Temperature-Triggered Nanotechnology for Chemotherapy:

Rapid Release From Lysolipid Temperature-Sensitive Liposomes

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ABSTRACT

Lysolipid temperature-sensitive liposomes (LTSLs) demonstrate enhanced release of encapsulated drug contents via grain boundary permeabilization when heated to their phase transition temperature, resulting in dramatic *in vivo* tumor toxicity. Dithionite ion permeability and doxorubicin release were measured for lysolipid and non-lysolipid containing membranes to characterize and attempt to determine the mechanism behind the lysolipid-generated permeability enhancement. Results indicate that a dramatic enhancement in permeability and drug release begins about two degrees below the calorimetric peak of the liposome thermal transition, and extends several degrees past it. Lysolipid appears to not desorb from the liposomes during heating, but remains in the membranes stabilizing long lasting pores through which small molecules and drugs can freely diffuse. This is the basis for the temperature-triggered nanotechnology for drug release.

Keywords: vesicle, drug delivery, phase transition, lipid, doxorubicin

1 INTRODUCTION

Despite advances in drug retention and evasion of the body's defenses, the therapeutic efficacy of many liposomes has been limited by low drug bioavailability within the tumor. To obtain controlled release, the carrier/drug relationship must change from the stable state (required for circulatory transport and delivery) to one of rapid instability and release at the tumor site.

The Needham laboratory has developed a thermal-sensitive liposome (lysolipid-containing temperature-sensitive liposome (LTSL)) that takes advantage of the anomalous permeability of lipid bilayer membranes at the transition temperature (T_m) due to melting grain boundaries [1], resulting in a formulation that releases trapped markers and drug contents at the phase transition much more quickly (tens of seconds) than in the absence of lysolipid, and in the range clinically attainable by mild hyperthermia (39-40°C) [2]. By incorporating a small amount (10 mol%) of water-soluble lysolipids, e.g., monopalmitoyl-phosphatidylcholine (MPPC), monostearoyl-phosphatidylcholine (MSPC), into dipalmitoyl-phosphatidylcholine (DPPC) liposomes prior to cooling from the liquid phase, the peak of the phase transition (T_m) upon remelting is shifted down slightly from ~41.9°C to 40.5°C and 41.3°C respectively and release/permeability is significantly enhanced.

The experiments presented here were designed to test two hypotheses regarding the mechanism behind the lysolipid-generated permeability enhancement. The first hypothesis was that, whilst trapped in the solid phase in ideal solid solution, upon heating to the transition and even past it, lysolipid remains in the bilayer and creates permeable or porous, stabilized defects in the membrane, for example, as headgroup lined pores. The second hypothesis was that, whilst trapped in the solid phase, the water-soluble lysolipid desorbs from the bilayer, as has been noted in micropipette experiments [3], entering the external media that is devoid of lysolipid, leaving behind vacancy defects in the partially melted solid/liquid interfacial regions through which small molecules can pass.

In order to distinguish between the two potential mechanisms listed above, and to more comprehensively characterize the nature of this enhanced membrane permeability, we have measured the permeability and release of the dithionite ion ($S_2O_4^{-2}$) and the chemotherapeutic doxorubicin (DOX), through LTSLs, as well as other pure lipid bilayers. The results from this investigation indicate that lysolipid does not readily desorb from the membrane upon heating to and through the transition, and supports the presence of lysolipid-stabilized pores in the membranes, probably at melting grain boundaries, that facilitate the rapid release of contents from the LTSL formulations upon heating to the phase transition region.

2 EXPERIMENTAL METHODS

All liposome samples, were prepared using thin film hydration methods and extruded through 100 nm filters using a Lipex Thermobarrel Extruder.

2.1 Dithionite Permeability Measurements

The dithionite ion, $S_2O_4^{-2}$, is a reaction intermediate in the nitro reduction of N-(7-nitro-2,1,3-benzoxadiazol) (NBD) to its corresponding amine, resulting in the irreversible quenching of NBD fluorescence and virtual elimination of the absorbance peak of NBD at 465 nm [4]. Incorporation of 1 mol% NBD headgroup labeled lipid into large, unilamellar vesicles (LUV, ~100 nm in diameter) results in a slightly asymmetrically labeled bilayer, with 54% of the NBD molecules residing on the outer monolayer and 46% residing on the inner monolayer due to curvature restrictions.

The addition of dithionite to the outside of the liposomes causes an immediate reaction with the NBD molecules on the exterior monolayer. Permeation of the dithionite ion through the bilayer results in a subsequent reaction with the inner monolayer. By monitoring the optical absorbance at 465 nm, measurements of the temperature-dependent dithionite permeability rate constant through pure and LTSL vesicles were made, as well as through palmitoyloleoylphosphatidylcholine (POPC, T_m ~2°C) vesicles as a measure of the temperature-dependent permeability of a non-phase transition lipid control. Using an exponential curve fit of relative absorbance measurements, ion permeability rate coefficients were calculated.

2.2 Lysolipid Enhancement Mechanism

To determine whether lysolipid desorption from the bilayer was responsible for the permeability enhancement, or if it was the presence of the single chain lipid, remaining in the membrane and creating *in situ* defects, dithionite permeability measurements were made following dialysis of the liposomes at a temperature at which the vesicles were liquid phase to allow for removal any desorbing lysolipid. Thin Layer Chromatography (TLC) and Proton Nuclear Magnetic Resonance Spectroscopy (NMR) measurements were used to determine if lysolipid desorbed during dialysis.

2.3 Doxorubicin Release Measurements

Doxorubicin (DOX) was loaded into liposomes using the pH gradient loading method described initially by Mayer et al. [5]. For pure DPPC liposomes, >95% drug loading was achieved reproducibly by incubating at 60°C for 10 minutes, and for the LTSLs, drug was loaded below the transition temperature of the liposomes (room temperature (23-25°C) overnight (20-24 hrs) for the DPPC:MPPC (10%) formulation, and at 37°C for 20 minutes for the DPPC:MSPC (10%) formulation). Small glass test tubes were filled with HEPES buffer at room temperature and 5 µl of DOX loaded liposomes were added. All test tubes were incubated at the temperature of interest using a circulating water bath.

For the first 5 minutes, starting at time zero, one test tube was removed every 20 seconds and immediately cooled below T_m (less than one second) to stop any subsequent drug release. Drug release at the experimental temperature was monitored over 30 minutes with a single tube removed every 60 seconds (5-18 minutes) and then every 120 seconds (20-30 minutes). In this manner, every test tube gave a single time point, all the way up to the last 30-minute time point. The fluorescence of each sample was then read separately and calculated as percent of drug release which was then converted into moles of drug released based upon the encapsulated volume and amount of drug loaded.

3 RESULTS

3.1 Dithionite Permeability

Figure 1 is a compilation plot of the permeability rate constants for DPPC, POPC, DPPC:MPPC (10%), and DPPC:MSPC(10%) through the temperature range, 30-52°C. The permeability rate constants for DPPC from 30-37°C (i.e. gel phase) are very slow, less than 0.0013/min, and then begin to increase near 39°C and reach a local maximum of 0.058/min at ~42°C (T_m). Permeability rate constants for POPC shown in Figure 1 represent dithionite ion permeation through an intact bilayer membrane that is in its liquid crystalline phase over the entire temperature range. It is interesting to note that the DPPC curve becomes coincident with the liquid phase POPC curve at temperatures above T_m, i.e., when both membranes are in their liquid phase, and that the anomalous peak in permeability at the DPPC transition (42°C) is only slightly higher than this liquid phase lipid at the same temperature.

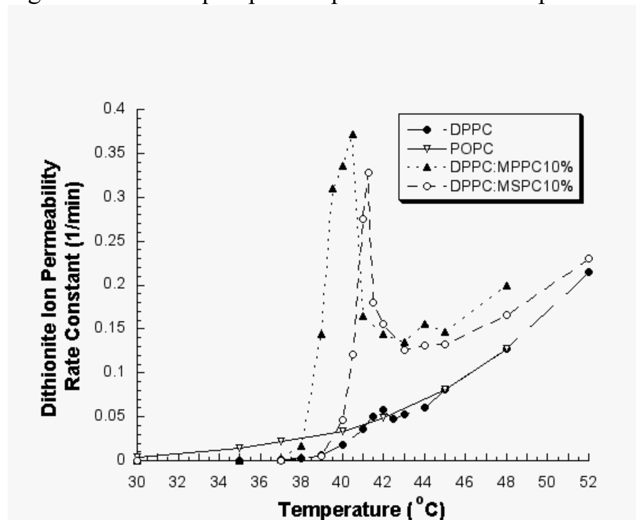

Figure 1: Compilation of dithionite ion permeability rate constants (1/minute).

The permeability rate constants for DPPC:MPPC(10%) and DPPC:MSPC(10%) in Figure 1 indicate minimal gel

phase permeability below 37°C, virtually identical to DPPC over the same temperatures. Permeability rate constants begin to increase at ~38-39°C and reach a peak at the membrane phase transition temperature (T_m), ~40.5°C and 41.3°C respectively. At higher temperatures above T_m, the rate constants through the liquid phase membranes drop below the phase transition maximum, but remain above the pure DPPC and POPC. All the liquid phase permeability rate constants appear to be approaching common values at temperatures in the range of 50°C and above.

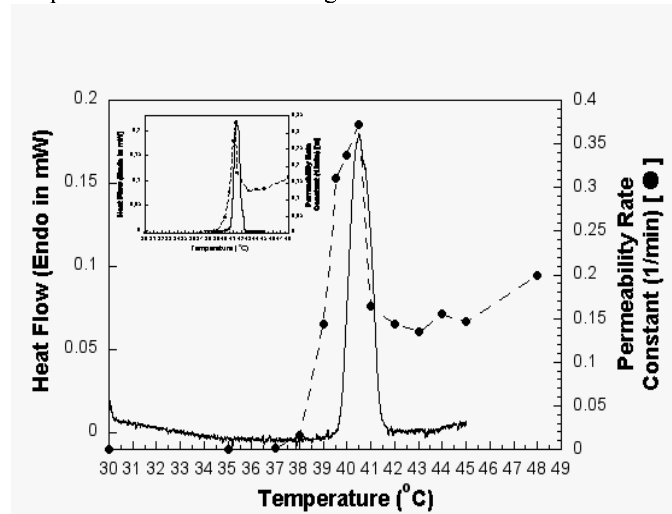

Figure 2: Calorimetric data and permeability rate constants for LTSLs.

A plot of ion permeability rate constants for DPPC:MPPC(10%) plotted together with DSC heat flow data is shown in Figure 2. Permeability begins to increase dramatically just above 38°C and is already almost a maximum at 39°C, yet the heat flow only starts to become significant (proportional to mass of lipid melted) near 39.5-40°C. The permeability rate constant peak coincides with the thermal T_m at 40.5°C. A similar plot for the DPPC:MSPC(10%) composition is shown in the inset of Figure 2.

Ion permeability rate constants of dialyzed samples were nearly identical to the non-dialyzed samples in both the gel (30-38°C) and liquid phase regions (42-48°C), as well as at the peak transition. These results indicate the presence of stable bilayer vesicles below and above the transition temperature. Furthermore, if lysolipid had desorbed, permeability would be expected to drop back down to the level of DPPC over the whole temperature range (gel and liquid phase). Results from the Thin Layer Chromatography and Nuclear Magnetic Resonance analyses of the dialyzed samples also suggested that lysolipid was not desorbing out of the membrane into the external dialysate.

3.2 Doxorubicin Release

Figure 3 shows the calculated Doxorubicin (DOX) release rates (moles/second) versus temperature for DPPC:DSPE-PEG(2000)(4%) liposomes, and the DPPC:MPPC(10%):DSPE-PEG(2000)(4%) and DPPC: MSPC(10%):DSPE-PEG(2000)(4%) temperature sensitive formulations. For DPPC, a slight peak in the DOX release rate is noted at T_m (42°C) which is ~5 times greater than the release rate at 37°C, demonstrating, however, only a modest rate enhancement at the transition temperature above the relatively impermeable solid phase. Release rates for the DPPC:MPPC formulation begin to increase substantially near 38°C, reaching an apparent maximum at 40°C that is ~650 times greater than its relatively impermeable gel phase and nearly 25 times above pure DPPC at the same temperature. Rates above 40°C could not be determined as 100% release of all contents occurred before the liquid phase temperatures were ever reached. Release rates from the DPPC:MSPC formulation were similar to DPPC through ~37°C and then rose considerably near ~39°C. At the phase transition, the release rate was 55 times greater than through gel phase membranes at the clinically relevant 37°C, and ~20 times greater than pure DPPC at T_m. At temperatures greater than T_m, the rates dropped back down in the liquid phase region, just slightly higher than for the pure DPPC liquid phase.

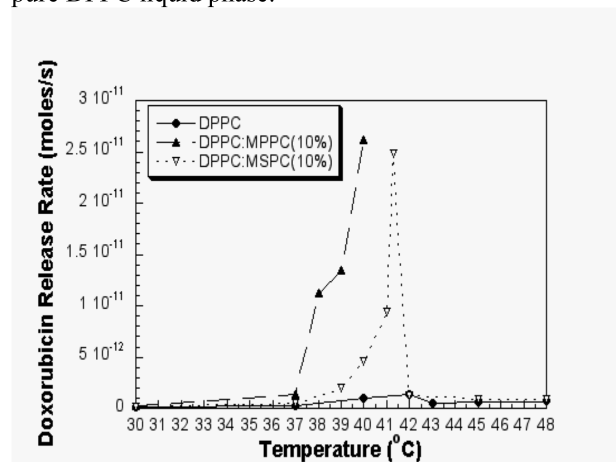

Figure 3: Doxorubicin release rates for DPPC and LTSLs.

4 DISCUSSION

The presence of lysolipid enhances the permeability of DPPC vesicles at the phase transition, presumably by stabilizing pores at melting grain boundary regions. For gel phase bilayers, the single chain PCs pack normally at the interface (same head group), but allow additional free volume in the hydrocarbon region that facilitate transmembrane transport and lower the activation energy barrier for ion diffusion through the bilayer.

As the membranes begin to melt, and grain boundary regions liquefy, lysolipid has the opportunity to form defect structures that stabilize pores along the highly compressible liquid-solid boundaries. As a result of their conical molecular shape (large head group, single chain), and tendency to form spherical micelles in aqueous solution,

these lysolipid molecules are well suited to form headgroup lined pores. Ions no longer have to interact with the low dielectric, hydrophobic region of the membrane, but can now pass through a headgroup lined, aqueous pore. Consequently, the permeability rates are dramatically higher than those of DPPC throughout the transition region. In the liquid phase region, lysolipid is still present in the membranes, and has not desorbed resulting in continued enhancement as a result of the presence of solid/liquid interfacial regions and lysolipid-stabilized pores that persist up to ~48°C. At temperatures above 48°C, theory predicts that no interfacial areas remain, and membrane permeabilities coincide, with the minor enhancement from the presence of lysolipid over pure DPPC at 52°C probably a result of increased free volume in the membrane.

The data in Figure 3 demonstrate a dramatic enhancement in the Doxorubicin release rates of the LTSLs above pure DPPC liposomes at the transition. In their transition region, the presence of grain boundaries and the presence of lysolipid pores suggest that the entire contents of the liposome, both the hydrophobic unprotonated DOX and the larger fraction of membrane non-permeable DOXH+, as well as the citrate buffer, are crossing the bilayer. As noted in the dithionite permeability data, drug permeability enhancement starts almost 2°C before the midpoint of the main calorimetric transition, adding additional support to the permeability enhancement of leaky membrane interfacial regions near 38°C.

Although experimental factors limited the ability to measure the true Doxorubicin release rates above the transition temperature, the results presented here provide important and clinically relevant data. In clinical treatment scenarios, liposomes can be introduced intravenously to the patient after heating the tumor to the desired temperature of a mild hyperthermia treatment, around 40-42°C (See Figure 4). The liposomes are then elevated from body temperature (37°C) to the hyperthermic temperature in the heated tumor region, even in the blood stream of the tumor, at a rate comparable to the experiments performed here.

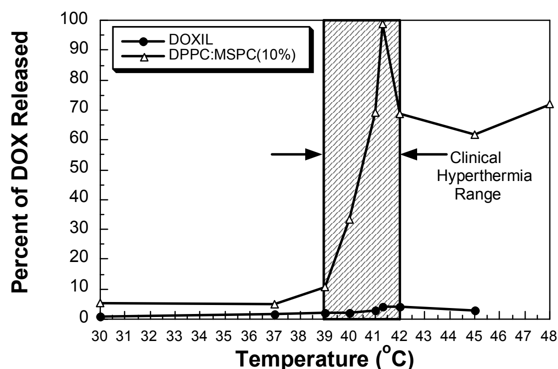

Figure 4: Dramatic enhancement in amount of Doxorubicin released from LTSLs vs. DOXIL® over clinical hyperthermia range.

Based upon these experiments, if liposomes, as they flow through the tumor vasculature, are exposed to temperatures in the range of ~40-42°C for as little as 40-60 seconds, significant amounts of drug (80-100%) would be released from each liposome passing through the heated region. Preclinical studies in window chamber and flank tumors have established that the mechanism of action is vascular shut down as a result of Hyperthermia to rapidly release Doxorubicin from the liposomes intravascularly as they pass through the blood vessels of the mildly heated tumor (See Figure 5). Thus, a new target for chemotherapy delivered via these rapidly releasing liposomes appears now to be vascular endothelia, in addition to cytotoxicity for cancer cells, as the rapid release of DOX during hyperthermia allows the drug to shutdown tumor blood flow while having only minor effects on normal microcirculation in subcutaneous tissues [6] (See Figure 5).

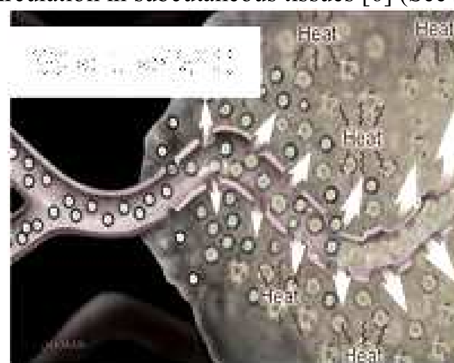

Figure 5: New paradigm for drug delivery - drug release in the bloodstream at the tumor site.

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