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Liposome Encapsulation of a Photochemical NO Precursor for Controlled Nitric Oxide Release and Simultaneous Fluorescence Imaging

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ABSTRACT: Described are photochemical studies of the nitric oxide precursors, trans-Cr(L)(ONO)2+ (L = cyclam = 1,4,8,11-tetraazacyclotetradecane, CrONO, or L = mac = 5,7-dimethyl-6-anthracenecyclam, mac-CrONO) encapsulated in phosphatidylcholine liposomes. The liposomes provide a means to maintain a localized high concentration of NO releasing complexes and are easily modified for in vivo targeting through self-assembly. Steady, controlled release of NO is seen after photolysis of the liposome-encapsulated CrONO as compared to the burst of NO release seen by the unencapsulated complex in oxygenated solutions. The quantum yields for photochemical NO release from liposome-encapsulated CrONO and mac-CrONO were determined in both oxygenated and anoxic solutions. The quantum yield for NO release in oxygenated solution for encapsulated CrONO was more than 5 times larger than that of unencapsulated CrONO, thus the net NO released after photolysis in oxygenated solutions is enhanced by encapsulation of CrONO in liposomes. Encapsulated mac-CrONO shows NO release after photolysis with low-intensity blue light. Furthermore, the fluorescence of mac-CrONO can be detected through the liposomes, thus allowing for development of theranostic NO delivery vessels where tracking and imaging can occur simultaneously with therapeutic NO release. This work provides insight into the development of multifunctional liposome constructs for disease theranostics.

KEYWORDS: nitric oxide release, theranostic, liposomes, photochemical, drug delivery

INTRODUCTION

There has long been interest in nitric oxide owing to its bioregulatory activity in a variety of physiological processes in both normal and disease states.1,2 The effect of NO is dictated by its concentration, where nM concentrations lead to vasorelaxation and higher concentrations up to μM are responsible for tumor suppression.3–10 NO also acts as a sensitizer leading to enhanced cell death when generated with γ-radiation.11–13 For therapeutic NO delivery then, it is critical to be able to control the concentration of NO released in the cell. With the intention of designing stimuli responsive NO releasing complexes for therapeutic applications, we have developed the NO precursor trans-Cr(cyclam)(ONO)2+ (“CrONO”, cyclam = 1,4,8,11-tetraazacyclotetradecane, see the Supporting Information) and related compounds that release NO only after irradiation with UV or visible light.14–18 Photochemical triggering of such a “caged” bioactive agent provides the ability to control the timing, dosage, and location of the NO release by controlling the timing, intensity, and location of light irradiation.19,20

As shown in Scheme 1, CrONO effectively releases NO with a high quantum yield after photolysis at 436 nm both in oxygenated aqueous buffer solutions and in anoxic media containing the biological reductant glutathione (GSH).17 Such photolysis-generated NO from CrONO is effective in activating soluble guanylyl cyclase and in triggering vasodilation in porcine coronary arterial rings.17 The CrONO complex does not show any acute toxicity, either alone or with low-intensity blue light exposure.18 Therefore the CrONO complex is a promising candidate for the controlled delivery of NO for therapeutic applications. However it remains a challenge to deliver the CrONO complexes to targets while maintaining concentrations that have therapeutic relevance, especially under in vivo conditions. A highly modular and biocompatible system employed for drug delivery is liposomes, where therapeutic materials are easily encapsulated at high concentrations21–25 and targeting capabilities are easily incorporated through the addition of modified peptides and proteins.26–29 In addition, the mechanical properties of the liposomes can be tailored to provide more robust vehicles for delivery of the CrONO complexes.30 Here, CrONO complexes are encapsulated in

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phosphatidylcholine (PC) liposomes, with the overarching goal of developing NO releasing theranostic constructs for cancer therapy and vasodilation: the physicochemical properties of the resulting structures are studied.

**EXPERIMENTAL SECTION**

**Synthesis of trans-Cr(cyclam)(ONO)$_2$BF$_4$ (CrONO).** trans-Cr(cyclam)(Cl)$_2$Cl was synthesized according to published procedures by Ferguson$^{31}$ and Poón et al.$^{32}$ Cyclam (1,4,8,11-tetrazacyclotetradecane, 98%) was purchased from Sigma. The trans-Cr(cyclam)(ONO)$_2$BF$_4$ was prepared from trans-Cr(cyclam)(Cl)$_2$Cl according to a method slightly modified from that of DeLeo et al.$^{33}$ Briefly, trans-Cr(cyclam)(Cl)$_2$Cl (50 mg) was dissolved in deionized H$_2$O (<5 mL) to which 20 equiv of NaNO$_2$ was added. The purple resulting orange solution was cooled slightly and NaBF$_4$ was allowed to precipitate overnight in the fridge. Orange Cr(cyclam)(Cl)$_2$BF$_4$ was dissolved in deionized H$_2$O (<5 mL) to which 20 equiv of NaNO$_2$ was added. The purple solution was refluxed in the dark for 2 h. After reflux, the resulting orange solution was cooled slightly and NaBF$_4$ was added. An orange precipitate started to form. The solution was allowed to cool and precipitate overnight in the fridge. Orange Cr(cyclam)(ONO)$_2$BF$_4$ was collected by filtration. Crude product was recrystallized from methanol. Yield: 75%. ESI-MS: 344.12. UV−vis in water: $\lambda_{\text{max}}(\epsilon)$ 475 (40), 336 (267).

**Synthesis of trans-Cr(mac)(ONO)$_2$BF$_4$.** trans-Cr(mac)-(ONO)$_2$BF$_4$ was synthesized according to a previously published procedure by DeRosa et al.$^{34,35}$ ESI-MS: 592. Absorbance and emission spectra are shown in Figure S1 in the Supporting Information.

**Liposome Encapsulation of CrONO and mac-CrONO.** Egg phosphatidylcholine (L-α-phosphatidylcholine, 99%) was purchased from Avanti Lipids. A phosphatidylcholine (PC) liposome, either 10 mM phosphate buffer saline pH 7.4 alone, or buffer solution. Care was taken to ensure that the light beam was below the tip of the electrode in the solution. After 100 s, an aliquot (200 μL) from the liposome sample stock solution was injected into the buffer solution. Care was taken to ensure that the electrode was not bumped when the injection was made. The current from NO electrode was calibrated by Griess assay, such that

$$p\text{A} = -1307 + 162\left[\text{NO}\right]$$

where the concentration of NO in solution is in nM.

**Nitric Oxide Analyzer.** Samples of the liposome-encapsulated CrONO and mac-CrONO as well as control liposomes were prepared in a y-shaped quartz cuvette by dilution of 300 μL of the stock sample into total 3 mL volume of 10 mM PBS pH 7.4. The sample was placed in a cuvette holder on an optical train equipped with a 470 nm LED light source (ThorLabs) and shutter. Helium was bubbled through the sample and into a Sievers nitric oxide analyzer (NOA 280b). Samples were stirred and bubbled with either helium or medical grade compressed air for 3 min in the dark at 25 °C to ensure equilibration. The samples were then photolyzed for a specific time (30 s, 60 s, and 90 s), and the NO generated was measured on the NOA. For total NO release, 100 μL of the stock solution of mac-CrONO was diluted into 3 mL total volume using 10 mM PBS pH 7.4. The sample was then photolyzed with the 470 nm LED until the signal returned to baseline. Integration of the signal gives a total of 3 nmol of NO released from the 100 μL aliquot. The NOA is calibrated as instructed in the user manual. Representative calibration curves are...
mole of NO = (area − 2.6)/6.8 × 10^{12}
for air atmosphere
mole of NO = (area − 2.6)/5.7 × 10^{12}
for helium atmosphere

## RESULTS AND DISCUSSION

To prepare the liposomal constructs, CrONO and macromolecular CrONO were dissolved in phosphate buffer saline (PBS) at pH 7.4 and the solution was used to dissolve a thin film of PC. The mixture was freeze–thaw cycled and then extruded through a liposome extruder to achieve constructs of approximately 120–130 nm in diameter as determined through dynamic light scattering (DLS). The unencapsulated CrONO was removed from the liposomes through dialysis. These constructs remain stable for a minimum of 2 weeks after dialysis, as determined by DLS (see Supporting Information).

The ability for NO to escape the lipid membrane of liposome encapsulated CrONO (Figure 1) was measured with an amperometric NO-specific electrode (Innovative Instruments) at 25 °C in pH 7.4 PBS. A high-pressure mercury lamp attenuated with a filter (λ_{ex} >350 nm, I_{ph} ~ 10^{18} photons/s) was employed as the light source. The CrONO complexes were successfully activated through the liposomes photochemically as determined through NO release (Figure 1). In contrast, no nitric oxide was detected upon similar photolysis of liposome solutions prepared without CrONO or from solutions of liposome encapsulated CrONO that were not irradiated. This demonstrated that the PC liposomes served as stable vessels for the nitric oxide precursor and once the NO is uncaged photochemically, it can readily diffuse across the lipid membrane.

The profile of NO released from the liposome-encapsulated CrONO after photolysis is distinctly different from that of unencapsulated CrONO. Unencapsulated CrONO shows an initial burst of NO followed by a rapid decrease in concentration despite continued irradiation. This is presumably due to the complex photochemistry of CrONO in oxygenated solutions where continued irradiation leads to formation of a CrIV intermediate that can be oxidized by O_2, leading to generation of superoxide as well as NO. The superoxide consequently scavenges NO, trapping it as peroxynitrite and leading to the decrease in NO concentration detected by the NO electrode, despite continued irradiation (see Scheme 1). This rapid decrease in NO concentration is not seen during photolysis of the liposome-encapsulated CrONO. Instead a nearly steady state is achieved where NO production is balanced by depletion processes (autoxidation, diffusion, etc.) with a very gradual decrease in concentration over time. This could be due to the increased permeability of the membrane to the NO gas as opposed to the CrONO itself or superoxide generated during the photolysis of the CrIV photoproduction. The enhancement of NO across the lipid bilayer could minimize scavenging of NO by the ionic superoxide inside the liposome. Finally, the liposome itself could act as a reducing agent, reacting with the superoxide generated or reducing the CrIV photoproduction to suppress superoxide generation analogous to the use of glutathione (GSH) (Scheme 1). This shows that liposome encapsulation of the CrONO complex can help facilitate controlled, steady release of NO during photolysis in oxygenated solutions. This would be critical for the application of CrONO as a therapeutic NO delivery agent, where a constant, steady release of NO at a specific concentration would be needed.

The quantitative release of NO by both the liposome encapsulated and unencapsulated CrONO under oxygenated and anoxic conditions was measured with a Sievers nitric oxide analyzer 280i (NOA). As shown in Figure 2, unencapsulated CrONO had a significant loss of net NO release in oxygenated solutions compared to anoxic solutions, corresponding to >90% loss of NO due to scavenging by superoxide. This dramatic loss is not observed for liposome-encapsulated CrONO (Figure 3). This supports our hypothesis suggesting that liposome encapsulation enhances the overall NO release by reducing scavenging of NO by superoxide presumably due to the differential partitioning of NO into liposome membrane or by reaction of the PC with any generated photoproducts (CrIV, superoxide). The quantum yields (QY) for net NO releases were also determined for both unencapsulated and liposome-
encapsulated CrONO and are shown in Table 1 (individual values with statistical analysis are shown in Table S1 and Table S3 in the Supporting Information). The QY for net NO release in oxygenated environments is much greater for the liposome-encapsulated CrONO (0.04 ± 0.01) compared to unencapsulated CrONO (0.006). It should be noted that, for unencapsulated CrONO in oxygenated environments, the NO release rapidly decreases during continuous irradiation, however this is not the case with the liposome-encapsulated CrONO sample (Table S2 in the Supporting Information). This shows that the liposome not only functions as a stable container for delivery of CrONO cargos but also enhances the rate and yield of effective NO released in oxygenated environments.

Another NO precursor we have previously described is the anthracene-labeled CrONO analogue,\textsuperscript{17} \textit{trans}-Cr(mac)-([ONO] \textsuperscript{2+} (mac = 5,7-dimethyl-6-anthracenyl-cyclam, mac-CrONO structure shown in the Supporting Information). The dye pendant to the CrONO moiety may have several functions. One is that when exciting with higher energy light it serves as an antenna to collect light and to sensitize photoreactions of the CrIII complex, thus enhancing the amount of NO released.\textsuperscript{16} By using an appropriate pendant dye, the NO release can be sensitized to occur after two-photon excitation with lower-energy light, allowing for greater tissue penetration for \textit{in vivo} NO release.\textsuperscript{19} Another function of the dye is that the anthracenyl fluorescence (see Supporting Information Figures S1 and S2) serves as a luminescent marker for the CrONO complex. While the sensitization can lead to more NO release, it is accomplished only by excitation with UV light that may be toxic to certain cell types.\textsuperscript{36} For these studies mac was employed solely as a luminescent marker for the CrONO, and NO release was triggered with lower-energy blue light. In this context, PC liposomes were prepared using identical procedures to encapsulate CrONO and mac-CrONO from \textit{trans}-[Cr(cyclam)(ONO)]\textsubscript{2}BF\textsubscript{4} and \textit{trans}-[Cr(mac)-(ONO)]\textsubscript{2}BF\textsubscript{4}, respectively.

Fluorescence measurements determined that the emission of the mac ligand was measurable across the lipid membrane (Figure S2 in the Supporting Information) although the light scattering from the liposomes lowered the intensity of the fluorescent signal. The NO released upon photolysis of the encapsulated mac-CrONO was quantitatively analyzed using a Sievers nitric oxide analyzer 280i (NOA). The samples were photolyzed for 30 s with a blue (470 nm) light emitting diode (Thor Laboratories), a low-intensity, portable light that has proved applicable for CrONO photolysis for \textit{in vitro} experiments (for example porcine arterial vasorelaxation).\textsuperscript{17} In this measurement, the integrated signal defines the amount of NO released. The results are shown in Figure 4.

As shown in Figure 4, even with a low-intensity visible (blue, 470 nm, 1.7 × 10\textsuperscript{15} photons/s) LED light source there is significant NO released during the 30 s photolysis of the encapsulated mac-CrONO. The amount of encapsulated mac-CrONO was quantitated by detection of the total NO released (Figure S8 in the Supporting Information). Assuming that two NOs were released by each complex as shown previously in this laboratory,\textsuperscript{38} this result corresponds to a concentration of roughly 15 µM of mac-CrONO in the liposome sample. This is in agreement with the measured concentration determined by absorbance, where the solution would be 16.3 µM (see Supporting Information).

The quantum yield for NO release from mac-CrONO inside the liposomes was also determined in both oxygenated and anoxic conditions as for CrONO (Table 1). Again, a larger QY

![Figure 3](image-url) Representative NOA trace showing the NO release after 30 s irradiation with a blue LED (470 nm, 1.7 × 10\textsuperscript{15} photons/s) of 300 µL aliquot of CrONO in liposomes in anoxic (blue) and oxygenated (orange) conditions in 10 mM PBS at pH 7.4, 25 °C. The integrated area is proportional to the amount of NO released, where moles of NO = area/6.8 × 10\textsuperscript{15} in air and moles of NO = area/5.7 × 10\textsuperscript{15} in He.

![Figure 4](image-url) NOA trace showing the NO release after 30 s irradiation by a blue LED (470 nm, 1.7 × 10\textsuperscript{15} photons/s) with helium bubbling in 10 mM PBS pH 7.4 at 25 °C: black, liposomes without any NO precursor; blue, liposome encapsulated mac-CrONO (2.7 µM). The integrated area is proportional to the amount of NO released where moles of NO = area/5.7 × 10\textsuperscript{15}.

<table>
<thead>
<tr>
<th></th>
<th>in Helium (µM)</th>
<th>in air (µM)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CrONO inside liposomes</td>
<td>0.31 ± 0.08\textsuperscript{a}</td>
<td>0.04 ± 0.01\textsuperscript{b}</td>
<td>&lt;0.0001</td>
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<tr>
<td>CrONO</td>
<td>0.28 ± 0.04\textsuperscript{a}</td>
<td>&lt;0.0006\textsuperscript{b}</td>
<td>&lt;0.0001</td>
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<td>p-value</td>
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<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>mac-CrONO inside liposomes</td>
<td>0.48 ± 0.02\textsuperscript{a}</td>
<td>0.04 ± 0.01\textsuperscript{b}</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>mac-CrONO</td>
<td>0.31 ± 0.03\textsuperscript{a}</td>
<td>N. D.</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}Determined at \textit{l} = 1.7 × 10\textsuperscript{15} photons/s, 470 nm LED irradiation. 
\textsuperscript{b}Determined at \textit{l} = 8.0 × 10\textsuperscript{15} photons/s, 436 nm irradiation, ref 15. 
\textsuperscript{c}Determined at \textit{l} = ∼10\textsuperscript{15} photons/s, using absorbance changes, 436 nm irradiation. See ref 16. 

Table 1. Comparison of QY for NO Release from Liposome-Encapsulated CrONO and mac-CrONO in Both Oxygenated and Anoxic Solutions
(0.04 ± 0.01) is determined for net NO release in oxygenated buffer solutions from the liposome-encapsulated mac-CrONO sample compared to the unencapsulated CrONO complex in solution (0.006), showing that the encapsulation of the NO-releasing complex can help facilitate controlled, steady release of NO across the liposome membrane. The amount of NO released during the photolysis of such liposome constructs using the low-intensity blue LED light corresponds to 1–10 nM concentrations of NO in solution, depending on the duration of irradiation (10–30 s). This is on the order of what is needed for therapeutic NO delivery, as it is nM concentrations that will activate soluble guanylyl cyclase, a primary pathway in bioregulation by NO.5,6

In summary, these studies have demonstrated that the photochemical NO precursors CrONO and mac-CrONO can be encapsulated into stable phosphatidylcholine liposomes and that photolysis of these in solution is an effective method for delivering NO. Liposomes are particularly attractive for use during therapeutic delivery of NO, as they allow for a controlled and steady release of NO as compared to CrONO free in solution, especially in oxygenated solutions. In addition, the modularity of self-assembly affords the ability to tune the mechanical properties of liposomes through chemistry7,8 and in vivo functionality through the incorporation of biomolecules.9,10,21,26–29,38

Furthermore, the encapsulation of both fluorescently labeled and NO releasing complexes, such as mac-CrONO, results in theranostic constructs that are effective as both a diagnostic and therapeutic agent. This work provides some fundamental insight for the development of self-assembled multifunctional liposome constructs for disease theranostics.

■ ASSOCIATED CONTENT

Supporting Information
Details of experimental procedures including characterization of the liposomes by DLS, structures of CrONO, mac-CrONO and PC as well as figures showing the absorption spectra of unencapsulated and encapsulated mac-CrONO. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

DLS, dynamic light scattering; CrONO, trans-Cr(cyclam)-(ONO)2; cyclam, 1,4,8,11-tetraazacyclotetradecane; GSH, glutathione; mac, 5,7-dimethyl-6-anthracenyl-cyclam; mac-CrONO, trans-Cr(mac)(ONO)2; NOA, nitric oxide analyzer; PC, phosphatidylcholine; PCs, phosphatidyl choline liposomes; PLp, photoluminescence; LED, light-emitting diode; QY, quantum yield

■ REFERENCES