LIPOSOMAL FORMULATIONS OF ALKYL NITRITES AND THEIR EFFICACY IN NITROSYLATION OF BLOOD

by

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LIPOSOMAL FORMULATIONS OF ALKYL NITRITES AND THEIR EFFICACY IN NITROSYLATION OF BLOOD

Abstract

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Blood nitrosylation therapy is a topic of recent discussion in the cardiovascular field. Its main concern is with the restoration of S-Nitrosothiols—an important signaling molecule governing oxygen delivery—to hemoglobin. Currently, there exists no simple continuous delivery system that can deliver nitrosylating agents to blood. This study examines the effectiveness of liposomes for delivering nitrosylating agents. Experiments were carried out to test the efficacy of liposomal formulations of two nitrosylating agents: ethyl nitrite and amyl nitrite. We examined the loading characteristics of the liposomes and by treating purified hemoglobin, red blood cells, and whole blood with liposomal formulations, investigated the controlling mechanism for delivery of ethyl and amyl nitrite. Our results show that a dose-dependent loading of the nitrosylating agents can be achieved in the liposomes. Further, while, in general, the liposomal formulations of these drugs were effective in increasing SNO levels of hemoglobin, the blood plasma plays a critical role in controlling the delivery. Overall, these results suggest that the key benefits of liposomes such as long circulating times and in vivo capabilities can be combined to form a powerful nitrosylating agent delivery system for clinical applications.
INTRODUCTION

Nitrosylation is a naturally-occurring process in the body that controls oxygen delivery and matches tissue perfusion with metabolic demand. The main signaling molecules involved in this process are S-nitrosothiols, which are generated when nitric oxide synthases (NOS) catalyze the production of nitric oxide (NO), which then reacts with cysteine thiol groups in hemoglobin (Hb). Cysteine thiols are unique because they contain an –SH group which readily reacts with NO to form –SNOs. Numerous reactions are known to occur on cysteine thiol side chains that affect protein structure and function. S-nitrosylated hemoglobin can deliver NO to tissues, leading to vasodilation and thereby allowing greater blood flow and oxygen delivery.

A lack of SNO in hemoglobin causes hypoxia, or reduced oxygen levels. Decreased SNO also reduces red blood cell deformability.

A recent development on the topic of nitrosylation is re-nitrosylation, which involves restoring SNO levels in stored blood and blood in organs or tissues that exhibits a decreased SNO level. Current methods of blood transfusion, while providing the chance of correcting diseased or life-threatening conditions, often exacerbate the problem with increased morbidity and mortality associated with stored blood. Decreased SNO levels cause hypoxia in tissue after blood transfusion and also result in the inability of red blood cells (RBCs) to effect hypoxic vasodilation. The decreased SNO levels in stored blood also decreases the RBC’s hypoxic vasodilatory capacity. Current studies in mice supplied with stored blood have shown decreased skeletal pO2 which results from a decreased SNO level. Restoring SNO-Hb levels can correct these problems in blood transfusions and organ transplantation. Additional benefits of re-nitrosylation include alleviation of inflammatory conditions to reduce tissue injury.
Alkyl nitrites (R-O-N+O) are alkyl esters of nitrous acid (H-O-N=O) and can be powerful nitrosylating agents. Ethyl nitrite (ENO), especially, has been shown to correct impairments in tissue perfusion and restore SNO-Hb levels\(^5\). Amyl nitrite (ANO) has been shown as an effective treatment for angina as early as 1867. While hemoglobin is the preferred target of these nitrites during therapy, SNO-Hb can exchange NO with other proteins and peptides (e.g. glutathione) as well.

Currently, there is no continuous controlled delivery system for alkyl nitrites. A major problem with most alkyl nitrites is that they are not very soluble in aqueous solutions nor are the stable in the presence of water. The purpose of this study is to examine the efficacy of liposomes as a drug delivery vehicle for alkyl nitrites. Liposomes are nanoparticles that allow for variable loading of drugs. Drugs previously abandoned due to their insolubility in water can be used in liposomes due to the hydrophobic outer region that can encapsulate hydrophobic molecules\(^6\). PEGylation of these liposomes has been shown to enhance circulation time as well as reduce non-specific tissue interaction\(^3\).

Our results show that different levels of alkyl nitrites can be incorporated into liposomes. When purified hemoglobin, red blood cells, and whole blood were treated with the liposomes, they show significantly increased SNO levels compared to controls. We conclude that liposomal alkyl nitrite formulations can be a valuable NO delivery system for preclinical applications.
BACKGROUND AND SIGNIFICANCE

Liposomes

Liposomes are nanoparticles (lipid particles in the nanometer size range that can be used to deliver drugs) that are used extensively as drug carriers. They consist of lipids; usually Dipalmitoylphosphatidylcholine (DPPC), cholesterol, and more recently polyethylene glycol (PEG). Figure 1 shows a diagram of a PEGylated liposome. Liposomes were discovered as early as 1965 as possible nanoparticles capable of storing various chemicals and starting in the 1970s they were explored as possible drug carriers\textsuperscript{23}. The encapsulation of drugs in liposomes has been shown to increase bioavailability/bioactivity of vaccines, glucocorticoids, and cancer drugs\textsuperscript{15}. Along with increasing efficiency, liposome formulations aim to reduce toxicity of drugs. Liposomes loaded with dexamethasone have shown to decrease levels of proinflammatory factors under inflammatory conditions as well as decreased phagocytosis and clearance by macrophages.

![Figure 1. Diagram of a PEGylated liposome\textsuperscript{18}.]
Liposomes, in contrast to micelles, microemulsions and solid-lipid nanoparticles, carry an interior water pool surrounded by a lipid bilayer. This facilitates high solubility of hydrophobic drugs in the bilayer, which normally would be taken up by the liver if not encapsulated in liposomes\textsuperscript{18}. The addition of cholesterol adds liposomal stability and prevents drug leakage\textsuperscript{15}. Ampiphilic drugs or partially hydrophilic/hydrophobic drugs can be encapsulated within the bilayer in liposomes, while hydrophilic drugs can be encapsulated within the aqueous center region.

Liposomes have a variety of uses in delivering drugs. The most common use is probably with chemotherapeutic drugs such as Doxil. Liposomes were first used in cancer treatment applications in the form of daunorubicin. The use of liposomes in chemotherapeutic applications offers several advantages. It is possible to control rates of drug release by altering lipid composition, stability, etc.\textsuperscript{18} It is also possible to target specific tissues by altering liposome composition\textsuperscript{24}. Liposomes also can bypass the liver and spleen, which take up most free drugs and severely cut down their bioavailability. They also alter the tissue distribution and rate of clearance of drugs. Liposomes allow for a greater amount of drug to remain in circulation, and for chemotherapeutic applications for example, in the tumor site. In applications of doxorubicin, administration of free DOX and Liposomal DOX (L-DOX) showed that 11% free DOX was present in urine, while 2.5% of L-DOX was present\textsuperscript{18}. This shows an increased bioavailability of liposomal formulations.

A major advantage of liposomes are their ability to reduce toxicity of drugs. Doxorubicin, for example, poses many adverse toxic effects such as myelosuppression, cardiac toxicity, along with nausea and vomiting. Liposomes can mediate these effects due to their
inability to cross certain cell barriers such as the endothelial lining in the heart\textsuperscript{32}. This lessens cardiac toxicity. This is significant in alkyl nitrite therapy because injection of free nitrite poses toxic effects such as nausea, vomiting, methemoglobinemia, and uncontrolled vasodilation\textsuperscript{32}, and the encapsulation of these nitrites in liposomes may lessen/eliminate these effects due to possible reduced toxicity. In L-DOX administration, the LD\textsubscript{50} decreased by at least half even in so called ‘leaky’ liposomes. In stabilized liposomes (those with cholesterol/PEG), there was almost a 6-fold decrease in LD\textsubscript{50}.

Traditional liposomes have been successful at delivering various drugs, but they possess the disadvantage of being rapidly removed from the blood by the mononuclear phagocyte system because they are recognized as ‘foreign’\textsuperscript{22}. The use of poly (ethylene glycol) (PEG) has been reported to be successful in drug delivery systems in general, so it has been considered for use in liposomal drug delivery. By tethering PEG chains to the surface of liposomes (PEGylation), circulation half-life was increased by reducing the uptake by the mononuclear phagocyte system\textsuperscript{15}.

<table>
<thead>
<tr>
<th>Liposome</th>
<th>Clearance rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEGylated liposomes</td>
<td>8.4 h</td>
</tr>
<tr>
<td>Pure phosphatidylcholine liposomes</td>
<td>0.47 h</td>
</tr>
</tbody>
</table>

\textbf{Figure 2.} Liposome clearance from blood as a function of the time after IV administration of DSPC and PEGylated liposomes. Adapted from [22].
PEGylation forms a hydrated barrier around the liposome which shields the nanoparticles from macrophage uptake. It has been shown that a dense polymeric ‘cloud’ is formed around the liposome, even at relatively low polymer concentrations. This is due to the multiple conformations PEG can take, due to its flexibility\(^\text{25}\). The covalent attachment of PEG with liposomes has also been explored in the delivery of insulin, which led to a low plasma glucose level in rats while enhancing oral delivery. The use of PEG and liposomes is essential in the delivery of alkyl nitrites, as in this study, since liquid nitrites are normally insoluble in water. PEGylation further increases circulation time and allows for more controlled and sustained release. The use of PEG makes liposome uptake by the body relatively insensitive to size and the clearance kinetics become dose-dependent. PEG mainly functions by excluding other molecules from the space immediately adjacent to the liposome surface\(^\text{18}\). Binding of blood plasma opsonins and recognition by macrophages is hindered due to steric hindrance and blocking of hydrophobic binding sites on the liposomes\(^\text{22}\).

The addition of PEG allows the liposomes to leak drug slowly, which contributes to the toxicity reduction with the use of liposomes. In PEGylated liposomes, the hydrophobic drug drug within the liposome core leaks in a similar manner to a slow infusion of drug. The slow leakage allows for a higher bioavailability; L-DOX has been shown to remain in tumor sites 24 hours after administration. Tests (Figure 2) have shown that liposomes containing PEG exhibit a clearance of only 15% of liposomes not containing PEG\(^\text{22}\).

Liposomes can also be used in targeted therapy by binding to antibodies or by injecting directly into the site of treatment. They also offer the advantage of lower dosing. Since more
of the drug is available to circulate, fewer administrations are required, which may also
decrease toxic effects or resistance acquired by repeated dosings. While the fabrication of
liposomes loaded with hydrophobic drugs is relatively simple (drugs loaded in lipid bilayer), the
use of highly hydrophilic drugs is a challenge. These drugs exhibit resistance to release from
liposomes. Some solutions may be using cleavable PEG that responds to pH changes and allows
for release from liposomes. This would affect the slow-release profile of the liposomes, which
still remains the best form of use.

S-Nitrosylation

Nitric Oxide (NO) is a naturally generated molecule in the body that functions in signal
transduction. It is generated by endothelial cells and causes relaxation of vascular smooth
muscle. The discovery of NO Synthases (NOSs) indicates various functions of NO\textsuperscript{14}. It is
recognized that NO-based protein modification is also effected by S-nitrosylation, which forms
the motivation of this study. S-Nitrosylation involves the coupling of an NO moiety to a reactive
cysteine thiol, which forms S-nitrosothiols. In the body, S-nitrosylation affects thousands of
different substrates, most of them proteins. It has also been found recently that S-nitrosylation
has direct correlation to the regulation of numerous signaling pathway in intact cellular
systems, and recent genetic evidence supports a diversity of regulatory roles for this protein-
modification reaction\textsuperscript{14}. There is also growing awareness that S-nitrosylation is a post-
translational protein modification process that is regulated precisely in time and space. It is a
main method by which the cardiovascular system is regulated through the regulation of protein
function\textsuperscript{8}. S-Nitrosylation can also promote or inhibit the formation of disulphide linkages
within or between proteins depending on thiol proximity and orientation.
In general, the NO moiety can be provided by NO itself, nitrite, or other NO\textsubscript{x} species (Figure 3). There are several transition metals that can promote S-nitrosylation in the body such as Cu and Zn, which catalyze the S-nitrosylation of hemoglobin by NO in solution. A principal source of NO in SNOs in mammalian cells is the heme iron-nitrosyl species (FeNO) that is formed initially from NO, which originates from NOS, low-mass SNOs or nitrite\textsuperscript{14}. S-Nitroso-glutathione (GSNO) is the main non-protein SNO in cells, which is thought to be in equilibrium with protein SNOs. GSNO can participate in protein S-nitrosylation through thiol-to-thiol transnitrosylation and can also function as a source of free-radical NO generated by hemolytic cleavage\textsuperscript{14}. 

S-Nitrosylation has been shown to convey or regulate physiological cellular signals. This includes signals that are transmitted along transduction cascades triggered by ligand-receptor interactions and the post-translational regulation that is exerted at several stages along signal-transduction pathways. It also functions uniquely to generate and deliver NO-derived bioactivity. For example, S-nitrosylation functions in the vasodilatory activity of erythrocytic hemoglobin14. S-Nitrosylation also affects the Hypoxia-inducible factor (HIFα) which has an important role in regulating cellular O2 homeostasis14. It has also been shown that the dysregulated S-nitrosylation of proteins in neurological, cardiovascular, and pulmonary systems

<table>
<thead>
<tr>
<th>Pathophysiology</th>
<th>SNO-protein</th>
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<tbody>
<tr>
<td>Alzheimer disease</td>
<td>Dynamin–related protein 1</td>
</tr>
<tr>
<td></td>
<td>Cyclin-dependent kinase 5</td>
</tr>
<tr>
<td></td>
<td>Protein disulfide isomerase</td>
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<tr>
<td></td>
<td>X-linked inhibitor of apoptosis</td>
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<td></td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>Stroke</td>
<td>Matrix metalloproteinase (MMP) 9</td>
</tr>
<tr>
<td>Heart failure</td>
<td>Ryanodine receptor 2</td>
</tr>
<tr>
<td>Pulmonary arterial hypertension</td>
<td>HIF1α</td>
</tr>
<tr>
<td>Diabetes (type 1)</td>
<td>Hemoglobin, Glukkokinase</td>
</tr>
<tr>
<td>Diabetes (type 2)</td>
<td>Insulin receptor β</td>
</tr>
<tr>
<td></td>
<td>Insulin receptor substrate 1</td>
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<tr>
<td>Blood transfusion: storage defect</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>Sickle Cell Anemia</td>
<td>Hemoglobin</td>
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Figure 4. Examples of dysregulated S-nitrosylation of proteins. Adapted from [8].

(Figure 4) are associated with pathophysiology.

The thiol Cys93 in the β-subunit of Hb is where S-nitrosylation occurs in blood. Oxygenation and deoxygenation of Hb promotes the S-nitrosylation and denitrosylation of β-Cys93. This allows red blood cells to deliver vasodilatory NO bioactivity along with O2 in accordance with local metabolic demand in the arterial periphery (known as hypoxic vasodilation). Systemic oxygen delivery is largely determined by microcirculatory blood flow and, to a lesser extent, by blood O2 content, which is a function of Hb O2 saturation and blood Hb concentration. Red blood cells have been investigated as a possible carrier for SNOs because of the access they provide to the microcirculation in the body that is dysfunctional in many cardiovascular diseases. Recent and accumulating evidence suggests that aberrant trans-nitrosylation by SNO-Hb exists in numerous pathologies. Conditions that disfavor the allosteric transition in Hb from the T to R state such as sickle cell anemia, or that promote deoxygenation of Hb (hypoxemia, acidosis) will be associated with reduced synthesis of SNO-Hb. This also relates to the impairment of hypoxic vasodilation exhibited by banked blood. Banked blood shows a rapid decrease in levels of SNO-Hb. Additionally, RBCs exposed to sustained hypoxia exhibit impaired pO2-coupled vasodilation in the lungs and are deficient in increasing blood oxygenation.

Recently, S-nitrosylation has been shown to have a role in pancreatic β-cell function in forming a protein complex that includes nNOS. Insulin-stimulated NO production through β-
cells results in the S-nitrosylation of glucokinase, which is a critical component of the transduction pathway that regulates insulin secretion in pancreatic β-cells. Through this mechanism, S-nitrosylation activates glucokinase. S-nitrosylation also functions to inhibit and activate many different enzymes and transcription factors such as Caspases, and NF-κB. This affects their function.

Current renitrosylation therapies

The Stamler group has investigated re-nitrosylation of blood through various methods. One method is the re-nitrosylation of stored blood through gaseous formulations of nitrite. This has been shown to reduce complications caused by transfusions of stored blood such as decreased pO₂ and decreased vasodilatory capacity. During this study, mice and anemic sheep were studied with both untreated blood and renitrosylated blood. The groups that received renitrosylated blood exhibited improved muscle pO₂, and improved blood flow to internal organs. The Stamler group also tested S-Nitrosylation therapy through inhalation in treatment of newborn rats. Inhaled nitrite seemed to improve post-natal alveolar development through inhibition of certain inflammatory factors. It is speculated that inhaled nitrite confers these benefits because postnatal alveolar development may be governed by S-Nitrosylation-dependent pathways.

Another method evaluated by the Stamler group is the use of a pediatric oxgenator. This requires blood ex-vivo. For large volume renitrosylation studies, packed RBCs were passed over a semipermeable membrane oxygenator system that contained gaseous ethyl nitrite (ENO) at 50 ppm in nitrogen. These RBCs were transfused into both anesthetized and awake anemic sheep. In hemodynamic studies in anemic sheep, this resulted in a restoration of...
systemic vascular resistance that was decreased by low SNO-Hb, and increased blood flow to critical internal organs such as the liver, kidney, adrenals, and spleen. In studies with awake sheep, transfusion of re-nitrosylated RBCs significantly increased tissue pO$_2$ values from baseline$^2$.

Non-human subjects that exhibit brain death also have been shown to have decreased NO bioactivity with resultant reductions in organ blood flow$^5$. Since the inability of SNO-Hb-depleted blood to facilitate hypoxic vasodilation has been well documented, the Stamler group has conducted experiments with inhalation of nitrite following brain death in swine subjects. The inhalation of nitrite resulted in enhanced tissue oxygenation and blood flow, and reduced markers of injury and inflammation. Inhalation of nitrite also reversed perturbations in blood flow to the kidneys and to other organs caused by brain death$^5$.

In cases of acute respiratory distress syndrome (ARDS), Nitric Oxide gas therapy has been used as an effective short-term oxygenation tool. Nitric Oxide (NO) has beneficial effects in the lungs as its vasodilatory actions are limited to the pulmonary vasculature. Inhaled NO selectively dilates blood vessels in only those lung segments that are actively participating in gas exchange$^{40}$.

The restoration S-nitrosylation capabilities is essential to bodily function. Since S-nitrosylation is a post-translational modification, it causes a conformational change in Hb structure, allowing for a release of NO to hypoxic areas in the body. S-nitrosylation is especially important to alkyl nitrite therapy, which delivers NO to the Cys93 thiol in Hb to form SNOs, similar to how the process occurs in the body, although the exact mechanism is unknown.
Nitrite therapy focuses on restoring the vasodilatory capability of the red blood cells through a restoration of SNO-levels, correcting many hypoxic conditions\textsuperscript{8,14}.

**Rationale**

Current studies involving alkyl nitrite inhalation therapy have limitations. Although inhaled alkyl nitrite has been shown to increase vasodilation and replenish SNO-Hb, it has not been thoroughly tested for effectiveness in the long term. Inhaled formulations of alkyl nitrites do not appear to circulate long in the body; studies have shown that more than 70\% of the inhaled NO appears in the urine within 48 hours of inhalation\textsuperscript{27, 28, 29}.

Inhaled NO-based therapies can also lead to side effects such as the formation of methemoglobin\textsuperscript{21}. It has been shown in a single-infant study that the adverse effects of methemoglobin formation with inhaled nitrite are exacerbated with existing hepatic failure, secondary respiratory arrest, and intractable hypotension, although a lower dose seems to correct this. This shows, however, that toxicity cannot be excluded with reference to inhaled nitrite therapy\textsuperscript{21}.

Inhaled NO has been used as a short-term oxygenation method in patients with ARDS. However, it is wrought with limitations. After the NO is inhaled and passes through the lungs and into the patient’s blood stream, its effects are quickly deactivated due to the formation of methemoglobin, and systemic hemodynamic effects are negated\textsuperscript{40}. While this may prevent a drastic decrease in blood pressure, it also does not help restore other bodily functions governed by SNOs, for example. It has also been shown that not all patients respond the same way to inhaled NO therapy. Some have an immediate response, while others exhibit a limited response\textsuperscript{40}. Additionally, for some patients, the positive response to inhaled NO appears to last
for only hours to days, while others respond positively for weeks\textsuperscript{40}. It has also been shown that doses of NO gas above 40 ppm did not provide any additional benefit in most patients\textsuperscript{40}. Multiple studies show that while NO provides short-term oxygenation, it does not reduce mortality, and it was not possible to wean patients from a ventilator\textsuperscript{40}.

Acute conditions such as serious cases of hypoxia may require higher dosage for a shorter time and chronic conditions may require lower dose for a longer time\textsuperscript{13}. Therapies such as GeNO inhaled drug delivery systems are being developed that may aid in both hospital and outpatient use. These could be useful for lower dosage for longer periods of time.

Injection of free nitrite as a nitrosylating agent poses toxic, unacceptable risks such as nausea, vomiting, methemoglobinemia, and uncontrolled vasodilation. Due to more controlled delivery and increased solubility, the encapsulation of nitrites in liposomes can lessen/eliminate many of the above shortcomings and provide better targeting of S-Nitrosylation pathways, specifically increasing SNO-Hb.

\textbf{MATERIALS AND METHODS}

\textbf{Materials}

Lipids (Cholesterol, 1, 2-Dipalmitoyl-sn-glycero-3-phosphocholine/DPPC, mPEG) were obtained from CordenPharma and Sigma-Aldrich (St. Louis, MO). Alkyl nitrites (ethyl nitrite 15-20% in
soln., amyl nitrite 96% in soln.) were obtained from Sigma-Aldrich. Whole human blood was obtained from the Case Comprehensive Cancer Center (Cleveland, OH). The center obtained the institutional review board approval for the procedure and supplied blood with no subject-identifiable information. The extruder used was from Northern Lipids (BC, Canada). Liposome DLS characterization was done at Dr. Karathanasis’ lab at the Laboratory for Nanomedical Engineering at Case Western Reserve University (CWRU).

We used hemoglobin, red blood cells (RBCs), and whole blood in nitrosylation experiments. In the following, we describe how we obtain RBCs, and hemoglobin from whole blood. Before SNO analysis, Hb was extracted from RBCs and whole blood as described below. The nitrolite machine used was from Dr. Johnathan Stamler’s lab at the Harrington Research Institute (Wolstein Research Building, Cleveland, OH).

**Dynamic Light Scattering (DLS)**

In order to confirm size distribution of the liposomes, DLS analysis was performed. When the light from the DLS machine hits the liposome particles, the light scatters in all directions. The scattering intensity fluctuates over time due to the particles undergoing Brownian motion, and the distance between the particles in solution is constantly changing over time. The scattered light then undergoes constructive or destructive interference by the surrounding particles, and with this intensity fluctuation, information is obtained about the time scale of the movement of the scattered particles. A dilution of each liposome sample was made to suppress particle-
particle collisions and to facilitate analysis. A distribution of particle size was obtained and an average mean diameter calculated.

**Liposome fabrication**

.0475g Cholesterol, .1285g DPPC, and .0257g of mPEG were weighed, and then dissolved in 1 mL of 100% ethanol in a vial. In experiments involving nitrosylation of hemoglobin, liquid formulations of ethyl or amyl nitrite were added to the lipid solution. In liposome loading tests, nitrite amounts equivalent to 4.5, 18, and 38 mM final concentrations were added to different lipid solutions. The solution was then mixed in a vial placed in a water bath at 60°C for 15 minutes. PBS was then added, and the solution was mixed for an additional 30 minutes. The mixed solution was then passed through an extruder eight times with a 200nm filter to ensure uniform size distribution. The newly fabricated liposomes (L-ENO and L-ANO) were then placed on dialysis for 2 days to remove ethanol.

**RBC Extraction and Purification**

Red blood cells are obtained from whole blood by removing plasma through centrifugation at room temperature. 0.5 mL of blood sample is placed in a micro centrifuge tube and spun at 2,000 g for 1 minute. Supernatant is drawn off and discarded. PBS/EDTA (0.5 mL, ~2x dilution) is added to tube and vortexed. The sample is then spun at 2,000g for 1 minute and supernatant is discarded. The PBS washing step is repeated 2 more times.

**Hemoglobin Extraction and Purification**

Hemoglobin was obtained by lysis of the purified RBCs. MilliQ purified water (1 mL) is added to the washed sample to lyse the RBCs. The sample is vortexed at max speed for 1 minute then
spun for 15 minutes at 20,000 g. The supernatant, which contains hemoglobin (Hb), is placed in a clean tube. The next step of the procedure involves purifying the hemoglobin. A vacuum flask was filled with G25 beads (1.5 g/sample) and filled with 400mL of PBS/EDTA. The flask was stoppered and attached to a vacuum line overnight. The G25 was resuspended by gently swirling the vacuum flask. A spin column, which contains the G25 beads prepared the previous day, is attached to a cut micro tube and put into a 50 mL centrifuge tube. Spin columns were filled with 8mL of G25 suspension and PBS was allowed to drain. This is spun at 1,000 g for 1 minute in a swinging bucket rotor to remove excess PBS in the column. The purpose of the G25 column is to separate small from large molecules. Through this process, all salts and other small molecules in the crude RBC lysates such as glutathione, nitrite, and GSNO are removed and retained in the column. Proteins, Hb in this case, run through the column and are collected. The excess PBS flow through is discarded and the column is attached to a clean micro tube. The lysate (200 µL) from the previous step is placed on top of the spin column evenly. The column is spun at 1,000 g for 5 minutes. The flow through, which is the purified hemoglobin, is collected.

Nitrosylation Experiments

Hemoglobin, RBCs, and whole blood were each incubated in a water bath at 37°C for 20, 40, and 60 minute time periods with ethyl or amyl nitrite liposome formulations (L-ENO, L-ANO, 38 mM). Protocol for hemoglobin purification was developed by Stamler et. al 34.
NO is cleaved from the donor compound (in our case O-NO from the nitrite) by photolysis (at 576 nm) in a vacuum, where it enters the gas phase. A stream of helium carries the NO to the detector, where it reacts with ozone to form NO2 (Schematic/formula in Figure 5). The NO2 is
in an electronically excited state, and releases a photon to return to the ground state. This is the chemiluminescence we measure.

We distinguish S-NO from other NO species (mostly Fe-NO) by treating an aliquot from each sample with mercury (Hg). Hg displaces NO from thiols in the form of nitrite, which goes largely undetected. Proteins are then desalted in physiological buffers to remove low-molecular-weight reactants. SNO levels are taken as the loss of signal caused by Hg. All SNOs are reactive to Hg, whereas FeNO is unreactive\(^{17}\). Area under peaks from an output graph are measured and, compared to a standard GSNO curve, and used to calculate NO.

*Calculate NO per heme*

The NO per Hb tetramer was calculated by first measuring heme concentration in the purified Hb sample using a spectrophotometer. 5 µL of Hb was added to a cuvette and diluted 200x with PBS. Absorbance reading was taken at 576 nm. Heme concentration was calculated using an extinction coefficient of 14.6 mM\(^{-1}\)*cm\(^{-1}\).

\[
\text{conc} = \frac{\text{Abs576} \times 200 \times 1000 \mu M}{14.6 \text{mM}^{-1}\text{cm}^{-1} \times 1 \text{cm}}
\]

To obtain 50µM heme in 2 mL (in a 1 mL cuvette) we used:

\[
\frac{\text{desired concentration}}{\text{measured concentration}} \times 2000\mu L = \text{volume needed}
\]

This was used to calculate total volume. Sample volume was calculated using:

\[
\text{for 2 mL} = \frac{7.3}{\text{Abs576}} \mu L
\]

This was subtracted from volume needed to calculate amount of PBS for dilution. A second absorbance reading was taken at 576 nm to verify the dilution:
\[ \text{conc} = \frac{\text{Abs}576 \times 1000}{14.6} \mu M \]

Samples were prepared by adding 20 µL 10% SDS to a 2 mL Hb sample and mixed. 10µL HgCl₂ was added to a separate 1.5 mL tube for FeNO measurements. To this tube, 990 µL Hb was added and mixed (1 mM final Hg²⁺ concentration).

Total NO readings were gathered using a nitrolite machine. SNO in the sample was calculated by measuring total NO (XNO) and Iron-bound NO (FeNO) and subtracting the difference. The SNO/1000Hb was calculated using the second absorbance reading at 576 nm and XNO measured by the nitrolite.

\[
\frac{(\text{peak area} - \text{intercept}) \times (\frac{100\mu L}{400\mu L})}{\text{slope}} \quad [\text{nM}]
\]

XNO: NO content from (-Hg) samples

FeNO: NO content from (+Hg) samples

SNO: XNO-FeNO

NO per heme was calculated by:

\[
\frac{\text{NO content}}{(\text{Abs}576 \times 17.1)} \quad [\text{NO/1000Hb}]
\]

A sample GSNO standard curve used for measurement is shown in Results.

Statistics

A standard Student’s t-test was conducted to measure statistical significance between Nitrosylation tests with Hb, RBCs, and whole blood and their respective controls. An Analysis of
Variance (ANOVA) was used to test variance in nitrosylation effects between the different treated groups (Hb, RBC, and whole blood). ANOVA was also used to test variance in loading in nitrite loading tests. Error bars in all graphs represent standard deviation.
RESULTS

Dynamic Light Scattering (DLS)

DLS measurements were taken to calculate a mean diameter of both L-ENO and L-ANO. In general, we observed that liposomes exhibited a statistically significantly lower diameter than the filter they were passed through would indicate. Filters used had a pore size of 200nm. The DLS measurement showed that L-ENO had a mean diameter of 143.6 ± 26.58 nm and L-ANO had a mean diameter of 153.57 ± 33.55 nm (Figure 6, panel B). A Student’s t-test showed a statistically insignificant difference between L-ENO and L-ANO average mean diameters (P > 0.05). The DLS machine measures mean diameter by creating a distribution based on a spectrum of particle sizes in Figure 6 (Panel A) below.

![Figure 6. Representative sample (A). DLS diameter measurements for L-ENO and L-ANO](image-url)
Nitrolite Readings

Samples were injected into a nitrolite machine to calculate total NO (XNO), FeNO, and SNO concentrations. NO was measured using area under peaks. A sample reading for an RBC test is shown below in figure 7.

Figure 7. Nitrolite Readings: (A) GSNO Standard for reference (B) –Hg (measure FeNO) (C) +Hg (measure XNO)
Nitrite Loading

Liposomes with different alkyl nitrite concentrations were made to test the loading efficiency of each alkyl nitrite (ENO and ANO) within liposomes. Each concentration used N=3 samples. The NO content of the liposomes were then measured. A Student’s t-test showed that at the 4.5 mM concentration of alkyl nitrites (ENO, ANO), there was an insignificant difference in liposomal NO content. There was a significant difference in liposomal NO content between the two alkyl nitrites at 18 mM and 38 mM. However, an ANOVA showed that for L-ENO and L-ANO respectively, there was a statistically insignificant difference between all three alkyl nitrite concentrations. There was inconclusive evidence to which alkyl nitrite is more efficiently encapsulated in liposomes, but results point to amyl nitrite being loaded more efficiently at the 38 mM concentration. Figure 8 shows a combined graph showing nitrosylation effects for L-ENO and L-ANO on Hb, RBCs, and whole blood.

![Figure 8. Combined loading graph](image)
Different dilutions (10x, 4x, and 2x) were made to test the optimal concentration for injection into the nitrolite machine by diluting L-ENO and L-ANO with PBS (Figure 9). It was determined that between these three dilutions, there was not a statistically significant difference in NO content when compared using a Student’s t-test.

**Figure 9.** Nitrite loading w/dilutions
Hemoglobin tests

Two different liposomal formulations (L-ENO, L-ANO), were tested for their hemoglobin nitrosylation efficacy. Each time point used N=3 samples. In Hemoglobin incubated with nitrite L-ENO, SNO/1000Hb levels increased from 1.129 ± 0.332 (control) to 3.191 ± 1.111 over 20 minutes of incubation (~3x increase); to 3.372 ± 2.65 over 40 minutes of incubation (2.6x increase), and to 4.3008 ± 1.2067 over 60 minutes of incubation (~4x increase) (Figure 10). In hemoglobin samples incubated with liposomal amyl nitrite, SNO/1000Hb levels increased from 4.923 ± .802 (control) to 7.543 ± .694 in a 20 minute incubation (~1.5x increase), to 9.237 ± .643 in a 40 minute incubation (~1.9x increase), to 7.65 ± .32 (1.5x increase) in a 60 minute incubation (Figure 10). Using a Student’s t-test, we saw that all incubation times led to statistically significant differences in SNO levels compared to controls.

![Graph](image_url)

**Figure 10. Hemoglobin: SNO concentration results for hemoglobin treated with liposomal nitrite.**
**Red Blood Cell (RBC) tests**

In RBC samples (N=3) incubated with liposomal ethyl nitrite, SNO/1000Hb levels increased from $1.0433 \pm 0.1596$ (control) to $1.80 \pm .32$ over 20 minutes of incubation (1.7x increase), to $2.043 \pm .567$ over 40 minutes pf incubation (1.9x increase), and to $2.22 \pm .32$ over 60 minutes of incubation (2.1x increase) (Figure 11). In RBC samples incubated with L-ANO, SNO/1000Hb levels increased from $2.273 \pm .355$ (control) to $6.033 \pm .975$ in a 20 minute incubation (2.6x increase), to $4.96 \pm .270$ (2.2x increase) in a 40 minute incubation, to $4.175 \pm .641$ in a 60 minute incubation (1.8x increase) (Figure 11). Similar to Hb tests, L-ANO showed higher SNO/1000Hb values compared to L-ENO, but again baseline values for L-ANO were higher. A Student’s t-test showed an insignificant increase from control to the 20 minute sample in L-ENO tests. All other incubation times in both L-ENO and L-ANO showed a significant increase from control (P<0.05).

![Figure 11. RBC: SNO concentration results for red blood cells with liposomal nitrite.](image-url)
Whole Blood tests

In whole blood samples (N=3) incubated with L-ENO, SNO/1000Hb levels increased from 2.54 ± .065 (control) to 3.86 ± .56 over 20 minutes of incubation, to 3.158 ± .077 over 40 minutes of incubation, to 5.763 ± .803 over 60 minutes of incubation (Figure 12). In whole blood samples incubated with L-ANO, SNO/1000Hb levels increased from 2.71 ± .46 (control) to 4.263 ± .465 in a 20 minute incubation, to 4.503 ± .467 in a 40 minute incubation, to 4.610 ± .503 in a 60 minute incubation (Figure 12). A Student’s t-test showed that there was an insignificant increase from control to the 20 minute sample in whole blood tests. Increases from the control to 40 and 60 minute samples were significant. There were no observable increases in heme concentration with the addition of L-ENO or L-ANO to whole blood when hemoglobin was extracted and measured. For Hb, RBCs, and whole blood, heme concentration varied between 1.21 mM-2.88 mM.

Figure 12. Whole Blood: SNO concentration results for whole blood treated with liposomal nitrite.
A combined graph (Figure 13) shows Hb, RBC, and Whole Blood test results for L-ENO (A) and L-ANO (B). An ANOVA test showed that there was a significant difference between Hb, RBC, and whole blood tests.

**Figure 13.** Combined Hb, RBC, Whole Blood nitrosylation test results for L-ENO (Panel A), and L-ANO (Panel B).

### Specificity

Determination of FeNO levels (NO bound to Iron in heme) demonstrated the specificity of NO binding from alkyl nitrites (L-ENO, L-ANO) onto Hb. We found that FeNO levels with untreated Hb was $0.1732 \pm 0.1095$ FeNO/1000Hb. This compares to $1.053 \pm 0.284$ FeNO/1000Hb in treated samples. A Student’s t-test shows a P value of 0.00745, showing a statistical significance between the the FeNO/1000Hb levels for untreated and treated samples.
DISCUSSION

An effective Nitrosylation therapy has many potential uses in hypertensive therapy, pulmonary dysfunction, blood transfusions and organ transplantation. Venodilation and vasodilation are the most important and well established effects of nitrites. At low doses, they have been shown to induce peripheral venodilation, redistributing blood flow from cardio-pulmonary circulation to the mesenteric and splanchnic vasculature. Nitrites have also been shown to lead to a fall in pulmonary artery and left ventricular end diastolic pressures and a reduction in preload. At high doses, it leads to arterial and arteriolar dilation, reducing afterload. These effects on preload and after-load lead to a reduction in oxygen demand; coupled with coronary dilation, leads to enhanced oxygen supply. Nitrites have been shown to also be much more potent vasodilators than nitroglycerine, which has been an established method for treating tissue hypoxia. When used in vivo, formulations of ethyl and amyl nitrite can offer advantages in transfusion and organ transplantation.

Current methods of blood transfusion exacerbate, rather than correct, issues such as lack of tissue oxygenation due to reduced SNO levels. Amyl Nitrite has been used for more than a century in the treatment of angina and has been shown to be an effect alternative to infusions of drugs such as isoproterenol. As discussed, nitrite formulations have been shown to restore SNO content to SNO-depleted tissue. Liposomal formulations build on this principle but add the advantage of solubility, as alkyl nitrites are insoluble or sparingly soluble in water. Liposomal nitrite formulations will also allow a liquid formulation to be injected directly into the body. Liposomes allow a greater loading capacity as well, along with a longer residence time compared to gaseous formulations.
DLS measurements were taken with L-ENO and L-ANO formulations showed that both formulations had liposomes with much smaller average mean diameter than their extrusion filters would indicate. In general, liposome particle size is expected to be smaller than the filter pores it is extruded through, however we observed much smaller diameters (~46-47 nm smaller) than filter pore size. This may be due to interfacial tension between the liposome and the surrounding area in which it is being extruded. Between L-ENO and L-ANO, there was an insignificant difference in average mean diameter (Student’s t-test). In conjunction with the nitrite loading data, it may be inferred that ENO is causing a reaction at the surface of the liposomes or within the bilayer, causing a decreased size. The size distribution of liposomal drug carriers is of key interest because size not only affects the vesicle’s in-vitro characteristics such as the amount of drug that can be accommodated, aggregation and sedimentation behavior, but also its in vivo behavior such as circulation time in the blood-stream upon i.v.-injection, and consequently also biodistribution.

Results from nitrite loading tests suggest that a dose-dependent loading can be achieved. However, the relationship between the loading concentration and the loaded levels was log-linear; higher loading concentrations led to substantially higher nitrite loading. If both ENO and ANO are encapsulated in the lipid layer, these results suggest higher resistance by the lipid layer for incorporating at low levels of nitrite. Further, our results also showed amyl nitrite to be much more efficient in loading than ethyl nitrite at lower concentrations of nitrite.

Figure 14 shows a proposed schematic for the delivery of NO from liposomal nitrite formulations to glutathione and cysteine thiol groups, the main site of S-Nitrosylation through nitrites. The main mechanisms that are not completely understood are the transport of nitrite
across the RBC membrane and the interactions of -ONO with plasma proteins and glutathione. There are some ideas about carrier proteins that function to deliver NO from alkyl nitrites such as AE1.

The increase in SNO-Hb levels in hemoglobin with the addition of liposomal nitrite in this study can be attributed to the binding of the NO from the nitrite O-NO bond to the –SH group of cysteine residues. It has been shown that the structure of the organic ‘R group’ residue (in this case, the O-NO group) is the part of the molecule that elicits the pharmacological effects. Incubation time affected the nitrosylation efficacy. Our results show that incubation times greater than 20 minutes are required for nitrosylation of hemoglobin. The liposomal formulations were also able to nitrosylate hemoglobin when incubated with RBC though the effect was not as pronounced as when hemoglobin was directly incubated with the liposomal
formulations. This can probably be attributed to the blood cell membrane causing less alkyl nitrite to reach the hemoglobin. Again, our results showed that incubation times greater than 20 minutes were required for effective nitrosylation. This is similar to results from experiments with hemoglobin, suggesting that the transport of –ONO across the RBC membrane is faster relative to the nitrosylation reaction with the –SH groups in Hb. In whole blood experiments, the liposomal formulations did increase SNO-Hb levels though the increases were much smaller. Our results indicating SNO-Hb levels of Hb > SNO-Hb levels of RBC > SNO-Hb levels of whole blood > SNO-Hb levels of control blood suggest that blood plasma has a major role in modulating the nitrosylation of SNO-Hb. Human Serum albumin (HAS) has also been shown to be a possible reservoir of NO and could be a clinically feasible biocompatible pharmacological agent. There is also evidence that shows plasma molecules such as glutathione can be nitrosylated, which can cause a decrease in the efficacy of liposomal formulations in nitrosylating Hb. While there is no pattern in regards to incubation time and SNO/1000Hb levels, the data seems to show that an incubation time higher than 20 minutes is required for significant increases in SNO. Future experiments will test which membrane proteins are being nitrosylated. Increases in SNO-Hb level were larger compared to gaseous nitrite experiments conducted by B. Yurcisin, et al.

Comparison to Nitroglycerin therapy and implications

Tests previously conducted in patients with labile and established hypertension showed inhaled amyl nitrite to be significantly more effective at reducing blood pressure—systolic, diastolic, and mean arterial pressures along with peripheral vascular resistance. Amyl nitrite
also increased cardiac output in labile hypertensive patients. Nitroglycerin (NTG) did not affect cardiac output and did not decrease peripheral vascular resistance. Amyl nitrite also seemed to enter circulation more rapidly compared to NTG. This could be attributed to routes of administration, but in general amyl nitrite was more effective. The use of liquid formulations will increase residence time and bioavailability of the nitrite. In our tests, it was shown that L-ENO was relatively a more effective nitrosylating agent than L-ANO.

One of the drawbacks of long-term treatment using NTG is the development of tolerance. Over time, tolerance develops rapidly to the hemodynamic and clinical effects of nitrates when they are administered continuously or at frequent intervals. It is proposed that this may occur through the decrease in activation of guanylate cyclase, which is stimulated by NTG when exerting its vasodilating effects and contributes to a reduction in metabolic conversion of NTG. It has been suggested that this is due to the depletion of intracellular sulphydryl cofactors such as cysteine. Findings suggests that the activation of endogenous neurohormonal systems such as the renin-angiotensin system, can limit pharmacological effects of NTG and cause tolerance. The only way to circumvent these tolerance effects is to administer NTG intermittently, which cannot provide continuous therapeutic effect and is frequently associated with intolerable side effects and rebound clinical events. The use of liposomal alkyl nitrites could allow for continuous delivery and higher dosing based on our results. This may offer an alternative that has fewer side effects and tolerance issues.

The effects of gaseous ethyl nitrite have been demonstrated in studies by Auten and others in studies on postnatal alveolar development in rats. It has been shown that Ethyl nitrite is significantly more effective at treating hypertension as well as hyperoxia-induced lung
inflammation. Much of Ethyl nitrite’s advantages are due to the fact that it forms S-Nitrosothiols (SNO) more readily than does Nitric Oxide (NO)\(^1\). Prevention of inflammation is a significant advantage of nitrites and has implications with liquid formulations as well, as in this study.

There are, of course, limitations to liposomal alkyl nitrite renitrosylation therapy. First and foremost, it has not been proven to be effective in real-world studies relating to reduced SNO levels. This is essential to taking the next step in developing a formulation that would be effective in treating various conditions associated with reduced SNO. Additionally, the mechanism of nitrosylation through nitrites is not completely understood, as stated earlier. The clarification of this mechanism would allow greater insight into developing a more effective liposomal alkyl nitrite formulation, if possible. We are not also completely sure of any adverse effects that treatment with liposomal alkyl nitrite could cause, especially those caused by current nitrosylation therapies. We can only hypothesize what may be alleviated with the encapsulation of alkyl nitrites within liposomes.

The results shown in this study offer major implications in cardiovascular research. The use of liposomal nitrite can correct issues previous methods have with residence time, continuous delivery, and toxicity, as well as offer variable loading capacity. This can lead to more effective treatment of cardiovascular diseases such as angina pectoris and diseased states such as sickle cell anemia, as well as increasing the number of organs available for donation. Liposomal nitrosylation therapy can also help reduce tissue injury, inflammation, and organ damage, as B. Yurcisin at the Duke Medical Center aims to achieve. Nitrites have also been shown to possess anti-thrombotic and anti-platelet effects, which may enhance their
therapeutic effects. Dysregulated S-nitrosylation has also been implicated as a cause or consequence of a broad range of diseases including asthma, cystic fibrosis, Parkinson disease, heart failure, and stroke. Re-nitrosylation through liposomal nitrite can have positive implications with regard to these diseased states.

FUTURE WORK

Currently, the mechanism of NO delivery through nitrite is not exactly known. From this study’s results, we can see that SNO levels are reduced in tests with RBCs and whole blood compared to hemoglobin. The cell membrane in RBCs and the plasma in whole blood is likely causing a decrease in penetration of nitrite/liposomes. The mechanism by which this happens is not known. Figure 14 shows a proposed schematic of how nitrosylation of hemoglobin occurs through the use of liposomal nitrite. There may be other molecules scavenging NO from the nitrite such as glutathione. In future experiments, it may be possible to fluorescently tag the liposomes to elucidate the mechanism of delivery. It is also important to test differences in hematocrit of different blood samples, since this will show why there is a variance in SNO/1000 Hb in controls. It is not known how exactly the liposome degrades in the presence of nitrite. Tests will need to be conducted to investigate the reactivity of ethyl nitrite at the liposome surface to see if ENO is causing any degradation of the lipids in the liposomes. This may be contributing to the decreased NO content observed with the nitrite loading tests. Further DLS tests with stored liposomes may show if the liposomes degrade over time in the presence of nitrite. It may also be possible to study the release kinetics of nitrite from the liposomes.
These tests will also allow investigation into how size affects function of liposomes in vivo. Liposomes of several different sizes loaded with nitrite can be fabricated to determine their effectiveness in improving blood flow. Doxorubicin exhibits slow-release kinetics; if nitrite exhibits the same kinetics, it would help to investigate more into the bioavailability and circulation time of the drug.

Tests can also be conducted with targeted liposomes using antibodies to deliver drugs to specific parts of the body in vivo. The addition of amyl nitrite to hemoglobin causes methemoglobin to form, which can be poisonous in excessive concentrations. Assays can be done to measure met-Hb concentration in Hb with the addition of liposomal nitrite. Excessive toxicity is not anticipated since liposomes are not known to leach out drug when fortified with cholesterol and PEG. The addition of PEG allows drug to be released slowly, so adverse effects should be prevented. Stability tests were conducted in this study in vitro, but it is necessary to tests stability of liposomal nitrite in vivo. Since liposomes exhibit around a 24 hour circulation half-life in the body, it should be possible to investigate the stability of liposomal nitrite in vivo. This may either be done by figuring out a possible mechanism of NO delivery or by measuring blood NO concentration 24 hours after administration of drug.

Studies can also be conducted in vivo to test liposomal nitrite use in anti-tumor treatment. Liposomal nitrite can utilize the Enhanced Permeability and Retention (EPR) effect in tumors to increase blood flow. Tumor tissue exhibits abnormalities such as aberrant vascular architecture, which could leave spaces in the tissue. The utilization of this effect addition to chemotherapeutic drugs could enhance drug delivery to the tumor.
REFERENCES