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Liposome Triggered Content Release Through Molecular Recognition of Inositol Trisphosphate

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Sydney E. Bottcher, †[a] Jinchao Lou†[a] and Michael D. Best*[a]

A stimuli-responsive liposomal platform that is selectively activated by inositol 1,4,5-trisphosphate (IP_3) over eleven other phosphorylated metabolites is reported. Dye release assays validated dose-dependent release of both hydrophilic and hydrophobic cargo driven by IP_3, showcasing the potential of this platform for triggered release and sensing applications.

Liposomes are spherical membrane bilayers that are versatile in terms of their ability to encapsulate cargo ranging from hydrophilic to hydrophobic. Due to their unique features, liposomes are highly effective nanocarriers for drug delivery applications, as evidenced by the > 15 formulations in clinical use.[1] Compared to free drugs, liposomal platforms exhibit optimized pharmacokinetics and reduced side effects. However, therapeutic potential could be further improved by achieving control over the timing and location of cargo release. Therefore, liposome-controlled release has been a vigorous area of investigation in which a number of both active (driven by external stimuli, i.e., light, ultrasound, heat) and passive (driven by diseased cell conditions, i.e., pH, enzyme, redox environment)[2] strategies have been reported. However, there are many challenges associated with these stimuli, including issues with delivering external stimuli and minimal differentiation between healthy and diseased cells. Liposomes additionally exhibit unfulfilled potential as sensors driven by small molecule recognition. While traditional molecular sensors typically only activate one sensor molecule per binding interaction, liposomes can release numerous encapsulated dye molecules to amplify signal and therefore sensitivity.[3]

To simultaneously expand the limited toolbox of stimuli for drug delivery applications and the prospects for liposomal sensor platforms, we report a unique approach for triggered cargo release by leveraging non-covalent binding interactions with target small molecules. To do so, we built upon recent work using synthetic lipid switches that undergo conformational changes driven by recognition events that perturb membrane packing and drive content release.[4,5] Our group has evolved these systems to progress from metal cation-responsive liposomes (calcium and zinc)[6,7] to liposomes that selectively release contents in the presence of adenosine triphosphate (ATP).[8] In the latter case, we showed that liposomal cell delivery was enhanced through pharmacological increase of intracellular ATP levels. In the current work we selected inositol 1,4,5-trisphosphate (IP_3) as a critical target for disparate applications. IP_3 is a key signaling molecule that releases calcium stores[9] and fluctuations in concentration from low nM to µM have been reported resulting from hydrolysis of the lipid phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) by phospholipase C.[10] On one hand, this variation in cellular abundance opens up the possibility of targeted delivery to cells based on IP_3 concentration. On the other hand, IP_3 remains a challenging target for sensor design and quantitation, which could be overcome through dye release triggered by IP_3 recognition. Toward both of these ends, we set out to develop an IP_3-responsive liposomal platform.

Building upon our ATP-responsive platform, which contains two zinc(II) dipicolylamine (ZnDPA) moieties for binding to phosphate groups,[11] we hypothesized that we may alter target selectivity by modulating lipid switch structural features. Based on reported IP_3 supramolecular hosts,[12] we designed switch **1** containing three ZnDPA moieties to accommodate the three phosphates of IP_3 (Scheme 1A). These are installed onto a rigid lipid scaffold that is expected to adopt a cylindrical lipid shape that favors formation of bilayer lipid membranes in the unbound form. However, **1**-IP_3 binding is envisioned to cause tris-ZnDPA headgroup constriction that induces conical shape known to perturb bilayer lipid packing and drive encapsulated cargo release (Scheme 1B).[13]

The synthesis of lipid switch **1** is shown in Scheme S1. With this compound in hand, we evaluated the triggered release

[a] Department of Chemistry, University of Tennessee, Knoxville, TN, 37996, USA
Email: mdbest@utk.edu
† Authors contributed equally
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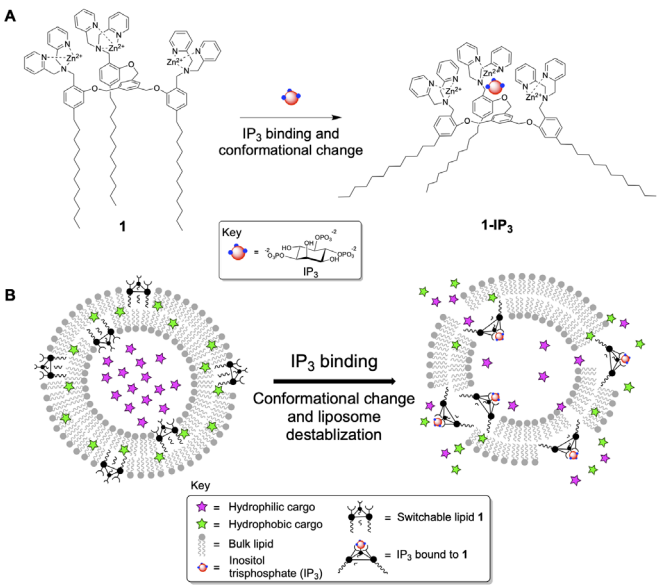

Scheme 1. Design for IP_3-responsive liposomes. **A.** Structure of lipid switch **1** and hypothetical conformational changes upon IP_3 binding. The three ZnDPA units on **1** can effectively bind to IP_3, leading to a cone-shaped non-bilayer forming lipid. **B.** Cartoon illustration of liposome cargo release triggered by IP_3 addition, in which the induced conformational change results in membrane disruption and content release.

properties of liposomes containing **1** utilizing fluorescence-based dye release assays. We first explored hydrophobic content release using Nile red (NR), a dye for which fluorescence is activated within a membrane environment and therefore deactivated when this molecule escapes the bilayer.[14] In this way, NR not only mimics the properties of common hydrophobic therapeutic cargo, but also enables signal transduction to evaluate release properties and sensing prospects. We first prepared unilamellar liposomes (2 mM) composed primarily of L-α-phosphatidylcholine (PC, mixed isomers from egg) doped with 0-20% lipid **1** as well as NR. Liposomes were prepared through film formation, hydration, freeze-thaw cycling and extrusion through 200 nm polycarbonate membranes. We then screened a variety of phosphorylated small molecule metabolites for NR release, including inorganic phosphate (Pi), inorganic pyrophosphate (PPi), adenosine diphosphate (ADP), adenosine monophosphate (AMP), D-fructose-6-phosphate (FP), D-fructose-1,6-bisphosphate (FBP), inorganic triphosphate (TPi) and the nucleotide triphosphates (ATP, cytidine (CTP), guanidine (GTP), and uridine (UTP) triphosphate).

Liposomes containing 20% of **1** were first tested. After an initial scan, liposomes were incubated with 1 mM of each analyte for five minutes before taking another reading. IP_3 induced the largest decrease in fluorescence signal (~45%) attributed to NR leakage (Figure 1A). There was some competition observed from other analytes, the greatest of which arose from inorganic phosphate treatment (~20% fluorescence decrease), but most analytes yielded minimal response, including ATP. For our ATP-responsive liposomes, we observed that decreasing the percentage of the lipid switch enhanced selectivity over similar molecules.[8] Accordingly, reducing the percentage of **1** to 10% (replaced by PC) resulted in enhanced selectivity, with IP_3 inducing ~20% fluorescence decrease, while the eleven other phosphorylated metabolites yielded minimal leakage (<5%). Control experiments were carried out with PC liposomes lacking **1** (Figure S1) or by titrating MilliQ purified water (MQ) into the **1**-liposomes (Figure 1A), none of which caused release. These results demonstrate that liposomes containing **1** are sensitized to IP_3 and that the selectivity can be tuned by adjusting the percentage of **1** within the membrane.

To further characterize IP_3-induced NR release, we carried out expanded titration experiments. PC-based liposomes doped with 0%, 5%, 10%, 15%, or 20% of lipid **1** (2 mM) were subjected to IP_3 titration. Dose-dependent NR release was observed with **1** present at greater than 10%. Specifically, 10% **1**-liposomes showed ~20% decrease in fluorescence intensity, which was increased to ~40% for 15% liposomes, but not further enhanced using 20% of **1** (Figure 1B). Pink precipitate

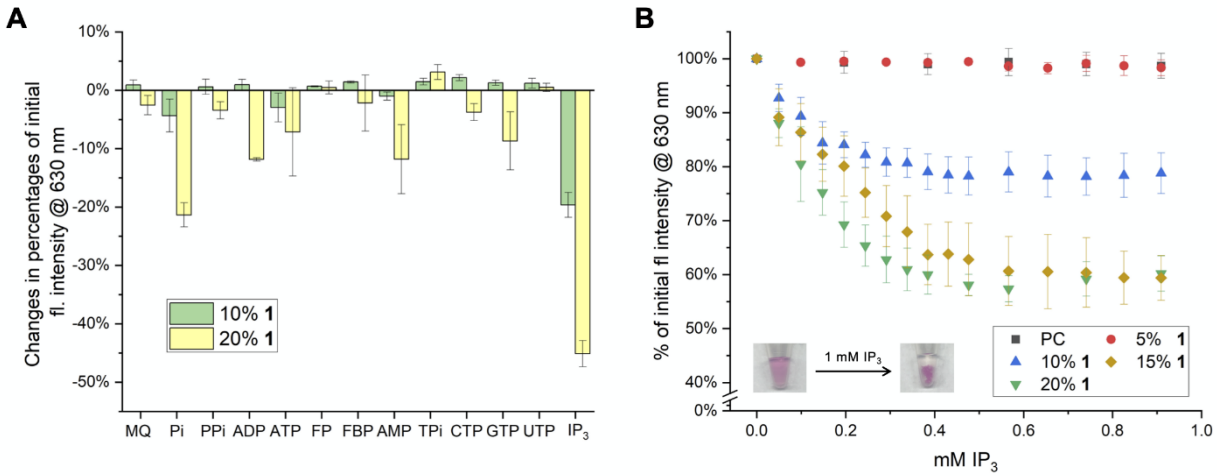

Figure 1. NR release results employing PC-based liposomes containing lipid switch **1**. **A.** Phosphorylated metabolite screens for **1**/PC liposomes. Liposomes (2 mM) were treated with 1 mM of each metabolite, with measurements taken after 5 min incubation. 20% **1**-Liposomes exhibited selectivity toward IP_3 (~45%) with inorganic phosphate showing ~20% release. 10% **1**-Liposomes showed enhanced selectivity toward IP_3 (~20% release with all other metabolites showing < 5%). **B.** Expanded IP_3 titration curves for liposomes containing **1**. Dose-dependent release correlating with **1** percentage was observed upon IP_3 addition. Error bars denote standard errors from at least three independent trials.

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was observed for 15% or 20% **1**-liposomes after IP_3 addition, indicating NR precipitation in line with prior reports.[6,15] PC control liposomes did not show any background release, nor did 5% **1**-liposomes, suggesting insufficient lipid switch in the membrane to induce release in the latter case. We also treated 5% **1**-liposomes with up to >5 mM IP_3 (Figure S2), and <5% release was observed, indicating that excess IP_3 did not yield activity. The correlation between release and **1** percentage indicates that both **1** and IP_3 are necessary for cargo escape. Studies of release kinetics also showed that both 10% and 20% **1**-liposomes reached a plateau in fluorescence signal within ten minutes, indicating rapid release (Figure S3). The stabilities of 0-20% **1**-liposomes encapsulating NR were also tested by measuring fluorescence over time. These were generally stable at 4 °C for five days, although minor decreases in fluorescence were observed (Figure S4).

Having confirmed lipid switch activity, we next determined whether this resulted from **1**-IP_3 binding using zeta potential (ZP) analysis, which measures particle surface charge. Here, **1**/PC-liposomes (0%, 10%, or 20%) were prepared in 1 mM HEPES buffer (pH = 7.4, containing 30 mM NaCl) and ZP values were measured before and after IP_3 addition (Figure 2). PC liposomes were slightly negatively charged, which is in line with literature.[16] IP_3 addition did not cause any changes in ZP for 100% PC liposomes. Both 10% and 20% **1**/PC-liposomes initially exhibited positive charge correlating with the percentage of **1** due to the ZnDPA units. After IP_3 incubation, we observed a significant decrease in ZP, indicating that **1**-IP_3 binding interactions diminished positive charge at the membrane surface, as expected. These results support that liposomes containing **1** undergo binding interactions with IP_3.

We characterized alterations to liposome properties that correlate with content release by probing changes in particle sizes caused by IP_3 using dynamic light scattering (DLS). The sizes of PC liposomes containing between 0% to 25% **1** were measured before and after IP_3 treatment. All liposomes pre-IP_3 showed uniform particle sizes with desired diameters of approximately 200 nm (grey bars in Figure 3), as expected from extrusion, indicating that these formulations are stable. After IP_3 treatment, while minimal changes in particle sizes were observed for PC control liposomes and 5% **1**/PC-liposomes,

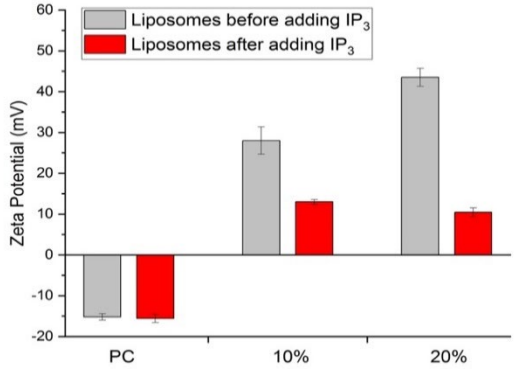

Figure 2. Zeta potential measurements for 0, 10, or 20% **1**/PC liposomes before and after adding IP_3. Both 10% and 20% liposomes exhibited positive charge initially, while IP_3 addition yielded a reduction in positive charge. PC liposomes showed virtually no ZP changes after IP_3. Error bars denote standard errors from three replicates.

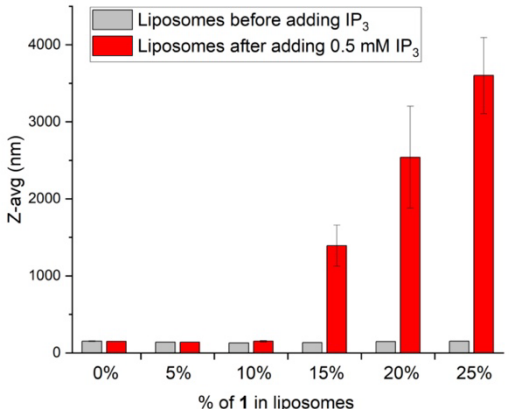

Figure 3. DLS results for 0-25% **1**/PC liposomes before (grey) and after (red) IP_3 treatment. Uniformly sized particles with desired diameters were initially formed in all cases. Upon IP_3 addition, liposomes containing 0%, 5%, or 10% **1** did not show significant size changes, while dramatic increases in particle sizes were observed for 15%, 20% and 25% **1**-liposomes. Error bars denote standard errors from at least three replicates.

dramatic increases in particle sizes were observed for 15-25% **1**-liposomes (Figure 3, red bars), which correlated with **1** percentages. These changes could also be influenced by charge since increased **1** can recruit more IP_3 to the liposome surface. Although 10% **1**-liposomes post-IP_3 addition did not show significant changes in average sizes, the polydispersity indeces (PDIs) of these samples increased significantly (Figure S5), indicating structural changes. Representative raw distribution curves for all DLS measurements are included in Figure S6. These DLS results demonstrate changes in liposome properties driven by IP_3 treatment only when **1** is present, which could be explained by alterations in lipid self-assembly by processes such as lipid reorganization (i.e., bilayer to inverted hexagonal phase) or fusion. Such processes would lead to larger particles. We additionally implemented confocal fluorescence microscopy as an alternate technique to probe the formation of larger aggregates over time. To do so, 1 mM 0% or 20% **1**/PC liposomes labeled with 0.08% rhodamine L-α-phosphatidylethanolamine (Rd-PE) were prepared and imaged before and after IP_3 addition. As shown in time-course videos S1-2 and Figure S7, both liposomes exhibited minimal initial fluorescence as their sizes were below the resolution limit of the microscope. After IP_3 treatment, the formation of ~2-3 μm aggregates was only seen for 20% **1**-liposomes.

Liposomes are also capable of encapsulating hydrophilic content within their aqueous core, which is of critical importance due to the emergence of RNA therapeutics.[17] Therefore, we evaluated hydrophilic cargo release using **1**-liposomes encapsulating the water-soluble dye sulforhohamine B (SRB). In this assay, SRB is encapsulated at high concentration such that its fluorescence is initially quenched due to collisional effects,[18] but SRB escape restores fluorescence through dilution, providing the benefit of fluorescence turn-on for sensing applications. Since SRB is non-specifically encapsulated, size exclusion chromatography (SEC) was employed to remove unencapsulated dye.

For these experiments, we formed liposomes by doping 10% of lipid switch **1** into a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-

phosphocholine (DPPC, 40%) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, 50%). DOPE is a non-bilayer lipid that can be incorporated into PC membranes[5] and we found that this destabilization was necessary for IP_3-induced release. The formation of stable liposomes was again confirmed by DLS (Figure S8). After an initial fluorescence scan, IP_3 (1 µL aliquots of a 50 mM solution) was titrated into the liposome solutions. After signal reached a plateau, the detergent Triton X-100 was added to lyse the liposomes and trigger complete content release to normalize results. When reporting data, fluorescence changes are plotted as a percentage of complete release following Triton X-100 treatment. **1**-Liposomes yielded ~40% dye release upon 8 mM IP_3 addition, while control liposomes lacking **1** treated with IP_3 or the same liposomes treated with MQ both showed minimal non-specific release (Figure 4 and representative raw spectra in Figure S9). These data support that release of polar cargo from the aqueous cores of liposomes containing **1** is mediated by IP_3, opening up potential applications in IP_3 sensing and targeted drug delivery/release using this platform.

In conclusion, we have designed and synthesized lipid **1** as an effective lipid switch for achieving IP_3-responsive liposomes. Building from our previous ATP-mediated switch, lipid **1** contains three ZnDPA units to cater to the structure of IP_3. Upon IP_3 treatment, **1** is designed to undergo a conformational change that invokes non-bilayer properties to disrupt membrane integrity and trigger encapsulated content release. Our data demonstrate that **1**-liposomes release both hydrophobic and hydrophilic content upon IP_3 addition. We also showed that the selectivity toward IP_3 can be tuned by adjusting percentages of **1** with NR release assay. This work further adds to the prospects of liposomal cargo release that could be achieved by leveraging the molecular recognition of biomolecules that are upregulated in disease states. Finally, IP_3 remains a difficult target to detect and quantify in biological systems. While conventional sensors suffer from poor sensitivity,[12] the major signal amplification caused by dye release could position the reported liposomes as effective sensors for these molecules.

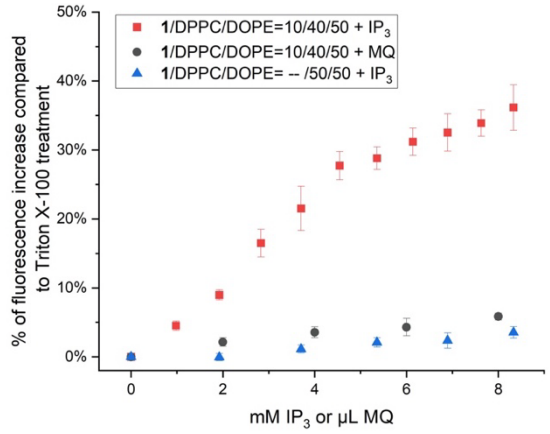

Figure 4. SRB release results for DPPC/DOPE liposomes with/without lipid switch **1** upon IP_3 treatment. Dose-dependent release was observed for liposomes containing 10% **1**, 40% DPPC, and 50% DOPE upon IP_3 titration, while control liposomes containing only DPPC and DOPE or **1**-liposomes titrated with MQ did not show background release. Error bars indicate standard errors generated from three independent trials.

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Conflicts of interest

There are no conflicts to declare.

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