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Palladium responsive liposomes for triggered release of aqueous contents

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ABSTRACT

Palladium (Pd) is a promising metal catalyst for novel bioorthogonal chemistry and prodrug activation. This report describes the first example of palladium responsive liposomes. The key molecule is a new caged phospholipid called Alloc-PE that forms stable liposomes (large unilamellar vesicles, ~220 nm diameter). Liposome treatment with $PdCl_2$ removes the chemical cage, liberates membrane destabilizing dioleoylphosphoethanolamine (DOPE), and triggers liposome leakage of encapsulated aqueous contents. The results indicate a path towards liposomal drug delivery technologies that exploit transition metal triggered leakage.

Introduction

The unique capabilities of palladium (Pd) to catalyze various bond making and breaking reactions makes it a promising choice as a catalyst for bioorthogonal chemistry and prodrug activation.[1–5] Recent work has reported a range of molecules and materials that deliver catalytic Pd to specific locations inside cells or living organisms.[1–7] A crucial experimental parameter is the Pd catalyst turnover number, which places an upper limit on the total number of caged molecules that can be activated in a designated time period. A variety of medical applications envision prodrug activation processes that produce a therapeutic dose only at the site of disease;[8] thus, raising the need for innovative Pd-activation systems that can generate a burst of active drug molecules within localized volume.

With this report, we introduce a conceptually new approach that is based on Pd-triggered release of aqueous contents from liposomes.[9–11] One way to create triggered-release liposomes is to develop chemically caged polar lipids that can be converted from a chemical structure that stabilizes a liposomal membrane into a bilayer destabilizing structure.[12–14] Chemically caged versions of 1,2-dioleoyl-*sn-glycero*-3-phosphoethanolamine (DOPE) are especially attractive. More specifically, it is well-established that *N*-acyl analogues of DOPE have a near-cylindrical amphiphilic shape and anionic charge that favours a bilayer assembly, but chemical cleavage of the *N*-acyl group produces DOPE which is a membrane destabilizing phospholipid.[14,15] Summarized in Scheme 1 are four types of stimulus (*i.e.*, light, redox, pH, enzyme activity) that have been previously used to transform *N*-acyl DOPE derivatives into DOPE, and produce liposome contents leakage.[12,13,14,16] We wondered if added Pd could be used as a chemical stimulus to uncage *N*-acylated DOPE, and we were drawn to the *N*-allyloxycarbonyl (alloc) derivative of DOPE that we designate as Alloc-PE (Scheme 1). To the best of our knowledge, this remarkably simple compound has never been described before.

Alloc-PE was prepared in one synthetic step (63% yield) by reacting DOPE with allyl chloroformate. The liposomes used in this study were large unilamellar vesicles (~220 nm diameter) composed of 100% Alloc-PE or 50:50 Alloc-PE/DOPE and readily formed using a standard lipid film hydration process followed by extrusion of the dispersion through porous polycarbonate membrane. The ability of $PdCl_2$ to completely convert Alloc-PE into DOPE was first proved by incubating Alloc-PE and $PdCl_2$ in THF/H_2O (4:1, v/v) for 1 h at 37 °C and using NMR spectroscopy to confirm the chemical change (Fig. S1). Proof that the reaction also occurred when the Alloc-PE was incorporated within liposomes was gained by treating liposomes composed of 100% Alloc-PE with 100 μM of $PdCl_2$ for 30 min at 21 °C and using ninhydrin to identify the amine group of liberated DOPE (Fig. S2). The most common chemical mechanism for Pd-promoted removal of an alloc group is a Tsuji-Trost reaction, that is, oxidative addition of Pd^0 to produce a π-allyl/Pd complex which fragments and regenerates the Pd^0 species.[17,18] There are literature reports that Pd^{2+} can associate with

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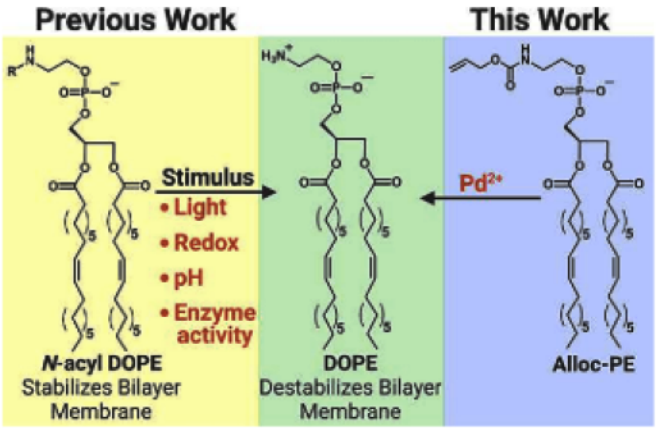

Scheme 1. Chemical structures of caged DOPE molecules. Created with BioRender.com.

anionic vesicles and be reduced to Pd^0,[19,20] but in the present vesicle system the identity of the putative reductant is not completely clear. Compounds with primary amine groups have the capacity to reduce $PdCl_2$ to Pd^0.[21] Therefore, the putative reductant could be the amine group in the DOPE. For consistency, all vesicle experiments in this study used the same buffer solution of 5 mM TES, 100 mM NaCl, pH = 7.4 with no added Pd^{2+} reductant.[5]

Initial leakage studies prepared liposomes with encapsulated carboxyfluorescein (CF), a water-soluble fluorescent dye that is self-quenched at the high concentration inside the vesicles (Fig. S3). These preliminary studies proved that Alloc-PE/DOPE (50:50) liposomes could retain aqueous contents for many days (Fig. S5). However, subsequent studies of Pd-activated CF leakage revealed an artifact – addition of $PdCl_2$ to an aqueous solution of CF induced significant quenching of CF fluorescence (Fig. S3) which introduced uncertainty into the Pd-triggered CF leakage assay. Therefore, we modified the fluorescent leakage assay, by employing the fluorophore/quencher pair of 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) and p-xylylenebis(pyridinium) bromide (DPX) which was compatible with the presence of $PdCl_2$ (Fig. S4). HPTS is a water soluble and membrane impermeant fluorophore, that is quenched when co-encapsulated at high concentration with dicationic DPX.[22] The HPTS fluorescence was generated by exciting at 413 nm which produces a pH independent fluorescence spectrum, whose intensity at 510 nm increases upon the dilution produced by liposome leakage and loss of collisional quenching efficiency by the co-encapsulated DPX (Fig. 1A).[9,23,24] As shown in Fig. 1B, there was very little HPTS/DPX escape from Alloc-PE/DOPE (50:50) liposomes over 7 days of storage. Thus, Alloc-PE/DOPE (50:50) liposomes were used to generate all the data in Fig. 1 (see Fig. S6 for a representative set of fluorescence spectra indicating HPTS/DPX leakage).

The plots in Fig. 1C compare HPTS/DPX release profiles from Alloc-PE/DOPE (50:50) liposomes in the presence or absence of 100 μM $PdCl_2$ and at 21 or 37 °C. As expected, there was more leakage at higher temperature.[25] Most experiments used a stock solution of buffered $PdCl_2$ that was prepared by an acidification/neutralization process, but we confirmed that the same rate of liposome leakage was a obtained using buffered stock solution of K_2PdCl_4 (Fig. S7).† Since, very high concentrations of divalent metal cations are known to promote leaky fusion of anionic vesicles containing *N*-acylated DOPE,[26] we compared liposome leakage induced by 100 μM of $PdCl_2$, $MgCl_2$, $ZnCl_2$, or $CaCl_2$ at 37 °C (Fig. 1D). The presence of $MgCl_2$ or $CaCl_2$ produced no measurable increase in liposome leakage over background; whereas $ZnCl_2$ increased liposome leakage by a factor of two, which was much smaller than the 8-fold increase in leakage induced by $PdCl_2$.

The following experimental results provide insight into the Pd-promoted liposome leakage mechanism; (a) The Dynamic Light Scattering (DLS) data in Fig. 1E showed that addition of $PdCl_2$ (100 μM) to the liposomes produced a large increase in average particle size and size dispersity. Moreover, the size increase could not be reversed by addition of the metal cation chelator EDTA, consistent with irreversible mixing of the liposome membranes. In contrast, DLS measurements of the same liposomes treated with equal amounts of $MgCl_2$, $ZnCl_2$, or $CaCl_2$ showed no change in particle size or size dispersity, indicating no metal cation induced liposome aggregation or fusion (Fig. S9). (b) The leakage data in Fig. 1F exhibited a near-linear relationship of Pd-induced leakage with the concentration of total polar lipid (20 to 80 μM) suggesting that leakage is promoted by liposome contact.[27–29] (c) A series of lipid probe dilution assays combined labelled Alloc-PE/DOPE (50:50) liposomes loaded with 1% 1,2-dipalmitoyl-*sn-glycero*-3-phosphoethanolamine-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD-PE) and 1% 1,2-dipalmitoyl-*sn-glycero*-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl (Rh-PE), with unlabeled target liposomes and treated the liposome mixture with $PdCl_2$. As described in Scheme S1, the lipid probe dilution assay exploits fluorescence resonance energy transfer (FRET) from the NBD-PE (FRET donor) to the Rh-PE (FRET acceptor). Intermembrane mixing dilutes the two probes and reduces the FRET efficiency, which lowers the ratio of Rh-PE /NBD-PE fluorescence intensities (see Fig. S10 for representative raw FRET data).[29] Control experiments show that the added $PdCl_2$ does not quench the NBD-PE or Rh-PE fluorescence (Fig. S11). The lipid mixing data is plotted as the change in % lipid probe mixing over time. As shown in Fig. 2A, unlabeled target liposomes comprised of Alloc-PE/DOPE (50:50) exhibited a moderate rate of Pd-induced lipid probe mixing (previous studies have shown that the probe dilution assay is insensitive to liposome aggregation without membrane mixing[30]). The lipid mixing assay was repeated using unlabeled target liposomes comprised entirely of zwitterionic 1-palmitoyl-2-oleoyl-*sn-glycero*-3-phosphocholine (POPC) and there was a faster rate of lipid probe mixing (Fig. 2B). This trend is consistent with a contact-mediated liposome perturbation process and reflects the reduced frequency that an anionic labelled Alloc-PE/DOPE (50:50) liposome will make physical contact with an anionic unlabeled Alloc-PE/DOPE (50:50) liposome (Fig. 2A) compared to physical contact with a zwitterionic unlabeled POPC liposome (Fig. 2B).[31] (d) Comparison of Fig. 1C and Fig. 2A clearly shows that Pd-induced leakage of liposome aqueous contents is a faster than the concomitant process of lipid probe mixing under essentially the same conditions and liposome composition.

Collectively, the leakage and lipid mixing results are consistent with Pd-promoted, chemical conversion of anionic Alloc-PE into zwitterionic DOPE. During early time points, the decreasing negative charge on the Alloc-PE/DOPE (50:50) liposome surface increases the frequency and life-time of liposome contact-events that mediate aqueous contents leakage.[27,28,31] On a slower time scale, the increasing fraction of DOPE within the Alloc-PE/DOPE liposomes promotes lateral phase separation within the membrane,[32,33] and eventually induces large membrane morphology changes such as lamellar to inverse hexagonal phase which enables extensive mixing of the lipid components[6,28].

In conclusion, we report the first example of Pd-responsive liposomes

[5] Switching from TES to phosphate buffer did not change the profile for $PdCl_2$-triggered leakage (Fig. S8) indicating that TES buffer is not essential as the Pd^{2+} reductant. Additional $PdCl_2$-activated liposome leakage experiments evaluated the effect of adding $P(Ph)_3$ as a Pd^{2+} reductant and observed slightly enhanced leakage in TES buffer, implicating Pd^0 as the species that removes the *N*-alloc cage. However, the enhancement in liposome leakage was modest and the effect of added $P(Ph)_3$ was not studied any further.

[6] While there is extensive mixing of lipid probes, a standard Tb^{3+}/dipicolinate assay produced no evidence for mixing of liposome aqueous contents which matches the observations of previous literature liposome studies that have exploited the capacity of DOPE to induce lamellar to inverse hexagonal polymorphism (see Ref. 28).

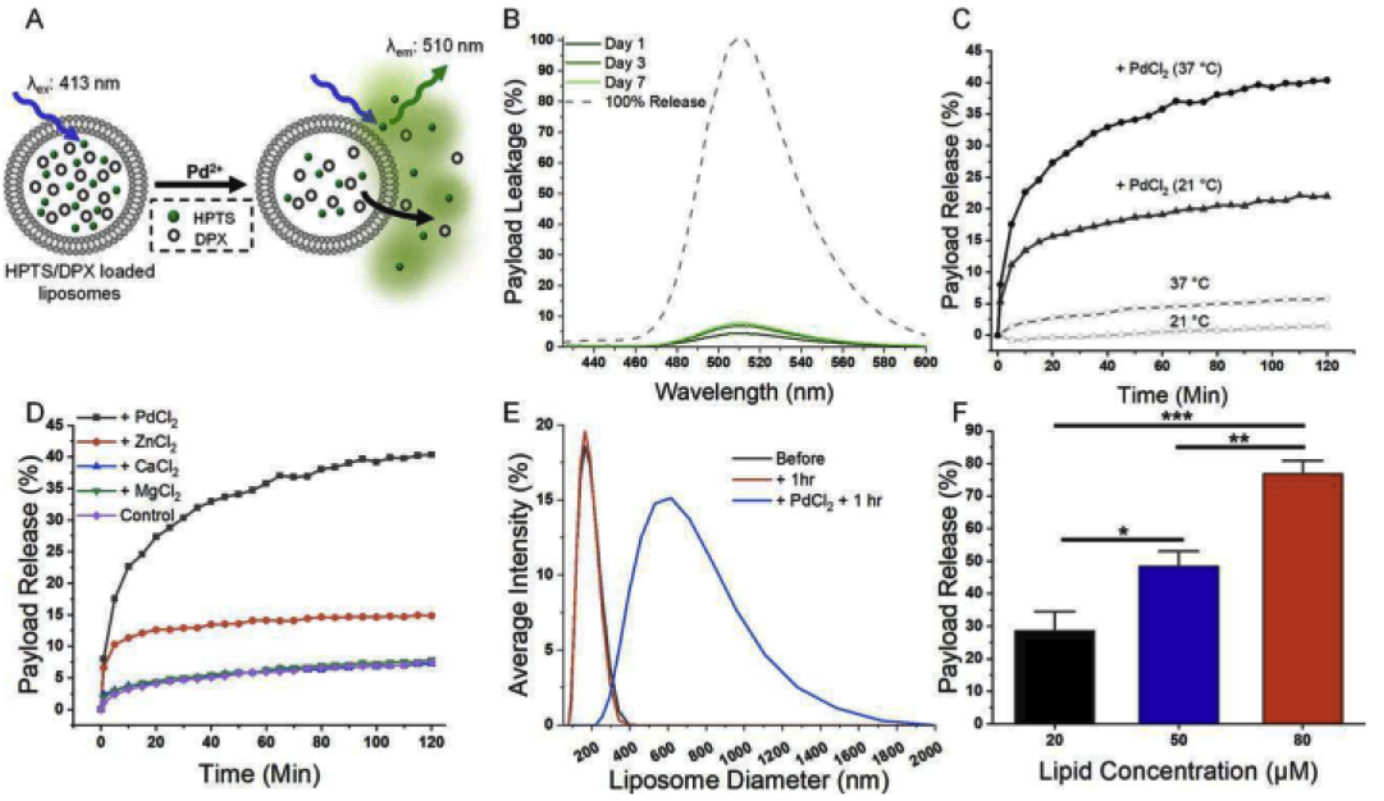

Fig. 1. (A) Schematic summary of liposome leakage assay using the fluorophore/quencher pair HPTS/DPX. (B) Extent of spontaneous HPTS/DPX (payload) leakage from **Alloc-PE**/DOPE (50:50) liposomes over one week of storage. (C) Percentage of HPTS/DPX (payload) release from **Alloc-PE**/DOPE (50:50) liposomes in the presence or absence of 100 μM $PdCl_2$ and at 21 or 37 °C. Data set is representative of triplicate experiments. (D) Percentage of HPTS/DPX (payload) release from **Alloc-PE**/DOPE (50:50) liposomes after addition of 100 μM $PdCl_2$, $MgCl_2$, $ZnCl_2$, or $CaCl_2$ at 37 °C. Data set is representative of triplicate experiments. (E) Three Dynamic Light Scattering (DLS) measurements of scattering intensity for **Alloc-PE**/DOPE (50:50) liposomes, before, one hour later, and one hour after addition of 100 μM $PdCl_2$ at 21 °C. Each DLS profile is representative of three or more independent studies. All liposome experiments in TES buffer (5 mM TES, 100 mM NaCl; pH = 7.4) with encapsulated HPTS (35 mM) and DPX (50 mM); unless stated otherwise total polar lipid concentration was 50 μM. (F) Percentage of HPTS/DPX (payload) release from **Alloc-PE**/DOPE (50:50) liposomes at 2 h after addition of 100 μM $PdCl_2$ at 37 °C. The only difference between experiments is the concentration of total polar lipid, which is 20, 50 or 80 μM. Data is the average of triplicate experiments with error bars representing ± SD. (HPTS λ_{ex}: 413 nm, λ_{em}: 510 nm) * = $p < 0.1$, ** = $p < 0.01$, *** = $p < 0.001$.

for triggered release of aqueous contents. Notable practical points are the straightforward one-step synthesis of the caged phospholipid, **Alloc-PE**, and the ready availability of $PdCl_2$ which makes it a very convenient chemical trigger for future mechanistic studies of bilayer destabilization using model and biological membranes.[15] Chemical precedence and preliminary evidence indicate the reactive palladium species to be *in situ* generated Pd^0, and one of the goals of ongoing work is to determine if added reductants or Pd-binding ligands enhance the membrane disruption effect†.[18,34,35] Another future goal is to expand the molecular design concept to different classes of caged polar lipids,[11] and transition metal catalysts beyond palladium.[36–39] Eventually, it may be possible to develop liposome-based drug delivery technologies based on transition metal promoted release. One possible scenario is a two-step treatment strategy that first targets a site of disease with a transition metal-antibody conjugate or alternatively an implanted material containing the transition metal catalyst,[1,6,7] and subsequently doses the patient with drug-containing transition metal-responsive liposomes for site specific release.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data (experimental procedures, synthesis and compound characterization, supplementary figures) to this article can be found online at https://doi.org/10.1016/j.bmcl.2023.129215.

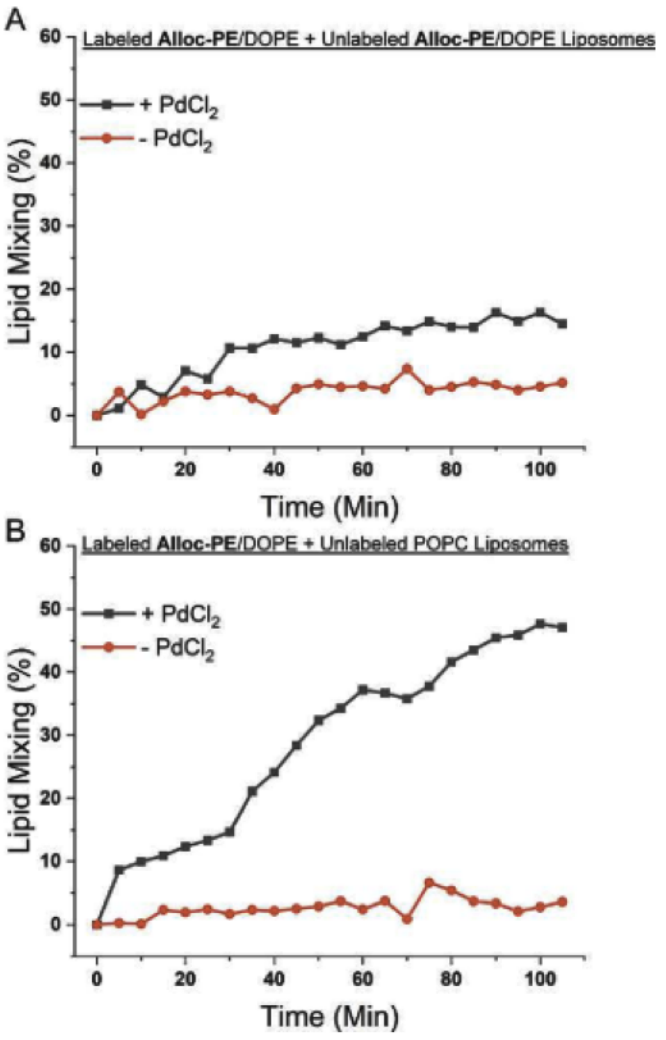

Fig. 2. Change in the % lipid probe mixing for a sample of labeled liposomes composed of **Alloc-PE/DOPE/NBD-PE/Rh-PE** (50:48:1:1, 20 μM total lipid) plus unlabeled, (A) **Alloc-PE/DOPE** liposomes (50:50, 80 μM total lipid); or (B) POPC (80 μM total lipid). Samples in 5 mM TES, 100 mM NaCl pH 7.4 buffer at 37 °C. In each case, an aliquot of buffer alone (-$PdCl_2$) or 100 μM $PdCl_2$ was added to each sample at time = zero, and fluorescence intensities were monitored over 105 min (ex: 464 nm). Data set is representative of duplicate experiments.

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