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COMMUNICATION

Copper-Responsive Liposomes for Triggered Cargo Release Employing a Piconilamide–Lipid Conjugate

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In this work, we report triggered content release from liposomes brought about by copper chelation to a synthetic lipid switch containing a picolinilamide headgroup. Fluorescence-based dye-leakage assays showcase release of carboxyfluorescein dye cargo upon copper treatment and for controlling liposomal release based on copper abundance. Our results additionally show that this platform is selective for copper and is accompanied by significant changes to liposome properties upon treatment with this ion.

Introduction

Liposomes are spherical artificial bilayer assemblies formed by hydrating phospholipids.[1] Their ability to encapsulate and enhance the delivery of therapeutic agents positions liposomes at the forefront of drug delivery. When encapsulated into liposomes, the pharmacokinetics of drugs are greatly improved due to increased solubility, stability, and lower cytotoxicity.[2] Despite significant advancements in the field, the benefits of liposomal nanocarriers could be further improved by controlling the site specificity of drug delivery and thereby minimizing off-target effects. To achieve this, liposome triggered release platforms using both passive and active release strategies have been developed. Passive release exploits internal stimuli including differences in pH, enzyme expression, and metabolite abundance between healthy and diseased cells, whereas active release strategies employ external stimuli such as ultrasound, light, and heat to effect triggered release.[3-5] While each approach comes with its own advantages and disadvantages, it would be beneficial to expand the toolbox further to realize the full potential of controlled liposomal release.

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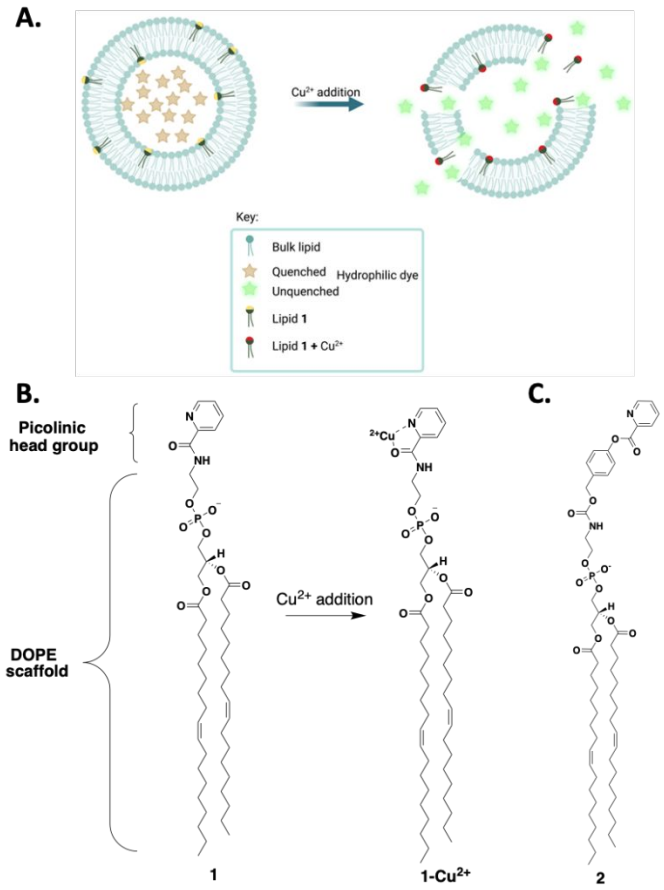
Through recent advances in the field, the enhanced abundance of chemical agents at diseased sites compared to healthy cells and tissues has recently emerged as a phenomenon that can be exploited for controlled cargo release or activated cell delivery.[6-12] A few metal ion-responsive liposome platforms have been developed in this context. For example, Zefirov and co-workers reported a bispidinone-based copper-responsive molecular switch to trigger liposomal content release.[13] Our group has developed distinct smart liposome platforms that respond to calcium or zinc using synthetic lipid switches that form non-covalent binding interactions with the respective metals that are designed to effect conformational changes in the lipids and thereby promote release.[14, 15] Nevertheless, the toolbox for ion-responsive liposomes remains small. In this work, we focused on copper as an intriguing disease-associated stimulus for liposomal release that has received minimal attention. Healthy serum copper concentrations range from 12-24 μM compared to 70-80 μM in the cerebrospinal fluid.[16] Changes in the labile-copper pool have been associated with ailments including Wilson's disease (WD), tumor growth, and Alzheimer's disease (AD).[16, 17] For instance, the serum concentration of copper has been found to be 54% higher in AD patients.[16] The development of copper-chelation therapy for various cancers and WD further illuminates the role of disrupted copper homeostasis in these ailments.[17]

Results and Discussion

To develop a copper-responsive liposome platform in this work (Scheme 1A), we exploited picolinic acid derivatives, which are effective for chelation of Cu^{2+} ions.[18] For example, picolinic esters have been developed as probes for imaging and tracking increased Cu^{2+} concentrations at diseased sites.[19-21] Through the course of this work, we pursued two prospective Cu-responsive lipid switches. Compound **1** contains the picolinyl moiety attached directly via an amide bond to a lipid scaffold composed of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine



Scheme 1. Design of copper-responsive liposomes. (A) Cartoon illustration of hypothetical model for content release from liposomes upon copper addition. (B) Structure and design of copper-responsive lipid **1** composed of picolinylamide head group moiety and DOPE scaffold. The picolinyl headgroup is included to chelate Cu^{2+}. (C) Structure of alternative lipid **2** designed for copper-responsiveness.

(DOPE) (Scheme 1B). Lipid **2** was also investigated, which instead contains a picolinic ester attached via a self-immolating linker to DOPE (Schemes 1C and S1B), a design that is similar to previously reported liposomes that respond to reactive oxygen species (ROS)[9] and enzymes.[7] In line with prior probes for detecting copper, we originally envisioned that copper chelation by these molecules could promote hydrolysis of the picolinoyl group to produce DOPE, which is a known non-bilayer forming lipid that destabilizes liposomal membranes to trigger content release.[22-25] However, as will be discussed further below, we showed that lipid **1** is effective for copper-driven liposome release even though our data indicate that picolinoyl group hydrolysis and DOPE production do not occur.

Lipid **1** benefits from a facile synthesis that was accomplished via a one-step amide coupling reaction between DOPE and picolinic acid (Scheme S1A). Lipid **2** required a lengthier 5-step synthesis to append the picolinyl group via a self-immolating linker (Scheme S1B).[19] Compound **2** more closely resembles prior copper sensing systems since its picolinyl group is linked via an ester to a phenoxy group. However, while this compound was synthesized successfully, during studies or storage, we routinely observed that **2** quickly underwent decomposition over time, as judged by TLC and NMR analysis. For this reason, we turned our focus to alternative compound **1** for the evaluation of content release properties upon its incorporation into liposomes.

For triggered release studies we employed the dye carboxyfluorescein (CF) for which cargo release from liposomes can be tracked via fluorescence assays.[13, 26, 27] The delivery of polar/hydrophilic contents using liposomes has taken priority in recent years due to the emergence of RNA therapeutics.[28, 29] CF can be encapsulated within the aqueous cores of liposomes at high concentrations such that it undergoes self-quenching. Membrane disruption and content release is then accompanied by dilution and an increase in fluorescence intensity. Initial liposomes were composed of 0-30% of lipid **1**, 10-15% L-α-phosphatidylcholine (PC, mixed isomers from egg or egg-PC), 50% DOPE, and 5-10% L-α- phosphatidic acid sodium salt (PA, from chicken eggs or egg-PA) to investigate hydrophilic content release properties, in line with formulations we have previously found to be successful for triggered release. Standard liposome preparation methods were utilized, which included thin lipid film formation, hydration, freeze-thaw, and extrusion through 200 nm polycarbonate membranes. Due to non-specific encapsulation of CF, a size-exclusion column was performed to remove unencapsulated dye. The formation of liposomes of the expected sizes was confirmed by dynamic light scattering (DLS) analysis, which will be discussed later in the context of particle size changes induced by copper. The fluorescence intensities of liposomal solutions were measured over time upon addition of copper chloride solution in Tris buffer, pH 7.4. Once the intensity reached a plateau, the detergent Triton X-100 was added to trigger complete release. The results are reported as a percentage of fluorescence increase upon Triton X-100 treatment.

Figure 1 shows changes in fluorescence intensity of liposomes containing **1** when subjected to copper chloride treatment. Liposomes containing 30% **1** resulted in a fluorescence increase that correlated with ~45% of the total release from Triton X-100 over a period of two hours, wherein the majority release occurred within the first twenty minutes, upon treatment with 1 mM copper chloride. One plausible explanation for release in this system is that the negatively charged PA component could drive liposome fusion based on electrostatic interactions with the positively charged lipid **1**-Cu^{2+} adduct resulting in content release. To explore this, we next conducted further experiments by switching from PA to 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), which is instead a positively charged lipid. Here, we observed that 30/15/50/5 **1**/PC/DOPE/DOTAP liposomes exhibited greater content release, yielding ~80% CF release upon 1mM Cu^{2+} addition and ~50% release with 0.75 mM Cu^{2+} treatment. Since liposomes containing DOTAP additive proved to be more effective at driving content release, this provides evidence that release is not driven by electrostatic attractions between oppositely charged liposomes. All Cu-responsive formulations proceeded rapidly, with release essentially complete within less than approximately 20 minutes.

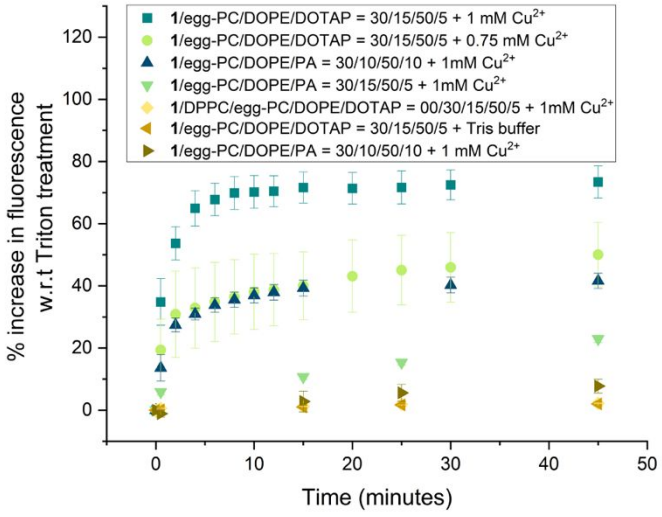


Figure 1. CF release profile over time for lipid **1** at 0-30% of lipid composition containing 50% DOPE, 5% DOTAP or 5-10% PA, 10-20% egg-PC, and 0-30% DPPC as the remaining percentage. Liposomes containing **1** at 30% with 5% DOTAP resulted in ~80% release over 2 h upon addition of 1 mM Cu^{2+}. Minimal release was observed for all control experiments. Error bars indicate standard error over triplicate experiments.

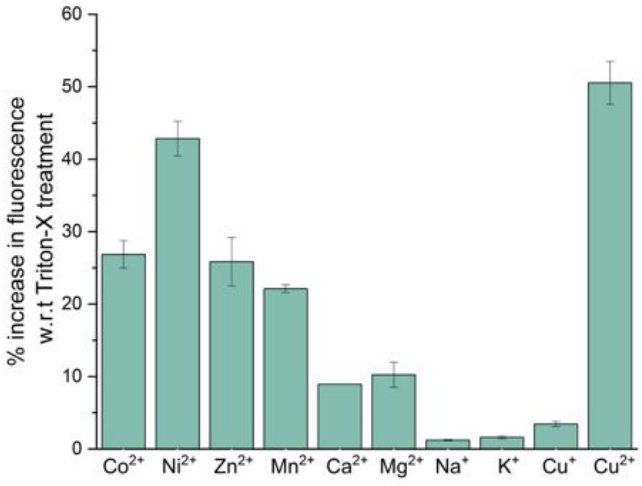


Figure 2. CF release results for 30% **1** liposomes in response to various biologically relevant metal ions. Liposomes were incubated with 1 mM of each metal, with measurements taken after 2h incubation to determine fluorescence increases caused by release. The system was selective for Cu^{2+} compared to other biologically relevant metal ions while Ni^{2+} afforded the highest interference. Error bars indicate standard error over triplicate measurements.

Despite the comparable kinetics, the variation in the amplitude of signal change supports that the extent of CF release is enhanced when liposomes are more active and are treated with higher concentrations of Cu^{2+}.

Control experiments were also conducted by either replacing lipid **1** with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) or addition of Tris buffer instead of copper chloride to liposomes containing **1**. Minimal release was observed for both of these control experiments. These results were also evident visually, as shown in Figure S1, wherein the yellowish-brown color of 30% **1** liposome solution changed to a more fluorescent green upon copper treatment suggesting dye leakage. No significant color change was observed for control experiments. It should be noted that we also observed a 23.13% ± 5.63% (averaged over three data sets) reduction in final fluorescence intensity upon addition of Triton X-100 to liposomes that had been treated with 1 mM Cu^{2+} compared to untreated controls, as well as a 2.65% ± 2.16% reduction for liposomes to which had been added 0.5 mM Cu^{2+}. This effect can be attributed to partial quenching of CF dye upon release from liposomes, brought about by coordination of Cu^{2+} to donor atoms in CF. While this effect diminishes the fluorescence intensity of each sample, it is not expected to affect the percentage change since we are comparing to Triton X-100 treated liposomes subjected to the same concentration of copper. Overall, these results show that compound **1** is effective as a copper-responsive lipid switch when incorporated into liposomes.

We next moved to conduct selectivity studies for liposomes containing **1** against other biologically relevant metal ions including transition, alkali, and alkaline earth elements. As depicted in Figure 2, alkali and alkaline earth metals exhibited significantly lower response, whereas intermediate interference was observed from ions including cobalt, zinc, and manganese. Nickel, on the other hand, showed the highest interference, which is consistent with results reported by Heffern and co-workers, wherein Ni yielded higher turn-on response than other metals tested for their caged imidazopyrazinone bioluminescent probe.[19] However, the serum concentrations of transition metals including cobalt and nickel are significantly lower (1.7-20.4 nM for Co and 4 – 800 nM for Ni)[30-31] than the concentrations tested (1 mM). In addition, Cu^{+} exhibited minimal response, thereby confirming that our system is selective for Cu^{2+} against Cu^{+}. It should also be acknowledged that the serum concentrations of Cu^{2+} ($\geqslant$ 80 µM for diseased tissues) are lower than those that yield release from this liposomal platform ($\geqslant$ 500 µM). Therefore, further enhancements in triggered release at low Cu^{2+} concentrations would be beneficial. Nevertheless, this platform serves as proof-of-concept for the development of Cu-responsive liposomes.

To determine whether liposomal assembly properties are altered during the release process, we next probed changes in liposome particle sizes before and after copper treatment through DLS measurements (Figure 3). All liposomes before copper treatment exhibited fairly uniform sizes below 200 nm, which is within the range expected following extrusion. An approximately two-fold increase in the average sizes of liposomes containing 30% **1** was observed upon treatment with 1mM Cu^{2+}, whereas a lesser size increase was observed for 1mM Cu^{2+}, addition to control liposomes lacking **1**. Similar changes were observed for the polydispersity indices of liposomes containing 0-30% **1** upon addition of 1mM copper chloride (Figure S2). The formation of larger lipid assemblies is in line with previous stimuli-responsive liposome designs and suggests that processes including fusion and/or lipid reorganization driven dye leakage.[7, 32]

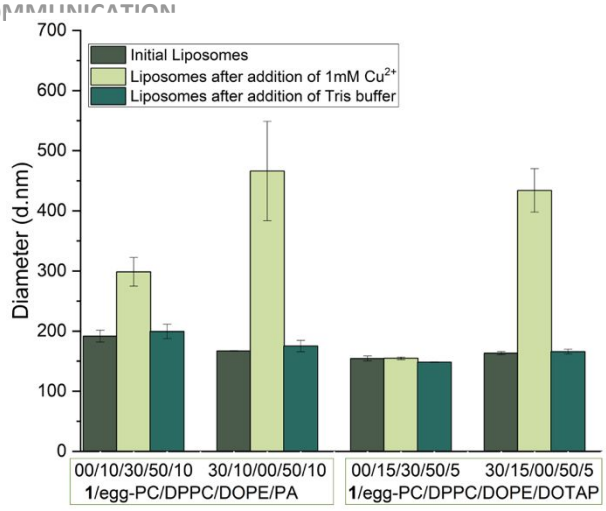


Figure 3. DLS analysis of liposomes containing 0-30% **1** upon addition of 1 mM copper chloride. A two-fold increase in size was observed upon addition of 1 mM Cu^{2+} to 30% **1** liposomes while lesser size changes were observed for liposomes lacking lipid **1**. Error bars indicate standard error over triplicate measurements.

We next conducted further experiments to confirm that copper binding is involved in driving release from liposomes. We first performed time-dependent NMR analysis by dissolving compound **1** in $CDCl_3/CD_3OD/D_2O$ (65/25/4; v/v), wherein after an initial scan, 0.3 mM copper chloride was added and spectra were taken over time (Figure S3). Broadening of signals in the aromatic region was observed, which became more significant with time (over a 12-hour period in total), and also complicated NMR analysis. Nevertheless, this supports that the picolinyl moiety is complexed to Cu^{2+}. While a potential mechanism for Cu-driven release from **1**-liposomes entails hydrolysis of the amide bond to produce DOPE, we did not observe the formation of the characteristic NMR peaks for this lipid during these experiments. To probe this further, we conducted mass spectrometry studies following treatment of **1** with copper in solution, after which we did not observe mass peaks corresponding to DOPE, but did detect the copper chelate of compound **1** (Figure S4).

Collectively, these data support that the formation of a **1**-Cu^{2+} chelate drives release. Our DLS data indicate that fusion is likely involved in release due to the formation of larger particles. Our data showing that replacement of PA with DOTAP led to enhanced release suggests that this process is not driven by simply electrostatic attractions between liposomes. Therefore, we theorize that the formation of the **1**-Cu^{2+} adduct causes release by perturbing membrane properties and driving fusion. It is also plausible that more than one molecule of **1** could interact with Cu^{2+}, which could also drive liposome fusion. Further experiments would be necessary to probe these details. Nevertheless, our data support that the binding of copper **1** is itself sufficient for driving cargo release in this system.

We also conducted stability assessments of CF-encapsulating liposomes in the absence and presence of synthetic lipid **1** via fluorescence-based leakage assays and DLS measurements (Figure S5). Minimal CF leakage was observed for 30% **1,** 10% PA liposomes that was comparable to control liposomes lacking **1** upon storage at 4°C over a period of 20 days. The liposomes were also found to be remarkably stable through DLS experiments with minimal to no size changes observed over the 20-day period.

Conclusions

In conclusion, we have developed a copper-responsive lipid **1**, which could be incorporated into liposomes for content release. Our results demonstrate that liposomes containing **1** release CF content upon complexation of Cu^{2+}. DLS measurements were conducted and supported the formation of liposomes of expected sizes from extrusion, which was accompanied by an increase in size and PDI upon copper treatment. Our triggered release system was also found to be selective towards Cu^{2+} compared to other biologically relevant metal ions including Cu^{+}, with some interference from similar ions such as Ni^{2+}. NMR and MS experiments indicated that formation of the **1**-Cu^{2+} complex was sufficient for driving release, presumably by modulating lipid properties and/or driving fusion. Liposomes containing 0-30% **1** with 10% PA as an additive were also found to be stable with minimum content leakage over a period of at least 20 days through DLS and fluorescence measurements. The development of copper-responsive liposomes provides a promising strategy for modulating drug release based on the labile copper pool, for which concentrations are known to increase in various diseased states. In this manner, the targeting of ions provides an opportunity to enhance control over drug release at diseased states.

Conflicts of interest

There are no conflicts to declare.

Acknowledgments

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