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Hypervolemic Infusion of *Lumbricus terrestris* Erythrocrucorin Purified by Tangential Flow Filtration

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Abstract

Background—The hemoglobin of the Earthworm *Lumbricus terrestris* (also known as erythrocrucorin, or LtEc) is a naturally occurring high molecular weight protein assembly (3.6 MDa) that is extremely stable, resistant to oxidation, and transports oxygen similarly to human whole blood. Therefore, LtEc may serve as an alternative to donated human red blood cells, however, a suitable purification process must be developed to produce highly pure LtEc on a large scale that can be evaluated in an animal model to determine the safety and efficacy of LtEc.

Materials and Methods—We used tangential flow filtration (TFF), an easily scalable and affordable technique, to produce highly pure LtEc from Earthworms. The purity, yield, methemoglobin level, viscosity, colloid osmotic pressure, O₂ binding equilibria, and ligand binding kinetics of the purified LtEc was measured *in vitro*. The purified LtEc product was then evaluated in hamsters using a hypervolemic infusion model to establish its basic biocompatibility and detect any changes in microcirculatory and systemic parameters.

Results—TFF was able to produce LtEc with high purity and yield (5–10 grams per 1000 worms). The purified LtEc product did not elicit hypertension or vasoconstriction when infused into hamsters.

Conclusion—LtEc may be easily purified and safely transfused into hamsters in small amounts (0.5–1.5 g/dL final concentration in blood) without any noticeable side-effects. Therefore, LtEc may serve as a very promising oxygen carrier for use in transfusion medicine.

Keywords

hemoglobin; erythrocrucorin; tangential flow filtration; oxygen carrier; red blood cell substitute; transfusion medicine

Introduction

In the United States alone, approximately 12 million red blood cell (RBC) transfusions are performed each year.¹ Donations are able to meet most of this demand, however, rare blood types are often unavailable and even the most common blood types are vulnerable to seasonal shortages. The situation is even worse in third world countries that lack proper screening techniques and/or storage facilities for donated blood. The World Health Organization has also reported that 20% of malaria deaths and 40% of deaths during childbirth in these countries could have been prevented with an adequate blood supply.

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Therefore, a significant worldwide demand exists for an alternative to donated blood which is affordable, safe, and available in large quantities.

Most research concerning alternatives to donated RBCs currently focus on the development of hemoglobin-based oxygen carriers (HBOCs). The precursor for HBOCs is usually mammalian hemoglobin (Hb), which may be purified from RBCs in a variety of ways.^{2,3} Unfortunately, Hb is susceptible to many undesirable side reactions when it is removed from the protective environment of the RBC. Hb tetramers rapidly dimerize in the bloodstream and extravasate through the walls of blood vessels and kidney tubules, causing tissue toxicity and renal damage, respectively.⁴ Whereas RBCs possess reducing enzymes that maintain Hb in its reduced form (Fe^{2+}), purified Hb is highly vulnerable to oxidation into the Fe^{3+} or Fe^{4+} forms.⁵ Free Hb in the bloodstream also reacts with nitric oxide (NO) to form nitrate, thereby eliciting vasoconstriction and systemic hypertension.⁶ Even though several HBOCs have shown good clinical potential⁷⁻⁹, none of them have been able to remedy all of these side-effects associated with removing Hb from the protective environment of the RBC.

Earthworms (*Lumbricus terrestris*) lack RBCs, yet they have a unique type of Hb called erythrocrutorin (LtEc). As a result, LtEc has adapted to function freely in the bloodstream. LtEc is a macromolecular assembly of 144 Hb subunits and 36 linker proteins¹⁰ which is extremely stable, even in the presence of 4 M urea (half-life = 24 hours).¹¹ It also has a lower rate of oxidation^{12, 13} than human Hb (HbA) and may reversibly bind NO rather than irreversibly reacting with it.¹⁴ LtEc has also been shown to effectively deliver O_2 in mice and rats without any major long term side-effects.¹⁵ All of these benefits make LtEc an attractive HBOC, especially since it does not require costly modifications (i.e. polymerization, surface conjugation, or encapsulation) like other HBOCs.

Even with all of these advantages, LtEc must be highly pure and readily available to be considered a viable alternative to donated RBCs. Crude Earthworm blood contains hemolysins which can rupture RBCs¹⁶ and clotting proteins which can aggregate and block capillaries.¹⁷ The clotting proteins (observed in *Eisenia foetida*, a close relative of *L. terrestris*) consist of a pair of clotting monomers (40 and 45 kDa) in the coelomic fluid that spontaneously aggregate when exposed to a protease in the worm's mucus. Therefore, clotting occurs any time the worm is injured and these proteins mix together.

Since LtEc is such a relatively large molecule, size exclusion chromatography (SEC)¹⁵ and ultracentrifugation¹⁸ have been previously used to purify it. Unfortunately, SEC is difficult to scale up and is limited by low yields, while ultracentrifugation requires expensive equipment and is energy intensive.

To overcome the disadvantages of SEC and ultracentrifugation, we decided to use tangential flow filtration (TFF) to purify LtEc. We have previously used TFF to purify several different types of mammalian Hbs² and found that TFF is an easily scalable process with relatively low costs. In our process (see Figure 1), clarified Earthworm homogenate is passed through a 0.22 μm TFF filter to sterilize it and remove large particles, then diafiltered with a 500 kDa TFF filter to remove impurities. This process is able to produce several grams of highly pure and functional LtEc from batches of 1,000 worms.

As a preliminary test of the safety of LtEc as a suitable transfusion solution *in vivo*, small amounts of LtEc were infused into healthy hamsters. Consecutive infusions of LtEc were used to raise the concentration of LtEc in the hamster's plasma from 0.5 to 1.5 g/dL (see Figure 2) while monitoring their microcirculatory and systemic parameters to detect any signs of vasoconstriction, hypertension, allergic reactions, or other complications associated

with the infusion of LtEc. Overall, our results show that LtEc appears to be a suitable HBOC that definitely warrants further animal and clinical studies.

Materials and Methods

Earthworm Blood Extraction

For each round of purification, 1,000 Canadian nightcrawlers (*Lumbricus terrestris*) were purchased from Wholesale Bait Company (Hamilton, OH). Worms were rinsed in tap water to remove dirt, then 2 L batches of worms were extensively washed with 20–30 L of tap water to remove as much mucus as possible. A blender was used to homogenize the worms (puree mode for ~10 seconds) and the homogenate was immediately centrifuged at 3,716 g for 40 min at 4°C. Solid debris were discarded and the cloudy red supernatant was centrifuged again at 18,000 g for 20 min at 10–15°C. The clear red supernatant (~2.0 L per 1,000 worms) was then put through filter paper to remove any remaining large particles.

Purification of LtEc

The earthworm homogenate was passed through two 0.22 µm TFF cartridges in parallel (1050 cm² surface area, Spectrum Labs, Rancho Dominguez, CA) at 480 mL/min until the majority of the sample volume was transmitted through the filter. The filter pores clogged several times during the filtration process (indicated by a clear filtrate) and were subsequently cleaned with deionized water. The red retentate of the 0.22 µm filter was stored at –80°C for future analysis. The 0.22 µm filtrate (1.8–2.0 L) was then concentrated on two 500 kDa TFF cartridges (1050 cm² surface area, Spectrum Labs) at a flow rate of 480 mL/min down to a final volume of approximately 200 mL. The 500 kDa retentate was then diafiltered by diluting it to 2.0 liters with buffer and concentrating it down to 200 mL a total of ten times. The retentate was diluted with 20 mM Tris buffer (pH 7.0) during the first eight rounds of diafiltration and modified lactated Ringer's buffer (115 mM NaCl, 4 mM KCl, 1.4 mM CaCl₂, 13 mM NaOH, 12.25 mM N-acetyl cysteine, 0.3% sodium lactate, pH 7.0) during the last two rounds of diafiltration. During the final round of diafiltration, the retentate was concentrated to 20–50 mL and sterilized by passing it through a 0.22µm syringe filter. The purified Ec was then stored at –80°C until needed. Some aggregation was observed after thawing the samples, but the aggregate was easily removed by centrifugation at 18,000 g for 10 minutes and filtration through a 0.22 µm filter. After each round of purification, all filters were rinsed and soaked in 0.2 M NaOH for 1 hour, then rinsed with distilled water and stored at 4°C.

SDS-PAGE Analysis

Samples were prepared for SDS-PAGE analysis by mixing them 1:1 with Laemmli sample buffer (0.5% β-mercaptoethanol) and incubating them at 95°C for 5 minutes. Ten microliters of each sample (25 µg of Hb) was loaded onto the gel (15% acrylamide resolving gel and 4% acrylamide stacking gel) and run at 115 V for 75 minutes. Gels were stained in R-250 Coomassie Blue (BioRad, Hercules, CA) overnight, then destained in destaining buffer (20% ethanol, 10% acetic acid).

Estimation of Yield and MetHb Level

The LtEc concentration and the percent of oxidized (Fe³⁺) LtEc (i.e. metHb) was measured using the cyanomethemoglobin method¹⁹. Each sample was centrifuged at 10,000 g for 5 minutes to remove any aggregates.

MALDI Mass Spectral Analysis

Samples were prepared for Matrix Assisted Laser Desorption Ionization (MALDI) by diluting them to 1 mg/mL, then mixing them 1:1:1 with 1 M HCl and a matrix solution of 50% v/v acetonitrile saturated with sinapic acid. Cytochrome C (12,361 g/mol), apomyoglobin (16,952 g/mol), and human serum albumin (66,437 g/mol) were used as molecular weight (MW) standards on a Bruker FLEX MALDI-TOF (Bruker Daltonics, Billerica, MA) and analyzed in the linear mode.

O₂ Equilibrium Curves

O₂ equilibrium curves for LtEc, bHb, and HbA were measured with a Hemox Analyzer (TCS Scientific, New Hope, PA). Approximately 20–200 µL of Hb sample (final concentration ~5 mg/mL) was diluted with 4.9 mL of Hemox Buffer (pH 7.4), 20 µL of Additive A (containing bovine serum albumin), 10 µL of Additive B, and 10 µL of an anti-foaming agent (TCS Scientific). The sample was saturated with O₂ by flushing it with compressed air (pO₂ = 148 mm Hg) and brought to a constant temperature of 37.0 ± 0.5°C. The samples were then flushed with pure nitrogen and the respective absorbances of oxy- and deoxy-Hb were recorded as a function of the solution pO₂ until the pO₂ decreased to 2.0 mm Hg. The O₂ equilibrium data was then fit to the Hill model (Equation 1) to obtain values for P₅₀ and *n*:

$$Y = \frac{Abs - A_o}{A_\infty - A_o} = \frac{pO_2^n}{pO_2^n + P_{50}^n} \quad (1)$$

Where *Y* is the fractional saturation of Hb, *A_o* is the absorbance at 0 mm Hg O₂ and *A* is the absorbance at full saturation. *P₅₀* is the partial pressure of O₂ (pO₂) at which the heme binding sites in the Hb sample are 50% saturated with O₂, while *n* is the Hill coefficient, a measurement of cooperativity in O₂ binding.

Fast Kinetics

O₂ dissociation rate constants were measured by rapidly mixing HbA, bHb, and LtEc samples (15 µM heme) with 1.5 mg/mL of sodium dithionite in an Applied Photophysics SF-17 microvolume stopped-flow spectrophotometer and deoxygenation was monitored at 437.5 nm in 0.1 M Tris buffer, pH 7.4 at 25°C as previously described in the literature.²⁰

The kinetics of carbon monoxide (CO) association with deoxygenated Hb was measured in a stopped-flow apparatus and the process was monitored at 437.5 nm in 50 mM Tris buffer, pH 7.4, at 25°C after mixing Hb and CO solutions in the presence of 1.5 mg/mL of sodium dithionite.

The kinetics of NO oxidation of oxyHb (also known as NO dioxygenation) was measured in a stopped-flow spectrophotometer. Before mixing the reactants, NO stock solutions (~ 2 mM) were prepared by bubbling NO gas through a deoxygenated solution of 1 M NaOH before saturating a deoxygenated 0.05 M Bis-Tris buffer, pH 7.0, in a gas-tight collection bottle at room temperature. This stock solution was then transferred to a gastight syringe for appropriate dilutions with deoxygenated Bis-Tris buffer. Solutions of air-equilibrated Hb were mixed with the NO solution and the conversion of oxyHb (Fe²⁺) to ferric Hb (Fe³⁺) was monitored by absorbance changes at 438 nm. The value of the bimolecular rate constant for this reaction is known to be extremely large, on the order of 1 × 10⁷ M⁻¹ s⁻¹, so the concentration of NO after mixing was kept low (~ 25 µM) to minimize loss of the reaction in the dead time of the instrument. Under these conditions, the rate of the autoxidation reaction of NO with dissolved O₂ is negligible compared with the rate of reaction with ferrous Hb.

The concentration of Hb in each experiment was kept at 0.5 μM to apply the pseudo-first-order approximation.

Viscosity and Colloid Osmotic Pressure

The viscosity of each solution was measured in a cone/plate viscometer DV-II Plus with a cone spindle CPE-40 (Brookfield Engineering Laboratories, Middleboro, MA) at a shear rate of 160 s^{-1} . The colloid osmotic pressure (COP) of each sample was measured using a Wescor 4420 Colloid Osmometer 47 (Wescor, Logan, UT). Measurements for viscosity and COP were taken at a protein concentration of either 50 or 100 mg/mL.

Animal Preparation

Infusions were made in 55 – 65 g male Golden Syrian Hamsters (Charles River Laboratories, Boston, MA) fitted with a dorsal skinfold window chamber. The hamster window chamber model is commonly used for microvascular studies in the unanesthetized state. The complete surgical technique has been previously described in the literature.^{21, 22} Arterial and venous catheters filled with a heparinized saline solution (30 IU/mL) were implanted into the carotid and jugular vessels. Catheters were tunneled under the skin, exteriorized at the dorsal side of the neck, and securely attached to the window frame. Animal handling and care followed the NIH Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the local animal care committee.

Inclusion Criteria

The microvasculature was examined 3 to 4 days after the window implantation surgery, and only animals passing the following systemic and microcirculatory inclusion criteria were used. Animals were considered suitable for experiments if: 1) systemic parameters were within normal range - heart rate (HR) > 340 beats/min, mean arterial blood pressure (MAP) > 80 mm Hg, systemic hematocrit (Hct) > 45%, and arterial O_2 partial pressure (p_{AO_2}) > 50 mm Hg, and 2) microscopic examination of the tissue in the chamber observed under 650 $\times$ magnification did not reveal signs of low perfusion, inflammation, edema, or bleeding.

Animal Experimental Setup

The unanesthetized animal was placed in a restraining tube with a longitudinal slit from which the window chamber protruded, then fixed to the microscopic stage for transillumination with the intravital microscope (BX51WI, Olympus, New Hyde Park, NY). Animals were given 20 min to adjust to the tube environment before any measurements were made. The tissue image was projected onto a charge-coupled device camera (4815, COHU, San Diego, CA) connected to a videocassette recorder and viewed on a monitor. Measurements were carried out using a 40 $\times$ (LUMPFL-WIR, numerical aperture 0.8, Olympus) water immersion objective.

Test Solutions

TFF-purified LtEc concentrations were adjusted to 10 g/dL. PolybHb solutions (MW 500 kDa) were prepared as previously described in the literature.²³ Two different PolybHb formulations, PolybHb 40:1 and PolybHb 50:1, were prepared by varying the molar ratio of glutaraldehyde:Hb during synthesis (either 40:1 or 50:1).²⁴ Human serum albumin (HSA, ABO Pharmaceuticals, San Diego, CA) was used as a negative control at matching protein concentrations.

Hypervolemic Infusion (Top-Load Protocol)

Animals were randomly divided into four experimental groups and assigned to a test solution. Three consecutive infusions of the test solutions increased the plasma protein

concentration by 0.5 g/dL, 1.0 g/dL, and then 1.5 g/dL from baseline. After each infusion, animals were allowed 20–30 min to stabilize prior to systemic and microvascular characterization. All infusions were performed intravenously at a flow rate of 100 μ L/min. Plasma Hb concentration was determined spectrophotometrically (B-Hemoglobin, Hemocue, Stockholm, Sweden). The use of the Hemocue to measure LtEc concentrations has not been fully validated, but the results obtained here should be useful since the spectra of the oxygenated forms of HbA and LtEc are quite similar. Similarly, the concentration of HSA was determined using spectrophotometric (Lambda 20 Perkin-Elmer, Norwalk, CT) analysis of the UV domain (280 nm). The infusion volumes of each test solution required to increase the plasma protein concentration by 0.5 g/dL were estimated in each animal prior to each infusion, based on the animal blood volume (BV, estimated as 7% of the body weight) and Hct, as $0.05 \times (1 - \text{Hct}) \times \text{BV}$, 5 min after infusion the plasma protein concentration was verified and increased if necessary.

Experimental Groups

Experimental groups were labeled based on the test solution infused: HSA, PolybHb 40:1, PolybHb 50:1, or LtEc. Hypervolemic (top-load) infusion responses for PolybHb 40:1 and PolybHb 50:1 have been previously published in the literature.²⁴ A total of twenty animals were used in the study and all animals tolerated the entire protocol without visible signs of discomfort. Five animals were assigned to each experimental group.

Systemic Parameters

MAP and HR were recorded continuously (MP 150, Biopac System; Santa Barbara, CA). Hct was measured from centrifuged arterial blood samples taken in heparinized capillary tubes. Hb content was determined spectrophotometrically (B-Hemoglobin, Hemocue, Stockholm, Sweden).

Blood Chemistry and Biophysical Properties

Arterial blood was collected in heparinized glass capillaries (50 μ L) and immediately analyzed for pO_2 , pCO_2 , base excess (BE) and pH (Rapidlab 248, Bayer, Norwood, MA). Blood samples for viscosity and COP measurements were withdrawn from the animal with a heparinized syringe at the end of the experiment. Blood viscosity was measured in a DV-II plus rheometer (Brookfield Engineering Laboratories, Middleboro, MA) at a shear rate of 150/sec, while the COP of the blood was measured using a 4420 Colloid Osmometer (Wescor, Logan, UT).

Functional Capillary Density (FCD)

Functional capillaries (defined as those capillary segments that exhibit RBC transit of at least a single RBC in a 45 s period in 10 successive microscopic fields) were assessed in a region measuring 0.46 mm^2 . The relative change in FCD from baseline levels after each infusion is indicative of the extent of capillary perfusion.²⁵

Microhemodynamics

Arteriolar and venular blood flow velocities were measured on-line by using the photodiode cross-correlation method (Photo Diode/Velocity Tracker Model 102B, Vista Electronics, San Diego, CA). The measured centerline velocity (V) was corrected according to blood vessel size to obtain the mean RBC velocity (V/R_v), where R_v represents the ratio between the blood vessel centerline velocity and the blood vessel average blood velocity based on data obtained in glass tubes.²⁶ According to Lipowsky and Zweifach²⁶, $R_v = 1.6$ for blood vessels between 15 and 90 μm diameter, but not for larger blood vessels. A video image-

shearing method was used to measure blood vessel diameter (D).²⁷ The blood flow rate (Q) was calculated from measured values as $Q = \pi \times (V/R_v) (D/2)^2$.

Statistical Data Analysis

Tabular results are presented as the mean $\pm$ standard deviation. Data within each group were analyzed using analysis of variance for repeated measurements (ANOVA, Kruskal-Wallis test). When appropriate, *post hoc* analyses were performed with the Dunns multiple comparison test. Comparison between samples was performed using two-way ANOVA (Hb plasma concentration and cross-link density); *post hoc* analyses were performed with Bonferroni post tests. Microhemodynamic data are presented as absolute values and ratios relative to baseline values. A ratio of 1.0 signifies no change from baseline, while lower and higher ratios are indicative of changes proportionally lower and higher than baseline (i.e., 1.5 represents a 50% increase from the baseline level). The same vessels and capillary fields were followed so that direct comparisons to their baseline levels could be performed, allowing for more reliable statistics on small sample populations. All statistics were calculated using GraphPad Prism 4.01 (GraphPad Software, Inc., San Diego, CA). Changes were considered statistically significant if $P < 0.05$.

Results

SDS-PAGE

Figure 3 shows SDS-PAGE analysis of samples from different points during the LtEc purification process. The retentate of the 0.22 μ m TFF filter, which was dark red, turbid, and highly viscous, is shown in lane 2 of the gel. Many impurities are present, along with a very dark band at the top of the gel. This dark band may correspond to a clotting protein aggregate that has also been observed in another Earthworm, *Eisenia foetida*.¹⁷ Indeed, when the 0.22 μ m retentate was diluted 10 $\times$ and centrifuged at 18,000 g for 30 minutes, the supernatant was clear red and a solid dark pellet was observed. The pellet was resuspended and centrifuged several times with water, then dissolved in 4 M urea and loaded onto a SDS-PAGE gel (results not shown). The gel revealed a faint pair of bands around 40 kDa, which corresponds to the MW of the clotting proteins observed in *E. foetida*. The 0.22 μ m filtrate, which was a clear light red, is shown in lane 3. This sample still has many impurities, but it appears that the high MW clotting aggregate was successfully removed by the filter. It is also worth noting that despite the red color of the samples used in lanes 2 and 3, the expected bands for LtEc that should occur around 16 and 33 kDa are not present.

The diafiltrated LtEc product is shown in lane 4 of the gel. The dark band at 35 kDa corresponds to the linker subunits of LtEc (estimated to be 24–32 kDa from amino acid sequences), while the bands around 15 kDa correspond to the five known Hb subunits of LtEc (A = 17,525 g/mol, B = 16,254 g/mol, C = 17,289 g/mol, D1 = 15,964 g/mol, and D2 = 15,997 g/mol). Only a few impurity bands are barely detectable in the purified LtEc sample at around 25 and 75–85 kDa. A sample of the 500 kDa TFF filtrate after the first round of diafiltration (lane 5) shows that the 500 kDa filter is able to effectively remove the impurities in the crude sample and essentially no impurities remain after 10 rounds of diafiltration (lane 6). However, when the final filtrate sample is concentrated 25 $\times$ (see lane 7), some faint impurities around 75 and 100–150 kDa are present.

MALDI and LC-MS

The MALDI mass spectrum of the pure LtEc product is shown in Figure 4. The observed peaks are identical to other LtEc spectra observed in the literature²⁸, showing defined peaks for the linker and globin subunits. Each linker (L1, L2, L3, and L4) appears near its expected MW (24–32 kDa), while the D1 and D2 subunits form a single peak around 16

kDa. Since the A, B, and C subunits are naturally cross-linked by disulfide bonds, they appear as a single trimer peak at around 52 kDa. Some residual tetramer (ABCD) is also seen near 68 kDa. These results are supported by tryptic digest and LC-MS data (data not shown), which positively identified peptide fragments from each of the LtEc subunits (A, B, C, D1, L1, L2, L3, and L4). Minor peaks are also barely visible around 40 and 45 kDa, which may correspond to the clotting protein monomers. Otherwise, no major impurity peaks are present in the MALDI spectrum.

Yield and MetHb Levels

The yield of pure LtEc was 4.8 ± 2.4 g per batch of 1,000 worms. We observed that transmission of LtEc through the $0.22 \mu\text{m}$ filter decreased over the lifetime of the filter, thereby limiting the final yield. After 3 runs, as much as 60% of the LtEc was trapped in the $0.22 \mu\text{m}$ retentate. Nonetheless, the level of metHb in the final LtEc sample was low (3.3–3.5%).

O₂-Hb Equilibrium Curves

The O₂-Hb equilibrium curves of purified LtEc samples are shown in Figure 5, while regressed values for the O₂ affinity (P_{50}) and Hill coefficient (n) are shown in Table 1. Native Hbs (HbA and bHb) and bHb polymerized at two different molar ratios of glutaraldehyde:Hb (PolybHb 40:1 and 50:1) in the low O₂ affinity state are included as positive controls. HbA displays the highest O₂ affinity (low P_{50}), since its allosteric effector (2,3 bisphosphoglycerate [2,3-BPG]) is removed during the TFF purification process. Since the O₂ affinity of bHb is not influenced by 2,3 BPG, it displays a higher P_{50} of 25.39 mm Hg, which is close to the measured value for bovine or human whole blood (26 mm Hg). The P_{50} of purified LtEc (26 mm Hg) is similar to bHb or human whole blood, however, its Hill coefficient is much higher (3.5–3.74) than HbA or bHb (2.43–2.45). The higher Hill coefficient of purified LtEc is reflected in the steep slopes of its O₂ equilibrium curve shown in Figure 5. The PolybHb samples both have much lower oxygen affinities (40–42 mm Hg), since they were polymerized in the fully deoxygenated state. Polymerizing Hb in the deoxygenated state locks it in a conformation that reduces its O₂ affinity.

Ligand Binding Kinetics

The kinetic rate constants of purified LtEc for O₂ release ($k_{\text{off}, \text{O}_2}$) and CO binding ($k_{\text{on}, \text{CO}}$) are also shown in Table 1. The rate constants of O₂ release vary only slightly between HbA, bHb, and LtEc. HbA has a slightly higher value for $k_{\text{off}, \text{O}_2}$ (38.72 s^{-1}), while bHb and LtEc are at 33.9 and 30.3 s^{-1} , respectively. The $k_{\text{off}, \text{O}_2}$ values for the PolybHb solutions are much higher (53.0 s^{-1}), which corresponds well with their lower O₂ affinities. While the CO binding rate constants are similar for HbA, bHb, and the PolybHbs (0.17 – $0.20 \mu\text{M}^{-1}\text{s}^{-1}$), the value for LtEc is significantly lower ($0.10 \mu\text{M}^{-1}\text{s}^{-1}$). It is also important to mention that the values shown in Table 1 for LtEc are almost identical to values for $k_{\text{off}, \text{O}_2}$ (32 s^{-1}) and $k_{\text{on}, \text{CO}}$ ($0.09 \text{ uM}^{-1}\text{s}^{-1}$) previously reported by Wiechelman, et al.²⁹

Figure 6 shows some key differences in how oxygenated HbA and LtEc interact with NO. When oxygenated Hbs from mammals are exposed to NO, the NO attacks the bound O₂ in a redox reaction that forms nitrate (NO_3^-) and oxidizes the heme group ($\text{Fe}^{2+} \rightarrow \text{Fe}^{3+}$). This reaction is very fast and may be tracked by monitoring the decrease in absorbance at a wavelength of 438 nm. As expected, Figure 6 shows a decrease in the A_{438} of the HbA sample over a rapid 10 ms time frame. The rate constant of oxidation in the presence of NO ($k_{\text{ox}, \text{NO}}$) was calculated from these data for HbA and found to be almost identical to the values of $k_{\text{ox}, \text{NO}}$ for bHb and the PolybHb samples (18 – $19 \mu\text{M}^{-1}\text{s}^{-1}$, see Table 1). In contrast, the LtEc sample shows an increase in absorbance over a much longer period of

time (1 second) when LtEc-O₂ is exposed to NO. Therefore, we were unable to obtain a value of $k_{ox,NO}$ for LtEc using this approach.

Viscosity and COP

The viscosities and COP values of LtEc, PolybHbs, and human whole blood are shown in Table 2. The viscosity and COP of LtEc both increase with increasing protein concentration. Interestingly, the viscosity and COP values of LtEc at 10 g/dL are only slightly less than the values for human whole blood. Meanwhile, the much higher MW PolybHb samples have viscosities that are much higher than human whole blood and COP values that are significantly lower.

Safety of LtEc

Table 3 shows some vital statistics for hamsters that were infused with the HBOCs PolybHb or LtEc. HSA, a naturally occurring non-vasoactive serum protein was also used as a negative control in this study. There were no significant differences in the baseline values of MAP, HR, or body weight for all animals. A slight decrease in hematocrit (Hct) was observed as the blood was diluted following infusion of each solution, as expected. The total Hb and plasma protein concentrations also increased with each infusion of LtEc or PolybHb, as expected. All of the animals survived and no significant immune or allergic responses were observed.

Vasoconstriction and Hypertension

Figure 7 shows the effects of purified LtEc and PolybHb solutions on MAP (a measure of hypertension) and arteriole diameter (a measure of vasoconstriction). At 1–1.5 g/dL, all transfusion solutions resulted in a significant increase in MAP relative to baseline. However, only the PolybHb solutions showed a significant increase in MAP relative to HSA at 1.5 g/dL and the increase in MAP associated with LtEc was considerably less than with the PolybHb's. Some vasoconstriction was observed with the PolybHb solutions, however, LtEc appeared to cause some slight vasodilation at higher plasma concentrations.

Arteriole Blood Flow and HR

Figure 8 shows the effects of PolybHb and LtEc on HR and arteriole blood flow. No significant effects on HR were observed until a plasma concentration of 1.5 g/dL was reached. At these higher concentrations, the PolybHb 40:1 solution displayed a significant decrease in HR relative to baseline. Meanwhile, the LtEc, PolybHb 50:1, and HSA solutions did not cause a significant decrease in HR relative to baseline. Interestingly, at a plasma concentration of 1.5 g/dL, both HSA and LtEc caused a significant increase in arteriole blood flow relative to baseline while the PolybHb samples appeared to reduce blood flow.

FCD

After hemorrhagic shock, reduced circulation volume and blood pressure may cause some capillaries to collapse. The resulting ischemia limits blood flow and O₂ delivery to vital tissues and organs and may cause significant problems. FCD is a measure of capillary collapse (higher FCD indicates less capillary collapse) that may be used to predict the effects of HBOCs in cases of hemorrhagic shock. Figure 9 shows that HSA, a common plasma expander which is used to restore FCD, causes a significant increase in FCD relative to baseline at 1–1.5 g/dL. On the other hand, PolybHb solutions appear to exhibit a lower FCD, especially at 1.0–1.5 g/dL. LtEc appears to have the same effect as HSA, significantly increasing FCD at 1.0 g/dL.

Discussion

Sample Purity and Identification

The SDS-PAGE gel in Figure 3 and the MALDI spectrum in Figure 4 show that the TFF purification process is able to yield highly pure intact LtEc from Earthworm homogenate. The MALDI spectra in Figure 4 and the tryptic digest/LC-MS results (data not shown) confirm the identity of all the Hb subunits (A, B, C, D1, D2) and the linker proteins (L1, L2, L3, L4) near their expected MWs, while no other major impurities were detected in solution. Some impurities may still be present, but are at extremely low concentrations and are non-detectable. Therefore, the purified LtEc should be sufficiently pure for transfusion studies in animals. Additional work will have to be done to measure batch-to-batch variability in the purity of the LtEc product and its stability during storage. So far, we have observed that the purity is consistent between batches (data not shown) under the conditions mentioned here. However, the final purity may be significantly decreased if higher concentrations of LtEc are used or the filters are compromised (either by rupturing or fouling the TFF membranes).

It is important to mention that we attempted to measure endotoxin in the LtEc samples with the Limulus Amebocyte Lysate (LAL) assay.³⁰ Even at the lowest dilutions, the LtEc solutions caused the LAL to coagulate (an effect which usually indicates the presence of endotoxin). However, we believe this to be a false positive, since LAL is known to also coagulate in the presence of high MW species like LtEc and no endotoxemia symptoms were observed in the animals. We are currently investigating other techniques to measure endotoxin levels in LtEc samples.

While our process is able to produce highly pure LtEc, it is still limited by the clotting aggregate proteins. Most of the aggregate is removed during the centrifugation steps in the purification protocol, however, enough remains to foul the 0.22 μm filter and decrease yields of LtEc over time. More work will have to be done to either completely remove the aggregate before 0.22 μm filtration or to prevent aggregation from happening at all.

Ligand Binding Properties

Figure 5 shows that purified LtEc is able to effectively bind and release O_2 , with a high Hill coefficient and O_2 affinity similar to human whole blood. The higher Hill coefficient observed in purified LtEc is due to the higher number of allosteric interactions between the 144 Hb subunits compared to the mammalian Hb controls (only 4 globin subunits). These characteristics indicate that LtEc should be a highly effective O_2 carrier *in vivo*.

The kinetic rate constants shown in Table 1 are quite interesting. It appears that the heme pocket in LtEc is more selective to certain ligands than HbA or bHb. While the rate constant for O_2 release are similar between the mammalian Hbs and LtEc, the rate constant for CO binding is significantly reduced. The crystal structures of HbA, bHb, and LtEc clearly illustrate that the heme binding pockets of LtEc are much more sterically hindered than HbA or bHb.¹⁰ Therefore, the lower CO binding rate constant may be caused by key amino acid residues which block or repel CO, while allowing O_2 to bind normally. The interaction between LtEc- O_2 and NO (Figure 6) is also quite different than HbA. Whereas NO quickly oxidizes the heme of HbA (as indicated by the decrease in A_{438}), LtEc- O_2 appears to exhibit a different response in the presence of NO. Others have suggested that NO may displace the bound O_2 in the Ec heme pocket, thereby avoiding oxidation.^{10,14} We have also observed that CO binding induces an increase in A_{438} similar to the one caused by NO (data not shown). It is unclear whether NO displaces bound oxygen in LtEc or simply has a lower rate of NO dioxygenation, but the results shown in Figure 6 suggest that HbA and LtEc interact with NO in significantly different ways. Additional studies will have to be done, both *in*

vitro and *in vivo*, to determine how LtEc interacts with NO and the pharmacodynamic effects of LtEc in animals.

Viscosity and COP

Table 2 clearly shows that the viscosity and COP of LtEc at physiologically relevant concentrations are similar to human whole blood. The higher viscosity of PolybHb solutions helps to limit vasoconstriction by stimulating NO release from the endothelium through a shear-induced mechanotransduction mechanism. This additional NO may then compensate for the NO scavenged by the PolybHbs and reduce vasoconstriction. It is also important to note that the COP of purified LtEc is much higher than the PolybHb solutions and closer to human whole blood. This is a definite advantage in cases of hemorrhagic shock, since the elevated COP of the LtEc will draw fluid into the capillaries and increase the circulation volume. Therefore, LtEc should be able to restore O₂ transport and maintain FCD.

Safety of LtEc in a Hamster Model

Figures 8 & 9 show that LtEc maintains HR, blood flow, and FCD at least as well as the HSA control. In contrast, PolybHb solutions exhibited slight reductions in each of those parameters. Figure 7 shows that LtEc did not cause a hypertensive or vasoconstrictive response. Instead, it seemed to slightly relax small blood vessels. These results suggest that LtEc may have a lower rate of NO dioxygenation than HbA, since it is widely accepted that NO scavenging causes vasoconstriction. Most importantly, no allergic reactions or side-effects were observed in hamsters at 0.5–1.5 g/dL LtEc and all of the animals survived the study. Pharmacokinetic parameters were not measured during this study, however, ongoing work in a hamster exchange transfusion model has shown that the circulation half life of LtEc is at least 12 hours. Additional pharmacokinetic data will be forthcoming. Altogether, these results suggest that LtEc may serve as a promising HBOC.

Further animal studies will need to be performed to fully determine the safety and efficacy of LtEc as an acceptable HBOC. First, larger scale transfusion experiments will need to be done in hamsters to determine the effects of higher concentrations of LtEc. LtEc should also be evaluated in guinea pigs, which have blood with a redox status more similar to humans. Finally, LtEc should be transfused into larger animals (pigs, dogs, etc.) and eventually tested in human clinical trials.

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Figure 1.

Purification of LtEc. After homogenization, samples were centrifuged at 3716 g for 40 min, then 18,000 g for 20 min and passed through filter paper to remove any remaining debris. The clarified homogenate was finally purified with 0.22 µm and 500 kDa TFF filters

Figure 2.

Hypervolemic infusion strategy for Hamsters. Each animal was infused with enough material to raise its plasma protein concentration to 0.5 g/dL, then given 30 minutes to recover. This process was repeated twice until the concentration of each material in the plasma was 1.5 g/dL

Figure 3. SDS-PAGE analysis of samples from the LtEc purification process. Lanes: (1) Protein MW ladder, (2) 0.22 μ m TFF retentate, (3) 0.22 μ m TFF filtrate, (4) 500 kDa Diafiltered LtEc, (5) Initial 500 kDa TFF filtrate, (6) Final 500 kDa TFF filtrate, (7) Final 500 kDa TFF filtrate, concentrated 25X

Figure 4. MALDI mass spectrum of LtEc, MW range of 10–75 kDa. M = monomer (D1 or D2 subunit), T = trimer (A, B, & C subunits), t = tetramer (A, B, C, & D subunits), L1, L2, L3, and L4 represent the linker proteins

Figure 5.

Oxygen equilibrium curves of purified LtEc and PolybHb samples with HbA and bHb as controls. Raw data are shown with dashed lines, while fitted curves are shown by solid lines. The y-axis shows the fractional saturation of Hb at various partial pressures of oxygen ($\log[pO_2]$, x-axis)

Figure 6. Interaction of NO with oxy-HbA and oxy-LtEc. Raw data are shown in red, while curve fits are shown in blue.

Figure 7.

Vasoactivity of LtEc in hamsters after hypervolemic infusion, as measured by mean arterial pressure and arterial diameter. Values which are significantly different from baseline (1.0) are marked with a (†), while values which are also significantly different from the negative control HAS are marked with a (†§)

Figure 8.

Heart rate and arterial blood flow in hamsters after hypervolemic infusion of LtEc and other materials. Values which are significantly different from baseline (1.0) are marked with a (‡), while values which are also significantly different from the negative control HAS are marked with a (†§)

Figure 9.

Effect of each LtEc, PolybHb's, and HSA on functional capillary density. Values which are significantly different from baseline (1.0) are marked with a (†), while values which are also significantly different from the negative control HAS are marked with a (†§)

Ligand binding properties of LtEc, bHb, HbA, and polymerized bHb (PolybHb 40:1 & PolybHb 50:1) including oxygen affinity(P_{50}), Hill coefficient (n), oxygen release rateconstant (k_{off,O_2}), CO binding rateconstant($k_{on,CO}$), and the NO oxidation rate constant in the presence of NO ($k_{ox,NO}$).

Table 1

Sample	P_{50} (mm Hg)	n	k_{off,O_2} (s^{-1})	$k_{on,CO}$ ($\mu M^{-1}s^{-1}$)	$k_{ox,NO}$ ($\mu M^{-1}s^{-1}$)
LtEc	26.09 ± 0.25	3.74 ± 0.17	30.3	0.10	<i>n/a</i>
HbA	12.09	2.43	38.7	0.20	18.5
bHb	25.39	2.45	33.9	0.20	18.3
PolybHb 40:1	40.75	0.84	53.0	0.17	19.0
PolybHb 50:1	41.5	0.87	53.0	0.18	18.9

Table 2

Molecular weight (MW), viscosity, and COP of LtEc and PolybHbs compared to human whole blood.

Sample	MW (kDa)	Viscosity (cP)	COP (mm Hg)
LtEc (5 g/dL)	3,600	2.10 ± 0.36	10.0 ± 2.0
LtEc (10 g/dL)	3,600	4.27 ± 0.96	14.0 ± 3.4
PolybHb 40:1 (10 g/dL)	5,494	7.2	5
PolybHb 50:1 (10 g/dL)	16,590	11.4	1
Human whole blood	<i>n/a</i>	4.50	19–24.5

Table 3

Systemic parameters at baseline, followed by the effects of consecutive transfusions of HSA, LtEc, and PolyHbs on hematocrit (Hct), total Hb concentration (Hb_{total}) and plasma protein concentration ($Protein_{plasma}$).

	HSA		PolybHb 40:1		PolybHb 50:1		LtEc	
	N	5	5	5	5	5	5	5
Baseline	MAP (mm Hg)	112 ± 8	108 ± 5	112 ± 8	112 ± 8	114 ± 7		
	HR (bpm)	435 ± 31	422 ± 24	440 ± 24	440 ± 24	428 ± 31		
	Hct (%)	48 ± 2	49 ± 2	48 ± 2	48 ± 1	48 ± 2		
	[Hb] (g/dL)	14.6 ± 0.7	14.9 ± 0.8	14.7 ± 0.8	14.7 ± 0.8	14.8 ± 0.6		
	Body weight (g)	65.6 ± 4.2	63.4 ± 4.2	68.1 ± 3.8	68.1 ± 3.8	67.3 ± 4.6		
0.5	Volume Infused (mL)	0.11 ± 0.03	0.12 ± 0.03	0.13 ± 0.05	0.13 ± 0.05	0.16 ± 0.07		
	Hct (%)	47 ± 2	48 ± 1	48 ± 1	48 ± 1	47 ± 2		
	g/dL Hb_{total}	14.0 ± 0.4	14.8 ± 0.6	14.8 ± 0.6	14.8 ± 0.6	14.9 ± 0.7		
	$Protein_{plasma}$ (g/dL)	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1		
	Volume Infused (mL)	0.24 ± 0.08	0.25 ± 0.09	0.26 ± 0.09	0.26 ± 0.09	0.28 ± 0.07		
1.0	Hct (%)	45 ± 1	46 ± 1	46 ± 1	46 ± 1	47 ± 1		
	g/dL Hb_{total}	13.6 ± 0.5	15.2 ± 0.5	15.3 ± 0.6	15.3 ± 0.6	15.1 ± 0.7		
	$Protein_{plasma}$ (g/dL)	0.9 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1		
1.5 g/dL	Volume Infused (mL)	0.41 ± 0.09	0.40 ± 0.09	0.41 ± 0.09	0.41 ± 0.09	0.46 ± 0.09		
	Hct (%)	44 ± 1	45 ± 1	45 ± 1	45 ± 1	46 ± 2		
	Hb_{total} (g/dL)	13.2 ± 0.5	15.9 ± 0.5	16.0 ± 0.5	16.0 ± 0.5	16.3 ± 0.5		
	$Protein_{plasma}$ (g/dL)	1.5 ± 0.2	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.2		
	Blood viscosity (cP)	4.2 ± 0.2	4.4 ± 0.2	5.0 ± 0.3	5.0 ± 0.3	4.7 ± 0.4		
	Plasma viscosity (cP)	1.1 ± 0.2	1.6 ± 0.2	2.0 ± 0.2	2.0 ± 0.2	1.8 ± 0.2		