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Editor's Summary

Oxygen on Demand

The clinical sequelae after prolonged oxygen deprivation can be serious, including cardiac arrest and brain damage. In these situations, patients are typically fed oxygen through a tube via the mouth. What happens when access to the lungs is impeded or delayed? Currently, few other options exist. In response, Kheir and colleagues have engineered microparticles that can be injected into the veins for systemic delivery of oxygen to all of the vital organs.

The lipidic oxygen–containing microparticles (LOMs) consist of a lipid shell and an oxygen gas (O_2) core, with an approximate diameter of 4 μm. These tiny particles were designed to mix with venous blood and deliver O_2 to oxygen-deprived hemoglobin —the molecule that carries oxygen to all tissues within the body. Kheir *et al.* first confirmed that the LOMs functioned as intended by mixing a foam suspension of the particles with human blood in tubes and measuring the rise in oxygenated hemoglobin. When administered intravenously to asphyxiated (and therefore hypoxemic) rabbits, the LOMs were able to maintain full-body oxygenation, normal blood pressure, and normal heart rate compared to control animals that only received a saline solution. The animals receiving LOMs also lived longer and did not experience any injury to major organs, such as liver and lungs.

This is an encouraging demonstration for critical care medicine situations, showing that animals can survive and remain healthy even after 10 to 15 min of complete asphyxia. Such short-term infusions could therefore serve an important therapeutic function for critically ill patients, but before you hear "LOMs, stat!" in the emergency room, additional studies will be needed to assess simultaneous removal of carbon dioxide buildup, LOM metabolism, and possible side effects from longer-term, continuous infusions.

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CRITICAL CARE MEDICINE

Oxygen Gas–Filled Microparticles Provide Intravenous Oxygen Delivery

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We have developed an injectable foam suspension containing self-assembling, lipid-based microparticles encapsulating a core of pure oxygen gas for intravenous injection. Prototype suspensions were manufactured to contain between 50 and 90 ml of oxygen gas per deciliter of suspension. Particle size was polydisperse, with a mean particle diameter between 2 and 4 μm. When mixed with human blood ex vivo, oxygen transfer from 70 volume % microparticles was complete within 4 s. When the microparticles were infused by intravenous injection into hypoxemic rabbits, arterial saturations increased within seconds to near-normal levels; this was followed by a decrease in oxygen tensions after stopping the infusions. The particles were also infused into rabbits undergoing 15 min of complete tracheal occlusion. Oxygen microparticles significantly decreased the degree of hypoxemia in these rabbits, and the incidence of cardiac arrest and organ injury was reduced compared to controls. The ability to administer oxygen and other gases directly to the bloodstream may represent a technique for short-term rescue of profoundly hypoxemic patients, to selectively augment oxygen delivery to at-risk organs, or for novel diagnostic techniques. Furthermore, the ability to titrate gas infusions rapidly may minimize oxygen-related toxicity.

INTRODUCTION

Low blood oxygen tension, or hypoxemia, occurs in a variety of critical illnesses, such as airway obstruction and acute respiratory distress syndrome, and is associated with increased mortality (*1*). When hypoxemia leads to cardiac arrest, resuscitated patients suffer poor neurologic outcomes (*2*). Severely hypoxemic patients are typically treated with inspired oxygen, intubation, and mechanical ventilation. When institution of these measures is delayed, complicated, or insufficient to provide adequate oxygenation, patients may suffer organ injury, cardiac arrest, or death (*3*). Injecting oxygen intravenously might raise venous and arterial oxygen tensions, permitting a safe window of time for definitive intervention, such as placement of a tracheal tube or institution of extracorporeal life support. For example, a patient with complex airway anatomy and difficulty maintaining oxygenation using basic airway maneuvers could avert a hypoxemic crisis during a prolonged intubation attempt. To date, safe and effective intravascular delivery of oxygen gas has not been realized.

In the early 1900s, intravenous administration of oxygen gas was used in attempts to relieve refractory cyanosis (*4*–*7*). Most reported that spontaneously breathing, cyanotic animals exhibited signs of pulmonary embolism at infusion rates in excess of 0.2 to 1 ml/kg per minute and required frequent pauses in the infusion (*4*, *5*); this infusion rate represented 10 to 25% of measured oxygen consumption in dogs. Infusion rates higher than this caused signs of hypotension, cardiac irregularity, increased cyanosis, and death, the latter attributed to pulmonary embolism and right ventricular failure. Adult humans exhibited a narrower therapeutic window, exhibiting signs of pulmonary embolism at infusion rates in excess of 0.25 ml/kg per minute (*6*, *7*). None of these studies documented an increase in oxygen content in the blood as a result of the intervention.

Gas-filled particles are commonly used as ultrasound contrast agents (*8*) and drug delivery vehicles (*9*). Such suspensions are typically designed to have prolonged circulation times and low gas fractions and thus are therefore not well suited for gas delivery. These particles have been manufactured by sonication (*10*), shearing (*11*), flow focusing (*12*), and extrusion (*13*) and are often composed of lipid or protein excipients. Particles containing perfluorocarbons (*14*) or hemoglobin (*15*) may circulate with high oxygen content and be used as blood substitutes, but rely on an intact airway-lung unit for oxygenation; they are therefore impractical for reversing severe hypoxemia in situations of airway or pulmonary catastrophe. Oxygenated perfluorocarbons can carry large volumes of dissolved oxygen gas but cavitate when mixed with blood, thus requiring ex vivo extracorporeal mixing for clinical use (*16*).

To address the important problems of critical hypoxemia and resulting tissue injury, we have designed lipidic oxygen–containing microparticles (LOMs) that contain an oxygen gas core surrounded by a semipermeable lipid monolayer. The lipid monolayer self-assembled around the oxygen gas, which was not bound to any substance. Each LOM served as a discrete circulating pocket of gas that delivered oxygen

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to hypoxic blood. We describe basic characteristics of the particles, their behavior when mixed with blood ex vivo, and their effects in two animal models of severe hypoxemia.

RESULTS

Particle characteristics

We manufactured LOMs from several candidate phospholipids and stabilizing surfactants (Fig. 1A and table S1) using a process of sonication, size isolation by flotation, and concentration by centrifugation (*17*). The lipidic oxygen suspension consisted of about 10^{10} particles per milliliter of suspension. The core of the particle was oxygen (O_2) gas, which was surrounded by a phospholipid shell consisting of amphipathic lipids (Fig. 1A). The membrane was stabilized by an amphipathic block copolymer. For example, in LOMs containing Poloxamer 188, the block copolymer consisted of poly(propylene oxide) and poly(ethylene oxide).

LOMs bring O_2 gas within close proximity of deoxyhemoglobin within erythrocytes. Hemoglobin provides an oxygen "sink" for large volumes of O_2 gas to diffuse down a concentration gradient and bind to deoxyhemoglobin until the surrounding blood is fully saturated with oxygen. As O_2 gas leaves the LOM, the gas core decreases in size markedly (Fig. 1A), breaking down into empty micelles containing the lipid monolayer, which is 2 nm thick (*18*). After intravenous injection, LOM suspensions mixed with venous blood, which was then oxygenated (Fig. 1B).

Mean particle diameter by optical scatter was 3.87 ± 1.94 (SD) μm, which did not change significantly when heated to body temperature [2.23 ± 0.48 (SD) μm; $P = 0.19$, Mann-Whitney] (Fig. 1, C and D). Electron microscopy of freeze-fracture replicas demonstrated a smooth surface without distinct domain boundaries (*18*) (Fig. 1E). At 4°C, suspensions composed of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and polyoxyethylene (100) stearyl ether (BRIJ 100) demonstrated a 0.85 ± 0.02% (SEM) loss per day (fig. S1A) but preserved their size distribution during a 100-day observation period (fig. S1D). Particles composed of DSPC with BRIJ 100 (table S1) or with 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[carboxy(polyethylene glycol)-5000] (DPPE-PEG5000) exhibited similar degradation curves ($P = 0.605$, nonlinear regression). The stability of suspensions stabilized using Poloxamer 188 was substantially higher than those containing either BRIJ 100 or DPPE-PEG5000 ($P < 0.0001$, nonlinear regression). Particles manufactured using DSPC and polyoxyethylene (40) stearate (PEG40S) or 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[carboxy(poly(ethylene glycol))-2000] (DSPE-PEG2000) exhibited the least stable suspensions ($P = 0.043$, between PEG40S and DSPE-PEG2000, and $P = 0.001$, between PEG40S and Poloxamer 188, nonlinear regression). These findings follow previous reports that resistance to gas flow across the lipid membrane is proportionate to the molecular weight of the stabilizing agent (*19*, *20*).

Particles composed of DSPC and Poloxamer 188 exhibited the smallest change in mean diameter ($P < 0.05$ compared to all groups, linear regression) and did not exhibit significant growth over the 100-day observation period ($P = 0.7681$, linear regression) (fig. S1D). The combination of DSPC and BRIJ 100 exhibited a mean diameter of 3 μm at the time of manufacture, which steadily increased over the observation period ($P < 0.0001$, linear regression). The combination of DSPC and Poloxamer 188 exhibited the lowest proportion of particles exceeding 10 μm ($P < 0.05$ compared to all other combinations, linear regression), and this fraction did not increase over time ($P = 0.9799$, linear regression) (fig. S1G). This cutoff is thought to permit transcapillary passage (*21*). Storage characteristics at room temperature and 37°C were also tested (fig. S1).

LOMs used for subsequent ex vivo and in vivo testing were composed of DSPC and BRIJ 100, and were diluted to gas fractions

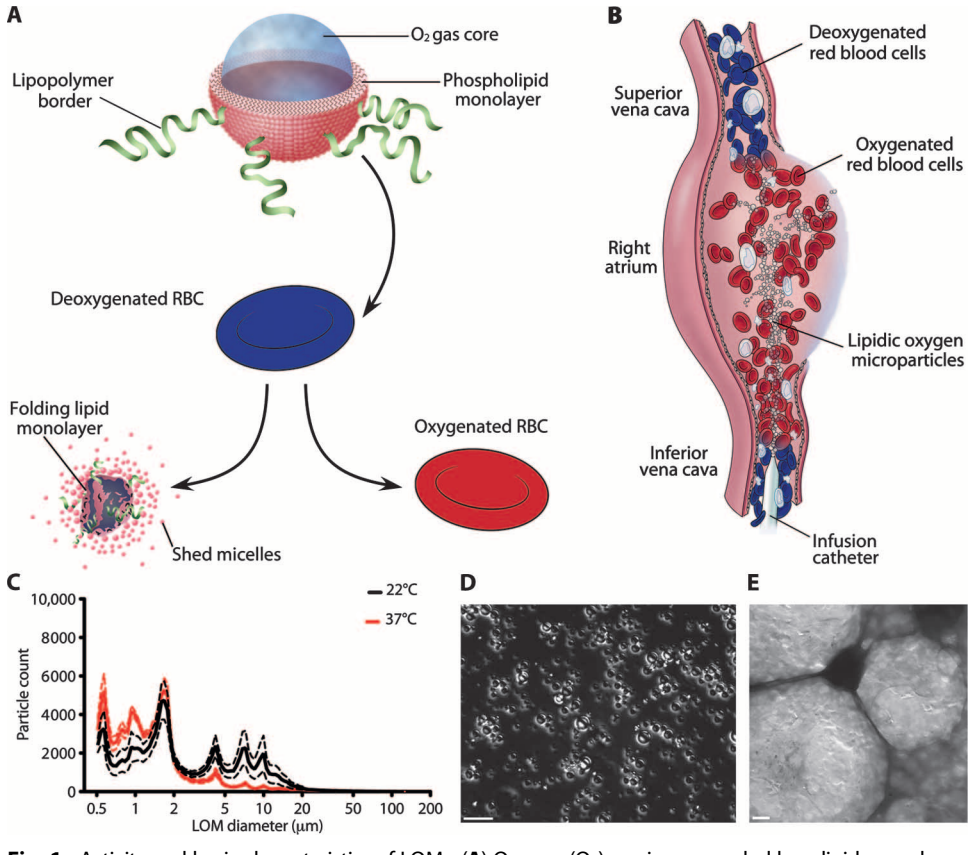

Fig. 1. Activity and basic characteristics of LOMs. (**A**) Oxygen (O_2) gas is surrounded by a lipid monolayer. As O_2 gas leaves the LOM and binds to erythrocytes, the shell breaks down by domain folding into multiple small vesicles. RBC, red blood cells. (**B**) Intravenously injected LOMs mix with blood, permitting oxygenation of systemic venous blood. (**C**) Particle size distribution by optical scatter of LOMs sized at 22°C and 37°C. Data are means ± SEM ($n = 4$ samples per temperature). (**D**) Light microscopy of LOMs. Scale bar, 10 μm. (**E**) Freeze-fracture electron microscopy of LOMs. Scale bar, 1 μm. LOMs in (C) to (E) were composed of DSPC and BRIJ 100.

between 50 and 90% (v/v) using Plasma-Lyte A, an intravenous fluid whose electrolyte composition is similar to that of plasma.

Suspension rheology

We studied the rheologic profile of LOM suspensions composed of DSPC and BRIJ 100 alone and when mixed with blood using parallel plate rheometry. Suspension viscosity increased exponentially with increasing gas fraction and exhibited shear thinning behavior (Fig. 2A). When mixed in a 1:1 (volume) ratio with blood, the viscosity of 90 volume % LOMs decreased two orders of magnitude ($P < 0.0001$, Wilcoxon matched pairs test), and that of 65 volume % LOMs (mimicking the admixture of LOMs and blood during in vivo experimentation) was similar to that of blood alone (Fig. 2A). As shear increased, the differences in viscosity became insignificant between 65, 80, and 90 volume % LOMs at stresses above 2.5 Pa [$P > 0.05$, two-way analysis of variance (ANOVA) with Bonferroni post test; Fig. 2A].

Complex viscosity of 90 volume % LOMs increased linearly with increases in temperature between 4 and 38°C ($P < 0.001$, linear regression; Fig. 2B). At a shear equivalent to infusion through a catheter (2000 s^{-1}), 65 volume % LOMs exhibited a viscosity about twice that of human blood [37.2 ± 5.3 (SD) mPa·s for 65 volume % LOMs versus 16.2 ± 0.9 (SD) mPa·s for blood], which was not a significant difference; viscosity of 80 and 90 volume % LOMs was significantly higher than that of blood (Fig. 2C). LOM yield stress increased exponentially with increasing volume % (Fig. 2D). There was no significant difference in yield stress between blood and 65 volume % LOM. Zero stress viscosity and infinite stress viscosity were also tested as a function of particle volume % (Fig. 2, E and F).

Ex vivo oxygen transfer

To characterize the release kinetics of O_2 gas from LOMs of varying gas fractions to blood, we desaturated human red blood cells using nitrogen gas and maintained them at 37°C under continuous motion on

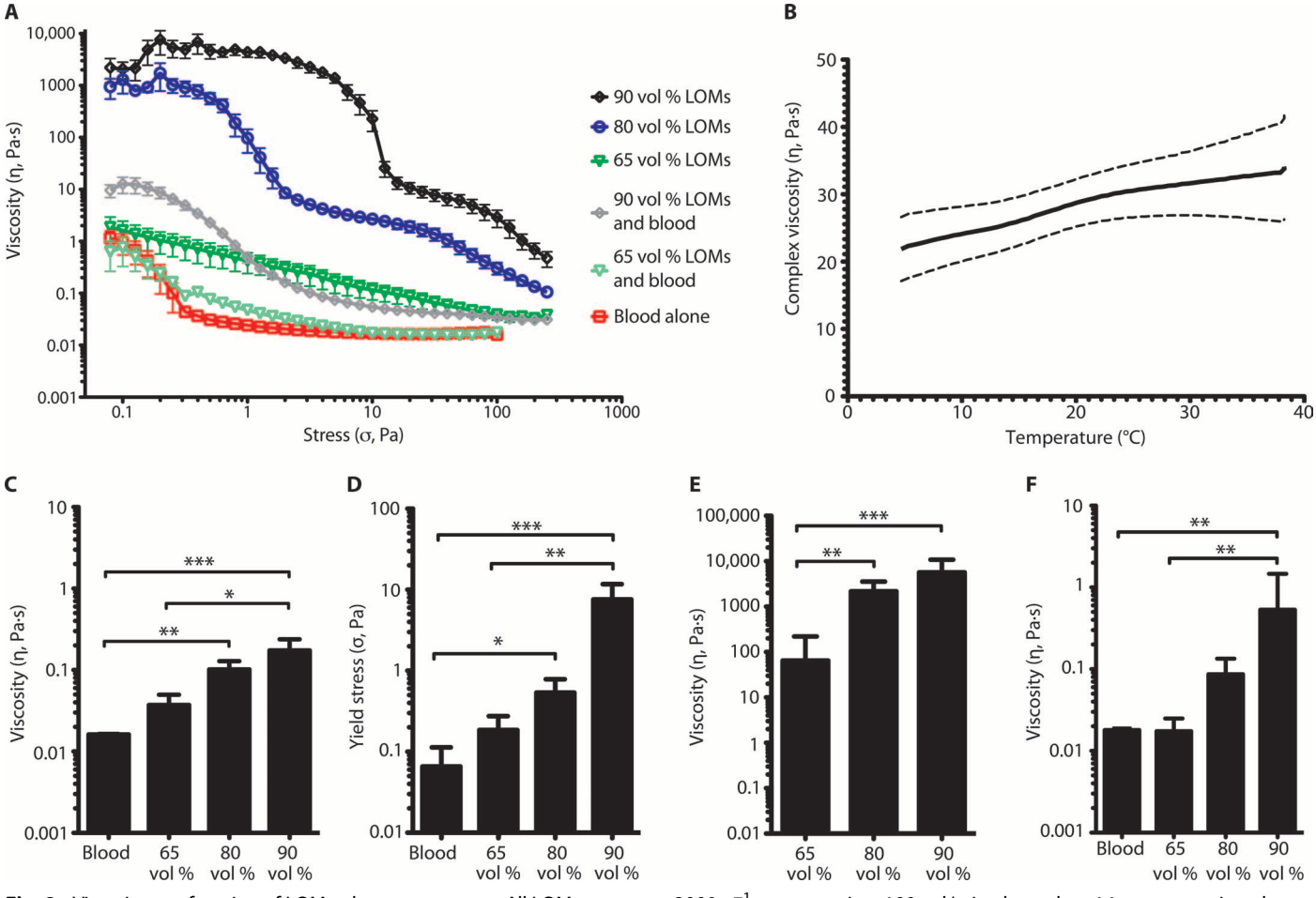

Fig. 2. Viscosity as a function of LOM volume percentage. All LOM suspensions were composed of DSPC and BRIJ 100. (**A**) Viscosity of LOM suspensions at various volume % either alone or mixed 1:1 (volume ratio) with blood during a stress sweep. Data are means ± SEM ($n = 8$ per group). (**B**) Complex viscosity of a 90 volume % LOM suspension as a function of temperature. (**C**) Viscosity of LOM suspensions at a shear rate of 2000 s^{-1}, representing 100 ml/min through a 14-gauge angiocatheter. (**D** to **F**) Yield stress (D), zero stress viscosity (E), and infinite stress viscosity (F) as a function of LOM volume %. In (B) to (F), data are means and error bars represent 95% CI ($n = 8$ samples per group). In (C) to (F), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Kruskal-Wallis test with Dunn's multiple comparison post test.

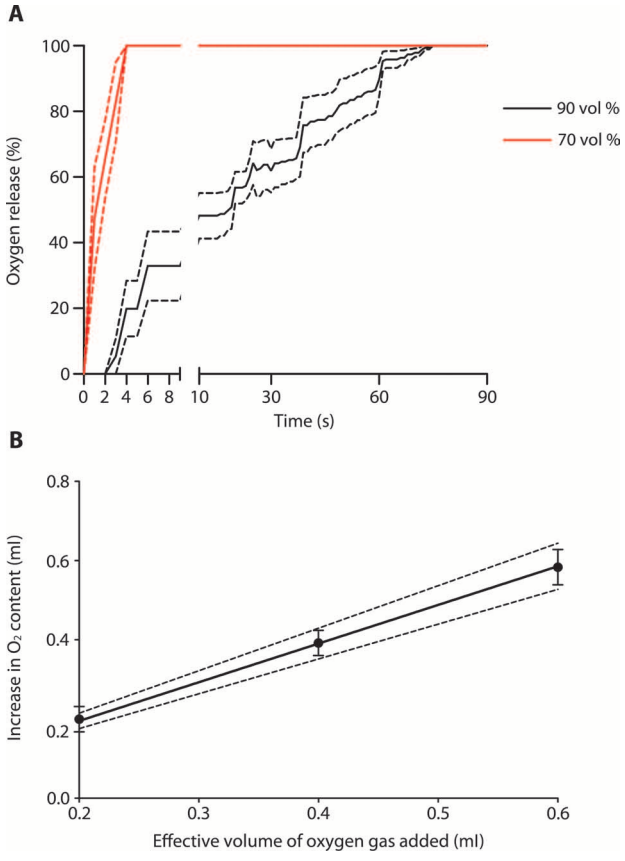

Fig. 3. In vitro characterization of oxygen release from LOMs. (**A**) Oxyhemoglobin concentration over time after addition of 70 or 90 volume % LOMs. Data are means ± SEM (n = 5 per group). (**B**) Oxygen content of blood samples versus the calculated volume of oxygen (contained within LOMs) added to each sample. Data are means ± SEM (n = 5 per group). Solid line, linear regression; dotted line, 95% CI of linear regression.

a stir plate. LOMs composed of DSPC and BRIJ 100 were added to the sample, and the rate of rise in oxyhemoglobin concentration was quantified. Complete transfer of oxygen from LOMs to blood was faster using 70 volume % [3.7 s; 95% confidence interval (CI), 3.1 to 4.7 s] than 90 volume % LOMs (71 s; 95% CI, 68 to 75 s) ($P < 0.0001$, linear regression; Fig. 3A). Owing to their viscosity, 90 volume % LOMs maintained a less fluid form and mixed slowly with blood, accounting for the slow oxygen transfer (movie S1).

To test the extent to which O_2 gas transferred from LOMs to human blood, we quantified the oxygen content of aliquots of venous human blood, and we added varying volumes of 50 volume % (composed of DSPC and BRIJ 100) LOMs to each aliquot. The increase in oxygen content was plotted against the volume of O_2 gas added (within the LOMs). The slope of the linear regression line was 0.9761 (95% CI, 0.8790 to 1.073) (Fig. 3B), indicating that the oxygen content of the blood increased by about 1 ml for each milliliter of O_2 gas (encapsulated by LOMs) that was added to the blood sample.

In vivo LOM administration during hypoxic ventilation

Using a prototype suspension composed of DSPC and BRIJ 100 and diluted to 52.7 ± 2.7 (SD) ml of O_2 gas per deciliter of suspension, we characterized the ability of injected LOMs to rapidly reverse hypoxemia in vivo. Rabbits were ventilated using 11% oxygen, producing arterial saturations of 65 to 70% without hemodynamic instability. After a 2-min period of observation, LOMs were hand-injected into a central vein over the course of 2 min, and the rate titrated to maintain target oxyhemoglobin saturations of about 90%. Oxyhemoglobin saturations rose to and maintained near-normal levels during infusion of the LOMs and then returned to near baseline upon termination of the injection (Fig. 4A). Concurrent with these changes, the fraction of oxygen in exhaled gas transiently rose during infusion (even exceeding inhaled gas fraction in some animals) (Fig. 4B), consistent with an increase in the oxygen content of pulmonary arterial blood. During some rapid injections, intact LOMs were noted in arterial blood. Mean arterial blood pressure increased during induction of hypoxemia and

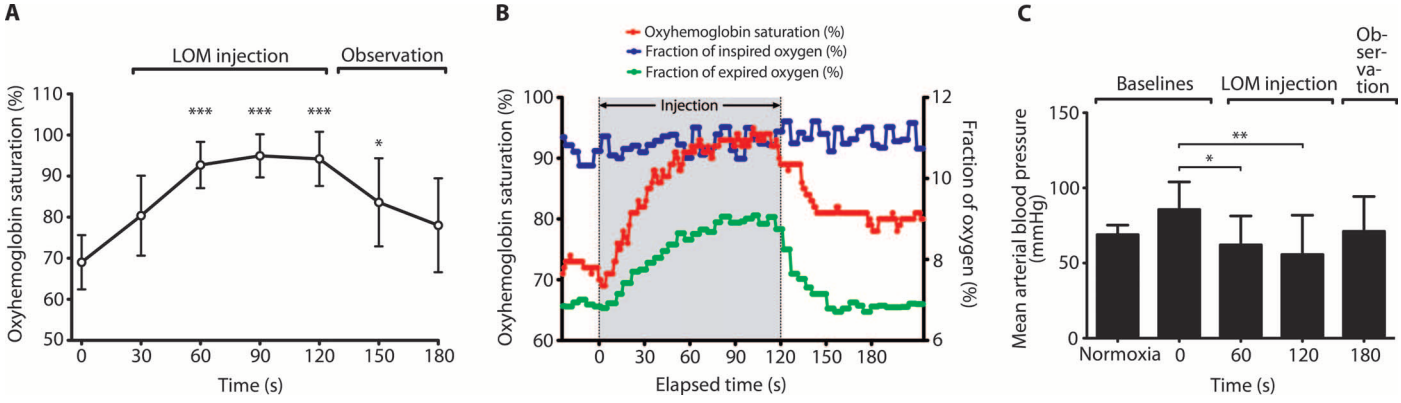

Fig. 4. Oxygenation and hemodynamic responses to hand injections of LOMs during hypoxic ventilation. (**A**) Oxyhemoglobin saturations by cooximetry in response to LOM infusions during hypoxic ventilation. Data are means ± 95% CI (n = 10 injections). *$P < 0.05$, ***$P < 0.001$, two-tailed Mann-Whitney test. (**B**) Oxygen saturation during induced hypoxia in rabbits in response to a single (n = 1) hand injection of LOMs for 120 s (red line). The fraction of oxygen in exhaled gas (representative of alveolar oxygen content during exhalation) is shown as a green line, whereas the fraction of inspired oxygen is in blue. (**C**) Mean arterial blood pressure during baseline, injection, and observation periods. Data are means ± 95% CI (n = 10 injections, n = 5 animals). *$P < 0.05$, **$P < 0.01$ relative to t = 0 s (hypoxic baseline), Friedman test with Dunn's multiple comparison post test.

returned to baseline during injection of LOMs (Fig. 4C). However, we noted transient hypotension during two injections, which resolved with downward titration of the injection rate.

In vivo treatment of prolonged asphyxia

To test the ability of LOMs to sustain full-body oxygenation for a longer time period (>10 min), we anesthetized and instrumented rabbits, including placement of a cuffed tracheal tube. After a period of observation on 21% inspired oxygen, rabbits were asphyxiated by clamp occlusion of the tracheal tube for 15 min. Animals were treated with either LOMs—titrated to an arterial oxygen tension greater than 30 mmHg (sufficient to maintain native circulation) (*22*)—or an isotonic fluid infused at a comparable rate. All animals experiencing a loss of circulation received continuous chest compressions and resuscitative medications but remained asphyxiated for 15 min. Surviving animals were supported for a 90-min observation period before sacrifice for tissue analysis.

Animals treated with LOMs received O_2 gas [3.96 ± 0.39 (SD) ml/kg per minute] via the infusion and were able to maintain the oxygenation goals stated above, despite asphyxia (Fig. 5, A and B). Relative to controls, LOM-treated animals demonstrated decreased metabolic acidosis, normal blood pressure and heart rate, and a lower incidence of cardiac arrest (prolonged survival) (Fig. 5, C to H). Hepatic enzymes aspartate aminotransferase (AST) and alanine transaminase (ALT), which are markers of liver injury, did not increase in LOM-treated animals but increased significantly in control animals within 90 min of asphyxia (Fig. 6A). There was no evidence of lung injury or of pulmonary embolism in LOM-treated animals by markers of oxygen diffusion, dead space ventilation, central venous pressure, gross inspection, or tissue histology (Fig. 6, B to D and F). There was no significant increase in hemolysis index [a spectrophotometric measure of plasma-free hemoglobin (*23*)] in LOM-treated or control animals (Fig. 6E). Oxygen content of the LOMs was 73.3 ± 2.5 (SD) ml per deciliter of suspension, and the surface area–to–volume ratio of the LOM population was $3.83 \times 10^6 \pm 3.59 \times 10^5$ (SD) m^{-1}. Animals in both groups received comparable volumes of fluid during asphyxia (LOM-treated, 21.6 ± 3.9 ml/kg; control, 18.6 ± 3.9 ml/kg; $P = 0.222$, Mann-Whitney). LOM-treated animals received lipid (about 0.2 g/kg).

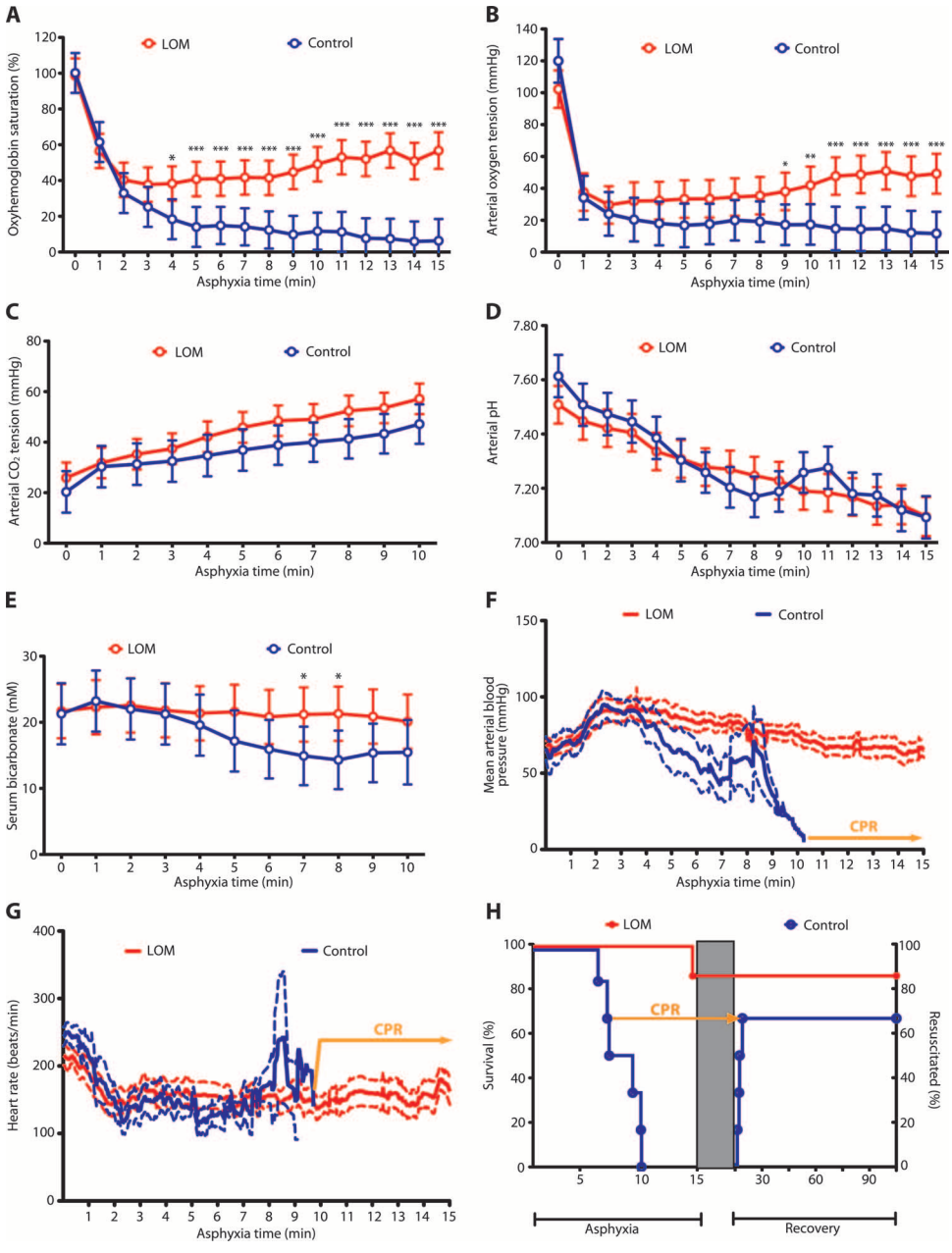

Fig. 5. In vivo effects of LOM infusion during asphyxia. (**A** to **G**) Various markers of LOM-treated ($n = 7$) and control ($n = 6$) rabbits were tested during 15 min of asphyxia, including oxyhemoglobin saturation (A), arterial O_2 tension (B), arterial CO_2 tension (C), arterial pH (D), serum bicarbonate levels (E), arterial blood pressure (F), and heart rate (G). In (A) to (E), data are means ± 95% CI. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (see Materials and Methods). In (F) and (G), data are means ± SEM. The blue lines end at 10.2 min because no animals treated as controls had spontaneous circulation after that time and received chest compression–only cardiopulmonary resuscitation (CPR) during the remainder of asphyxia. (**H**) Kaplan-Meier plot of animals experiencing cardiac arrest during asphyxia (left; $P = 0.0002$, log-rank test), restoration of mechanical ventilation (shaded box), and subsequent recovery and observation (right).

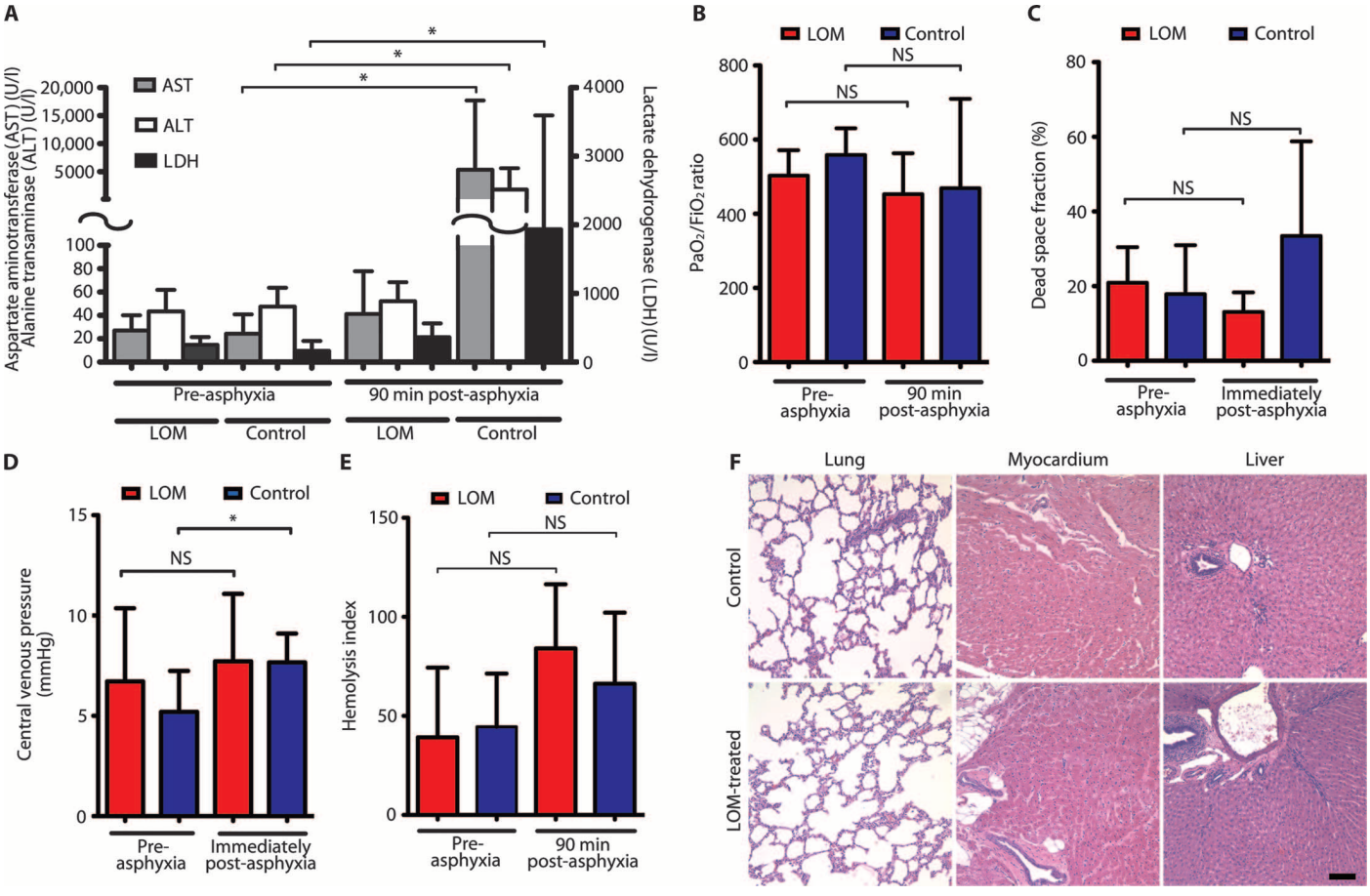

Fig. 6. Indices of injury after asphyxia. (**A**) Liver enzyme concentrations in animals before (n = 7 LOM-treated, n = 6 controls) and 90 min after (n = 5 surviving LOM-treated, n = 4 surviving controls) asphyxia. (**B**) Ratio of arterial oxygen tension (PaO_2) to fraction of inspired oxygen (FiO_2) before and 90 min after the end of asphyxia in LOM-treated (n = 6) and control (n = 4) animals. (**C**) Dead space fraction in LOM-treated (n = 6) and control (n = 4) animals before and immediately after asphyxia. (**D**) Central venous pressure in LOM-treated (n = 6) and control (n = 4) animals before and immediately after asphyxia. (**E**) Hemolysis index in both LOM-treated (n = 5) and control (n = 4) groups. For (A) to (E), data are means with error bars that represent 95% CIs. *P < 0.05, two-tailed Mann-Whitney test. NS, not significant. (**F**) Hematoxylin and eosin staining of representative lung, myocardium, and liver of animals undergoing a 15-min asphyxia period followed by a 90-min observation period. Scale bar, 400 μm.

DISCUSSION

Hypoxemia remains an important cause of morbidity and mortality among critically ill patients and can be acutely life-threatening. We have created a complex fluid that stores O_2 gas within concentrated microparticles and can be used to administer oxygen intravenously to the bloodstream. This approach may provide rescue oxygenation to patients with acute airway or lung failure, affording time for definitive intervention and avoiding cardiac arrest.

Here, we demonstrated that the size of LOMs can be controlled, such that a very small proportion exceed 10 μm in diameter, and that in some formulations this fraction did not increase over time, as can occur in lipid-water emulsions by Ostwald ripening (*24*). LOMs with the most stable shelf-lives exhibited 20% loss in 2 weeks. Shelf-life may be improved by increasing lipid acyl chain length [to create higher shell resistance (*19*, *20*)], by adding stabilizing agents, or possibly by freeze-drying the suspension. We were able to manufacture LOMs containing up to 90 volume % O_2 gas. A suspension of this concentration would be ideal to support oxygenation in that it would minimize the fluid phase administered; however, these suspensions are exceedingly viscous and do not mix well with blood (but do so at 60 to 70 volume %). Although we lowered viscosity to 50 to 75 volume % by dilution, mechanical preshearing or other viscosity-lowering agents, such as mannitol, were not investigated. Notably, the apparent viscosity of infused LOMs decreases at high shear conditions within the catheter, in addition to a dilution effect when mixed with blood.

When mixed with blood ex vivo, we found that oxygen transferred from the LOMs to deoxyhemoglobin within seconds and that virtually no O_2 gas was retained within LOMs in the presence of deoxyhemoglobin. When surrounding blood becomes fully saturated with oxygen, it is possible that LOMs persist intact in circulation until reaching deoxyhemoglobin, as evidenced by the presence of intact LOMs in the arterial circulation during rapid intravenous injections. During hypoxic ventilation in vivo, we demonstrated that an injection of LOMs reverses hypoxemia within seconds, providing rapid changes in pulse oximetry and measured oxyhemoglobin concentrations. We

found a low incidence of reversible hypotension during these rapid hand infusions of LOMs, which may have been related to unrefined suspensions (suspension impurities), lipid toxicity, embolism of LOMs in the pulmonary arteries—although we did not note contemporaneous alterations in heart rate or central venous pressure—or myocardial stretch. Although hypoxic ventilation as a cause of hypoxemia does not occur clinically, the increase in exhaled oxygen content represents a process of "back diffusion," in which oxygen diffuses from oxygenated pulmonary arterial blood to the hypoxic alveolus. Clinically, this effect would be minimal in patients with hypoxia due to lung injury or airway obstruction, and can be mitigated by administration of 100% oxygen to the tracheal tube.

The asphyxia study was intended to emulate a possible clinical use of the formulation. For example, LOMs could be infused by paramedics, clinicians in the emergency department and intensive care unit, or anesthesiologists to stabilize a hypoxic patient during a difficult intubation attempt. We found that LOMs provided O_2 gas (about 4 ml/kg per minute) for 15 min, supplied the animals' full oxygen consumption, and decreased the incidence of cardiac arrest and organ injury from asphyxia. LOM infusions were hemodynamically well tolerated in this model, possibly owing to the lower, more controlled infusion rate. We did not note signs of pulmonary embolism, including an increase in dead space ventilation, tachycardia, hypotension, or free gas upon necropsy.

Several aspects of the LOMs make them suitable for emergency gas delivery. First, they are portable and injectable into a vein without preparation or advanced medical training, making them ideal for broad use in emergency situations. Gas transfers efficiently owing to the high surface area/volume ratio and fluid-like properties, which are important for mixing in the venous bloodstream. They are size-limited to allow safe transcapillary passage (*21*) of intact LOMs. By preventing a direct interface between blood and O_2 gas, the LOMs avoid hemolysis (*25*), as evidenced by a low hemolysis index after the asphyxia experiment. LOMs are efficient one-way carriers of oxygen, so that diffusion of other gases into the particle is disfavored by the Laplace overpressure imposed by the lipid shell, which imposes low resistance to gas egress (*20*), and the presence of deoxyhemoglobin, which creates an oxygen sink. Finally, they can be manufactured using excipients that are safe during rapid injection and categorized as "generally recognized as safe (GRAS)" by the U.S. Food and Drug Administration. Some of these excipients are described in table S1.

There are several limitations to this technique that may dictate their clinical use. First, a volume of fluid must be administered along with the gas phase in the LOM suspension. Although the volume of gas infused rapidly becomes bound to hemoglobin and decreases 800-fold in volume, the fluid phase remains in circulation. In the asphyxia study performed here, the volume of fluid administered to support oxygenation for 15 min would be clinically acceptable in most patients. Nevertheless, the quantity of lipid may be further minimized by the use of alternative substances, such as dextran (*26*), to manufacture the microparticles. This is likely to limit the total volume of O_2 gas that can be infused using LOMs, although measures can be taken to increase the volume % of gas in the injectate. We found viscosity to be limiting in this regard and believe that it will be important to improve the rheologic profile of concentrated LOMs. Second, because LOMs do not recirculate in the body as do blood substitutes, they must be continuously infused to provide oxygen. (However, this same property makes them uniquely suited to reverse hypoxemia when oxygen diffusion in the lungs is impaired, which blood substitutes cannot.) The high volume of oxygen consumption coupled with the large infusion volumes required to meet them may limit the clinical application to short-term therapies and diagnostic studies. Short-term infusions would nevertheless fill an important therapeutic gap for critically ill patients. Third, LOMs do not remove carbon dioxide. The rate of rise of carbon dioxide in the absence of ventilation is about 3 to 4 mmHg per minute (*27*), affording a window of time for intervention before acidosis becomes life-threatening. Hypercarbia can be temporarily addressed using buffering solutions. Finally, because our follow-up studies were of limited duration, we have not drawn conclusions regarding the long-term risks of LOMs, including blood and tissue damage, metabolic fate of LOM excipients, and free radical injury. Considering the short half-life of a LOM infusion, titration of the infusion rate to low-normal oxygen tensions may minimize hyperoxic injury.

Clinical applications abound for LOMs, which package O_2 gas at 50 to 90 volume %. Because suspensions may be injected intravenously to deliver critical volumes of oxygen during asphyxia, these particles could decrease the likelihood of hypoxemic cardiac arrest. An injection of LOMs may be used to treat patients in cardiac arrest, raising the circulating oxygen content and improving tissue oxygen delivery. Other applications include infusing LOMs during profound hemorrhagic shock, which may raise circulating oxygen content (even in cases of severe lung injury) and prolong the "golden hour" of trauma, or infusing LOMs briefly to reverse severe carbon monoxide poisoning. The ability to raise venous saturations may also stabilize infants with severely cyanotic congenital heart disease. A noninvasive application would be topically applying the oxygenated particles to improve wound healing. The inclusion of other gases into the particles may broaden the clinical use of this technique further.

MATERIALS AND METHODS

LOM suspension preparation and characterization

DSPC, DSPE-PEG2000, and DPPE-PEG5000 were obtained from NOF Corporation. PEG40S, BRIJ 100, and 0.9% sodium chloride solution were obtained from Sigma. Poloxamer 188 was obtained from Spectrum Chemicals & Laboratory Products. The different combinations of these excipients are shown in table S1. Base lipids (10 mg/ml) and surfactants were hydrated in 0.9% sodium chloride. LOMs were manufactured with a customized apparatus that included sonication, size isolation by flotation, and concentration by centrifugation (fig. S2).

Particle size distribution

Particle size distribution was determined by light obscuration (Accusizer 780A, Particle Sizing Systems). LOM suspensions (10 μl) were directed into a jacketed 30-ml flask under low convective motion. To image particles, we performed differential interference contrast and phase microscopy using a Zeiss Axiovert 200M inverted microscope illuminated with a halogen light source.

Freeze-fracture transmission electron microscopy

Samples were mounted on copper planchets for double replica technique and frozen with slushy nitrogen for rapid freezing. The fracturing process was carried out in a Balzers BAF 400T at −150°C

and vacuum of 6×10^{-7} mbar. Samples were etched at −90°C for 90 s. The exposed fracture planes were shadowed with Pt for 30 s at a 45° angle and carbon for 45 s at 2 kV and 90 mA. The replicas were cleaned with various concentrations of NaClO and rinsed in deionized water, and then imaged with a JEOL 2100 transmission electron microscope.

Quantification of particle behavior under storage conditions

Concentrated lipid suspensions were collected into sealed 13-ml graduated conical tubes. For each combination in table S1, five tubes were filled and sealed for storage at 4°C. Loss of particle suspension was assessed by evaluating the height of remaining suspension within each tube. The percentage of original particles remaining at each time point was calculated as follows: (volume remaining$_{\text{day } x}$)/(starting volume$_{\text{day } 1}$).

Rheometry measurements

Aliquots (2 ml) of donated human packed red blood cells and LOM suspensions at 65, 80, and 90 volume % (n = 8 per group) were studied with a 40-mm parallel plate geometry (AR 2000ex, TA Instruments). The steady-state flow viscosity was measured as stress increased from 0.1 to 10,000 μN·m. Temperature was maintained at 25°C by a Peltier plate. No conditioning step was used. We also performed a temperature ramp (4 to 38°C) of 90 volume % LOMs using constant oscillatory flow (1 Hz and 1% strain).

In vitro study of oxygen transfer kinetics

Donated human blood (50 ml, hemoglobin 20 g/dl, pH 7.40) was desaturated with nitrogen gas and maintained at 37°C on a heated plate. Under continuous stirring, LOMs of either 70 or 90 volume % were added to the blood. Oxyhemoglobin concentration was monitored continuously (COBE Spectrum oximeter), and the rate of change was compared between concentrations by linear regression.

In vitro dose-response curve

Aliquots (10 ml) of human red blood cells were distributed into 15-ml glass test tubes. Baseline hemoglobin, oxyhemoglobin saturation by co-oximetry, and oxygen tension were quantified (Radiometer ABL 80 Co-Ox Flex, Radiometer America) for calculation of oxygen content. Varying volumes of 90 volume % LOMs were added to each test tube, which were inverted, and oxygen content was measured again. Change in oxygen content was calculated for each test tube and compared between doses by linear regression. This experiment was conducted at room temperature.

In vivo testing of LOMs during hypoxic ventilation

The following protocols were approved by the Institutional Animal Care and Use Committee at Children's Hospital Boston. Female New Zealand white rabbits (4 to 5 kg) were acclimatized with free access to food and water for 3 days before the procedure. Animals (n = 5; weight range, 4.1 to 4.8 kg) were sedated with a fentanyl infusion (50 μg/kg per hour) and intermittent pancuronium (0.1 mg/kg, intravenously). Instrumentation included tracheal intubation, femoral artery, and vein cannulations. Animals were ventilated with pressure control ventilation, peak inspiratory pressure of 25-cm H_2O, positive end-expiratory pressure of 5-cm H_2O, respiratory rate of 10 breaths per minute, and an inspired oxygen fraction (FiO_2) of 21% (SERVO-i, Maquet Inc.). Pulse oximetry, heart rate, and arterial blood pressure were continuously monitored (SurgiVet veterinary monitor, Smiths Medical). Analysis of inhaled and exhaled gases was performed via sidestream analysis (Datex-Ohmeda, GE Healthcare). After a 15-min period of observation, hypoxia was induced using ventilation with 11% oxygen via the ventilator.

Once a stable level of hypoxia was reached (saturations, ~65%), about 80 ml of 50 volume % LOMs was hand-injected over 120 s into the femoral vein. The injection rate was adjusted to achieve pulse oximeter saturations of >90%. After this period, the injection was discontinued, and animals were monitored for an additional 2 min. Arterial blood gases were drawn every 30 s and analyzed by co-oximetry. Animals were then recovered on 40% FiO_2, and the experiment was repeated once per animal.

In vivo testing during prolonged asphyxia

New Zealand white rabbits (n = 13; weight range, 4.1 to 4.7 kg) were sedated, paralyzed, intubated (with a cuffed tracheal tube), ventilated, and monitored as described above. For each LOM-treated animal, six 140-ml syringes were prepared as described above and infused in parallel with a syringe pump (NE 1600, New Era Pump Systems). Animals were asphyxiated by placement of an occlusive clamp on the endotracheal tube, and the ventilator was disconnected. Care was taken to ensure that the cuff of the endotracheal tube was inflated and that animals were not making spontaneous breathing efforts during the asphyxia period.

Animals were treated with infusions of either LOMs or control solutions. Treatment group allocation was determined by convenience (availability of lab staff to manufacture the LOMs). Infusions were initiated concurrent with onset of asphyxia. Infusion rate in the LOM group was initiated at 4.5 ml/kg per minute and titrated in 10% increments to maintain an arterial oxygen tension (PaO_2) of at least 30 mmHg, measured continuously by indwelling fiber-optic probe (Oxford Optronix). Control animals received infusions of Plasma-Lyte A at a rate equivalent to the aqueous phase, or 1.3 ml/kg per minute, until cardiac arrest ensued, at which point infusions were stopped. We treated animals in the control group with proportionate infusion of fluid phase administered to LOM-treated animals to account for any resuscitative effect that volume administration may have. We used Plasma-Lyte A without the lipid constituents in the control group in case rapid infusion of the lipid suspension had detrimental effects. Animals experiencing cardiac arrest [defined as mean arterial blood pressure (MABP) of <10 mmHg (*28*)] received continuous chest compressions and advanced life support medications, as per American Heart Association guidelines (*29*).

At the end of the asphyxial period, mechanical ventilation was restored with 100% oxygen until return of pulsations (in animals receiving chest compressions) and then titrated downward to achieve arterial saturations of >92%. Animals achieving return of spontaneous circulation after relief of asphyxia were treated with standard intensive care management, including inotropic support (dopamine, 2 to 10 μg/kg per minute, intravenous infusion) to maintain MABP of at least 40 mmHg during the follow-up period. Hyperthermia was avoided by passive ambient cooling (goal, 34 to 35°C). Animals were sacrificed 90 min after the end of asphyxia for lab and histology sampling.

Measurements included time from onset of asphyxia to onset of cardiac arrest and arterial blood gas sampling including co-oximetry drawn after each minute of asphyxia; hemodynamics (heart rate, MABP, and central venous pressure) measured continuously; and

PaO_2/FiO_2 ratio, hepatic transaminases, and hemolysis index (Roche Cobas E501, Roche Diagnostics) before and 90 min after asphyxia. Dead space fraction was calculated as V_d/V_t = ($PaCO_2$ − end tidal CO_2)/$PaCO_2$ before and immediately after asphyxia; $PaCO_2$ is arterial carbon dioxide tension. Formalin-fixed tissue from all animals was paraffin-embedded and stained with hematoxylin and eosin for review by a pathologist (M.W.L.) blinded to treatment group.

Statistical analysis and modeling

In fig. S1, we used generalized estimating equations to account for repeated measures within each experimental replicate, which are a class of statistical methods that adjust for the structure of temporally correlated data to yield proper estimates and SEs of regression parameters (*30*). In Fig. 5, A to E, the relationship between outcome and duration of asphyxia was modeled with linear mixed-effects models with indicator variables at each time point and random intercepts for each animal to account for repeated measurements within animals. Overall differences between treatment groups over time were assessed via interaction terms, with differences at specific time points indicated on the graphs. In Fig. 5F, the relationship between MABP and asphyxia time was modeled with a "broken stick" linear mixed-effects model with random intercepts and slopes for each animal. The model allowed for a change in intercept and slope at 195 s, the time point that minimized the Akaike information criterion.

SUPPLEMENTARY MATERIALS

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Fig. S1. LOM stability and growth at various storage temperatures.
Fig. S2. Apparatus used for LOM manufacture.
Table S1. Combinations of lipid excipients used in LOM manufacture.
Movie S1. Oxygen transfer kinetics from LOMs to blood.

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M.A.B., and E.J.S. designed the manufacturing apparatus. J.N.K., L.A.S., J.H.R., and K.J.B. manufactured suspensions and completed in vivo experiments. B.K.W. and K.E.M. assisted with in vivo experiments. D.A.G. performed statistical analyses and interpreted data. D.C.B. performed TEM imaging. M.W.L. completed histologic analyses. M.A.B. and A.L. provided insights into lipid structure and compositions. C.B. oversaw hemolysis data. F.X.M. provided mentorship, experimental guidance, and analysis and edited the manuscript. **Competing interests:** The institutions of J.N.K., M.A.B., E.J.S., L.M.T., and F.X.M. have filed patents on the composition, manufacturing process, and clinical uses of LOMs described in this manuscript. **Data and materials availability:** Correspondence and requests for materials should be addressed to J.N.K.

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