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Exit-strategies – smart ways to release phospholipid vesicle cargo†

Denia Mellal and Andreas Zumbuehl*

This highlight describes recent trends in fundamental phospholipid research towards possible future drug delivery technology. In particular it focuses on synthetic phospholipids and their vesicular constructs and describes selected "smart" ways to release cargo from liposomes. Various chemical and physical release triggers are discussed such as temperature changes, application of ultrasound, enzyme degradation, changes in pH, redox reactions, photochemical reactions, as well as the effects of shear stress on vesicles.

1. Introduction

Liposomes or vesicles are made from one or more concentric lipid bilayer spheres entrapping an aqueous volume.[1] Liposomes can carry drugs in the vesicle's water cavity, the hydrophobic bilayer or attached to the polar lipid surface.[1] This versatility makes liposomes natural drug delivery vehicles and soon after the discovery of vesicles[2] the first drugs were encapsulated.[3] Currently, 13 liposomal drug delivery formulations are approved by the FDA and a similar number of formulations are in various stages of clinical trials.[3]

Department of Chemistry, University of Fribourg, Chemin du Musée 9, 1700 Fribourg, Switzerland. E-mail: andreas.zumbuehl@unifr.ch; Tel: +41 26 300 8794

† This manuscript is dedicated to Tobias Valentin who was born the day after we finished writing the paper.

The volume of a standard liposome with a diameter of 100 nm is surprisingly small: 400 zeptolitres (400×10^{-21} L).[4] To put that in relation: a vesicle that was loaded with a 2 mM solution of doxorubicin[5] will optimally contain 500 molecules of the drug.[4] If used in a smart fashion, a liposome will therefore deliver only a limited amount of a drug and will not flush the human organism with sextillion (10^{21}) copies of a molecule as we currently do. Through decoration of the outer membrane of a liposome, vesicles can be kept in the blood circulation for longer periods of time leading to tumor (outer rim) uptake through the enhanced permeability and retention effect and eventual release of the vesicle cargo.[3] Besides this passive approach, it should be possible to actively trigger drug release from liposomes.[6] Here, we could take advantage of the fact that liposomes are self-assembled from roughly 10 000 molecules[4] and that the vesicle composition can easily be modified in the formulation stage.[7] Several possibilities are mentioned in the

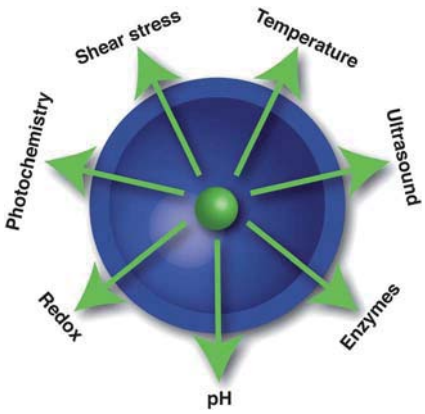

Fig. 1 This highlight describes various ways to actively release a cargo molecule from a phospholipid liposome. The release triggers are either based on endogenous changes found in the human body (changes in temperature, enzymatic concentration, pH, redox, and shear stress) or changes that are applied exogenously (ultrasound or photoreaction pulses).

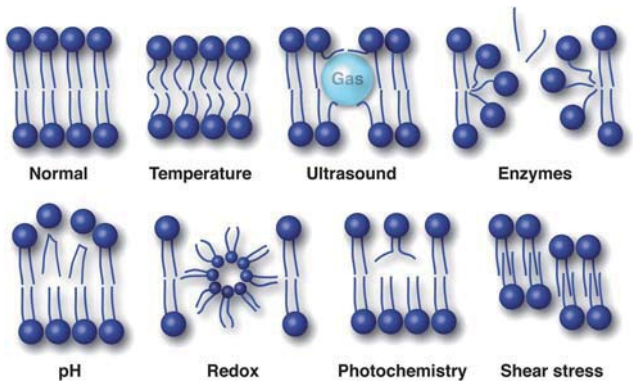

Fig. 2 Schematic view of the effects of various release-triggers. Membrane permeability will be affected if the temperature is raised above the T_m, if gas bubbles disorder the hydrophobic core of the membrane, if tails or headgroups are chemically removed from the phospholipids, if the shapes of phospholipids are changed by reducing the size of the headgroup or by inducing a significant kink in the fatty tails, and if membrane defects are attenuated by the application of shear forces. The same conformational and constitutional changes on the phospholipids can be induced by different triggers.

following paragraphs, highlighting the potential of artificial phospholipids (see Fig. 1).

2. Triggered release from liposomes

2.1. Temperature as trigger

Lipid bilayers undergo a gel phase-to-liquid phase transition at a specific temperature (see Fig. 2 for a schematic overview of the discussed effects).[8] The phase transition (T_m) is influenced by the torsional angle found between neighboring CH_2 groups in the *n*-alkane part of the phospholipids with the gel phase being represented by an all-anti-periplanar (or all-*trans*) conformer and the liquid phase showing several *gauche* interactions leading to a reduced alkane packing density.[8] At the T_m a coexistence of gel-phase lipid domains in liquid phase bilayers is found similar to ice floating in already molten water.[9] At the phase boundaries between solids and liquids, lipid chain packing defects and therefore lateral density fluctuations ensue, resulting in a maximum of the passive membrane permeability at the T_m: the membrane becomes leaky.[10–12]

The T_m not only depends on the lipid chain conformation but can also be influenced by the headgroups of the phospholipids with phosphatidylethanolamine (PE) headgroups showing significantly higher T_m compared to phosphatidylcholines (PC).[8] Different phospholipids therefore show different passive transbilayer transport characteristics.[13] If various phospholipid types are mixed during vesicle preparation[7] the bilayer T_m can be tuned and differences in temperature can then be used as a trigger to release cargo from liposomes.[9] A particularly nice example is a vesicle consisting of DPPC : HSPC : Chol : DPPE-PEG2000 mixed in a molar ratio of 50 : 25 : 15 : 3 leading to a $T_m = 41.9$ °C.[14] In other words: mild hyperthermia of 42 °C will induce drug release. This opens promising new routes for minimally invasive cancer treatment: the vesicles were loaded both with a gadolinium-MRI tracer and the anticancer agent doxorubicin. Using MR-guided high-intensity focused ultrasound hyperthermia can be induced locally to deep-seated solid tumors.[14]

The challenge lies in finding a liposomal system that releases its cargo rapidly in order to minimize the time a patient needs to spend in a machine.[15] Unfortunately, liposomes will reseal if the temperature drops below T_m and once more the passive transbilayer permeability drops significantly, *i.e.* the hyperthermia conditions need to be maintained for the duration of the treatment.[9] A possible solution to the slow release mentioned is to simply destroy the vesicular envelope using lower frequency ultrasound.

2.2. Ultrasound as trigger

High-frequency ultrasound (>1 MHz) mainly leads to thermal effects as stated in Section 2.1. In contrast, low-frequency ultrasound has a profound mechanical effect on a bilayer membrane such as acoustic cavitation.[16] An ultrasound wave will cause the growth and oscillation of a gas bubble leading to high strain exhibited on the membrane. If the gas nuclei are formed in the hydrophobic region of a liposomal membrane, it can lead to transient membrane pore formation and drug release.[16] The effects can be amplified by the presence of lipids containing unsaturated fatty acyl chains that form less densely packed bilayers. Furthermore, an effective absorption of ultrasound by a phospholipid bilayer membrane is maximized at the T_m.[16]

Using HSPC : Chol : DSPE-PEG2000 in a molar ratio of 51 : 44 : 5, it was shown that several types of encapsulated drugs were almost completely released from the vesicles applying low-frequency ultrasound for less than 3 minutes.[17] The sensitivity towards ultrasound can be increased by using non-bilayer forming lipids such as DOPE.[18] Additionally, liposomes can be filled with ultrasound-sensitive emulsions such as perfluorocarbons.[19] Ultrasound will induce a perfluorocarbon

phase change from liquid to gaseous and the continued application of ultrasound will cause the cavitation of the gas bubble leading to vesicle membrane disruption.[19]

2.3. Enzymes as trigger

Roughly half of the phospholipids found in a biomembrane are prone to induce a lamellar to inverted hexagonal membrane phase change.[20] Such a transition leads to the formation of a lipidic transmembrane pore. Phosphatidyl ethanolamine headgroups are less hydrated than their phosphatidyl choline counterparts. This leads to an increase of the Israelachvili lipid packing factor and a lipid shape change from cylindrical to conical.[8] The laminar bilayers membrane reacts to this change by locally inducing an inverted hexagonal phase, *i.e.* a lipidic transmembrane channel.

An even more dramatic change can be seen by the action of phospholipase C on glycerophospholipids leading to diacylglycerols (see Fig. 3 for lipids discussed throughout this text).[21] At 37 °C one diacylglycerol can influence the packing of 15 neighbouring phosphatidylcholine or 98 phosphatidylethanolamine lipids.[22] This number has to be put into relation with the 100 molecules found in an inverted micelle that will ensue, when diacylglycerols induce a lamellar to inverted hexagonal membrane phase transition.[22] Having phospholipids that are prone to form inverted hexagonal phases alongside with phospholipids that are forming lamellar phases leads to an equilibrium that might tilt to either point depending on the ratio between the lipids.[20]

The phospholipase PLA_2 superfamily includes a broad range of enzymes defined by their ability to catalyze the hydrolysis of the ester bond at the *sn*-2 position of phospholipids, providing a free fatty acid and a lysolipid, having a cone-shaped packing factor.[8] Therefore, the action of PLA_2 on a glycerophospholipid leads to the formation of pores and is a common pathway of action of neurotoxins.[23] On the other hand, secretory PLA_2 enzymes are often overexpressed in human tumors and the enzyme might be utilized as a targeted trigger for liposomal delivery of cancer drugs.[24] Interestingly, the incorporation of a lipopolymer (DPPE-PEG2000) or a short chained DCPC both significantly increase the rate of PLA_2 action.[24]

Artificial phospholipids were synthesized containing a non-hydrolyzable ether bond in the *sn*-1-position and a standard acyl ester in the *sn*-2-position. Liposomes formulated from these lipids were equally prone to PLA_2 degradation at *sn*-2 as their natural bis-ester counterparts.[25] However, the ether-lysolipid

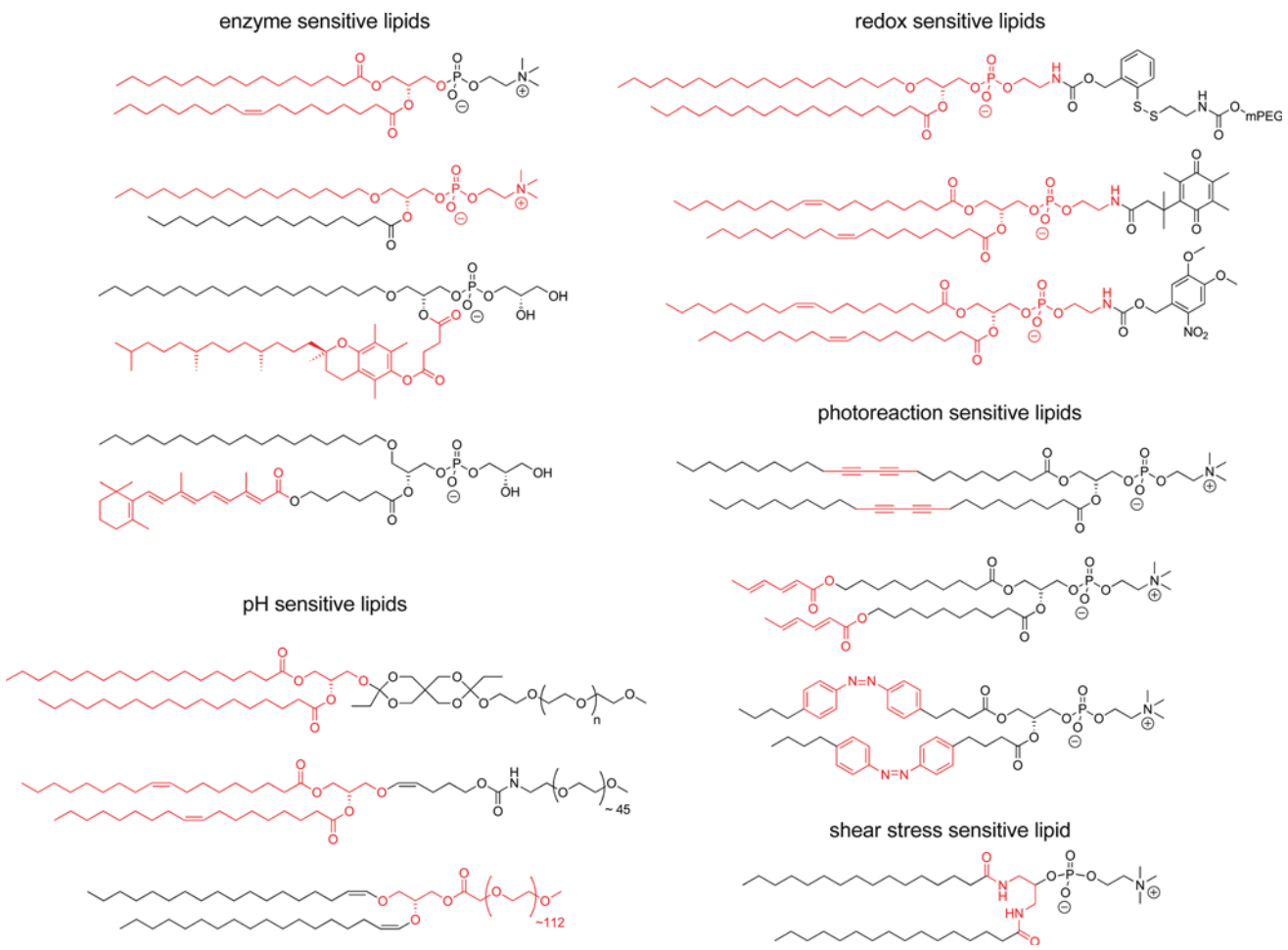

Fig. 3 A selection of "smart", reactive phospholipids and their triggers. The parts of the molecules that are important for inducing liposome cargo release are highlighted in red.

formed strongly resembles the anti-tumor agent edelfosine. This lysolipid is highly toxic due to its detergent-like character once the non-toxic phospholipid is unmasked by enzymatic action.[25,26]

Another system used secretory PLA_2 sensitive retinoid-phospholipids for the design of liposomal drug-delivery systems.[27] Here, the lipid backbone of the prodrug contains either an *sn*-1-ester or the metabolically more stable *sn*-1-ether function. A C6-linker has been incorporated between the glycerol backbone of the lipids and the retinoids to confer more flexibility to the retinoid-phospholipid prodrugs in the $sPLA_2$ active center of the enzyme and thereby enhancing its activity with a complete prodrug hydrolysis within 24 h.[27] Similarly, α- and δ-tocopheryl succinates, two potent cancer cell growth inhibitors, were linked to the *sn*-2 position of a phospholipid backbone.[28] Due to their membrane permeability, a non-covalent incorporation into a liposomal drug delivery system would not be possible. However, it turned out that the incorporation of the tocopherol derivatives made the prodrug a poor substrate for secretory PLA_2-triggered release.[28]

2.4. pH changes as trigger

The microenvironment of a cancer has a lowered pH value (pH = 6–7) compared to the healthy surrounding tissue.[29,30] Similarly, in the endocytotic pathway of any cell, the pH value inside the endosomal compartment drops to 6.5 in the early endosome, 5.0–6.0 in the late endosome, and 4.0–5.0 in the lysosome.[31] Clearly, such changes in proton concentrations that range over 3 orders of magnitude should be able to serve as a trigger for liposomal drug delivery. The typical pH-sensitive liposomal formulations include cone-shaped phospholipids like DOPE and a weak acid such as cholesteryl hemisuccinate. However, these formulations loose their fusogenicity in serum, due to opsonisation. Protection of the liposomal envelope with a PEG coating seems to significantly lower the responsiveness of the system[32] and other approaches for pH-sensitive drug delivery are needed.[33]

A classic amongst fast-acting pH-labile protecting groups are orthoesters. They have been cleverly placed as linkers between a distearoyl glycerol scaffold and a polyethylene glycol polymer headgroup.[34] *In vitro*, the polymer coating is serving as a spacer between vesicles. Upon hydrolysis of the orthoester, the unshielded vesicles can come closer to each other and form inter-membrane stalks leading to lamellar-to-inverted hexagonal phase changes and cargo burst release.[29] The inter-membrane contact is essential as initially only 16 molecules (PEs) on each membrane are involved in stalk formation.[22] The resulting inverted micelle itself initially only contains 100 molecules.[22]

Vinyl ethers undergo an acid-catalyzed hydrolysis. Incorporated in low ratios (1–5%) into DOPE liposomes, the vinyl ether lipids were able to stabilize a lamellar vesicular system until the vinyl ether bonds were hydrolyzed, leading to the disintegration of the liposome.[35] A significantly faster reacting system was found when the acid-labile vinyl ether or ketene acetal bond was relocated to the lipid headgroup.[36] The DOPE : vinyl ether lipid 90 : 10 liposomal mixtures are stable for at least 2 days at pH = 7.5 and carry a PEG coating against opsonisation.[36] Currently, they represent one of the most promising triggerable vesicular drug delivery systems.[37]

2.5. Redox reactions as trigger

The cytoplasm is a more reducing environment than the blood plasma.[29] This is mainly due to a high sulfhydryl concentration, *e.g.* of glutathione.[29] Therefore, dithiol reduction would be a viable way to release a cone-shaped phospholipid from a cylindrical precursor molecule. For instance, an *ortho*-dithiobenzyl-linked mPEG-DSPE was completely reduced by cysteine within 30 min.[38] This resulted in a 100% release of an entrapped fluorescent marker in 10 min only from a vesicle formulated from PHPC : cholesterol : dithiol lipid in a molar ratio of 95 : 5 : 3.[38]

In combination with the enhanced permeability and retention effect, liposomes could be exposed to the upregulated quinone reductase enzyme found near cancerous tissue.[39] Therefore, a quinone phospholipid was synthesized that, upon reduction of the quinone to a hydroquinone, would undergo a cascade reaction to set free a molecule of DOPE.[39] The formulated vesicles were stable for at least a week, and, as expected, upon dithionite reduction the entrapped calcein was released in about 5 hours.[39] An improved formulation including DOPE and small amounts (≤3%) PEG2000-DOPE significantly shortened the cargo release to 30 min only.[40]

2.6. Photo-reactions as trigger

Light can be used in three different ways in order to release a cargo from phospholipid vesicles: lipids can be uncaged, polymerized, or they can undergo *trans*/*cis* isomerization.[41] This leads to an interesting liposomal toolbox for topical applications (due to the low tissue penetration of light) and mainly biochemical applications.[6,42]

The use of photocleavable amine protecting groups is an attractive means to mask the headgroup of a PE phospholipid and inducing a lamellar phase preference of the molecule.[43] The irradiation of 6-nitroveratryloxycarbonyl protected lipid with a 150 W UV light lead to an ~90% deprotection of the amine in 20 min.[43] Applied to a vesicular system, a beautiful first order rate release of entrapped calcein was noted, unfortunately its applications are limited due to photobleaching effects.

The light-triggered activation of polymerizable phospholipids leads to solid domains within liquid phospholipid membranes. Similar to the temperature effects seen in Section 2.1, this translates into increased membrane permeability at the phase boundaries.[41] When a liposomal formulation of cholesterol : bis-SorbPC : DOPC : PEG200-DOPE in a molar ratio of 40 : 30 : 15 : 15 was exposed to UV light for 5 min, the bis-SorbPC was completely polymerized and this leads to a 200-fold increase of membrane permeability for water-soluble compounds.[44] Diacetylene lipids such as ($DC_{8,9}PC$) are classical polymerizable phospholipids.[45] DPPC : $DC_{8,9}PC$ vesicles were formulated at a molar ratio of 86 : 10 and loaded with doxorubicin.[46] Raji cells and liposomes were irradiated at

514 nm for 5 min which resulted in half of the cells being killed in this short period of time.[46]

Bis-azo phospholipids undergo wavelength-dependent *trans*/*cis* isomerization under UV light. This transition has a direct influence on membrane packing.[47] Indeed, DPPC : cholesterol : bis-azoPC formulations at a molar ratio of 69 : 25 : 6 showed an complete loss of entrapped calcein when exposed to light at 470 nm for 30 seconds.[48] In a follow-up study it was shown that cargo-release could be triggered even by a single (10 ns!) laser pulse.[49]

An additional release pathway leads through the incorporation of a non-lipidic photosensitizer into the liposomal membrane such as trisulfonated aluminum phthalocyanine[50] or aluminum phthalocyanine disulfonic acid[51] leading to a singlet-oxygen mediated oxidation of lipid double bonds and continuous release of cargo molecules.[51]

2.7. Shear stress as trigger

The differences in shear-stresses in the human body could be used as a drug delivery trigger, as has been reported very recently.[52,53] This purely physical trigger is interesting in situations where biological targeting is not viable, *e.g.* in the case of atherosclerosis where the inflammation signals of the plaque are not different from other inflammation signals found in the body.[54] On the other hand, standard wall-shear stresses measured in the blood stream are around 1.5 Pa and rise more than one order of magnitude close and at the site of a blood vessel constriction.[55]

In order to use phospholipid vesicles as shear-sensitive drug delivery devices, two major obstacles must be overcome. First, spherical vesicles will only shear at around 40 Pa, a value that is too high for *in vivo* applications; this calls for non-spherical, faceted particles.[56,54] Second, a vesicle formulation is needed that is not-releasing its cargo when at rest but releases its contents when shaken. Natural phospholipid vesicles are either leaky in both cases (eggPC) or do not release in both cases (DPPC or 16:0 SM).[52]

Recently, faceted self-assembly systems based on catanionic lipids have gained significant interest in the soft matter community.[57,58] The combination of a high bilayer stretching energy and a lower bending energy leads to a high Föppl–van-Kàrmàn ratio and the formation of non-spherical particles.[59] A synthetic 1,3-diamidophospholipid, Pad-PC-Pad, has been reported where the fatty acyl tails are spaced in the non-natural 1,3-fashion compared to the natural 1,2-constitution.[60] This possibly leads to easier membrane leaflet interdigitation, which would impose a high bilayer stiffness.[61] Indeed, vesicles formulated from Pad-PC-Pad are non-spherical and show a lenticular morphology.[52]

Vesicles formulated from pure Pad-PC-Pad were not releasing the entrapped 5(6)-carboxyfluorescein when left untouched on the bench. As soon as the vesicles were shaken, either by hand or by vortex shaker, 40% of the cargo was released within one minute.[52] Additionally, *in vitro* assays showed a significant difference of vesicle cargo release when the liposomes were exposed to an artificial blood circulation containing either a "healthy" or "stenosed" plastic artery model.[52]

3. Conclusions

During the past years, chemical lipidology has led to a large variety of synthetic phospholipids. Each of these lipids was formulated into vesicles that reacted to different external stimuli, significantly enhancing the potential of liposomal drug delivery. The power of the vesicles lies in the self-assembly approach that allows for simple mixing of different non-toxic liposome components. Through the synthesis of additional types of phospholipids, a new generation of "smart" vesicles becomes accessible, and hopefully will have an impact as future delivery tools in biology and medicine.

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