Research Article

Arenicola marina extracellular hemoglobin: a new promising blood substitute

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The need to develop a blood substitute is now urgent because of the increasing concern over Europe's BSE outbreak and the worldwide HIV/AIDS epidemic, which have cut blood supplies. Extracellular soluble hemoglobin has long been studied for its possible use as a safe and effective alternative to blood transfusion, but this has met with little success. Clinical trials have revealed undesirable side effects–oxidative damage and vasoconstriction–that hamper the application of cell-free hemoglobin as a blood substitute. We have addressed these problems and have found a new promising extracellular blood substitute: the natural giant extracellular polymeric hemoglobin of the polychaete annelid *Arenicola marina*. Here we show that it is less likely to cause immunogenic response; its functional and structural properties should prevent the side effects often associated with the administration of extracellular hemoglobin. Moreover, its intrinsic properties are of interest for other therapeutic applications often associated with hemorrhagic shock (ischemia reperfusion, treatment of septic shock and for organ preservation prior to transplantation). Moreover, using natural hemoglobin is particularly useful since recombinant DNA techniques could be used to express the protein in large quantities.

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Abbreviations: **AmHb**, extracellular hemoglobin of *Arenicola marina*; **COP**, colloid osmotic pressure; **Hb**: hemoglobin; **HbA**, adult human hemoglobin; **HBOC**, hemoglobin-based oxygen carriers; **HBL**, hexagonal bilayer; Hp, haptoglobin; **i.v.**, intravenous; **i.p.**, intraperitoneal; **L**, linker chain; **MetHb**, methemoglobin; **RBC**, red blood cell; **ROS**, reactive oxygen species; **(R)SNO**, (reactive) S-nitrosothiols species; **SOD**, superoxide dismutase activity

1 Introduction

Interest in the use of extracellular soluble hemoglobin (Hb) as a blood substitute first appeared in the literature in 1933 [1]. Attempts at human transfusion with Hb solutions occurred in the late 1940s, but met with little success [2]. Patients had anaphylactic symptoms, severe renal toxicity, and hypertension. Nowadays, the current designs that serve as prototypes for oxygen therapeutics are: (i) modified stroma-free Hb of human [3], animal [4] or recombinant origin [5], where the oxygen affinity and stabilization can be controlled by a combination of cross-linking, site-directed mutagenesis and microencapsula-

tion, and (ii) perfluorochemicals [6]. Through several different Hb-based oxygen carriers (HBOCs) that have been developed, studies have demonstrated that the administration of relatively large quantities of dissolved mammalian Hb or Hb derivatives may lead to a variety of undesirable toxic side effects [7]. Remarkable progress has been made in the use of cell-free Hb as blood substitute [3]. Significant problems remain, however, including susceptibility to oxidative inactivation [8] and propensity to induce vasoconstriction [9].

We have considered these problems and have found a new promising extracellular blood substitute that will eliminate these unwanted effects. In light of these concerns and inherent problems, we looked directly at natural extracellular polymeric Hb of the polychaete annelid *Arenicola marina* (AmHb) as a potential blood substitute [10]. AmHb is easily available and purified to a homogeneous product avoiding costly synthetic steps. It is easy to store and less likely to cause immunogenic responses since cell membranes are not present and the Hb is not glycosylated [11].

The giant extracellular hexagonal bilayer Hbs (HBL-Hbs) found in most terrestrial, aquatic, marine and deep sea annelids are ~3.6-MDa complexes of globin and non-globin linker chains, which represent a summit of complexity for oxygen-binding heme proteins [12]. A model of the quaternary structure of AmHb-HBL has been proposed by Zal and collaborators [11]. The studies indicated the existence of ten subunits: eight of which are globins, including two monomers (M; ~15 kDa) and five disulfide-bonded trimers (T; ~49 kDa). The remaining two chains are linkers (L), that are disulfide-bonded to form homo and hetero dimers (~50 kDa). These latter polypeptide chains are essential for maintaining the integrity of the Hb molecule [13, 14]. Three and six copies of each of the two monomers subunits and one copy of the trimer form a dodecamer subunit (D) with a mean mass close to 200 kDa [11, 15]. Twelve such complexes of globin chains are linked together by 42 linker chains to reach the total mass of 3648 ± 24 kDa [11].

With the design of HBOCs, the question of molecular weight restriction remains, and the advantages of high molecular weight Hb found in more primitive organisms were used as the basis for polymerizing human Hb (HbA) with glutaraldehyde [16]. Thanks to their high molecular weight, these artificially polymerized Hbs should exhibit limited access to the vascular space and increase vascular retention time compared to intracross-linked Hb tetramers. However, they have a wide distribution of molecular weight. Preliminary experiments have been performed with the naturally polymerized *Lumbricus terrestris* Hb (LtHb). Mice and rats undergoing exchange transfusion with LtHb revealed no apparent behavioral and physical changes [17].

Here we report the structural and functional properties of AmHb, which are consistent with the requirements of the new generation of blood substitutes [18, 19]. These properties should prevent kidney damage, reduce vasoconstrictor effect and oxygen radical formation, often associated with the administration of dissolved mammalian Hb. Structural analyses of AmHb under simulated human physiological conditions (*in vitro*) revealed its dissociation into high molecular weight and functional subunits, while *in vivo* experiments performed on mice revealed no apparent behavioral or physiopathological changes and showed not over allergic response. Moreover, AmHb has interesting intrinsic properties for other therapeutic applications often associated with hemorrhagic shock, such as clinical situations involving ischemia reperfusion, treatment of septic shock and for organ preservation prior to transplantation. Finally, using a naturally occurring Hb is particularly useful since recombinant DNA techniques could be used to express the recombinant protein in large quantities [20]. The results reported here should help getting round the worldwide blood shortage.

2 Materials and methods

2.1 Extraction and purification of AmHb

Individual *Arenicola marina* were collected at low tide from a sandy shore near Roscoff (Penpoull beach), Nord Finistère, France, by the crews of the marine station facilities. Blood samples were withdrawn from the lugworm's ventral vessel and purified as described [20, 21]. *Arenicola* saline buffer is composed of 4 mM KCl, 145 mM NaCl, 0.2 mM $MgCl_2$ and 10 mM HEPES/0.1 M NaOH, pH 6.8. This buffer is compatible with *in vivo* experiments and AmHb structural integrity [21]. It was used for all analyses unless specified otherwise. The resulting samples were kept either frozen (–40°C) or under liquid nitrogen until use.

2.2 Gel filtration LC

AmHb was diluted in lyophilized human plasma (Sigma) dissolved in 10 mM HEPES buffer at 37°C and pH 7.4 at a concentration of 20 mg/mL. Analytical gel filtration was performed on 50 µL of injected sample on a 1 × 30-cm Superose 6-C (fractionation range from 5 to 5000 kDa; Amersham Biosciences Biotechnology) using a high-pressure HPLC system (Waters, Milford, MA, USA). The elution (flow rate: 0.5 mL/min) was monitored with a photodiode array detector (Waters 2996) over the range 250–700 nm. Chromatographic data were collected and processed by the Empower software (Waters). The percentages of each subunit were determined by integrating the chromatogram at 414 nm (characteristic of heme) with the Empower software.

2.3 Multi-angle-laser-light-scattering

Interaction of Hbs with haptoglobin (Hp) was followed by multi-angle-laser-light-scattering measurements performed with a DAWN EOS system (Wyatt Technology Corp., Santa Barbara, CA, USA) directly on-line with the HPLC system. AmHb and cell-free HbA (Sigma) were incubated in the presence of 1 equivalent of human Hp (Sigma) diluted in *Arenicola* buffer at pH 7.4 for 1 h at room temperature before analysis.

2.4 ESI-MS

Electrospray data were acquired on a Q-TOF II (Waters). Peak *D* and *A* (Fig. 1) were collected after 3 h incubation at 37°C, desalted by washing against 10 mM ammonium acetate, repeated ten times on an ultrafiltration device (Amicon-30kDa, Millipore) at 4°C. The analyses under denaturing conditions [11] and non-denaturing conditions [22] were performed as previously described. The multiple charged data produced by the mass spectrometer on the *m/z* scale were converted to the mass scale using Maximum Entropy-based software (MaxEnt) or manually as described [22].

The interaction of S-nitrosothiol species (RSNOs) with AmHb free cysteine was followed by ESI-MS under denaturing conditions. A stock solution of CysNO was synthesized as previously described [23] just before the analysis. Desalted AmHb was incubated with CysNO at a molar ratio of 100:1 free cysteine, and the resulting solution analyzed just after the mixing.

2.5 Spectrophotometric analysis of Hb

The methemoglobin (MetHb) formation was determined spectrophotometrically (UV mc2, SAFAS, Monaco) in the Visible region (450–700 nm). Spectra were measured for samples equilibrated with NO in 1-mL sealed vial. Cell-free HbA and AmHb were incubated with 1 equivalent of NO *per* heme, diluted in *Arenicola* buffer at pH 7.4 at room temperature. NO was generated as previously described [24]. Deoxygenation of Hb (when required) was achieved by adding an excess of dithionite under N_2 atmosphere.

Spectra were measured in the presence of reactive oxygen species (ROS). Fully oxygenated AmHb (Oxy AmHb) was incubated with 8 equivalents of KO2 [a substrate for superoxide dismutase (SOD) activity] *per* heme diluted in *Arenicola* buffer at pH 7.4 at room temperature to test the possible degradation of AmHb by its reaction with superoxide anions.

Spectra were measured for samples in the presence of 5 mM β-NADH (Sigma) diluted in *Arenicola* saline buffer at 37°C and pH 7.4 (simulated human physiological conditions). AmHb was oxidized in the presence of 100 mM K_3FeCN_6 for 20 min, resulting in MetAmHb spectra. The sample was desalted and reduced in the presence of 5 mM β-NADH for 20 h to give the resulting spectra of MetAmHb + β-NADH.

2.6 Oxygen-binding properties

Oxygen equilibrium curves were determined on 3-μL samples using a thermostated diffusion chamber [25] linked to cascaded Wösthoff gas mixing pumps (Bochum, Germany). The diffusion chamber was placed in the light path of a spectrophotometer (Hitachi U1100) at 436 nm. Oxygenation data based on at least three equilibrium steps between 0.2 and 0.8 fractional saturation (Y) were converted to Hill plots {log [Y/(1 – Y)] against log PO_2, where PO_2 is the oxygen partial pressure} for the estimation of the half-saturation oxygen partial pressure (P_{50}) and Hill's cooperativity coefficient at half-saturation (n_{50}).

2.7 *In vivo* experiments on mice

2.7.1 *General state experiments*

These were performed on ten C57BL/6JIco (C57) and ten DBA/2JIco (DBA) mice strains (Charles River, Como, Italy). Blood volume of 200–500 μL were removed from the retro-orbital plexus of the eye sinus of these mice (about 20 weeks of age) while under ether anesthesia, in accordance with the Institution's guidelines (Biotrial A35-238-1, investigator 3518). The blood of each mouse was centrifuged, and the plasma was separated for re-injection with AmHb. An equivalent of desired volume, containing 50% purified AmHb in autologous plasma (n=4 C57 and n=4 DBA), 50% purified AmHb in mouse isotonic saline (n=4 C57 and n=4 DBA) of AmHb, was infused through the tail vein. Control mice (n=1 C57 and n=1 DBA) were transfused with vehicle (*Arenicola* saline buffer). The volume of the substitute was essentially the same as the initial volume removed from the mouse. The concentration of AmHb administrated was calculated to be equivalent to the concentration of heme removed from mouse. One DBA and one C57 mice were challenged after 4 weeks. The mice were observed for 18 weeks.

2.7.2 *Evaluation of proallergenic potential in mice*

The experiments were carried out using male BP/2 mice (Centre d'Elevage R. Janvier, B.P. 55, Le Genest-Saint-Isle, France) weighing 27–37 g (6–8 weeks old) at the beginning of the experiment. In a first set of experiments, conscious mice were sensitized (D_0) by intraperitoneal (i.p.) injection with 0.4 mL of a 4 mg/mL suspension of $Al(OH)_3$ followed by intravenous (i.v.) administration (0.1 mL/10 g body weight, through caudal vein) of either 0.9% NaCl, *Arenicola* buffer (vehicle of AmHb), 600 mg/kg AmHb, 500 μg/kg ovalbumin. After 21 days (D_{21}), each mouse was anaesthetized with sodium pentobarbitone (60 mg/kg i.p.) and a blood sample was collected by cardiac punc-

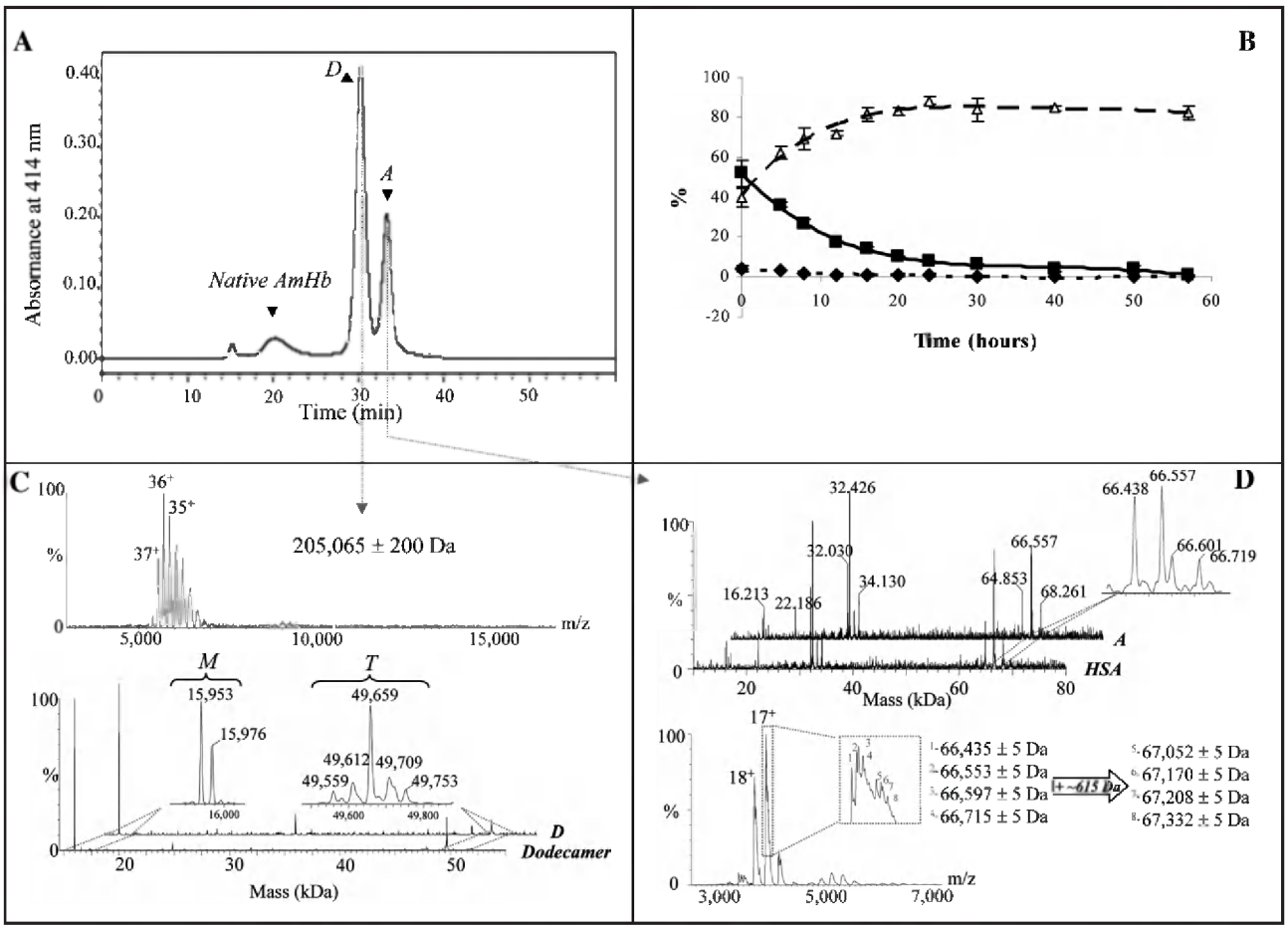

Figure 1. Dissociation of transfused AmHb. **A** Native AmHb dissociates into two major heme-containing subunits *D* and *A* as revealed by gel filtration elution profile at 414 nm of native AmHb immediately after exposure under human physiological conditions. **B** Kinetics of dissociation of native AmHb and *D*, and kinetics of formation of product *A*. The dissociation of native AmHb (♦) is almost complete within the initial time of our first measurement (~5 min). Product *D* (■) remains present for at least 50 h and its dissociation is directly correlated with formation of product *A* (Δ). Results are means ± SEM of five individual experiments. **C** Product *D* corresponds to AmHb dodecamers. ESI-MS analysis of *D* isolated by gel filtration after 3-h incubation. *Top*: *D* has a calculated molecular mass of 205 065 ± 200 Da measured by means of ESI-MS multicharged spectra under non-denaturing conditions. *Bottom*: *D* has a subunit composition identical to AmHb dodecamers according to the deconvoluted spectra obtained under denaturing conditions. The inset shows the characteristic subunit composition of AmHb dodecamers: the two globin monomers M and the five disulfide-bounded globin trimers T. **D** Product *A* corresponds to hemin-HSA. ESI-MS analysis of *A* isolated by gel filtration after 3-h incubation. *Top*: Deconvoluted spectrum of *A* under denaturing conditions overlaid with spectrum of commercial HSA (Sigma). No AmHb subunits were detected. The components observed correspond to four HSA isoforms (inset, ~66.8 kDa) and their dissociation into di-domains (~33.4 kDa) under ESI-MS conditions, as previously observed [57]. *Bottom*: ESI-MS multicharged spectra of the same sample under non-denaturing condition. The inset shows a close-up of 17^+ multicharged peak revealing the presence of eight components. They correspond to the four HSA isoforms and four new components with masses increased by ~615 Da, which corresponds to the molecular mass of one hemin scavenged by HSA.

ture. The collected blood was centrifuged to separate the serum. Aliquots of serum were stored at –20°C before analysis of IgE levels.

In a second set of experiments, conscious mice were first sensitized (D_0) by i.p. injection with 0.4 mL of a 4 mg/mL suspension of $Al(OH)_3$ followed by i.v. administration (0.1 mL/10 g body weight, through caudal vein) of either HEPES buffer or 600 mg/kg AmHb or 500 μg/kg ovalbumin. This procedure was repeated 7 days later (D_7, challenge). At 7 days after this second injection (D_{14}), each mouse was anesthetized with sodium pentobarbitone (60 mg/kg i.p.) and a blood sample was collected by cardiac puncture. The collected blood was centrifuged to separate the serum. Two aliquots of serum were stored at –20°C before analysis of both IgE and IgG2a levels.

Serum IgE and IgG2a antibody titers were measured by ELISA according to Ledermann *et al.* [26] and Emson *et al.* [27] respectively. We tested statistical significance of differences between conditions by ANOVA.

3 Results and discussion

3.1 Dissociation of AmHb

To investigate a possible influence of the human physiological environment on AmHb, we followed its structural integrity *in vitro* by gel filtration and ESI-MS when diluted in human plasma, pH 7.4 at 37°C for 60 h (Fig. 1). After 1 h incubation, AmHb-HBL almost completely dissociated, and two major heme-containing subunits (absorbing at 414 nm), designated *D* and *A* (Fig. 1a) were observed. *D* has a molecular mass of ~205 kDa as determined by ESI-MS under non-denaturing conditions and a subunit composition corresponding to the dodecamer subunit of AmHb-HBL [11, 15] (Fig. 1c). *A* was not composed of any AmHb subunits (Fig. 1d), but its absorbance at 414 nm (Fig. 1a, b) and its UV-visible spectrum was characteristic of the presence of oxidized heme. *A* has been identified as metalbumin by ESI-MS (Fig. 1d), with AmHb hemin being scavenged by HSA. The kinetic of dissociation of AmHb revealed that the dodecamers remained circulating in the plasma for at least 50 h (Fig. 1b). The dissociation of AmHb was not specific for human plasma because the same analyses on mice and rat plasma revealed a similar behavior. The dissociation of AmHb is explained by the slightly basic pH and the low salts content of plasma. Our previous studies have shown that AmHb dissociates at a slightly alkaline pH ($7.0 < pH < 8.0$) if salts concentration is lower than the physiological concentration in *A. marina* blood [20, 21]. This phenomenon is accelerated by the temperature of 37°C, which is considerably higher than that encountered by *A. marina* (~15°C).

Even if AmHb-HBL dissociates into the dodecamers, the size of AmHb-HBL (~25 nm for ~3600 kDa) [11] and its dodecamers (~9 nm for ~205 kDa) [11] should offer the benefits of reduced renal clearance. Indeed, glomerular capillaries form highly efficient barriers that prevent the loss of macromolecular substances with sizes > 3.6 nm [28]. On the other hand, AmHb should avoid extravasations into the interstitium between endothelial and smooth muscle cells where Hb is able to scavenge NO, thereby altering the distribution of NO and resulting in hypertension [29, 30]. Moreover, the transfusion of AmHb-HBL, even if it dissociates into its dodecamers, should not significantly increase the normal colloid oncotic pressure (COP) because polymerization has also the advantage of lowering it. Maintaining a proper COP (human: 20–25 mmHg) is vital since it regulates the exchange of fluids between the intra- and extravascular spaces.

3.2 Functional properties: a high oxygen affinity

We investigated the influence of human physiological conditions on AmHb functional properties. Oxygen binding molecules are usually compared by means of their oxygen dissociation curve, which gives access to characteristic parameters such as the O_2 affinity (P_{50}, the partial pressure of oxygen necessary to saturate half of the oxygen binding sites and consequently the higher the P_{50}, the weaker the oxygen is bound), cooperativity (n_{50}), Bohr effect and temperature effect (ΔH). In contrast to the vertebrate Hb, where oxygen affinity is decreased by organic phosphates contained in the red blood cells (RBCs) [31], the affinity of the high molecular-weight extracellular annelid respiratory proteins is not affected by organic phosphates, but is increased by inorganic divalent cations [32]. For AmHb, Mg^{2+} and Ca^{2+} exert the same effect, slightly increasing the affinity at pH 7.5 for concentrations higher than 9 mM [32], while in human plasma their concentration are lower than 5 mM. Although the affinity of AmHb is high under *A. marina* physiological conditions (P_{50} = 2.6 mmHg, Table 1), it decreases when diluted under human physiological ones (P_{50} = 7 mmHg, Table 1). The Bohr coefficient ($\Delta \log P_{50}/\Delta pH$) of *A. marina* blood is normal (negative) relatively high (0.96 at 15°C, Table 1). It decreases when AmHb is diluted under human physiological conditions (0.5 at 37°C, Table 1). This decrease is explained by the increase of the temperature. The calculated ΔH (~19 kJ/mol, Table 1) reveals that the temperature effect on the oxygen affinity of AmHb under human physiological conditions is not very pronounced. Its cooperativity is not affected (n_{50} = 2.5, Table 1). These results show that the overall functionality of AmHb is not affected under human physiological conditions. It has been generally accepted that HBOCs should have properties resembling those of human blood with a P_{50} between 22 and 28 mmHg [31]. Current commercial blood substitutes were designed with this principle in mind. The affinity of AmHb under human physiological conditions (~7 mmHg, Table 1) is lower than the affinity of human blood (~23 mmHg under similar experimental conditions, Table 1) and slightly lower than the affinity of free HbA (~11.6 mmHg under similar experimental conditions, Table 1). However, recently, Winslow and collaborators [19, 33] have presented studies performed on an artificial capillary suggesting that the typical vasoconstriction induced by HBOCs is at least partially due to the over oxygenation of tissue resulting in abnormally high O_2 delivery [33]. This has the potential to produce an autoregulatory response in which vasoconstriction causes increased vascular resistance and reduced flow when excess of oxygen is sensed. These analyses have resulted in an unexpected finding, which suggests that increased diffusive O_2 delivery by low-affinity blood substitute paradoxically decreases O_2 uptake by tissue because of vasoconstriction [34], and that the presence of a low oxygen affinity cell-free Hb *in vivo* may not necessarily lead to a corresponding high level of O_2 delivery to the tissues [34]. To avoid this counteracting control mechanism, the rate of O_2 transfer by RBCs under normal conditions should be mimicked, which can be accomplished by limiting the facilitated diffusion of O_2 as oxyhemoglobin (OxyHb). This

Table 1. Functional properties and SOD activity[a)]

	Arenicola marina blood [51][b)]	Human blood[c)]	AmHb	HbA[c)]	RBCs
P_{50} (mmHg)	2.6 ± 0.46	26–30 [52] **23**[d)] **(*n*=1)**	**7.05 ± 0.93**[d)] **(*n*=9)**	8–18 [49] **11.6**[d)] **(*n*=1)**	–
n_{50}	2.5	2.7 [52] **2.75**[d)] **(*n*=1)**	**2.54 ± 0.23**[d)] **(*n*=9)**	2.3 – 3.0 [52] **2.45**[d)] **(*n*=1)**	–
Bohr coefficient[e)]	–0.96 at 15°C	**n.d**	**–0.5**[d)]	**n.d**	–
ΔH (KJ mol–1)[e)]	–35 (10° < T < 15°C) –18 (15° < T < 25°C)	**n.d**	**–19**[d)]	**n.d**	–
SOD activity (U/mg Hb)		12.1 ± 1.7 [40]	**3.53 ± 0.02 (*n*=3)**[f)]	–	1–2 [53]
CN inhibition			**100%**[g)]	–	–
Fe (atom/molecule)			156 [11]	–	–
Cu (atom/molecule)			**3.58 ± 1.17 (*n*=5)**[h)]	–	–
Zn (atom/molecule)			**5.13 ± 0.75 (*n*=5)**[h)]	–	–

a) The results obtained for this article are in bold and they are expressed as the results ± SEM; n.d.: not determined.
b) The experiments were performed under *A. marina* physiological conditions: 15°C, pH 7.4.
c) Only one measurement was performed to have a comparable result on the same instrument.
d) The oxygen equilibrium binding data were collected in dissolved lyophilized plasma (Sigma) in 10 mM HEPES, pH 7.35 at 37°C for a final heme concentration of 40 mg/mL. The Bohr coefficient was measured over the pH range 7.2–7.6, and the temperature effect from 33° to 41°C, which corresponds to the range of values encountered in human, including pathological cases.
e) The Bohr coefficient and temperature sensitivity of hemoglobin are calculated as described [54].
f) The SOD activity was determined using the Flohé and Ötting method [55] adapted for 96-well microplate measurements, for AmHb diluted into *Arenicola* buffer.
g) AmHb were incubated for 10 min with 50 mM KCN prior to the addition of xantine oxidase.
h) The Cu and Zn contents were determined by inductively coupled plasma-MS as described [56].

finding suggested that blood substitutes with low P_{50} and low macromolecular diffusion coefficients may more closely imitate the oxygen delivery profile of human RBCs in the microcirculation, and may be more appropriate for blood-like oxygen transport [19, 33]. On the basis of this new paradigm, the next generation of HBOCs should possess high oxygen affinity with a low P_{50} ranging from ~5 to 10 mmHg [19] as it is the case for AmHb under human physiological conditions.

3.3 AmHb NO scavenging

The interaction of NO with proteins is known to play a crucial role in several different physiological systems, ranging from blood pressure regulation to neurotransmission. NO (the "endothelium-derived relaxing factor", EDRF) or a labile compound releasing NO (such as RSNOs) have the capacity to interact with Hb on the ferrous heme and free cysteine, respectively [29, 35]. Unusual biological feedback mechanisms have been observed in animals, including man, in which the majority of HBOCs cause hypertension as the result of blood vessel constriction [9]. The physiological mechanisms governing this response may be numerous and are incompletely resolved. Currently, at least two distinct theories have been proposed to explain the phenomenon, both implying separate approaches for development of clinical products. While low oxygen affinity HBOC has previously been suggested as a possible explanation [34], another key hypothesis is NO scavenging by cell-free Hb [29, 35]. Cell-free Hb possibly extravasates into the interstitium between endothelial and smooth muscle cells, thereby altering the distribution of NO. Using AmHb as a blood substitute should prevent these unwanted reactions, because AmHb-HBL (~25 nm for ~3.6 MDa) [11], and even its dodecamers (~9 nm for ~205 kDa) [11], cannot extravasate through the endothelium [30]. Moreover, we observed that the dissociated products of dodecamers are immediately oxidized, resulting in the dissociation of heme and degradation of the apoglobin [21]. This means that even if the dissociated products extravasate, they will not be able to scavenge NO. On the other hand, in human blood, when NO binds to the heme iron, it can be involved in redox reactions with the metal ion, leading to the production of MetHb, nitrate, and the formation of additional ROS. In contrast, RSNOs do not react with the heme [35]. They rather participate in transnitrosation reactions with the sulfydryl groups of Hb. We investigated the reactions that occur on exposure of AmHb to NO and RSNO using UV-visible spectrophotometry and ESI-MS, and compared the results with those of cell-free HbA. The presence of NO led to the formation of MetHb for cell-free HbA solution, while the light-absorption spectrum of AmHb was not as significantly affected over the same period of time (Fig. 2a). It has been proposed that *in vivo,* NO preferentially binds to the very small amount of deoxy-

genated heme of Hb that is present under physiological conditions (about 1%) to yield a nitrosyl complex. This proposal is based on the hypothesis that NO binds to the R-state HbA at least 100 times faster than to the T-state [36]. Figure 2a reveals that NO bound very rapidly to DeoxyHb (Hb[FeII]), forming nitrosyl-Hb complexes Hb[FeII]NO. The oxygenation of this solution caused the rapid decay of the nitrosyl-Hb to MetHb for cell-free HbA, a conversion which is much slower for AmHb for which the nitrosyl complex Hb[FeII]NO is relatively stable. Our results revealed that NO does not provoke oxidation to any physiologically significant degree compared to cell-free HbA. Its transfusion should therefore not be responsible for side-effects associated with the formation of MetHb.

The native extracellular Hb of the lugworm, which colonize intertidal areas, presents free cysteines on their globin chains scavenging H_2S (HS-) for detoxification purposes [37]. We explored the potential mechanisms for S-nitrosylation of AmHb-HBL. Indeed, AmHb-HBL posses a total of 124 free cysteines; 1 *per* globin monomers and trimers, *e.g.*, 10 *per* AmHb dodecamers and none on the linkers subunits, as determined by ESI-MS [11]. The NO group of RSNOs possesses a nitrosonium (NO^+) character that distinguishes it from NO itself, and enables it to elicit responses that NO cannot. These results suggest that the free cysteines of globin monomers are more accessible than those of trimers. AmHb, following incubation with S-nitrosated cysteine (CysNO), was directly analyzed by ESI-MS. ESI-MS technology made it possible to directly detect the formation of NO adducts on globin monomers a_1 (15,950 Da) and a_2 (15,974 Da) of AmHb (Fig. 2b). The transnitrosylation was not observed for the trimers, which all have one free cysteine potentially capable of binding NO. AmHb can transfer NO, while the release of NO from SNO-Hb has the potential to bring about reactions normally associated with free NO. Several pathological conditions have been associated with abnormalities in NO generation. Elevated NO levels are produced during septic shock, severe hemorrhage and endotoxemia, provoking vasodilatation and hypotension [38], whereas abnormally low NO concentrations have been measured *in vivo* during ischemia/reperfusion [39]. Thus, our results have implications for the rational design of AmHb blood substitutes as NO scavenger [38] or therapeutic NO donor [39], depending on the pathology.

3.4 SOD activity

One of the original characteristics of AmHb is its intrinsic SOD-like activity (Table 1). This SOD-like activity has been measured to be 3.53 ± 0.02 U/mg Hb (Table 1). The total inhibition of AmHb SOD activity by cyanide (Table 1) revealed that superoxide scavenging possibly involved Cu and Zn atoms in AmHb, such is the case for Cu-Zn-SOD. This is confirmed by the analysis of the metal con-

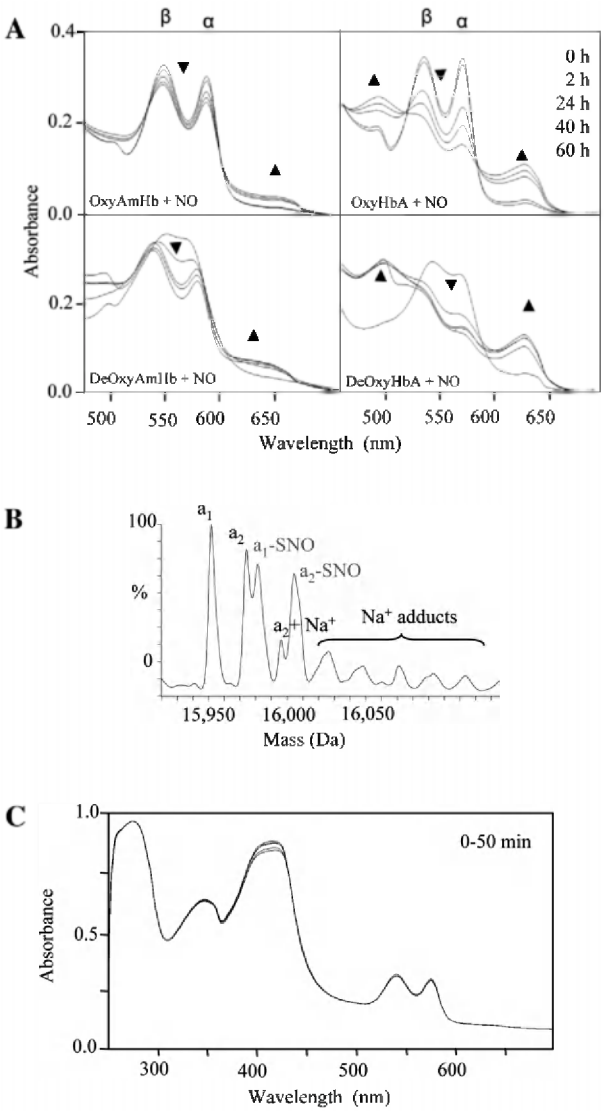

Figure 2. NO, RSNOs and KO_2 interactions with AmHb. **A** Interaction of NO with heme of Deoxy- and OxyAmHb compared to cell-free HbA. Conversion of HbA to MetHbA (HbFe[III]) occurred instantaneously upon incubation of Oxy- and DeoxyHbA with NO as previously reported [35]. There is little (if any) formation of MetAmHb when either Deoxy or OxyAmHb is incubated with NO over 60 h. Conversion of DeoxyAmHb (AmHb[FeII]) to nitrosyl-complex AmHb[FeII]NO is formed instantaneously, explaining the flattened appearance of the spectra of AmHb at t0. **B** Interaction of S-nitrosothiols with AmHb. Conversion of the monomers a_1 and a_2 of AmHb to a_1-SNO and a_2-SNO is observed upon incubation of S-nitrosocysteine (CysNO). Of the four main intense species present on the deconvoluted spectra, the first is the a_1 globin (15,953 Da) and the second the a_2 globin (15,976 kDa), whereas the third and the fourth had a mass of 15,979 and 16,003 kDa with a shift of +29 units compared to that measured for a_1 and a_2 globins, respectively, indicating the addition of a single NO moiety to the protein. The major peaks are accompanied by higher mass satellites of lower intensity. These are adducts of Na^+ added as $NaNO_2$ to produce CysNO. **C** Evaluation of the oxidation of OxyAmHb by oxydation after incubation with superoxide anions. The presence of KO_2 (substrate for SOD activity [58]) did not oxidized AmHb over a 50-min exposure. The reactivity of AmHb with superoxide anions does not modify AmHb, as expected for a SOD-like activity.

tent, which revealed the presence of two to five atoms of Cu and five to six atoms of Zn *per* mole of AmHb (Table 1). Although the SOD activity determined for the human RBCs and reported in the literature (1–2 U/mg Hb, Table 1) exhibits differences (which could be explained by the variability between individuals) [40], it is slightly lower than the AmHb SOD activity. These results revealed that AmHb has an important antioxidant activity as SOD protects the tissue from the harmful effects of superoxide radicals to a certain degree. OxyHbA is known to undergo a slow, but spontaneous intramolecular oxidation-reduction reaction, in which the heme iron is oxidized into the ferric form and the oxygen is reduced into superoxide. Within the erythrocytes, the antioxidant systems prevent deterioration of Hb and loss of its heme, which has its own cytotoxicity [41]. In the absence of these enzymes, as is the case when extracellular AmHb is administrated directly in the plasma, the oxidation of AmHb could serve as a source of ROS whose toxicity will be averted by AmHb SOD activity without oxidation of the hemoglobin (Fig. 2c). On the other hand, reperfusion of ischemic tissues with oxygen-carrying fluids, as in strokes, myocardial infarction, organ transplantation, severe sustained hemorrhagic shock and other conditions, can result in the release of superoxide, oxygen radicals that eventually exhaust the antioxidant capacity of RBCs and lead to tissue injuries. In those situations, the administration of exogenous SOD has been tested in several experimental models. The results are controversial because of the short half life of native SOD (6 min). However, the protective effect of SOD against warm ischemia/reperfusion injury can be greatly improved by binding the enzyme to a polymer [42]. Thus, in these clinical situations, AmHb will be particularly useful because it combines oxygen carrying properties with SOD activity [8].

3.5 Resistance to autoxidation

One of the universal problems among RBC substitutes is their short life span. The plasma residence times for HBOCs range from approximately 12 h for cross-linked Hb to about 2 days for PEG-Hb, compared with a mean residence time of 120 days for RBCs [3]. This explains why HBOCs are currently used in conjunction with, or as a bridge to, transfusion. Acellular Hbs are particularly susceptible to oxidation and denaturation. Oxidized acellular Hb can undergo further degradation through hemichrome formation, leading to released heme-iron and globin chain precipitation, which has the potential to cause endothelial and surrounding tissue damage. An interesting property of extracellular Hb of annelids is their resistance to autoxidation [12]. UV-visible spectrometry and size exclusion chromatography were used here to assess the *in vitro* stability under simulated human physiological conditions (Fig. 3a). AmHb in the presence of human plasma dissociates almost immediately into dodecamers (Fig. 1). The dodecamer subunits diluted in the plasma, which contains non-enzymatic reducing components, in the reduced state (Fe^{2+}) for up to 50 h as indicated by the very limited change in the UV-visible light absorption spectrum (Fig. 3a, *D*). Obviously, longer plasma retention times are desirable, but in most applications involving trauma and surgery, a 24–48 h half-life is sufficient.

Purified AmHb also exhibited long-term redox stability under storage conditions (4°C) up to 30 days and less than 5 days at 20°C as revealed by the light-absorption spectrum (Fig. 3b), which was not significantly altered over these periods of time. On the other hand, purified AmHb can be stored either at –40°C or in liquid nitrogen until needed. No changes in its properties are apparent with these stored preparations over at least several months.

An enzymatic reduction may bypass oxidation and increase the half-life of AmHb. To evaluate the protective reducing properties of compounds usually associated with Hb redox stability, the effect of β-NADH on the rate and extent of MetHb reduction was investigated at 37°C (Fig. 3c). AmHb visible spectra were only slightly altered after 144 h at 37°C in the presence of β-NADH, whereas the AmHb converted entirely into MetHb under similar conditions in the absence of β-NADH (Fig. 3c, up). Redox conversion back to the reduced form by β-NADH was characterized by the elimination of the 630-nm charge-transfer band and the re-established spectral maxima at 576 and 540 nm characteristic of reduced AmHb (Fig. 3c, down). β-NADH prevents the formation of MetHb at 37°C and can also reduce MetHb without the need of another electron carrier. Thus, the toxic side reactions that are commonly encountered during reperfusion experiments, as well as during clinical trials of HBOCs, can be minimized for AmHb by a co-administration of one or more specific agents such as β-NADH. These results revealed that the reducing agent should play an effective role in long-term storage of AmHb, and serve as reducing agents in the *in vivo* exchange.

3.6 Interaction with plasma proteins

When large quantities of extracellular Hb are present in the circulation, as is the case when an Hb solution is used as a blood substitute, the spontaneous oxidation to MetHb leads to an increased production of free hemin, free iron and peptides, resulting from the hydrolysis of the globin. Hemin, the free heme oxidized to the ferric state, is potentially toxic since it intercalates in lipid membranes and catalyzes the formation of hydroxyl radicals and the oxidation of low-density lipoproteins, thereby contributing to the observed side effects [41]. Hemopexin and HSA are pathological transporters of heme and with Hp, in the first line of defense against intravascular heme-mediated oxidative damage. We investigated the interaction of Hp and HSA with AmHb dissociation products.

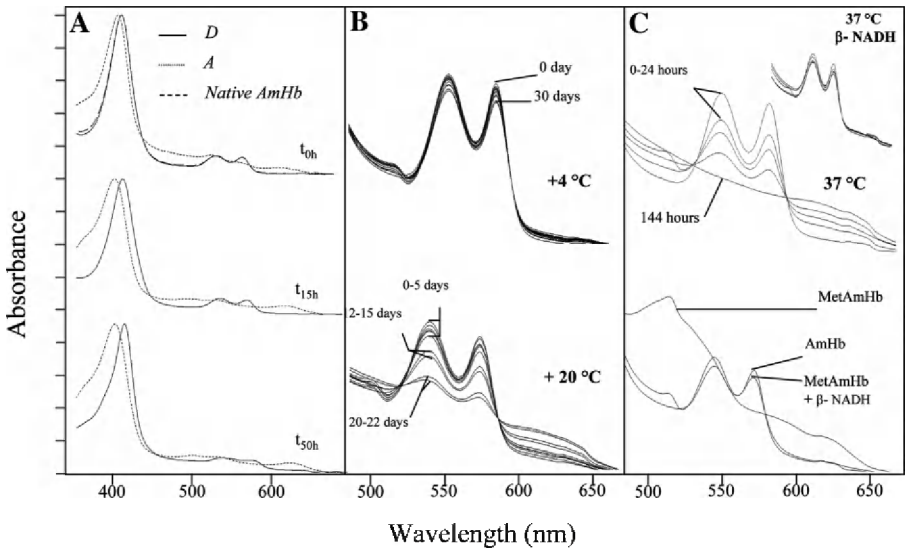

Figure 3. Resistance to autoxidation of AmHb. **A** Product *D* is resistant to autoxidation under human physiological conditions over 50 h. The profile of *A* is characteristic of MetHb and confirms the scavenging of hemin by HSA to form metalbumin. **B** There is little (if any) MetAmHb formation under storage conditions at 4°C over 30 days and at 20°C over 5 days. Conversion of AmHb to MetAmHb is observed at 20°C after 12 days. **C** Resistance to autoxidation increased with β-NADH. *Top*: The presence of β-NADH at 37°C prevents the conversion of AmHb to MetAmHb over 144 h. *Bottom*: MetAmHb is reduced back to OxyAmHb in the presence of β-NADH.

Hp is involved in promoting the clearance of plasma Hb, and is depleted from plasma during elevated hemolysis. The Hp-Hb complex is then engulfed by the macrophage and degraded by the spleen and liver. The binding of Hp to cell-free HbA is among the strongest of all known non-covalent protein-protein interactions, preventing loss of iron and regulating the renal threshold for Hb [43]. One problem of using extracellular Hb as blood substitutes is their possible scavenging by Hp in order to be cleared from the circulation. We used gel filtration-multi-angle-laser-light-scattering techniques to evaluate the binding capacity of human Hp with AmHb-HBL and its dissociation products. Cell-free HbA was used as a positive control. The results revealed that Hp does not scavenge either the AmHb-HBL or the dissociated products of AmHb, whereas the formation of the Hp-HbA complex was apparent (Fig. 4). While the combination of Hp with Hb is not species specific (human Hp can combine with various mammalian Hbs) [44] and the nature of the binding site is still under discussion [45, 46], it has been reported that Hp only binds to the oxygenated Hb and has a much higher affinity to the αβ dimer of HbA. AmHb does have an original dissociation process [21]; it dissociates into the functional dodecamers that further degrade into the unstructured apoprotein and hemin. Hp recognizes neither AmHb-HBL nor the dodecamers because of their high molecular weight [45]. It does not recognize the dissociated products because they are deoxygenated and unstructured [45]. No aggregates (Hp-AmHb) should be formed when AmHb will be transfused to human. However, the dissociated products, which might be toxic, will not be scavenged either.

HSA is the most abundant protein in the plasma. Its most important physiological function is to maintain the COP and the pH of blood. However, the protein is characterized by its remarkable ability to bind a broad range of endogenous and exogenous small hydrophobic molecules. The toxicity of hemin is largely averted though the scavenging action of hemopexin [47] and HSA [48], which bind hemin with high affinity and transport it to various tissues for intracellular catabolism. Previous observations (Figs. 1, 3a, *A)* revealed the formation of metalbumin following the dissociation of AmHb dodecamers, and this has been confirmed by the ESI-MS multicharged spectra of *A* under non-denaturing conditions (Fig. 1d, bottom). While we still do not know how the degraded apoglobin is metabolized, we have shown that the hemin is scavenged by HSA, thus probably preventing any toxic side effect.

3.7 *In vivo* experiments on mice

3.7.1 General behavior

We examined whether the administration of AmHb affected the body weight and the overall behavior of C57 and DBA mice, which are amongst the most studied inbred strains in behavioral pharmacology. After i.v. administration of AmHb (previously diluted in plasma or the physiological serum), the animals were all alive after 18 weeks. They all grew normally with increasing body weights (C57: 7.8 ± 0.7 g, n=10 and DBA: 9.6 ± 0.7 g, n=10 in 18 weeks). Their general health was normal, which in-

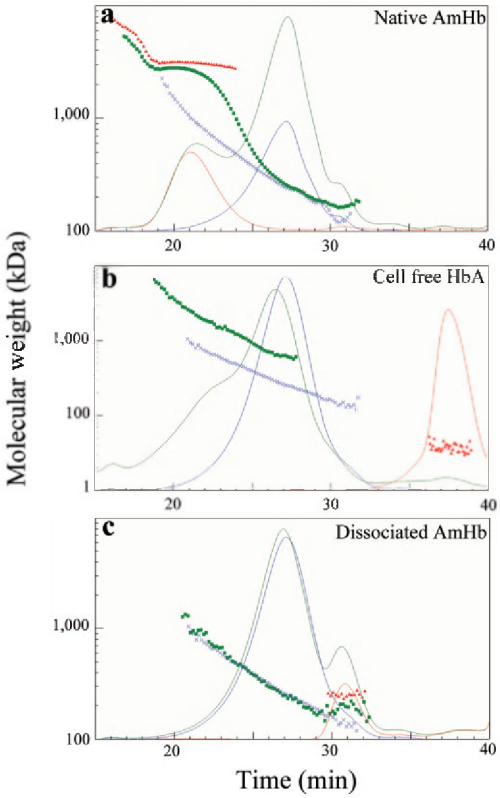

Figure 4. Interaction with Hp. (a) Native AmHb (red) is not scavenged by human Hp (blue). The mixed solution refractive index profile (solid green curve) is the sum of both the independent profiles. The molecular mass of the mixed solution (green square) is the average for native AmHb (red triangle) and human Hp (blue cross). (b) Cell free HbA (red triangle) is scavenged by human Hp (blue cross). The formation of HbA-Hp complex is revealed by an increase of the molecular mass (green square) and a new peak (solid green curve) at a lower retention time (*i.e.*, higher molecular mass). (c) Dissociated AmHb products (including dodecamers) (red triangle) are not scavenged by Hp (blue cross). The mixed solution profile (green square) is the sum of both the independent profiles.

dicates that AmHb had no apparent toxicity. Similar experiments carried out by Hirsch and collaborators [17] showed that mice and rats, partially transfused with *Lumbricus terrestris* extracellular Hb, do not exhibit any special behavior and/or pathological trouble after the transfusion over 10 months of observations.

3.7.2 Antigenicity and immunogenicity

The use of AmHb as an artificial blood substitute may trigger an immune response. To study this potential effect, we studied the antibody response after one and two i.v. administrations of clinically relevant doses of AmHb to evaluate if an immune response is likely to occur. The antibody titers (IgE and IgG2a, Fig. 5a, b) showed that AmHb may weakly activate the immune system, but this effect is not statistically different from the vehicle ($p<0.05$), in contrast to the response observed for ovalbumin for which 33% of the mice died after two i.v. administrations (Fig. 5b). Immunizing doses of AmHb produce antibody titers but at a non-significant level, keeping in mind that the mice used in this study are hyper-responsive (BP/2 strains) and easily produce antibody.

It may seem surprising that upon immunogenic challenges, no significant antibody formation could be detected. There is an abundant evidence that the antigenicity of human cell-free Hb is low and is decreased by polymerization [49]. The notion is raised that antigen recognition mechanisms resulting in antibody formation cannot be activated by a protein of this size, but their immunological relevance has not been entirely clarified [49].

4 Concluding remarks

4.1 A promising blood substitute

All the results presented here show the promise of the use of AmHb as a useful model system for developing therapeutic extracellular blood substitutes [10, 20]. Indeed, AmHb-HBL and its dodecamer subunits have all the properties, which are currently under investigations to develop a safe blood substitute. AmHb-HBL is a naturally highly polymerized protein, has a high molecular weight that should prevent extravasations into the endothelium and through the glomerular capillary, and it should induce only a small increase of COP. AmHb has a relatively high oxygen affinity, which fulfill the new requirement of the next generation of blood-substitutes, probably allowing vasoconstrictive effects to be avoided [19]. Its functional properties are not affected when dissolved in simulated human physiological conditions, thus AmHb should deliver sufficient oxygen to tissues to allow effective oxygenation without renal and other organ toxicity. High-affinity blood substitutes may also have additional advantages from a stability perspective over their lower affinity counterparts. Studies by Nagababu *et al.* [50] have shown that the anti-oxidative processes used by many commercial blood substitute manufacturers to lower oxygen affinity can actually lead to increased heme degradation and autoxidation. Moreover, AmHb may deliver oxygen more effectively than RBCs in some situations by accessing into regions not accessible to RBCs. For example, during coronary artery balloon angioplasty procedures, AmHb may still be delivering O_2 to tissues, whereas circulation of RBCs is obstructed. Although the structural integrity of AmHb is affected, the dissociated products–dodecamers–exhibit redox stability under simulated human physiological conditions as long as their structure is maintained, satisfying a circulatory retention time of at least 50 h. This fits the requirement of blood substitute knowing that the dodecamer half-life could be increased in the presence of β-NADH. HBOCs are developed to be used in case of acute preoperative blood loss and, espe-

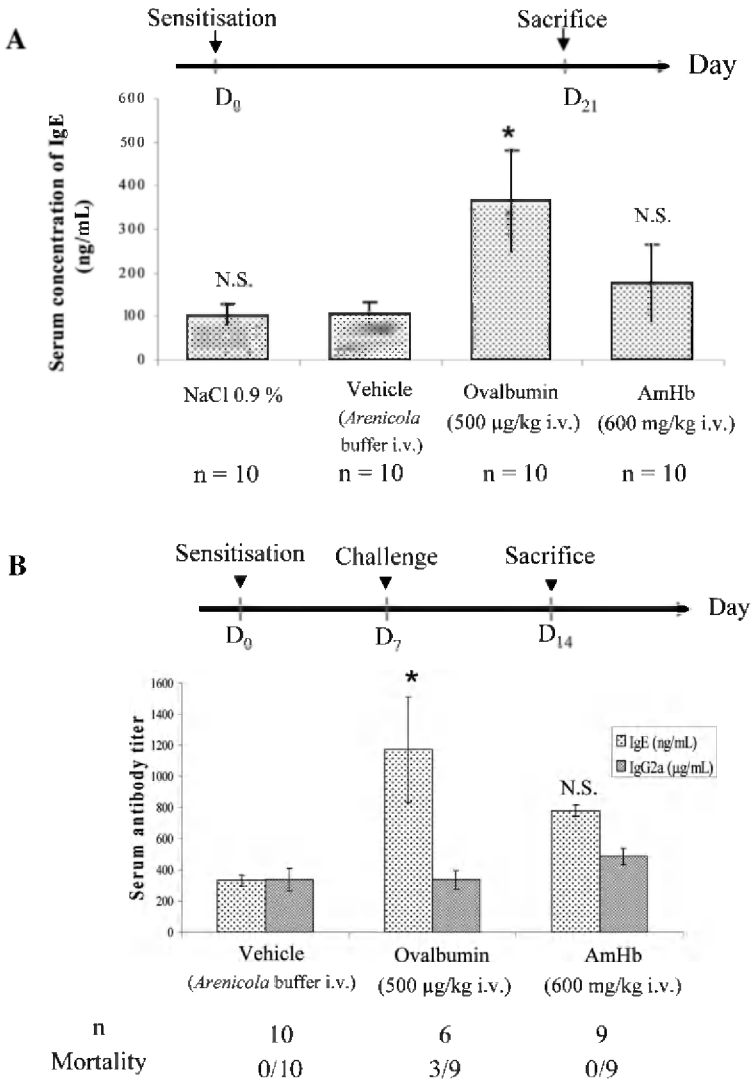

Figure 5. *In vivo* experiments on mice. **A** IgE serum levels in mice treated with 0.9% NaCl and vehicle were similar, demonstrating that the vehicle of AmHb is not proallergenic by itself. In contrast, treatment with ovalbumin led to a 3.4-fold increase in IgE level 3 weeks after the initial administration (1171 ± 339 ng/mL compared to 331 ± 37 ng/mL in the vehicle-treated group). AmHb very slightly increased IgE levels, but this effect did not reach statistical significance when compared to the vehicle-treated group. Data are means ± SEM, n=10 male mice *per* condition, *p<0.05, N.S.: not significant, *versus* vehicle-treated group. **B** In the group challenged at D_7 with ovalbumin, three mice out of nine died 15 min after administration. The remaining mice killed 7 days later (D_{14}) showed a marked increase in IgE level (1171 ± 339 ng/mL compared to 331 ± 37 ng/mL in the vehicle-treated group), whereas IgG2a were unchanged (337 ± 57 µg/mL compared to 340 ± 72 µg/mL in the vehicle-treated group). The second administration of AmHb at D_7 did not induce mortality. IgE and IgG2a levels at D_{14} were moderately increased when compared to vehicle-treated group (779 ± 36 ng/mL and 487 ± 52 µg/mL, respectively). Data are means ± SEM, n=9–10 male mice *per* condition, *p<0.05, N.S.: not significant, *versus* vehicle-treated group.

cially, for trauma patients, and none of the current products would be optimal in patients with chronic anemia. The blood group antigens of RBC membranes requires careful cross-matching and typing before donor blood can be administrated. AmHb is extracellular and not glycosylated [11], therefore a time-consuming cross-matching or typing is not required and AmHb can be given immediately on the spot to anyone. AmHb will be useful to bridge temporary shortages of RBC products or in situations where lacks of time or special conditions make use of RBC products impractical. The hemin resulting from its degradation is scavenged by HSA and, even if the way of metabolism of apoglobin has not been determined, *in vivo* experiments on mice have revealed that its administration is non-toxic. Indeed, the lack of severe morbidity or mortality in mice model partially exchanged with the AmHb is the foremost observation. Surprisingly, exchanged mice challenged with AmHb exhibited no apparent behavioral and pathological changes, and showed no over allergic response. Further *in vivo* investigations have started on rats. They are focused on deleterious aspects such as those occurring in cardiac and renal functions. AmHb is readily available, easily purified as a homogenous product, and exhibits extraordinary stability under the proper storage conditions. Immediately effective and easy to handle, AmHb used as a blood substitute should avoid the problems of blood shortage, transmission of infectious diseases, and the problem of blood compatibility.

4.2 Novel therapeutic applications

There are also additional therapeutic applications for the use of AmHb as an artificial oxygen carrier. AmHb NO scavenging should be useful for the treatment of NO-induced hypotension or hypertension. On one hand, NO

gradually released by SNO-AmHb can actively prevent endothelium dysfunction. It is known that exogenous NO exhibits a negative feedback on the production of NO [39]. On the other hand, treatment of septic shock will take advantage of the ability of AmHb to scavenge NO on its heme or free cysteine without toxic effect. High-affinity Hb may also be useful as a perfusion component in organ preservation prior to transplantation. Indeed, in the storage of donor organs, the circulation half-life limitation does not apply. An additional interesting property of AmHb is its intrinsic SOD activity. This property is sought in second generation modified Hb, for which strategies to combat autoxidation reactions of Hbs are evolving by cross-linking [8] trace amounts of catalase and SOD to Hb. This should prevent both the occurrence of potentially harmful heme protein-associated free radical species and the release of Hb degradation products that may exacerbate ischemia-reperfusion injury. AmHb will therefore be very useful as a blood substitute in ischemia-reperfusion.

4.3 Recombinant expression

Finally, AmHb-HBL is characterized by self-association properties, which allow its reconstruction *in vitro* [20, 21]. The fact that AmHb dissociates into a functional and relatively stable dodecamer when transfused into mammals, directed our analysis to a simplified recombinant expression [20] and to rebuild the dodecamers rather than the whole molecule to use as a blood substitute.

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