifuged again at 15,000 g for 30 minutes at 4°C. The crude LtEc solution was clarified with a 0.2 µm TFF membrane with a surface area of 790 cm² (Spectrum Labs, Rancho Dominguez, CA). The 0.2 µm filtrate containing LtEc and other impurities was then diafiltered with a 500 kDa molecular weight cut-off (MWCO) TFF filter for 10 consecutive rounds. In each round of diafiltration, the sample was concentrated to 50 mL and then diluted to 500 mL with 20 mM Tris (pH 7.0, 4°C).

Glutaraldehyde Cross-Linking

Cross-linking reactions (see Figure 1) were conducted at increasing molar ratios of glutaraldehyde to heme, including 0:1, 1:1, 2:1, 4:1, 8:1, 16:1, 32:1, 64:1, 128:1, 256:1, 512:1, 1024:1. In each reaction, the heme concentration was held constant at 0.25 mM while the glutaraldehyde concentration increased (e.g. an 8:1 ratio corresponds to a reaction of 2 mM glutaraldehyde with 0.25 mM heme). Concentrations of LtEc were estimated by measuring the sample absorbance at 541 nM and using an extinction coefficient of $\epsilon = 13.8$ mM heme⁻¹cm⁻¹. All cross-linking reactions were conducted at room temperature (~20°C) for 1, 2, or 4 hours in 20 mM HEPES buffer. Most reactions were conducted with oxygenated LtEc, but the T state gLtEc samples shown in Figure 7 were prepared by first adding 1 mg/mL sodium dithionite prior to cross-linking and confirming deoxygenation via UV-Vis spectroscopy. The reactions were all quenched with 1 M Tris buffer (pH 8). The Schiff base formed by glutaraldehyde could then be reduced with sodium borohydride (NaCNBH₃), but this reduction step did not have a significant effect on thermal stability (data not shown).

Polyacrylamide Gel Electrophoresis (PAGE)

The extent of intermolecular cross-linking between the LtEc subunits was determined by loading the cross-linked samples on a 12% Polyacrylamide gel and running it in a pH 9.0 buffer (0.05 M Tris, 0.38 M glycine, 0.2% SDS). All samples were diluted to 7 mM heme in 20 mM Tris buffer (pH 7.0) and mixed in 1:1 ratio with Laemmli buffer containing β -mercaptoethanol, then incubated for 10 minutes at 95°C. Gels were run at 30 Volts for 10 minutes to separate any excess salts, then the voltage was increased to 125 volts for 1–2 hours. Finally, the gels were stained overnight in 0.25% Brilliant Blue R (Sigma Aldrich, B0149) and destained with a solution of 10% acetic acid, 20% ethanol, 70% water.

Dynamic Light Scattering (DLS)

The effective diameter of the gLtEc molecules were measured via Dynamic Light Scattering (DLS) using a NanoBrook 90Plus Particle Size Analyzer from Brookhaven Instruments (Holtsville, New York). Native LtEc and the cross-linked samples were diluted to ~20 mM concentrations in plastic cuvettes and three scans were run at 25°C and averaged to obtain effective diameter measurements.

Thermal Shift Assay

SYPRO Orange dye was used to measure the melting temperature of the cross-linked LtEc samples. In this assay, SYPRO binds to hydrophobic residues that are exposed as the protein denatures and provides a fluorescent signal that can be used to detect protein denaturation at high temperatures. The 5,000x SYPRO Orange dye stock (ThermoFisher, Cat. #S6650) was diluted 25-fold in DMSO, then 45 μ L of the diluted SYPRO was mixed with 5 μ L of LtEc samples at 4 μ M heme in 10 mM HEPES buffer (pH = 7.4). To prevent the oxidation of the heme iron during the assay (a phenomenon which has previously been shown to reduce thermal stability in other Ecs²⁹) the antioxidant ascorbic acid was also added to the samples at a final concentration of 1 mg/mL. All samples were then transferred to MicroAmp Optical 96-well plates and analyzed with an Applied Biosystems (Foster City, CA) 7300 qPCR machine. Specifically, the sample temperature was increased 1°C per minute from 30–89°C while fluorescence was monitored at 600 nm. The melting temperature of each sample was then determined by locating inflection points ($y'' = 0$) in the fluorescence data.

HEMOX Analysis

A HEMOX Analyzer (TCS Scientific, New Hope, PA) was used to measure the oxygen equilibrium curves of the gLtEc samples. Samples were diluted in Hemox buffer prior to each run, then sparged with air until the partial pressure of O₂ (pO₂) reached ~150 mm Hg. The samples were then sparged with pure N₂ until the pO₂ decreased to ~2 mm Hg while spectroscopy was simultaneously used to measure the percent O₂ saturation (Y) of the hemoglobin samples. The oxygen affinity (P₅₀) of each sample was estimated as the pO₂ at which the hemoglobin was 50% saturated with O₂ (Y = 0.5). Hill coefficients (n) were then calculated using Equation 1.

$$\log \left(\frac{Y}{1-Y} \right) = n \log \left(\frac{pO_2}{P_{50}} \right) \quad (\text{Eqn. 1})$$

Oxidation Levels and Rates

Oxidation levels and rates were measured using UV-Vis spectroscopy. Absorbance spectra were recorded from 500–700 nm and then Equation 2 was used to calculate the percentage of reduced hemoglobin (%Fe²⁺). This equation utilizes an isosbestic point at 405 nm and the absorbance at 415 nm, which decreases as LtEc oxidizes from Fe²⁺ to Fe³⁺. Absorbance values for pure LtEc:Fe²⁺ and LtEc:Fe³⁺ were obtained by mixing LtEc with the reducing agent dithiothreitol (DTT) and the oxidizing agent potassium ferricyanide, K₃Fe(CN)₆, respectively.

$$\% Fe^{2+} = \frac{\left(\frac{A_{415}}{A_{405}} \right)_t - \left(\frac{A_{415}}{A_{405}} \right)_{LtEc:Fe3+}}{\left(\frac{A_{415}}{A_{405}} \right)_{LtEc:Fe2+} - \left(\frac{A_{415}}{A_{405}} \right)_{LtEc:Fe3+}} \quad (\text{Eqn. 2})$$

For each oxidation assay, Ecs were first diluted to A₄₁₅ = 1.0 in either 20 mM Tris (pH 7.0) or Ringer's Modified Lactate Solution (115 mM NaCl, 0.3% sodium lactate, 12.25 mM N-acetyl L-cysteine, 1.4 mM CaCl₂, 4 mM KCl, pH 7.0) and then sterilized using a 0.2 μm sterile syringe filter in a biological safety cabinet (BSC). Samples were then aliquoted and stored in the dark at room temperature. Absorbance spectra were taken daily for 2 weeks and then used to estimate oxidation levels using Equation 2. Since the oxidation plots shown in Figure 5 appeared to be linear, a single exponential decay model (Equation 3) was used to estimate oxidation rates (k_{ox}).

$$\ln \left\{ \frac{[Fe^{2+}]}{[Fe]_{total}} \right\} = -k_{ox}t \quad (\text{Eqn. 3})$$

Analytical Size Exclusion Chromatography

A BioRad Enrich SEC 650 column (24 mL, 300 mm bed height) on a BioRad NGC chromatography system was equilibrated with 20 mM Tris buffer at the desired pH (e.g. pH = 8, 9, or 10). Cross-linked LtEc samples were then thawed and diluted to 75 μM (heme) in 20 mM Tris buffer at the corresponding pH. The samples were then loaded onto the column and eluted with Tris buffer at a constant flow rate of 0.2 mL/min. Protein elution was detected by monitoring the absorbance of the column effluent at 280 nm.

Statistical Analysis

All statistical analyses were performed using R Studio software (Boston, MA) or by simultaneous, paired T tests in Microsoft Excel. All analyses conducted in R Studio were parametric analysis of variance (ANOVA) and significant differences were determined using Tukey's Honestly Significant Difference following tests of normality and homogeneity of variances. Statistical significance was defined as $p < 0.05$.

Results and Discussion

Thermal Stability of gLtEc

Significant aggregation was observed during the cross-linking reactions at glutaraldehyde:heme (G:H) ratios of 512:1 & 1024:1. Consequently, those G:H ratios were not tested further, but the effects of lower molar ratios (1:1–256:1) on the thermal stability (T_m) of gLtEc are shown in Figure 2. At all G:H ratios, the reaction appears to be complete in less than an hour, since reaction time (1, 2, or 4 hours) does not have a significant effect on T_m for any of the ratios. Based on this observation, a reaction time of 1 hour was used to prepare samples for all of the remaining assays in Figures 3–7. It is also important to mention that the amount of oxidized LtEc (Fe^{3+}) did not significantly increase during any of the cross-linking reactions, as shown in Table 1).

While reaction time did not influence T_m , increasing the G:H ratio significantly increased thermal stability. Indeed, even the lowest G:H ratio of 1:1 significantly increased the T_m of LtEc from 57.3°C to 59°C. The melting temperatures of the remaining gLtEc samples continued to increase logarithmically up to the G:H ratio of 64:1 ($T_m = 68^\circ\text{C}$), but no further increase in melting temperature was observed at 128:1 or 256:1. In contrast, a slight decrease in T_m was observed at a ratio of 512:1 (slight aggregation was also observed at this G:H ratio).

Alkaline Dissociation

Native LtEc exists primarily as a high MW hexagonal bilayer (3.6 MDa) that quickly elutes from the SEC column after 45 minutes (see Figure 3). However, increasing the pH to 9 or 10 induces dissociation of the HBL into globin dodecamers, linker trimers, and other smaller components, which elute after longer times (50–75 minutes). Cross-linking LtEc at a G:H ratio of 2:1 significantly decreases the amount of dissociation observed at pH 9 and 10, while the gLtEc cross-linked at an 8:1 ratio shows no signs of dissociation at all. Similar results were also observed at higher G:H ratios (32:1 and 128:1, data not shown), with only a single fraction eluting at 45 minutes. Therefore, it appears that glutaraldehyde cross-linking prevents dissociation of LtEc at alkaline pH.

Structure and Size of gLtEc

After observing that glutaraldehyde cross-linking increased the thermal stability of LtEc and prevented its dissociation at pH 9–10, we sought to determine the effects of glutaraldehyde on the structure and size of LtEc. Figure 4 shows a PAGE analysis of five cross-linked gLtEc samples (G:H ratios of 0:1, 2:1, 8:1, 32:1, 128:1). While the native LtEc sample only exhibits the expected banding pattern with individual linker subunits (25–32 kDa) and

globins (at 10–14 kDa), smeared bands at higher MWs are observed in the gLtEc samples. At the higher G:H ratios of 32:1 and 128:1, the individual subunit bands become progressively lighter and more sample appears to be retained in the well. This change in banding patterns suggests that the individual subunits are cross-linked by glutaraldehyde, thereby producing peptides with higher MWs and some products that are too large to enter the PAGE gel (> 250 kDa).

To determine whether glutaraldehyde also increases the overall size of the gLtEc particles by forming cross-links between separate hexagonal bilayer (HBL) structures, the effective diameter of the gLtEc samples was measured with dynamic light scattering (DLS, Figure 4). First, it is important to mention that although the actual diameter of the LtEc HBL is 30 nm, the effective diameter measured by DLS is much larger (85 nm). Nonetheless, cross-linking LtEc with glutaraldehyde did not have a significant effect on effective diameter at any of the G:H ratios tested (up to 256:1). There was also no significant difference in the polydispersity index of native LtEc and the gLtEc samples ($PDI = 0.34\text{--}0.35$). Therefore, it appears that the glutaraldehyde cross-links formed within and between the subunits of LtEc, not between separate HBLs.

Oxidation Rate

The oxidation rates of native HbA, native LtEc, and the gLtEc samples in Tris and Ringer's buffers at 20°C are shown in Figure 5 and Table 2. In Tris buffer, HbA showed very little oxidation ($0.55 \times 10^{-3} \text{ hr}^{-1}$), while the oxidation rate of native LtEc was significantly higher ($3.2 \times 10^{-3} \text{ hr}^{-1}$). The highest oxidation rate was observed at the lowest G:H ratio (2:1, $k_{ox} = 12.4 \times 10^{-3} \text{ hr}^{-1}$), but the oxidation rates of the other gLtEc samples progressively decreased as the G:H ratio increased. Finally, at the highest G:H ratio of 128:1, there was no significant difference between the 128:1 gLtEc sample and native LtEc.

In contrast, the oxidation trends observed in Ringer's Modified Lactate Solution differed significantly from those observed in Tris buffer. Ringer's Lactate contains the antioxidants sodium lactate and N-acetyl L-cysteine, which decrease the oxidation rate of LtEc (but not HbA).³⁰ For example, HbA exhibited a much higher rate of oxidation ($11.34 \times 10^{-3} \text{ hr}^{-1}$) in Ringer's Solution. In contrast, the oxidation rates of LtEc and all of the gLtEc samples significantly decreased by 2–3 orders of magnitude in Ringer's Solution. There was also no significant difference between the oxidation rates of native LtEc and most of the gLtEc samples, except for the 2:1 gLtEc sample, which showed a slight, yet significant, increase in oxidation rate ($0.07 \times 10^{-3} \text{ hr}^{-1}$) compared to native LtEc ($0.04 \times 10^{-3} \text{ hr}^{-1}$).

Oxygen Equilibria

The effects of glutaraldehyde cross-linking on the oxygen equilibria of gLtEc samples at 25°C and 37°C are shown in Figure 6 and Table 3. As expected, the P_{50} of native LtEc is 32.5 mm Hg at 37°C, which happens to be similar to the P_{50} of human red blood cells.⁴ Interestingly, cross-linking the gLtEc samples increased their oxygen affinity in a glutaraldehyde-dependent manner, with the highest oxygen affinity (i.e. lowest P_{50}) observed at a G:H ratio of 128:1 ($P_{50} = 19.3 \pm 0.3$ mm Hg at 37°C). The cooperativity (as

indicated by the Hill coefficient, n , in Table 3) of the gLtEc samples also decreased significantly as the G:H ratio increased.

The decrease in cooperativity and increase in O_2 affinity observed with the gLtEc samples may be due to the gLtEc subunits becoming locked in the R state conformation, since they were cross-linked in the presence of O_2 . Indeed, the oxygenated R state of HbA has a much higher O_2 affinity than the deoxygenated T state, which would explain the increase in O_2 affinity observed in the gLtEc samples at high G:H ratios. To test this theory, LtEc samples were also cross-linked in the deoxygenated T state, which has a lower affinity for O_2 and higher P_{50} (Table 3 and Figure 7). Indeed, the P_{50} of the gLtEc synthesized in the T state (24.6 mm Hg) was significantly higher than the corresponding R state gLtEc (19.3 mm Hg), but still lower than native LtEc (32.6 mm Hg).

Our results show that cross-linking significantly increases the oxygen affinity (and decreases the P_{50}) of the gLtEc samples, but cross-linking the 128:1 gLtEc in the deoxygenated T state provides a modest, yet significant, increase in P_{50} . A similar, but much more drastic, effect has also been observed when bHb is cross-linked with glutaraldehyde.³¹ Bovine Hb cross-linked in the R state has a very high O_2 affinity ($P_{50} = 1.84$ mm Hg), while bHb cross-linked in the T state has a very low O_2 affinity ($P_{50} = 41.16$ mm Hg) relative to native bHb ($P_{50} = 27$ mm Hg). Both types of cross-linked bHb also display a much lower cooperativity ($n = 0.7$ – 1.0) than native bHb ($n = 2.84$). It is currently unclear whether high or low O_2 affinity would be more effective *in vivo*, but it is important to note that increasing the O_2 affinity of a hemoglobin can decrease its nitrite reductase activity, thereby limiting its ability to generate nitric oxide *in vivo*, which may be an important factor in alleviating vasoconstriction due to NO scavenging.^{32,33} Nonetheless, it is interesting to note that the P_{50} of glutaraldehyde cross-linked Hbs can be increased to 25 mm Hg by cross-linking them in the T state. In addition, Figure 7 shows that the P_{50} of gLtEc can also be tuned by varying the G:H ratio.

Conclusion

Native LtEc is already a highly stable protein, with intermolecular disulfide bonds that prevent dissociation and a melting temperature of 57°C. However, our results show that glutaraldehyde cross-linking reactions can further improve the thermal and structural stability of LtEc. Indeed, cross-linking LtEc at a glutaraldehyde:heme ratio of 128:1 for 1 hour at room temperature yields a gLtEc product with a higher melting temperature ($T_m = 68^\circ\text{C}$) and resistance to alkaline dissociation at pH 10. Most importantly, the gLtEc samples are still able to transport O_2 , albeit with a higher O_2 affinity and lower cooperativity. Low G:H ratios did increase oxidation rates in Tris buffer, but the oxidation rates of all the gLtEc samples were much lower (and virtually negligible) in the antioxidant-containing Ringer's Modified Lactate Solution, which is more likely to be used for transfusions. All of these properties suggest that gLtEc may be an attractive blood substitute for deployment in battlefield scenarios. Lastly, it is also worth mentioning that a PEGylated LtEc developed by Roche et al has a dramatically increased circulation half-life (66.8 ± 1.8 hr) compared to native LtEc (18 ± 0.8 hr).³⁴ Taken together, both LtEc derivatives demonstrate that LtEc can be synthetically modified to make it an even more attractive blood substitute.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH NHLBI Grant 1R15HL133880-01A1.

References

1. Kelly JF, Ritenour AE, McLaughlin DF, et al. Injury severity and causes of death from Operation Iraqi Freedom and Operation Enduring Freedom: 2003–2004 versus 2006. *J Trauma*. 2008; 64(2 Suppl):S21-6-7. [PubMed: 18376168]
2. Kim-Shapiro DB, Lee J, Gladwin MT. Storage lesion: role of red blood cell breakdown. *Transfusion*. 2011; 51(4):844–851. [PubMed: 21496045]
3. Buehler PW, Alayash AI. Oxidation of hemoglobin: mechanisms of control in vitro and in vivo. *Transfus Altern Transfus Med*. 2007; 9(4):204–212.
4. Elmer J, Zorc K, Rameez S, Zhou Y, Cabrales P, Palmer AF. Hypervolemic infusion of *Lumbricus terrestris* erythrocytes purified by tangential-flow filtration. *Transfusion*. 2012; 52(8):1729–1740. [PubMed: 22304397]
5. Brandt JL, Frank NR, Lichtman HC. The effects of hemoglobin solutions on renal functions in man. *Blood*. 1951; 6(11):1152–1158. [PubMed: 14869374]
6. Cole DJ, Drummond JC, Patel PM, Reynolds LR. Hypervolemic-hemodilution during cerebral ischemia in rats: effect of dapsirin cross-linked hemoglobin (DCLHb) on neurologic outcome and infarct volume. *J Neurosurg Anesthesiol*. 1997; 9(1):44–50. [PubMed: 9016440]
7. Chang TMS. Stabilisation of enzymes by microencapsulation with a concentrated protein solution or by microencapsulation followed by cross-linking with glutaraldehyde. *Biochem Biophys Res Commun*. 1971; 44(6):1531–1536. [PubMed: 5160711]
8. Zhang N, Jia Y, Chen G, Cabrales P, Palmer AF. Biophysical properties and oxygenation potential of high-molecular-weight glutaraldehyde-polymerized human hemoglobins maintained in the tense and relaxed quaternary states. *Tissue Eng Part A*. 2011; 17(7–8):927–940. [PubMed: 20979534]
9. Jahr JS, Moallempour M, Lim JC. HBOC-201, hemoglobin glutamer-250 (bovine), Hemopure (Biopure Corporation). *Expert Opin Biol Ther*. 2008; 8(9):1425–1433. [PubMed: 18694360]
10. Chen J-Y, Scerbo M, Kramer G. A review of blood substitutes: examining the history, clinical trial results, and ethics of hemoglobin-based oxygen carriers. *Clinics (Sao Paulo)*. 2009; 64(8):803–813. [PubMed: 19690667]
11. Harrington JP, Wollocko H. Pre-clinical studies using OxyVita hemoglobin, a zero-linked polymeric hemoglobin: a review. *J Artif Organs*. 2010; 13(4):183–188. [PubMed: 21127928]
12. Harrington JP, Wollocko J, Kostecki E, Wollocko H. Physicochemical characteristics of OxyVita hemoglobin, a zero-linked polymer: liquid and powder preparations. *Artif Cells Blood Substit Immobil Biotechnol*. 2011; 39(1):12–18. [PubMed: 20626229]
13. Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. *JAMA*. 2008; 299(19):2304–2312. [PubMed: 18443023]
14. Helms C, Kim-Shapiro DB. Hemoglobin-mediated nitric oxide signaling. *Free Radic Biol Med*. 2013; 61:464–472. [PubMed: 23624304]
15. Schechter AN, Gladwin MT. Hemoglobin and the paracrine and endocrine functions of nitric oxide. *N Engl J Med*. 2003; 348(15):1483–1485. [PubMed: 12686706]
16. Royer WE, Sharma H, Strand K, Knapp JE, Bhayravhatla B. *Lumbricus erythrocytes* at 3.5 Å resolution: architecture of a megadalton respiratory complex. *Structure*. 2006; 14(7):1167–1177. [PubMed: 16843898]

17. Martin PD, Kuchumov AR, Green BN, et al. Mass Spectrometric Composition and Molecular Mass of *Lumbricus terrestris* Hemoglobin: A Refined Model of its Quaternary Structure. *J Mol Biol.* 1996; 255(1):154–169. [PubMed: 8568863]
18. Xie Q, Donahue RA, Schneider K, et al. Structure of chain d of the gigantic hemoglobin of the earthworm. *Biochim Biophys Acta - Protein Struct Mol Enzymol.* 1997; 1337(2):241–247.
19. Chiancone E, Bull TE, Norne JE, Forsen S, Antonini E. Studies on Erythrocrurin V. Nuclear Magnetic Resonance Quadrupole Relaxation Study of Sodium, Calcium and Chloride Binding. *J Mol Biol.* 1976; 107:25–34. [PubMed: 12370]
20. Harrington JP, Kobayashi S, Dorman SC, Zito SL, Hirsch RE. Acellular invertebrate hemoglobins as model therapeutic oxygen carriers: unique redox potentials. *Artif Cells Blood Substit Immobil Biotechnol.* 2007; 35(1):53–67. [PubMed: 17364471]
21. Hirsch RE, Jelicks LA, Wittenberg BA, Kaul DK, Shear HL, Harrington JP. A First Evaluation of the Natural High Molecular Weight Polymeric *Lumbricus terrestris* Hemoglobin as an Oxygen Carrier. Jul.2009
22. Elmer J, Palmer AF, Cabrales P. Oxygen delivery during extreme anemia with ultra-pure earthworm hemoglobin. *Life Sci.* 2012; 91(17–18):852–859. [PubMed: 22982347]
23. Rousselot M, Delpy E, Drieu La Rochelle C, et al. Arenicola marina extracellular hemoglobin: a new promising blood substitute. *Biotechnol J.* 2006; 1(3):333–345. [PubMed: 16897713]
24. Harrington JP, Hirsch RE. Analysis of the acid and alkaline dissociation of earthworm hemoglobin, *Lumbricus terrestris*, by front-face fluorescence spectroscopy. *Biochim Biophys Acta.* 1991; 1076:351–358. [PubMed: 2001383]
25. Chiancone E, Vecchini P, Fanelli MRR, Antonini E. Studies on erythrocrurin: II. Dissociation of earthworm erythrocrurin. *J Mol Biol.* 1972; 70(1):73–76. [PubMed: 5073352]
26. Zhu H, Ownby DW, Riggs CK, et al. Assembly of the Gigantic Hemoglobin of the Earthworm *Lumbricus terrestris*. *J Biol Chem.* 1996; 271(47):30007–30021. [PubMed: 8939947]
27. Carvalho FAO, Carvalho JWP, Alves FR, Tabak M. pH effect upon HbGp oligomeric stability: Characterization of the dissociated species by AUC and DLS studies. *Int J Biol Macromol.* 2013; 59:333–341. [PubMed: 23648596]
28. Poli AL, Moreira LM, Imasato H. Autoxidation of giant extracellular hemoglobin of *Glossoscolex paulistus*: molecular mechanism and oligomeric implications. *Spectrochim Acta A Mol Biomol Spectrosc.* 2011; 82(1):306–315. [PubMed: 21824807]
29. Carvalho JWP, Santiago PS, Batista T, et al. On the temperature stability of extracellular hemoglobin of *Glossoscolex paulistus*, at different oxidation states: SAXS and DLS studies. *Biophys Chem.* 2012; 163–164:44–55.
30. Muzzelo C, Neely C, Shah P, Abdulmalik O, Elmer J. Prolonging the shelf life of *Lumbricus terrestris* erythrocrurin for use as a novel blood substitute. *Artif cells, nanomedicine, Biotechnol.* 2017; 20:1–8.
31. Cantu-Medellin N, Vitturi DA, Rodriguez C, et al. Effects of T- and R-state stabilization on deoxyhemoglobin-nitrite reactions and stimulation of nitric oxide signaling. *Nitric Oxide.* 2011; 25(2):59–69. [PubMed: 21277987]
32. Roche CJ, Talwar A, Palmer AF, Cabrales P, Gerfen G, Friedman JM. Evaluating the capacity to generate and preserve nitric oxide bioactivity in highly purified earthworm erythrocrurin: a giant polymeric hemoglobin with potential blood substitute properties. *J Biol Chem.* 2015; 290(1):99–117. [PubMed: 25371199]
33. Grubina R, Basu S, Tiso M, Kim-Shapiro DB, Gladwin MT. Nitrite Reductase Activity of Hemoglobin S (Sickle) Provides Insight into Contributions of Heme Redox Potential Versus Ligand Affinity. *J Biol Chem.* 2008; 283(6):3628–3638. [PubMed: 18056715]
34. Jani VP, Jelvani A, Moges S, et al. Polyethylene Glycol Camouflaged Earthworm Hemoglobin. *PLoS One.* 2017; 12(1):e0170041. [PubMed: 28099525]

Figure 1.

Glutaraldehyde cross-linking reaction. Left to Right: The aldehyde groups of glutaraldehyde spontaneously react with free amines (e.g. lysine) on the protein's surface to form intra- or intermolecular cross-links with Schiff bases. To prevent cleavage of this bond at acidic pH, the Schiff bases can be subsequently reduced with a strong reducing agent (e.g. NaCNBH₃).

Figure 2.

Effects of cross-linking on the thermal stability of LtEc. The melting temperatures of the gLtEc samples steadily increase as the G:H ratio increases up to 64:1, then it plateaus and starts to decrease at 512:1. Extending the reaction time past one hour did not have a significant effect on T_m . *Significant differences compared to 0:1 control and defined as $p < 0.05$ calculated via simultaneous, paired T tests.

Figure 3. Analytical SEC of native LtEc and gLtEc samples at alkaline pH (8, 9, and 10). Each sample shows at least one peak that elutes at around 45 minutes, which corresponds to the complete hexagonal bilayer structure (HBL) of LtEc. The native LtEc and 2:1 gLtEc samples also show peaks that elute at longer times and are indicative of dissociated subunits/complexes.

Figure 4.

PAGE (left) and DLS (right) analysis of gLtEc samples. Left: While native LtEc only shows the expected globin (10–15 kDa) and linker subunit (25–32 kDa) bands on a 12% acrylamide gel, gLtEc samples display bands at higher MW. Right: No significant change in the effective diameter was observed between LtEc and gLtEc samples in DLS experiments.

Figure 5.

Oxidation of gLtEc samples in Tris and Ringer's Modified Lactate Buffers. Samples were incubated at 20°C over the course of 10 days and %Fe²⁺ was determined spectroscopically. Each data point represents averages from three independent samples.

Figure 6. Representative oxygen equilibrium curves of gLtEc samples at 37°C in Hemox Buffer. The oxygen saturation of the sample (y-axis) is shown at different partial pressures of oxygen (x-axis).

Figure 7.

Oxygen affinity (P_{50}) of gLtEc samples. Left: Effects of G:H ratio and temperature (25°C or 37°C) on the P_{50} of native LtEc and gLtEc in Hemox buffer. Right: Differences in oxygen affinity between gLtEc samples synthesized in the oxygenated R state and the deoxygenated T state at G:H ratios of 8:1, 32:1, and 128:1. In both plots, an asterisk (*) indicates that the value is significantly less than the value observed for native LtEc, while a cross (†) indicates a significant difference between the gLtEc samples synthesized in the T and R states. Differences in effects of G:H ratio and temperature on P_{50} calculated using parametric ANOVA and Tukey's Honestly Significant Difference. Differences in P_{50} between T and R states calculated via simultaneous, paired T tests. Significant differences defined as $p < 0.05$.

Table 1Percentage of Fe²⁺ in gLtEc Samples

Glut:Heme	%Fe ²⁺
2:1	97.0 ± 1.5%
8:1	97.1 ± 0.3%
32:1	96.3 ± 1.4%
128:1	95.0 ± 0.8%

Table 2

Oxidation Rates of gLtEc Samples at 20°C

k_{ox} (hr ⁻¹ ×10 ³)	Buffer	
	Tris	Ringer's
HbA	0.55 ± 0.04	11.3 ± 2.1
LtEc	3.2 ± 0.5	0.04 ± 0.05
2:1	12.4 ± 3.6 ^a	0.07 ± 0.04 ^a
8:1	11.3 ± 3.4 ^a	0.04 ± 0.04
32:1	7.1 ± 1.0 ^a	0.03 ± 0.03
128:1	3.2 ± 1.3	0.05 ± 0.05

^aSignificantly higher than native LtEc calculated via simultaneous, paired T tests. Significant differences defined as $p < 0.05$.

Table 3

Oxygen Affinity (P_{50}) and Cooperativity (n) of gLtEc Samples at 37°C

G:H Ratio	LtEc	R State				T State		
		2:1	8:1	32:1	128:1	8:1	32:1	128:1
P ₅₀ (mm Hg)	32.6±0.2	32.4±0.2	30.3±1.2	25.0±0.3	19.3±0.3	28.6±1.6 ^a	27.6±1.5	24.6±1.9 ^a
n	1.87±0.01	1.80±0.01	1.93±0.04	1.79±0.05	1.62±0.04	2.24±0.07 ^a	1.96±0.30	1.34±0.18

^aSignificant difference observed between gLtEc samples cross-linked in the T and R states calculated via simultaneous, paired T tests. Significant differences defined as $p < 0.05$.