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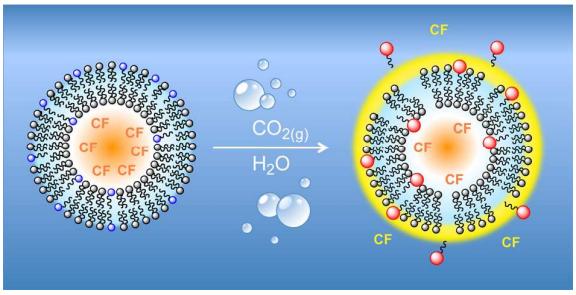

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CO_2-triggered release from switchable surfactant impregnated liposomes

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Incorporation of an amidine-based switchable surfactant into the lipid membrane of a liposome produces a system that is capable of triggered release upon *in situ* exposure to CO_2. The amount of liposomal contents released is dependent on the concentration of switchable surfactant incorporated. A mechanism of the release is proposed.

Liposomes have been widely investigated for their potential as drug delivery systems in order to decrease toxicity and increase efficacy of current therapeutic drug regimes.[1] The current generation of approved liposome/drug formulations employ passive diffusion release methodologies, making the regulation of delivery difficult.[2] Recently, various methods for providing greater control over the release of their contents have been investigated using 'stimuli-sensitive' liposomes.[3] These possess either a structural modification to the lipid itself,[4] or the inclusion of an additional constituent, which is able to induce release.[5] A change in conditions or an externally applied source of energy is used to trigger release, for example temperature,[6] pH,[7] light,[8] oscillating magnetic field[9] or ultrasound.[10] The use of triggered release systems in a physiological system requires the system and the trigger to display good biocompatibility. Carbon dioxide (CO_2) is a potentially attractive trigger for initiating release from a drug delivery system due to its benign nature, and it is generally present in biological systems in significant quantities as a key metabolite present in the respiratory system and at sites of high metabolism. Furthermore, CO_2 displays a high degree of membrane permeability and high localised levels of CO_2 in the body can be related to metabolic diseases.[11]

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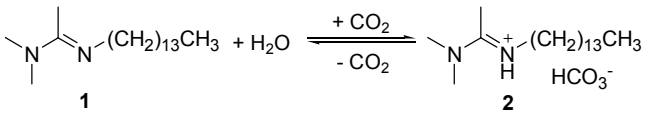
Electronic Supplementary Information (ESI) available: [Experimental procedures are documented including the synthesis and characterisation of surfactant molecules, measurement of critical micelle concentrations, liposome preparation, fluorescence assay procedures, and dynamic light scattering measurements]. See DOI: 10.1039/c000000x/

One possible avenue in the development of controlled solute release from liposomes is the incorporation of a switchable surfactant. A switchable surfactant can be reversibly converted, typically via a chemical[12] or photochemical[13] trigger, into a 'prosurfactant' whose amphiphilic nature is greatly reduced with an associated increase in lipophilicity (Scheme 1). This allows the prosurfactant to reside in the lipid bilayer, until 'switched' to release liposomal contents.[14] A number of systems have been previously described incorporating switchable surfactants with weakly acidic (anionic),[15] weakly basic (cationic),[16] or acid-cleavable headgroups.[17] The switching regimes of these systems are primarily induced by the direct addition of acid.

Of all the classes of switchable surfactants the alkyl-amidines have drawn much interest due to their ability to rapidly form and break emulsions upon the addition of $CO_{2(g)}$, presumably through the *in situ* formation of carbonic acid (Scheme 1).[12] The CO_2 responsive amidine headgroup has also recently been utilised in polymeric vesicles by Yan *et al.*, which can be triggered to induce expansion and contraction of the vesicle, leading to release of its contents. Significant leakage and cessation were achieved with the administration of CO_2 and argon, respectively, over an hour timescale.[18]



Scheme 1. CO_2 induced interconversion between prosurfactant (**1**) and the surfactant (**2**).

As phospholipid-based vesicle systems have been widely studied due to their high biocompatibility and stability, alongside their current acceptance in clinical practise, it is reported herein preliminary release studies for a system that incorporates the amidine prosurfactant (**1**) into the lipid bilayer of a liposome, which

upon the addition of either $CO_{2(g)}$ or $HCl_{(aq)}$ induces a switch to the surfactant form and leads to the rapid release of the encapsulated fluorescent carboxyfluorescein (CF), where the degree of leakage can be tuned through small modifications in the liposome formulation (Figure 1).

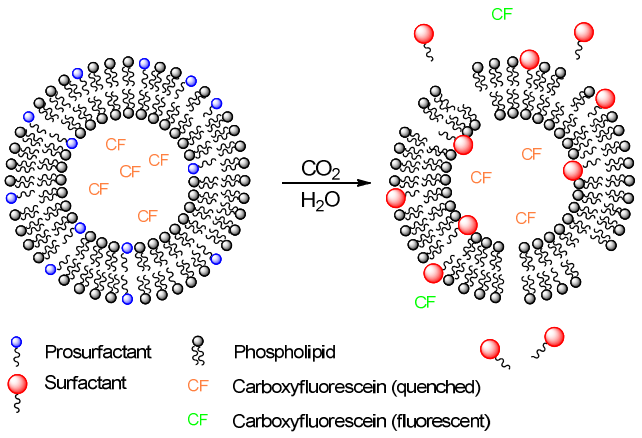


Figure 1. CO_2-induced release of CF from liposomes impregnated with amidine-based switchable surfactants.

The amidine surfactant, *N'*-tetradecyl-*N,N*-dimethyl-acetamidinium chloride (**2**), and its prosurfactant form, *N'*-tetradecyl-*N,N*-dimethylacetamidine (**1**), were synthesised using a method adapted from Harjani *et al.* (Scheme 1).[11] The critical micelle concentration (CMC) of **2** with either a chloride or bicarbonate counterion was determined, by conductivity, to be 2.8 ± 0.1 and 3.5 ± 0.2 mM, respectively. The apparent pKa of octylamidine was previously found to be 12.2 and is thought be comparable to the pKa of **1**.[12] Additionally, the lipophilic forms of pH sensitive amphiphiles are known to be stabilised within a bilayer with a subsequent reduction on the order of one pKa unit.[13] Accordingly, the initial pH of the system was set to 12.4 to ensure a significant portion of the prosurfactant was present.

To ensure that the majority of the prosurfactant protonates upon pH decease, the initial and final target pHs were selected to be 12.4 and 7.4 respectively. Assuming the pK_a of the prosurfactant in the membrane to be approximately 11.2, this would allow for 94% percent to be in the prosurfactant form prior to switching, and approximately 100% in the surfactant form subsequent to the pH decrease. Compound **1** was incorporated into liposomes consisting of 100:5 mol/mol ratio of the lipid 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol (chol) at 0, 5, 10 and 20 mol% of DPPC. Liposomes were formed by extrusion through a 200 nm polycarbonate membrane and the size confirmed by dynamic light scattering (DLS). Incorporation of **1** was found to increase membrane permeability and higher concentrations were found to be unviable, causing spontaneous CF leakage.

The triggered release of CF from the liposomes was monitored using fluorescence spectroscopy. Briefly, CF was encapsulated at a high, fluorescence quenching, concentration (100 mM in 20 mM Tris at pH 12.4) within unilamellar liposomes (~ 200 nm diameter) using film hydration and membrane extrusion. Free CF was separated from the liposomes by size-exclusion chromatography using a SephadexTM G-100 stationary phase. CF leakage from liposomes results in increasing fluorescence which was related to the amount of CF released.

At pH 12.4 all aforementioned suspensions were found to exhibit minimal fluorescence over time, suggesting that the integrity of the liposome bilayer is not unduly compromised by the incorporation of small amounts of **1**. The pH was then reduced to 7.4, by adding 100 µL of the stock liposome solution (pH 12.4, 1 mM) to 1.9 mL of Tris buffered (20 mM) Milli-Q™ water. After 250 s this resulted in a total of 6% CF release from control liposomes, whereas, the incorporation and subsequent switching of **1** caused a maximum of approximately 20 and 35% of the total CF to be released, when switched with CO_2(g) and HCl(aq) respectively (Figure 2A). No significant release was observed in the absence of these triggers (see supporting information)

The amount of CF released was found to be dependent on the percentage of compound **1** incorporated. This is thought to be due to greater amounts of **2** causing more significant bilayer disruption inducing a phase change, during which CF leaks, as the DPPC rearranges to accommodate the protonated headgroup. Unexpectedly, a further increase in **1** to 20% caused a decrease in CF release. The degree of protonation is expected to be similar in each case, given that the prosurfactant is a minor component of the system. This would suggest that an increased concentration of prosurfactant/surfactant reduces the permeability of the lipid bilayer to CF.

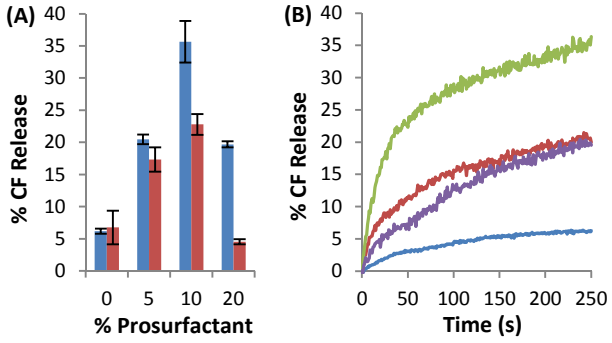


Figure 2. (A) Total release after 250 s induced by HCl (blue) and CO_2 (red). (B) Percentage CF release from DPPC liposomes with the following prosurfactant/lipid ratios; 0 % (blue), 5 % (red), 10 % (green) and 20 % (purple) induced by a change in pH from 12.4 to 7.4 over 250 s.

The rate of release was monitored over 250 s, as no appreciable increase in the percentage release was observed after this time when measured for 30 minutes. It was observed that the majority of the CF is released within the first 75 s (Figure 2B), after which the rate of release significantly decreases to a constant rate that is similar for all systems incorporating compound **1**. This suggests an equilibrium has been reached and CF release is due to passive diffusion across the bilayer.

Compound **1** was also 'switched' by bubbling $CO_{2(g)}$ through the various aforementioned liposome suspensions until a pH of 7.4 was achieved. Total CF release with respect to the concentration of **1** exhibited the same general profile as that observed via 'switching' with the addition of $HCl_{(aq)}$, with a maximum CF release at 10% incorporation of **1**. The percentage of CF released by $CO_{2(g)}$ addition was less than the 35% released with $HCl_{(aq)}$ (Figure 2A and 3A), which could be attributed to the different counterion.

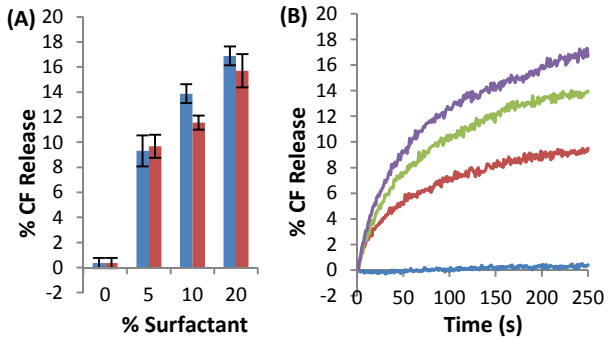


Figure 3. (A) Total release at 250 s from the addition of **2** with either a chloride (blue) or bicarbonate counterion (red). (B) Percentage CF release from DPPC liposomes at pH 7.4 via the addition of **2**.Cl in the following percentages relative to the concentration of DPPC; 0 (blue), 5 (red), 10 (green), 20 (purple) over 250 s.

The nature of the counterion has been shown to affect the CMC, as previously stated, and other physical parameters such as counterion binding, liposome solubilisation and leakage.[14,15] To further elucidate the mechanism of release, CF release was also induced from DPPC/Chol liposomes (0% **1**) at pH 7.4 by the addition of **2** at concentrations corresponding to the 5, 10, and 20% prosurfactant incorporation (Figure 3A). Unlike the systems in Figure 2, CF release was proportional to an increase in surfactant concentration. Maximum release via the external addition of **2** was also found to be significantly less (17% vs. 35%) when compared to systems where **1** was 'switched-on' to form **2** within the bilayer. These differences are thought to arise because, when added externally, **2** must first partition between the aqueous medium and the membrane before bilayer disruption can occur. This would lead to a smaller amount of surfactant that is incorporated into the bilayer relative to the system where the surfactant is 'switched-on' *in situ*. Consequently, less membrane disruption would occur, therefore inducing less CF leakage.

The requirement for the surfactant to partition is also thought to be the reason why the release profiles show a slower decline in the rate of CF release and, at least initially, a similar rate of release with different surfactant concentrations (Figure 3B). Interestingly, the nature of the counterion has a more profound effect on CF release when the profurfactant is incorporated into the lipid membrane and 'switched on' *in situ*, as opposed to external addition.

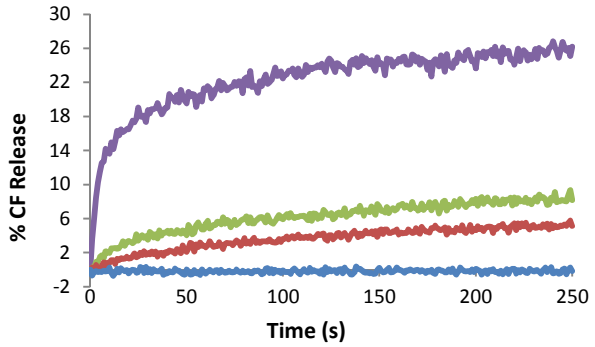


Figure 4. Percentage CF release from liposomes incorporating 10 % of **1** after a pH change from 12.4 to 7.6 (purple), 8.0 (green), and 8.5 (red), or at 12.4 (blue).

The effect of final pH on the behaviour of CF release was also investigated. DPPC liposomes incorporating 10% of compound **1** were added to unbuffered water to give final pH values of 7.6, 8.0, 8.5 and 12.4 (Figure 4). An increase in final pH resulted in a decline in both the initial rate and total amount of CF released. This result suggests that the leakage is controlled by the degree of protonation of the prosurfactant.

To determine if the surfactant can induce repeated release events, the system was 'switched' back and forth between pH 12.4 and 7.4, through the addition of small volumes of concentrated HCl and NaOH. After the initial change in fluorescence, no significant change was observed after the second pH switch (Figure 5), even allowing for small changes in the ionic strength resulting from the switching events. This supports the supposition that CF release is a result of 'switching' the prosurfactant and is not simply due to equilibration of the proton gradient across the lipid bilayer. The lack of release after a second switching cycle also suggests that the surfactant is no longer located in the lipid bilayer, but rather exists in solution as solubilised monomers, as the concentration of **2** is far below the CMC. The CF release observed presumably occurs during the reorganisation of the phospholipid bilayer in response to the change in membrane composition. The reason for the decreased rate of CF release exhibited by the 20% prosurfactant system (Figure 2) is unclear, however it suggests a more rapid reorganisation of the bilayer, presumably due to its different initial organisation.

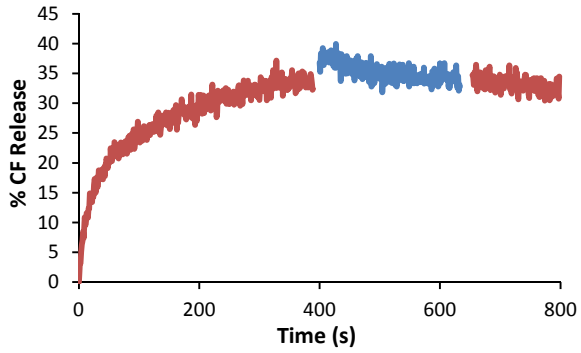


Figure 5. Compilation of the traces of two switching cycles, where the system is switched from pH 12.4 to 7.4 (red), back to 12.4 (blue), and finally to 7.4 (red), applied to the 10 % system over 800 s. The discontinuities are the result of removing the sample cuvette from, and replacing it back into, the spectrometer.

The difference in CF release behaviour observed with different counterions (Figure 2A) may be attributed to the counterion becoming part of the membrane composition, as the counterion is expected to be closely associated with the membrane when the prosurfactant is switched *in situ*. Dynamic light scattering measurements also confirm that there is no significant change in the size of the liposomes after the 'switching' event (see supporting information), thus further suggesting that leakage is due to a reorganisation of the membrane rather than solubilisation of the liposome itself.

Conclusions

In summary, a new method of releasing encapsulated solutes from liposomes has been presented. This consists of incorporating the lipophilic prosurfactant **1** into CF loaded liposomes. A decrease in pH presumably protonates **1** producing amphiphilic surfactant **2**,

causing destabilisation of the bilayer and the release of CF. The percentage CF released at all ratios of **1** investigated show a significant increase compared to the control with maximal release exhibited with 10 % incorporation of compound **1**. This technology would be amenable to various systems comprised of different switchable surfactants and lipid compositions, which are currently under study, providing a diverse range of potential new liposome payload release triggers.

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