Thermoresponsive Liposomes?

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Comment on "A Liposomal System Capable of Generating CO_2 Bubbles to Induce Transient Cavitation, Lysosomal Rupturing and Cell Necrosis"

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cavitation · drug delivery · liposomes · NH_4HCO_3 · osmotic gradient

Sung et al. have reported temperature-sensitive liposomes encapsulating ammonium bicarbonate (ABC), supposedly releasing their payload above 42 °C owing to ABC decomposition, subsequent CO_2 bubbles generation, and transient cavitation. In an attempt to reproduce the system, insurmountable difficulties were encountered. We believe the excessively strong transmembrane osmotic gradient and the generation of CO_2 bubbles already at 37 °C make it impossible to control cargo release via hyperthermia. Despite exchanging correspondence with Sung et al. and travelling to their laboratory to work cooperatively, the system could not be reproduced. We learnt that several procedures differed substantially from those in the original article and that critical aspects (i.e. appropriate purification) had been overlooked, invalidating the characterization and release experiments performed. The results presented herein cast doubts on the thermoresponsiveness of the liposomes described by Sung et al.

Our group has long been interested in controlled release systems for pharmaceutical applications. Back in 2012, Sung et al. reported liposomes encapsulating ammonium bicarbonate (ABC) that would rupture and release the payload above 42 °C.[1] The release mechanism was said to be mediated by the temperature-induced decomposition of ABC, which would generate CO_2 bubbles and cause transient cavitation. The liposomes were also said to induce cell death via necrosis in the absence of cytotoxic drugs by disrupting the cells' lysosomal membranes. Naturally, the system's thermoresponsiveness combined with its benignity at physiological temperature but cytotoxicity at 42 °C, caught our attention. Moreover, hyperthermia is a suitable trigger to control treatments in time and space, since it can be easily modulated externally (as opposed to physiological stimuli, such as pH). Follow-up publications by Sung et al. described applications of increasing complexity where the ABC liposomes were used for localized delivery of chemotherapeutics and in vivo tumor targeting with aptamer-conjugated or FRET-guided versions of the vesicles.[2] We intended to apply the system in our work but encountered insurmountable technical obstacles. Protocol clarifications kindly provided by Sung et al. did not aid the reproduction of the work; therefore, to understand the root cause for the irreproducibility, we travelled to Sung's laboratory to work jointly. Numerous discrepancies were noted with the original protocol published by Sung et al., and most importantly, the purification of the liposomes was found to be ineffective. Residual ABC and calcein were highly abundant in the liposomal suspension, compromising the validity of the calcein release kinetics estimated by Sung et al. Indeed, when the liposomes were purified further, the release of calcein at 42 °C did no longer differ from that at 37 °C. Liposomes prepared following the methods specified by Sung et al. lack the thermoresponsive properties described in their 2012 paper. Essentially, in our hands neither the extent nor the rate (profile) of cargo release from the ABC liposomes could be reproduced, and we attribute this to several reasons for which we provide evidence below.

In their original publication, Sung et al. measured the cumulative release of encapsulated calcein at different temperatures, and reported that at 42 °C about 50 % of the encapsulated calcein was released after 30 min and approximately 80 % by 180 min. However, in our hands the liposomes produced (average diameter 188 ± 17 nm for ABC liposomes, 206 ± 51 nm for negative control liposomes and 151 ± 4 nm for positive control liposomes) only achieved 5 % release after 30 min at 42 °C, which increased to merely 17 % after 180 min (Figure 1 a). The modest release became even more apparent when compared to a well-established temperature-sensitive system, used herein as a positive control. Such formulation consisted of phospholipids (transition temperature 40–43 °C) and lysolipids (phospholipids lacking alkyl chains),[3] and accordingly it released nearly all cargo at 42 °C in contrast to only 15 % at 37 °C (Figure 1 b). Temperature-insensitive liposomes hydrated with PBS instead of the ABC solution were used as negative control, and these did not release any of the encapsulated calcein at any of the temperatures tested (Figure 1 c). Equivalent results were obtained as well with a different encapsulated cargo of

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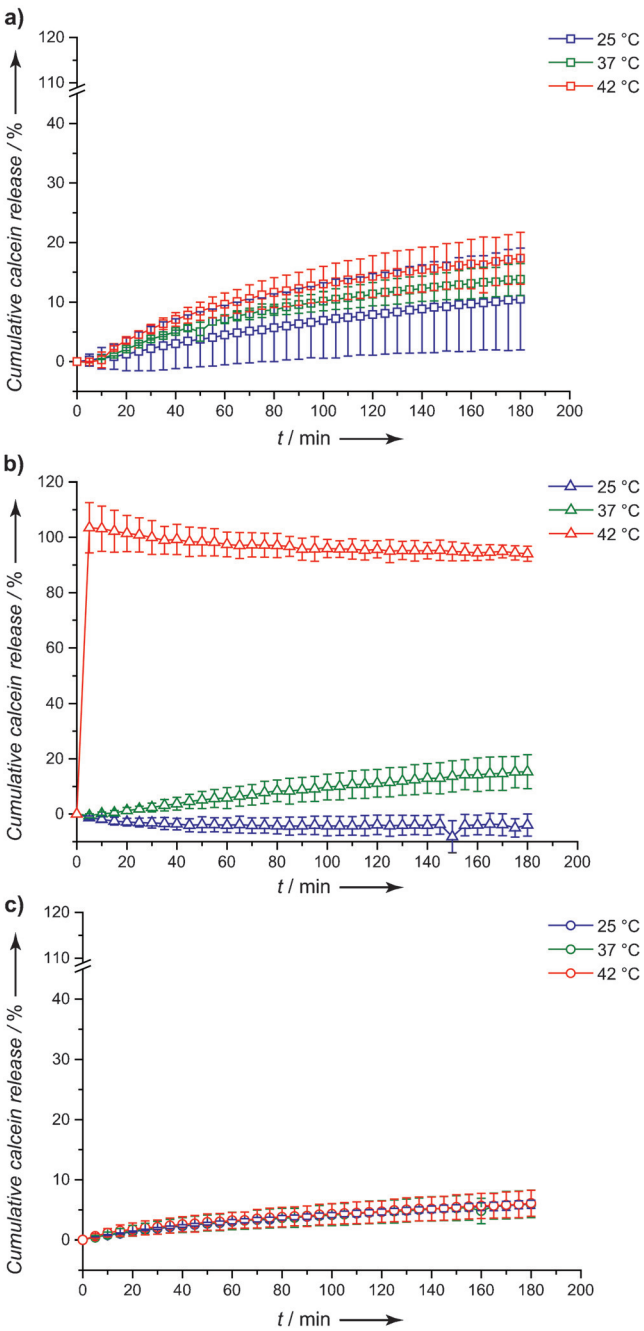

Figure 1. Release of encapsulated calcein as a function of time. The release of calcein from a) ABC, b) positive control, and c) negative control liposomes was measured at 25, 37, and 42 °C over 180 min (mean ± SD, $n = 3$).

smaller molecular weight, the fluorescent dye carboxyfluorescein (CF) (Supporting Information, Figure S1).

To investigate whether the ABC liposomes produced in our laboratory would perhaps have a higher release-triggering temperature, the release of calcein was measured as a function of temperature ranging from 25 to 50 °C. Whereas the positive control liposomes exhibited a sigmoidal profile with an abrupt release within the expected temperature window leaking essentially all their cargo at 41 °C, the ABC liposomes showed a very gradual release profile that barely reached a modest

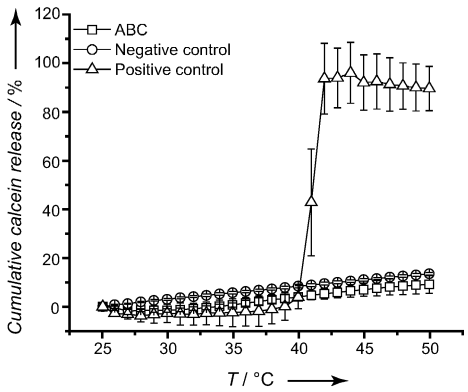

Figure 2. Release of encapsulated calcein as a function of temperature. Fluorescence intensity was measured as temperature was increased 0.1 °C min^{-1}. Data were normalized to the fluorescence obtained after incubating the samples with Triton X-100, which was considered as 100 % release (mean ± SD, $n = 3$).

15 % leakage at 42 °C (Figure 2). Experiments with CF instead of calcein revealed the same results (Figure S2).

These results led us to question the sensitivity of the ABC liposomes to temperature, since in our hands they behaved similarly at 37 and 42 °C. Indeed, a freshly prepared solution of ABC generated macroscopically visible CO_2 bubbles already at 37 °C (Figure 3), suggesting the encapsulation of

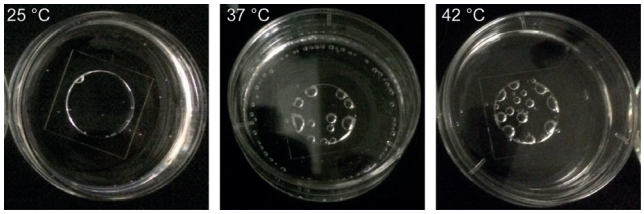

Figure 3. Generation of CO_2 bubbles from ABC. Freshly prepared ABC solution (2.7 M) was incubated in petri dishes for 15 min at 25 °C, 37 °C, and 42 °C, after which images were acquired with a digital camera. Representative images of three independent experiments are shown.

ABC could not yield a temperature-sensitive system. Our observations are in line with a 1994 patent[4] in which an ammonium transmembrane gradient was exploited to load amphiphilic drugs into liposomes. The patent described that the mechanism relied on the acidification of the liposomes' core, which occurred upon exchange of the surrounding buffer for one without ammonia.

To reestablish the equilibrium upon the buffer-exchange step, the encapsulated ammonia exited from the liposome core in its deprotonated form, leaving behind a proton which ultimately acidified the vesicles' lumen. Such decrease in pH in turn causes the remaining carbonate to decompose into water and CO_2, generating bubbles from the initially encapsulated ABC solution.[4] Hence, from this earlier work and also from our results it is clear that CO_2 bubbles would be generated as soon as the buffer exchange would take place during the liposomes' preparation. This would imply that the transient cavitation that Sung et al. reported would not be

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a phenomena controllable through hyperthermia, but through the timing of the buffer exchange.

Furthermore, based on the high concentration (2.7 M) at which ABC was encapsulated in the published protocol and considering its substantially higher osmolality compared to the PBS used for the buffer exchange (4460 ± 139 vs. 297 ± 2 mOsm kg^{-1}, as measured with a freezing point osmometer), we hypothesized that the preparation method would establish an excessively strong osmotic gradient across the liposomal membranes that would compromise their stability. The stability of the ABC liposomes was therefore studied by measuring the efficiency with which they retained the encapsulated cargo during the purification procedure. Calcein release was measured after inducing the liposomes' rupture by addition of Triton X-100. While the negative control liposomes exhibited a 5-fold increase of fluorescence (11-fold with encapsulated CF) after addition of the detergent, the ABC liposomes only increased by 3-fold (4-fold with CF). This suggests that the ABC liposomes' membrane integrity would have already been compromised before the addition of the detergent, and that part of the initially encapsulated calcein had already leaked out of the vesicles at the time of the experiment.

Hence, in our hands the ABC liposomal system described by Sung et al. lacked the thermoresponsive properties that motivated us to work with it. More importantly, owing to the strong transmembrane osmotic gradient, the method used by Sung et al. yielded unstable liposomes that could not retain the encapsulated cargo.

Thus, the results herein presented cast serious doubts on the liposomes' potential value as nanocarriers with controlled release and question the follow-up publications in which Sung et al. applied the system.[2] Certainly, simple drug delivery systems controllable by hyperthermia are of great interest to the drug delivery field and would be a valuable contribution. We therefore felt compelled to communicate the obstacles we have faced in reproducing this work to peers who might be considering investing time on this system.

Experimental Section

Liposomes preparation: Liposomes were prepared by the lipid-film rehydration method. The lipids used were 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), hydrogenated soybean phosphatidylcholine (HSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (ammonium salt) ($DSPE\text{-}PEG_{2000}$) (Lipoid, Ludwigshafen, Germany), cholesterol (CHOL) (Fluka, Buchs, Switzerland), 1,2-di-*O*-octadecenyl-3-trimethylammonium-propane (chloride salt) (DOTMA) and 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (MSPC) (Avanti Polar Lipids, Alabaster, AL). The formulations used for the ABC-encapsulating liposomes and the negative control (temperature-insensitive) liposomes were HSPC:CHOL:DOTMA (57.1:38.1:4.8 mol %; 63.8 mM total lipid), whereas for the positive control (temperature-sensitive) liposomes the formulation was DPPC:$DSPE\text{-}PEG_{2000}$:MSPC (86.5:3.9:9.6 mol %; 5 mM total lipid). Briefly, lipids dissolved in chloroform were dried in small vials and kept in a desiccator overnight. For the negative and positive control liposomes, calcein (Sigma–Aldrich, Buchs, Switzerland) was dissolved at 200 mM in 1 M NaOH and subsequently diluted to 50 mM in PBS pH 7.4 (Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, NaCl 87 mM and KCl 2.7 mM). For the ABC-encapsulating liposomes, ABC was first dissolved at 2.7 M in milli-Q water at 4 °C and then 50 mM calcein was dissolved in the ABC buffer. The dried lipid films were rehydrated with the corresponding solutions containing calcein. In the case of negative control and ABC liposomes, the liposomes were prepared via sonication in an ultrasonic bath at 40 kHz (Elma Transsonic Digital S, Singen, Germany) in order to dissolve the lipid film completely. Intervals of 5 s (sonication on and off) were applied for 15 min. The formed liposomes were then dialyzed (100 kDa cut-off Float-A-Lyzer, Spectrum, Rancho Dominguez, CA) 1:2000 (v/v) against PBS pH 7.4 for 24 h at 4 °C with 3 × dialysis buffer change according to the original publication.[1] Since the dialysis did not completely remove the unencapsulated calcein, the liposomes were further purified with a G-25 column (GE Healthcare, Little Chalfont, UK). The positive control liposomes underwent vortexing and 7 freeze-thaw cycles after lipid film rehydration. The final liposome suspension was extruded 10 × through two stacked 200-nm polycarbonate membranes (Sterlitech, Kent, WA) and the unencapsulated dye was removed with a G-25 column. The size of the liposomes was determined after sonication using laser diffraction (Mastersizer® 2000, Malvern Instruments, Malvern, UK). For the positive control liposomes, size was determined after extrusion using dynamic light scattering (DelsaNano®, Beckman Coulter, Indianapolis, IN).

Calcein release from liposomes: Cumulative release curves were recorded at excitation 490 nm and emission 520 nm with a Cary Eclipse fluorescence spectrophotometer (Agilent, Santa Clara, CA) equipped with a thermostatted multicell holder. The samples were diluted 1:100 (v/v) with PBS and measured in quartz glass fluorescence cuvettes (Hellma Analytics, Mühlheim, Germany). Values obtained were normalized to the fluorescence measured after Triton X-100 treatment (see below). For the cumulative release curves as a function of temperature, fluorescence measurements were recorded at increasing temperatures (0.1 °C min^{-1}) starting from 25 °C until 50 °C. For the cumulative release curves as a function of time, single measurements were recorded every 5 min over a period of 180 min at the constant temperatures 25 °C, 37 °C, and 42 °C.

The fold increase in fluorescence intensity upon Triton X-100 treatment was measured with a plate reader (Tecan, Männedorf, Switzerland). Equal volumes of liposomes and Triton X-100 1 % (m/v) (Sigma) were incubated for 10 min at 65 °C to rupture the liposomes' membrane and release the encapsulated calcein. The mixtures were then diluted in PBS 1:10 (v/v) and the fluorescence was measured. The values obtained were normalized to non-treated liposomes.

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Conflict of interest

The authors declare no conflict of interest.

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