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

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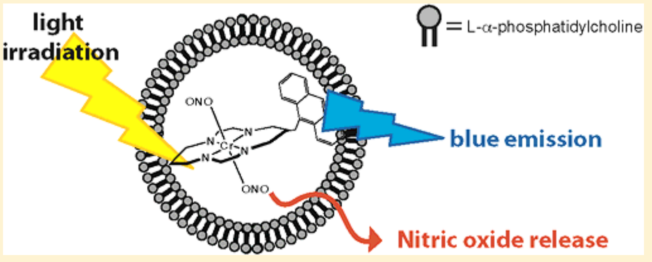
Supporting Information

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-anthracenylcyclam, 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 concentrations[21−25] 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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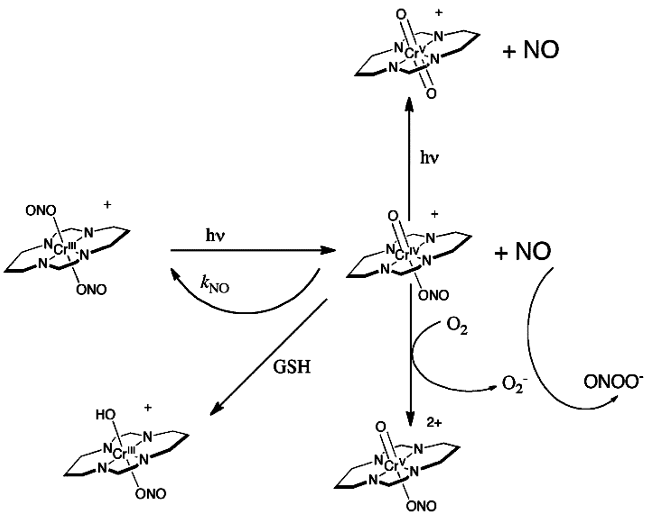
Scheme 1. Photochemistry of CrONO



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

■ EXPERIMENTAL SECTION

Synthesis of *trans*-Cr(cyclam)$(ONO)_2BF_4$ (CrONO). *trans*-Cr(cyclam)$(Cl)_2$Cl was synthesized according to published procedures by Ferguson[31] and Poon et al.[32] Cyclam (1,4,8,11-tetrazacycltetradecane, 98%) was purchased from Aldrich, and trichlorotris(tetrahydrofuran)chromium(III) was purchased from Sigma. The *trans*-Cr(cyclam)$(ONO)_2BF_4$ 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_2O (<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)$(ONO)_2BF_4$ was collected by filtration. Crude product was recrystallized from methanol. Yield: 75%. ESI-MS: 344.12. UV–vis in water: λ_{max} (ε) 475 (40), 336 (267).

Synthesis of *trans*-Cr(mac)$(ONO)_2BF_4$. *trans*-Cr(mac)-$(ONO)_2BF_4$ 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) film was prepared by dissolving 8 mg of PC in $CHCl_3$ in a glass centrifuge tube and dried under N_2 flow. The PC film was placed overnight in a vacuum oven to remove residual solvent. The PC film was hydrated with 3 mL of warm (50 °C) solution, either 10 mM phosphate buffer saline pH 7.4 alone, buffer with CrONO (1 mM), or buffer with mac-CrONO. The samples were incubated in a water bath at 50 °C for 10 min and then vortexed to ensure even hydration. The samples were frozen in liquid N_2 and then thawed to 50 °C in a water bath. Five cycles were performed to homogenize the PC, and then the solution was extruded 10× through 100 nm membranes, at 37 °C. The samples were stored refrigerated at 4 °C and dialyzed in a 50K MWCO membrane against 10 mM PBS (pH 7.4) in the dark. The 10 mM PBS was changed 4× over a 3 day period. The liposomes (with CrONO and mac-CrONO), final volume of 3 mL, were stored in the dark at 4 °C until use. The absorbance and emission spectra of the liposomes with mac-CrONO are shown in Figure S2 in the Supporting Information. The size of the liposomes before and after photolysis was measured using dynamic light scattering. Dynamic light scattering determined that the liposomes were stable for a minimum of 2 weeks. At 2 weeks, the constructs with and without CrONO measured 118 nm and 116 nm, respectively.

For the quantitative NO experiment, the control liposomes were 138.6 nm in diameter. The liposomes with CrONO were 134.4 nm in diameter. The liposomes with mac-CrONO were 131.9 nm in diameter (Figures S5–S7 in the Supporting Information).

The concentration of Cr(III) complex in the final stock solution was determined to be ~15 μM (see Supporting Information). This corresponds to ~0.02 mg of complex (CrONO MW = 430.92 g/mol, mac-CrONO MW = 562.92 g/mol) encapsulated in 3 mL. So, using 8 mg of lipid in 3 mL of PBS, 0.02 mg of the Cr complex can be encapsulated, corresponding to approximately 1% encapsulation efficiency. Higher concentrations of lipids can potentially encapsulate more complex.

Nitric Oxide Detection. *Electrode.* The NO-specific electrode was polarized in phosphate buffer for at least 24 h prior to use. When ready for analysis, the NO electrode was allowed to equilibrate in 3 mL of 10 mM phosphate buffer saline pH 7.4 at 25 °C in a quartz cuvette for ~15 min while stirring until a stable baseline was reached. At that point the light (>350 nm selected from a Hg arc lamp) was turned on to irradiate the sample. Care was taken to ensure 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

$$pA = -1307 + 162[NO]$$

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 280i). 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

$$\text{moles of NO} = (\text{area} - 2.6)/6.8 \times 10^{12}$$
for air atmosphere

$$\text{moles of NO} = (\text{area} - 2.6)/5.7 \times 10^{12}$$
for helium atmosphere

■ RESULTS AND DISCUSSION

To prepare the liposomal constructs, CrONO and mac-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 ami-

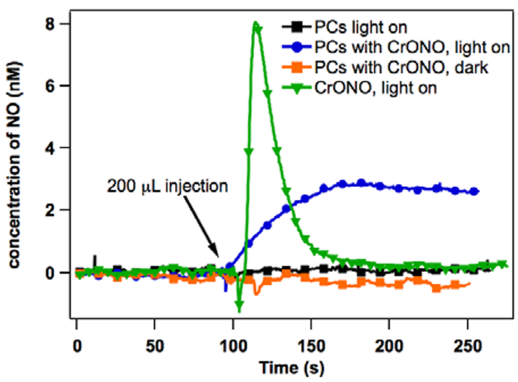


Figure 1. Concentration of NO in solution as determined by a NO-specific electrode for solutions containing liposome encapsulated CrONO. The initial solution was 3 mL of 10 mM PBS at pH 7.4 and was irradiated continually with visible light from a high pressure Hg lamp (>350 band-pass filter, $I_0 \sim 10^{18}$ photons/s). At ∼100 s a 200 μL sample of the liposome solution was injected. The sample containing CrONO showed a strong signal indicating NO generation (blue), while the liposome solution without CrONO did not (black). The same experiment in the dark did not show any NO production (orange). The NO release is compared to that seen for unencapsulated CrONO (200 μM) (green).

700 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, $I_0 \sim 10^{18}$ 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 Cr^{IV} intermediate that can be oxidized by O_2, leading to generation of superoxide as well as NO.[18] 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).[18] 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 after oxidation of the Cr^{IV} photoproduct. The enhanced partitioning of NO across the lipid bilayer could minimize scavenging of NO by the ionic superoxide inside the liposome. Finally, the liposome itself could also act as a reducing agent, reacting with the superoxide generated or reducing the Cr^{IV} photoproduct 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

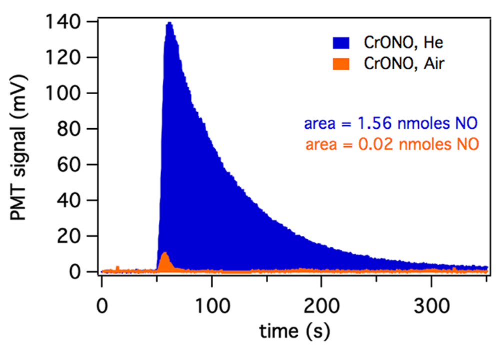


Figure 2. Representative NOA trace showing the NO release after 5 s irradiation at 436 nm with a Hg lamp (8.0 × 10^{15} photons/s) of 300 μM CrONO in anoxic (blue) and oxygenated (orange) conditions in 15 mM phosphate buffer pH 7.4 at 25 °C. The integrated area is proportional to the amount of NO released, where moles of NO = area/6.8 × 10^{12} in air and moles of NO = area/5.7 × 10^{12} in He.

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 the 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 (Cr^{IV}, superoxide). The quantum yields (QY) for net NO releases were also determined for both unencapsulated and liposome-

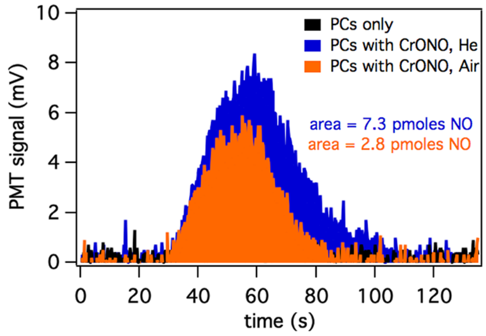


Figure 3. Representative NOA trace showing the NO release after 30 s irradiation with a blue LED (470 nm, 1.7×10^{15} photons/s) of 300 μL aliquot of CrONO in liposomes in anoxic (blue) and oxygenated (orange) conditions in 10 mM PBS at pH 7.4, 25 °C. The integrated area is proportional to the amount of NO released, where moles of NO = area/6.8×10^{12} in air and moles of NO = area/5.7×10^{12} in He.

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.

Table 1. Comparison of QY for NO Release from Liposome-Encapsulated CrONO and mac-CrONO in Both Oxygenated and Anoxic Solutions

	in Helium	*in air*	
	QY	QY	*p-value*
CrONO inside liposomes	0.31 ±0.08[a]	0.04±0.01[a]	*<0.0001*
CrONO	0.28 ±0.04[b]	<0.006[d]	*<0.0001*
p value	0.3	*<0.0001*	
mac-CrONO inside liposomes	0.48 ±0.02[a]	0.04 ±0.01[a]	*<0.0001*
mac-CrONO	0.31 ±0.03[c]	N. D.	

[a]Determined at $I_0 = 1.7 \times 10^{15}$ photons/s, 470 nm LED irradiation. [b]Determined at $I_0 = 8.0 \times 10^{15}$ photons/s, 436 nm irradiation, ref 15. [c]Determined at $I_0 = \sim 10^{15}$ photons/s, using absorbance changes, 436 nm irradiation. See ref 16. [d]Determined at $I_0 = 4.0 \times 10^{15}$ photons/s 436 nm irradiation. The QY is changing rapidly under these conditions, see ref 17 and Table S2 in the Supporting Information. The value reported is the highest value determined.

Another NO precursor we have previously described is the anthracene-labeled CrONO analogue,[17] *trans*-Cr(mac)-$(ONO)_2^+$ (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 Cr^{III} complex, thus enhancing the amount of NO released.[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 *in vivo* NO release.[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.[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 *trans*-$[Cr(cyclam)(ONO)_2]BF_4$ and *trans*-$[Cr(mac)(ONO)_2]BF_4$, 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 *in vitro* experiments (for example porcine arterial vasorelaxation).[17] In this measurement, the integrated signal defines the amount of NO released. The results are shown in Figure 4.

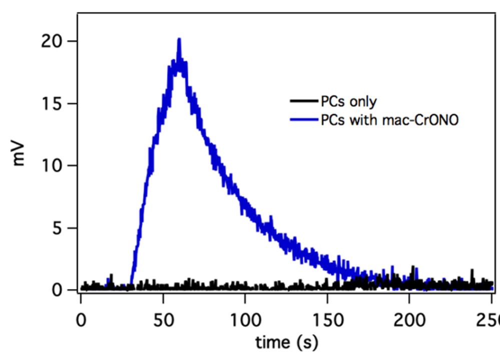


Figure 4. NOA trace showing the NO release after 30 s irradiation by a blue LED (470 nm, 1.7×10^{15} photons/s) with helium bubbling in 10 mM PBS pH 7.4 at 25 °C: black, liposomes without any NO precursor; blue, liposome encapsulated mac-CrONO (2.7 μM). The integrated area is proportional to the amount of NO released where moles of NO = area/5.7×10^{12}.

As shown in Figure 4, even with a low-intensity visible (blue, 470 nm, 1.7×10^{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,[18] 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

(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.[37]

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 chemistry[30] and *in vivo* functionality through the incorporation of biomolecules.[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, phosphatidyl choline; PCs, phosphatidyl choline liposomes; PL, photoluminescence; LED, light-emitting diode; QY, quantum yield

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