Review

Biophysical Properties of *Lumbricus terrestris* Erythrocruorin and Its Potential Use as a Red Blood Cell Substitute

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Abstract: Previous generations of hemoglobin (Hb)-based oxygen carriers (HBOCs) have been plagued by key biophysical limitations that result in severe side-effects once transfused *in vivo*, including protein instability, high heme oxidation rates, and nitric oxide (NO) scavenging. All of these problems emerge after mammalian Hbs are removed from red blood cells (RBCs) and used for HBOC synthesis/formulation. Therefore, extracellular Hbs (erythrocruorins) from organisms which lack RBCs might serve as better HBOCs. This review focuses on the erythrocruorin of *Lumbricus terrestris* (LtEc), which has been shown to be extremely stable, resistant to oxidation, and may interact with NO differently than mammalian Hbs. All of these beneficial properties show that LtEc is a promising new HBOC which warrants further investigation.

Keywords: red blood cell substitute; hemoglobin; erythrocruorin; oxygen carrier

1. Extracellular Hemoglobins: A New Paradigm

As of 2011, the only hemoglobin (Hb) based oxygen carriers (HBOCs) that have entered phase III clinical trials are polymerized human [1–4] and bovine [5–8] Hb (PolyHb) as well as poly(ethylene glycol) surface-conjugated human hemoglobin (MP4, Sangart Inc., San Diego, CA, USA) [9–12]. MP4 is currently undergoing clinical trials, but the PolyHbs have been discontinued due to indications of

increased mortality and other complications [13]. The major problems associated with these HBOCs (instability, oxidative stress, and nitric oxide (NO) scavenging) can be directly attributed to removing Hb from the protective environment within the red blood cell (RBC). The RBC has enzymes to prevent oxidation [14–16], a cell membrane to reduce interactions with NO [17], allosteric effectors to modulate O_2 delivery [18], and high Hb concentrations that minimize dimerization of the Hb tetramer [19].

Since mammalian Hbs purified from RBCs are burdened with so many problems, extracellular Hbs from other organisms may be better suited for use in HBOC development. This special class of Hbs, known as erythrocruorins (Ecs), are found in organisms which lack RBCs (most annelids [20], some mollusks [21] and insects [22]). Consequently, Ecs have already adapted to the harsh conditions in the bloodstream with unique structural and functional modifications that make them attractive natural HBOCs. This review will focus on the unique properties of Ec from the Earthworm *Lumbricus terrestris* (LtEc).

2. Structure and Stability of LtEc

Ecs come in a wide variety of shapes and sizes, including the spherical Ec of *Riftia pachyptila* (~400 kDa) [23], the hexagonal bilayer (HBL) Ecs of *L. terrestris* [24] or *Arenicola marina* [25], and the huge cylindrical Ec of the clam *Cardita borealis* (12 MDa) [26]. These Ecs are all held together by covalent disulfide bonds and strong electrostatic or hydrophobic forces within large subunit interfaces. Therefore, they are not susceptible to dissociation at low concentrations like mammalian Hbs, which lack intermolecular disulfide bonds [27].

LtEc consists of a macromolecular assembly of 144 globin subunits and 36 linker chains (Figure 1) [24,28,29]. There are 5 types of globins (A, B, C, and D_1 or D_2) [30,31] and 4 types of linkers (L1, L2, L3, and L4) [32,33]. Each globin subunit has a single intramolecular disulfide bond and a structure that is more similar to myoglobin than mammalian Hb subunits [28]. Each subunit also contains a heme group, which binds oxygen (O_2) and even contributes to subunit association by forming hydrogen bonds with adjacent subunits through propionate groups [29]. The A, B, and C subunits also have intermolecular disulfide bonds which form an ABC trimer. The ABC trimer and D monomer self-associate through electrostatic and hydrophobic interactions to form the ABCD tetramer [34]. Next, the $A_3B_3C_3D_3$ dodecamer spontaneously forms from three ABCD tetramers through disulfide bonds. The dodecamer is hemi-spherical and has a structure that is reminiscent of the spherical (double dodecamer) Ec of *R. pachyptila* (RpEc) or *Oligobrachia mashikoi* (OmEc), suggesting that LtEc may have also been spherical at some point during its evolution [23,35,36].

The linker chains are not required for dodecamer formation [37], but they are necessary to form the complete hexagonal bilayer structure of LtEc. Initially, three linker chains self-assemble to form a linker trimer. The linker chains are degenerate, meaning that several combinations of L_1, L_2, L_3 or L_4 can create the trimer. In fact, the minimum requirement for linker trimer formation is only a binary mixture of L_1 or L_2 with L_3 or L_4 [37]. The purpose and origin of the degeneracy in the linker and globin subunits are not known and any possible effects of different subunit compositions will need to be considered in future studies. The linker trimer is held together by numerous disulfide bonds and strong hydrophobic interactions within a coiled coil domain [24]. The linker trimer also has large low density lipoprotein (LDL) domains which strongly bind the dodecamer to form the protomer. Finally,

12 protomers assemble through interactions between the coiled coil domains of the linker trimers to form the hexagonal bilayer structure of LtEc, which has a molecular weight (MW) of approximately 3.6 MDa and a diameter of 30 nm as shown in Table 1 [24]. To put these numbers into context, human Hb (HbA) has a MW of 0.064 MDa and a diameter of 5 nm [27].

Figure 1. Assembly of *Lumbricus terrestris* erythrocruorin (LtEc). LtEc consists of 5 globin subunits (A, B, C, D_1', and D_2) and 4 linker chains (L_1, L_2, L_3, or L_4). The subunits self assemble into an ABC trimer that pairs with a D monomer to form the ABCD tetramer which then associates with two more tetramers to form the dodecamer. Three linker subunits form a linker trimer which binds the dodecamer to form a protomer. Finally, 12 protomers assemble into the hexagonal bilayer structure of LtEc, which has a MW of 3.6 MDa and a diameter of approximately 30 nm [24]. HbA [27] and myoglobin (Mb) [38] are shown to the right to provide a sense of scale.
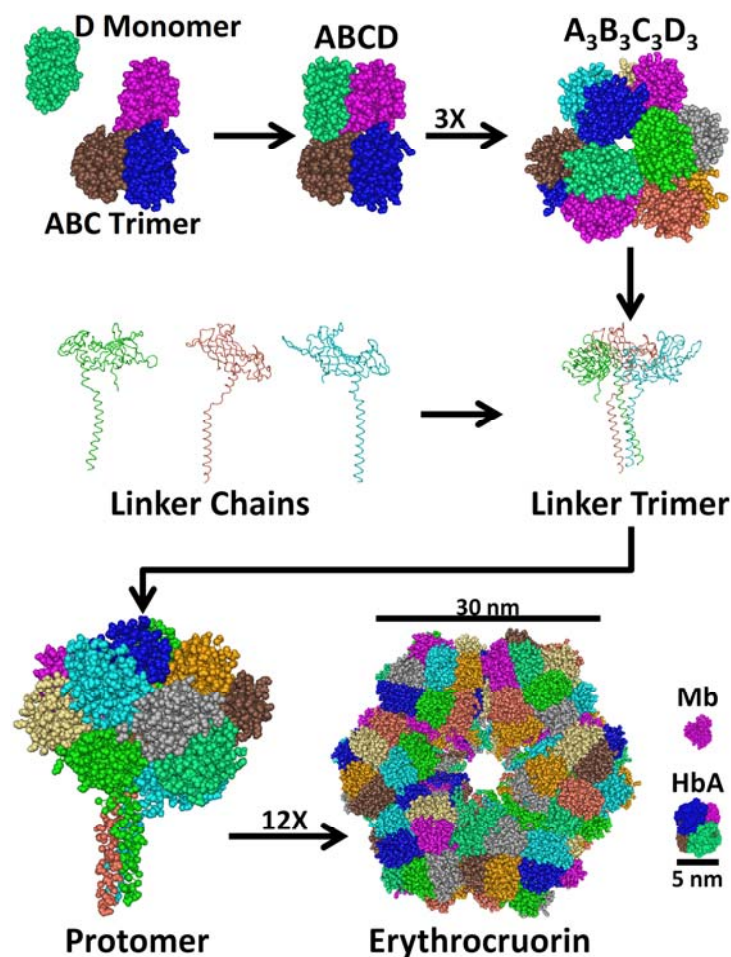

Table 1. Size, molecular weight (MW), O_2 affinity (P_{50}), and cooperativity (calculated as the constant *n* from the Hill Equation) of HbA, AmEc, LtEc, and human RBCs.

	MW (kDa)	Diameter (nm)	P_{50} (mm Hg)	*n* (---)
HbA	64	5	11	2.7
AmEc	3,600 [39]	30 [39]	2.6	2.5 [39]
LtEc	3,600	30	28 [40]	3.7
RBC	---	8,000	26 [41]	2.75 [41]

Several other elements also contribute to the structure of LtEc. Approximately 50 calcium ions (Ca^{2+}) are bound at various sites throughout LtEc. Copper and zinc atoms are also bound to LtEc [42]. The Ca^{2+} ions increase the stability of LtEc and help it resist unfolding at high temperatures [34,43]. Barium (Ba^{2+}) has similar effects and addition of EDTA (which chelates divalent cations) decreases the thermal stability of LtEc [43]. LtEc is also extremely stable in the presence of chemical denaturants, exhibiting a half-life of 28 hours in 1.75 M urea [34]. However, LtEc is prone to subunit dissociation at alkaline pH (>8.0) [44]. In the oxidized (Fe^{3+}) form, LtEc is also susceptible to higher rates of hemin release than oxidized HbA (LtEc = $20–40 \times 10^{-3}$ min^{-1}, HbA = 7.7×10^{-3} min^{-1}) [45]. It is important to mention that some Ecs may be unstable *in vivo*. For example, the marine worm *A. marina* expresses an Ec (AmEc) which is adapted to a high ionic strength and quickly dissociates into dodecamers in human plasma, which has relatively low ionic strength [39]. In contrast, LtEc comes from the terrestrial Earthworm and is stable at the ionic strength of human blood [40].

3. O$_2$ Transport by LtEc

Human blood and LtEc bind and release O_2 in a similar fashion (see Table 1). The O_2 affinity or P_{50} (pO_2 at which half of the hemes are saturated with O_2) of human blood (26 mm Hg) is almost identical to LtEc (28 mm Hg) [40]. This is in contrast to pure HbA and AmEc, which both have significantly lower P_{50} values (higher O_2 affinities) than human blood. The O_2 affinity of HbA decreases when it is purified from human blood due to the removal of its allosteric effector 2,3-DPG [18]. The allosteric effector of LtEc is Ca^{2+}, which increases the O_2 affinity of LtEc and is available in the bloodstream. Other divalent cations, like Ba^{2+}, Sr^{2+}, and Mg^{2+}, have a similar effect on the O_2 affinity of LtEc [46,47]. The relatively high O_2 affinity (low P_{50}) of AmEc is probably another effect of its exposure to low ionic strength buffers or an adaptation to the low O_2 environment in which *A. marina* is found [39].

Cooperative oxygen binding is a unique trait of Hbs in which small changes in one subunit (*i.e.*, ligand binding) affect the conformations and ligand affinities of adjacent subunits. This phenomenon allows Hbs to become saturated with O_2 in the lungs, hold onto it in the arteries, then release it in large amounts in the arterioles and capillaries. The cooperativities of HbA, AmEc, and blood are all around 2.5–2.7 under physiological conditions. The cooperativity of LtEc is relatively higher under physiological conditions (3.7), due to the increased number of subunit interactions within the LtEc dodecamer. In fact, the maximum cooperativity of LtEc is 7.9 at 25 °C and pH 7.7 with 25 mM $CaCl_2$ [48]. The effects of cooperativity also appear to be mostly within the dodecamers and only slightly (if at all) transmitted between dodecamers [49].

As previously mentioned, the LtEc dodecamer spontaneously forms in the absence of the linker chains. Interestingly, isolated dodecamers and ABCD tetramers have O_2 affinities and cooperativities similar to LtEc in its full form. The isolated ABC trimer and D monomer, however, have significantly higher O_2 affinities and lower cooperativities. Therefore, the linker chains are not required for O_2 transport and appear to simply increase the stability and size of LtEc [49].

4. Autoxidation of LtEc

Oxidation of the heme iron ($Fe^{2+} \rightarrow Fe^{3+}$) is an inevitable side-effect of O_2 transport for all Hbs. After O_2 binds to the heme iron, it can strip away an electron and escape the heme pocket, forming the

pro-oxidant superoxide (O_2^-) and oxidized Hb (metHb, Fe^{3+}). MetHb can be further oxidized to the ferryl form (Fe^{4+}) and/or generate toxic hemichrome and other free radicals which greatly increase lipid oxidation in cell membranes and overall oxidative stress [50]. The size, structure, and amino acid composition of the heme pocket all have significant effects on the rate of Hb autoxidation. Large heme pockets allow O_2^- to easily escape [51,52], while aromatic amino acids (*i.e.*, tyrosine or phenylalanine) within the heme pocket stabilize O_2^- and reduce oxidation rates [52].

The heme pockets of LtEc are much smaller than HbA heme pockets [24,27]. Each subunit of LtEc also has phenylalanine or tryptophan residues which are not present in the heme pockets of HbA subunits [24]. These differences are clearly expressed in the redox potentials of HbA and LtEc (see Table 2). The redox potential of a species is a measure of how likely it is to accept or donate electrons. Species with positive redox potentials are more likely to accept electrons (reduction), while negative redox potentials indicate that a species is more likely to donate electrons (oxidation). The redox potential of HbA is negative (−50 mV), whereas LtEc has a highly positive redox potential (+112 mV). Therefore, LtEc is much less likely to undergo autoxidation than HbA [53–55]. In fact, experiments have shown that the autoxidation rate of LtEc (<0.010 h^{-1}) is lower than HbA (0.014 h^{-1}) [53]. LtEc may also be reduced by reducing agents that are found in the bloodstream (ascorbic acid or glutathione), while HbA is not as easily reduced [53,54].

Table 2. Autoxidation rates and redox potentials of HbA, LtEc, and AmEc.

	k_{ox} (h^{-1})	E_o (mV)
HbA	0.014 [39]	−50 [53]
LtEc	≤0.010	+112 [53]
AmEc	0.005 [56]	+63 [53]

Divalent cations also influence oxidation of LtEc. For example, Ba^{2+} and Ca^{2+} both reduce the rate of LtEc autoxidation. Sr^{2+} and Mg^{2+} have a similar, yet less significant effect [43]. The Cu and Zn atoms which are bound to LtEc also appear to have some superoxide dismutase (SOD) activity. SODs are a family of enzymes which react with O_2^- to form water, thereby preventing formation of harmful H_2O_2 from O_2^-. The SOD activity of LtEc is approximately 10% of the human SOD enzyme, but any intrinsic anti-oxidant activity is beneficial from an HBOC development perspective [57].

5. Interactions Between LtEc and other Ligands

Hbs are known to bind, transport, and/or react with several other ligands besides O_2. For example, Hbs bind both O_2 and carbon monoxide (CO), but release CO much more slowly than O_2. This competitive inhibition of O_2 binding is the reason CO is a poisonous gas. LtEc can also bind CO and its subunits appear to have varying affinities (either high or low) for CO [58,59].

The interactions between Hb and NO have recently become crucially important with respect to HBOC development. Mammalian Hbs have been shown to catalyze a NO dioxygenation reaction in which O_2 and NO react to form NO_3^- and metHb. The metHb increases oxidative stress, but the elimination of NO can have much more significant effects *in vivo* [60]. NO is a signaling molecule which regulates the diameter of blood vessels, relaxing them at high concentrations (vasodilation) and constricting them at low concentrations (vasoconstriction). Vasoconstriction also increases blood

pressure and causes harmful systemic hypertension. Therefore, reducing NO dioxygenation is a high priority in HBOC design.

Mutagenesis studies in HbA have shown that mutations that reduce autoxidation also reduce the rate of NO dioxygenation. For example, substituting large apolar or aromatic residues (leucine, tryptophan, or phenylalanine) within the heme pocket or charged amino acids (glutamine) near the heme pocket entrance greatly reduces the rate of NO dioxygenation in oxy-HbA [60,61]. Both LtEc and AmEc have naturally occurring phenylalanine and tryptophan residues within their heme pockets [24,25]. There have not yet been any studies which focus specifically on the interactions between Ec's and NO, but there have been suggestions that Ec's may have a significantly reduced rate of NO dioxygenation relative to HbA [24,39,62]. However, it is important to mention that these studies were not conclusive and more experiments must be done to determine the exact nature of the interactions between Ec's and NO.

6. Availability and Economic Analysis of LtEc

A wide array of different Ec's have been discovered with unique characteristics, such as extreme heat tolerance or H_2S transport [36,63,64]. However, since a large amount of Ec will be needed to meet the global demand for a RBC substitute, the host organism must be readily available. Most of the Ec's in the literature are extracted from deep sea worms, which may be difficult to grow at an industrial scale. Fortunately, LtEc is extracted from terrestrial earthworms which are readily available at a low cost (~$75/1,000 earthworms) from many commercial sources. It is important to mention, however, that many different species of terrestrial earthworms have been discovered which might be grown as easily as *L. terrestris*. For example, the Ec of the Brazillian earthworm *Glossoscolex paulistus* (GpEc) has been extensively studied [65–72] and is an interesting alternative to LtEc that may also be commercially viable.

Any Ec product must also be ultrapure to be considered a viable HBOC candidate. The purification strategy used for Ec production must be efficient to keep the costs of the Ec product comparable to a unit of donated blood (estimates vary from $200–$1,000) [73]. A few different Ec purification processes have been developed, including ultracentrifugation [49], size exclusion chromatography (SEC) [40], and tangential flow filtration (TFF) [62]. Ultracentrifugation is simple and quick, but requires expensive equipment and may not produce ultrapure material. SEC produces ultrapure Ec, however, it is limited by low yields and is difficult to scale up. In contrast, TFF can easily produce large amounts of ultrapure LtEc (5–10 g/1,000 worms) and is easily scalable. Using the TFF LtEc yield, assuming a unit of donated human blood contains approximately 40–80 grams of HbA, and omitting operational costs (which should be low for a TFF process), we can estimate that it would take at least 8,000 worms to produce a "unit" of LtEc with a cost of $600 [62]. A more thorough economic analysis would be needed before producing any Ec on an industrial scale, but this estimate suggests that LtEc may be a slightly more expensive, yet affordable, alternative to donated blood.

7. Preliminary Animal Studies with Ec's

Preliminary experiments have been conducted in which small amounts of LtEc [40] and AmEc [39] have been injected into healthy mice and rats. AmEc quickly dissociated after mixing with plasma *in*

vitro, but caused no noticeable side-effects *in vivo* and all animals were healthy 18 weeks after injection. *In vitro* experiments also indicated that AmEc is not scavenged by haptoglobin, a serum protein which strongly binds to free HbA and clears it from the bloodstream.

Injection of LtEc into mice and rats also lacked any side-effects. Most importantly, no immune response was observed even after repeated injections of LtEc [40]. We have also recently transfused small amounts (0.5–1.5 g/dL) of LtEc into hamsters without any side-effects. In fact, we have observed that transfusion of LtEc causes slight vasodilation instead of the vasoconstriction caused by other HBOCs. These are only preliminary results, yet they suggest that LtEc may have a negligible rate of NO dioxygenation. LtEc also has a colloid osmotic pressure (14 mm Hg) that is higher than some HBOCs (e.g., polymerized bovine Hb, 1–5 mm Hg), but similar to human whole blood (19–24 mm Hg) [62]. Further studies with larger doses of LtEc will have to be done to accurately determine the efficacy and safety of LtEc, but all of the results presented here show that LtEc and other Ec's are intriguing class of HBOC.

8. Conclusions

Altogether, these results suggest that LtEc may be a superior HBOC, since it appears to avoid many of the problems associated with other HBOC's. It is highly stable, resistant to oxidation, and may have a negligible rate NO dioxygenation. It also has beneficial antioxidant properties which should minimize oxidative stress *in vivo*.

Despite all of the promising properties of LtEc, it is important to note that LtEc is still far away from clinical evaluation. Larger exchange transfusion studies need to be done in a series of animals (hamsters, guinea pigs, pigs, *etc*.) to determine if higher doses of LtEc pose any significant risks or side-effects. Since LtEc will be used in large doses, we must also determine the clearance mechanisms through which LtEc is eliminated *in vivo* to anticipate any toxic accumulation of heme or free iron. Models of hemorrhagic shock must also be used to simulate the effects of LtEc in emergency scenarios. *In vitro*, more work must be done to determine the exact nature of the interactions between LtEc and NO and other physiologically important ligands or effectors. The effects of H_2O_2 on LtEc oxidation and stability must also be determined to predict the effects of transfusing LtEc into patients with sepsis. The effects of storage conditions (pH, temperature, and buffer formulation) on LtEc must also be investigated to determine if/how LtEc can be kept for long periods of time. Once all of these questions are answered, human clinical trials with LtEc may begin.

Acknowledgements

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