Modifying the Release Properties of Liposomes Toward Personalized Medicine

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ABSTRACT: Surfactant–liposome interactions have historically been investigated as a simplified model of solubilization and breakdown of biological membranes by surfactants. In contrast, our goal was to utilize surfactants to modify the encapsulation and release properties of liposomes. The ability to manufacture one liposomal formulation, which could be modified by the addition of a surfactant to support a wide range of release profiles, would provide greater flexibility than manufacturing multiple batches of liposomes, each differing in composition and with its own specific release profile. A liposomal ciprofloxacin formulation was modified by the addition of various surfactants. These formulations were characterized in terms of liposome structure by cryo-TEM imaging, vesicle size by dynamic light scattering, drug encapsulation by centrifugation–filtration, and *in vitro* release (IVR) performance. The addition of polysorbate 20 or polysorbate 80 to liposomal ciprofloxacin, in a hypotonic environment, resulted in a concentration-dependent loss of encapsulated drug, and above 0.4% polysorbate 20, or 0.2% polysorbate 80, a modified IVR profile as well. This study demonstrates that the encapsulation and release properties of a liposomal formulation can be modified postmanufacture by the addition of judiciously chosen surfactants in combination with osmotic swelling of the liposomes and may support a personalized approach to treating patients. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:1851–1862, 2014

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INTRODUCTION

There are a range of formulation technologies that can be used to modulate the release properties of pharmaceutical drugs, including liposomes.[1] Liposomes are phospholipid vesicles composed of one or more lipid bilayers surrounding an aqueous core and can vary in size ranging from approximately 20 nm to 10 μm.[2] Liposomes as drug delivery vehicles are now well accepted with more than 10 approved liposomal products on the market.[3] A wide variety of drugs have been formulated into liposomes including small molecules, peptides, and nucleic acids; hydrophilic drugs are generally dissolved in the aqueous compartment, whereas hydrophobic drugs are associated with the lipid bilayers.[1,2] The liposome composition, lamellarity, and the manufacturing process can each influence the physicochemical properties of a liposomal product, including its drug release kinetics. Although there are a wide number of variables in play to produce liposomal formulations with differing pharmacokinetics, it would be complex and costly to market multiple liposomal compositions to cover a broad range of release profiles. A liposomal formulation which could be easily modified postmanufacture to produce different release profiles has the potential to provide greater flexibility in treatment.

In the present study, we were interested whether the release properties of a liposomal ciprofloxacin for inhalation (CFI) could be modified by the addition of a surfactant to the drug product postmanufacture. One could envision a scenario in which this simple procedure is performed by a doctor or a patient, from a kit containing ampules with different concentrations of surfactant, or different surfactants, to provide a formulation tailored to the specific needs of that patient. This could prove less costly, and less complex, than developing and manufacturing multiple, unique liposomal formulations which differ in composition to cover a broad range of release profiles. However, to our knowledge, this represents a new paradigm as surfactant has not previously been reported to be useful in the context of modifying the release properties of a liposome formulation, once it has been manufactured. Of course, under this new personalized medicine approach, new hurdles will arise such as how to identify the appropriate release profile for each individual, but that is not the focus of this paper.

There is an extensive history of detergents or surfactants being used to solubilize biological membranes to allow for elucidation of membrane structure and function.[4] The ability of surfactants to solubilize phospholipids, specifically, has been reviewed.[5] As surfactant is added to phospholipids, surfactant initially partitions between the solution and the phospholipid bilayers and the bilayer permeability may increase without loss of structure.[5] Once the phospholipid bilayers become saturated with surfactant, the addition of more surfactant leads to the formation of mixed micelles of surfactant and phospholipid until all of the remaining surfactant-saturated bilayers are converted to mixed micelles. Any further addition of surfactant leads to a decrease in the size of the micelles as they become

Abbreviations used: CF, carboxyfluorescein; CFI, ciprofloxacin for inhalation; Cryo-TEM, cryogenic transmission electron microscopy; DLS, dynamic light scattering; DRCFI, dual release ciprofloxacin for inhalation; IVR, *in vitro* release; PC, phosphatidylcholine; TEA, triethylamine.

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more dilute in phospholipid content. Vesicle–surfactant systems have frequently been characterized by monitoring their light scattering properties, with maximum turbidity generally associated with the surfactant-saturated bilayer state[6] followed by a rapid decline in turbidity once the bilayers are completely solubilized by surfactant.[7–12]

Liposomes have also been used as a simplified model of biological membranes. Interest in the development of antimicrobials that would function by disruption of the bacterial membrane spurred a better understanding of the interaction of surfactant-like molecules with liposomes.[13,14] During this same time period, liposomes were also being investigated as drug delivery vehicles in their own right, so knowledge of the factors which affected the timing and rate of release of the encapsulated drug, including interaction with biological fluids, was important. After *in vivo* administration, liposomes come into contact with many natural amphiphiles present in physiological fluids. Surfactants were used as a simplification to the complex biological milieu, allowing for characterization of drug release from liposomes in the presence of surfactant.[15]

The observation that surfactants can interact with liposomes to cause leakage of encapsulated drug was extensively investigated in the 1970s and 1980s, but new insights into the mechanism of surfactant-induced liposomal breakdown and the kinetics of release of the entrapped agent [carboxyfluorescein (CF)] were just recently described.[6] Upon the addition of solubilizing levels of surfactant, there is a complete breakdown of liposome structure because of the direct interactions of the surfactant molecules with the lipids, resulting in complete release of encapsulated drug in less than a second.[6] At lower surfactant concentrations, surfactant molecules can cooperatively assemble into structures which form pores in the liposomes, again allowing for almost instantaneous release of a portion of the encapsulated agent, but these holes then close limiting further release.[6] Nagawa and Regen[13] demonstrated that for some combinations of liposomes and surfactant, the encapsulated agent was released from all vesicles with increasing surfactant concentration. For other combinations, there was a catastrophic rupture in which a subset of the vesicles rapidly released their entire encapsulated payload, whereas others were unaffected.[13] Although there are many reports of loss of drug from liposomes in response to surfactant addition, none have shown that the surfactant can be used to modify the pharmacokinetic properties and thus the *in vitro* release (IVR) profile of the liposomes.[6,13–17]

A mechanistic understanding of the interactions of surfactants with liposomes is generally well understood; however, interest in utilizing surfactants to modify the release properties of liposomes for therapeutic purposes, which is the focus of this paper, has not been explored. Rather than using the strongly solubilizing surfactants, like Triton X-100, we chose surfactants that are common to many pharmaceutical formulations, for example, the polysorbates, to generate liposomal formulations with modified release properties. We also studied other nonionic surfactants including poloxamers and spans, which are closely related structurally to the polysorbates. It was expected that these "milder" surfactants may not be sufficiently disruptive to the liposomes at low concentrations to yield formulations with significantly modified properties. To overcome this challenge, we evaluated the combination of addition of surfactant along with dilution of the liposomal formulations with water, to produce a hypotonic environment. The resulting osmotic swelling of the liposomes might enhance the ability of the surfactant to intercalate or associate with the liposome membrane and thus alter its drug encapsulation and release properties. This paper describes liposomal formulations of ciprofloxacin with modified encapsulation and release properties because of the addition of surfactant under hypotonic conditions. In line with the goal of personalized medicine, to tailor a product to an individual's needs so that it releases the "right amount of drug at the right time," this strategy may provide more flexibility than the alternative of manufacturing multiple batches of liposomes differing in composition to cover a broad range of desired release profiles.

MATERIALS AND METHODS

Materials

Free ciprofloxacin (FCI), 20 mg/mL in an acetate-buffered aqueous formulation at pH 3.3 and CFI, 50 mg/mL in a histidine-buffered aqueous formulation at pH 6.0 were from Aradigm Corporation (Hayward, California). (The ciprofloxacin concentrations are expressed in terms of ciprofloxacin hydrochloride.) Polysorbate 20 was purchased from VWR Int. (West Chester, New Jersey). Polysorbate 80, sorbitan monolaurate (Sp20), sorbitan monooleate (Sp80), poloxamer L44, and poloxamer L62 were a gift from Croda, Inc. (Edison, New Jersey). HEPES, free acid was purchased from Avantor (Center Valley, Pennsylvania). Sodium chloride was obtained from Amresco (Solon, Ohio). Sodium acetate was purchased from Sigma–Aldrich (St. Louis, Missouri). HPLC grade methanol was purchased from Fisher Scientific (Fair Lawn, New Jersey) and triethylamine (TEA) was purchased from JT Baker (Center Valley, Pennsylvania). Donor adult bovine serum was obtained from HyClone (Logan, Utah). Nanosep centrifugal filtration devices, 10 K and 30 K Da molecular weight, were obtained from Pall Corporation (Ann Arbor, Michigan). Deionized water was used for all studies.

Preparation of Liposomal Ciprofloxacin

Ciprofloxacin for inhalation is an aqueous dispersion of unilamellar liposomes of approximately 80 nm containing hydrogenated soy phosphatidylchocline (PC) and cholesterol. The preparation of CFI has been reported previously.[18] Briefly, multilamellar liposomes are extruded through membranes to produce unilamellar liposomes which are then actively loaded with ciprofloxacin.[19,20] Any unencapsulated ciprofloxacin is removed by diafiltration resulting in >99% encapsulated ciprofloxacin at a target concentration of 50 mg/mL. Dual-release CFI (DR-CFI) is an equivolume mixture of FCI and CFI resulting in approximately 70% encapsulated and approximately 30% FCI.

Addition of Surfactant to CFI

In the first series of experiments, CFI was diluted from 50 mg/mL to 30, 20, 15, 12.5, or 10 mg/mL with histidine buffer (HB: 25 mM histidine, 145 mM sodium chloride buffer, pH 6.0) and polysorbate 20 to achieve a final polysorbate 20 concentration of 0.1%, 0.2%, 0.4%, 0.8%, 1.2%, 1.6%, or 2.0% (w/v). A second series of experiments was identical to the first except that the CFI was diluted with water, instead of HB, to determine whether osmotic swelling might play a role in enhancing the effect of the addition of surfactant. In a third experiment, the order of dilution was varied to determine whether the properties of these preparations depended upon the order of addition of water and surfactant to the 12.5 mg/mL CFI preparations. In

a fourth experiment, after 30 min equilibration, the 30 mg/mL CFI preparations containing various levels of surfactant were diluted with water to 10 mg/mL CFI to determine whether the final encapsulation state was dependent upon the timing of the preparation steps. In all of these experiments, the encapsulation state was expressed as the mean of duplicate values.

Another series of experiments explored the use of various surfactants, including Sp20, Sp80, poloxamer L62, poloxamer L44, and polysorbate 80, to modify the properties of liposomal ciprofloxacin, CFI. In these studies, the formulations contained 12.5 mg/mL CFI (i.e., diluted fourfold with water to produce a hypotonic environment) in 0.01%, 0.05%, 0.1%, 0.5%, or 1% (w/v) of each surfactant. A final experiment evaluated the reproducibility of this effect across three different batches of CFI, upon the addition of polysorbate 20 in a hypotonic environment. In this study, 50 mg/mL CFI was diluted fourfold with water, and 1% polysorbate 80 to a final concentration of 12.5 mg/mL CFI in 0%, 0.05%, 0.1%, 0.2%, 0.3%, 0.4,% 0.5%, or 0.6% (w/v) polysorbate 80.

In all experiments, the CFI samples containing surfactant were allowed to equilibrate for at least 30 min to provide adequate time for the surfactant to associate with the liposomes. No further change in the properties of these formulations was observed when samples were equilibrated for longer periods of time (data not shown). For each of these preparations, the vesicle size distribution and drug encapsulation states were determined. For a number of the polysorbate 20 preparations, cryogenic transmission electron microscopy (cryo-TEM) images and IVR profiles were also obtained.

Vesicle Size

Each CFI or CFI plus surfactant sample was diluted with isotonic saline to a concentration of approximately 1 mg/mL CFI (2 mg/mL liposomes), and 0.5 mL was transferred to a disposable culture tube (Kimble Glass Inc. (Vineland, NJ)) for vesicle size analysis using a Submicron Particle Sizer Autodilute Model 370 (Nicomp (Santa Barbara, CA)). The following instrument parameters were selected: temperature: 23°C; viscosity: 0.933; refractive index: 1.333; intensity set point: 300 kHz; channel width: 10 μs; scattering angle: 90; run time: 5 min; mode: vesicle; Gaussian distribution. The mean and SD of the vesicle size distribution were recorded.

Drug Encapsulation

Nanosep Omega centrifugation devices (Pall Corporation) with modified polyethersulfone membrane filters of 10,000 or 30,000 molecular weight cutoffs were used to separate free drug from liposomal encapsulated drug. Each sample was diluted 10-fold into acetate buffer (50 mM sodium acetate, 145 nM NaCl, pH 4.0) and 400 μL was transferred to the centrifugation device and centrifuged for 18 min at 8100*g* (10,000 rpm). A centrifugation time of 18 min and a speed of 8100*g* were chosen to ensure that there was an adequate volume in the filtrate to transfer to HPLC vials without risk of rupture to the membranes because of a too high centrifugation force. These conditions were previously shown to provide quantitative recovery.[21] (Subsequently, it was found that centrifugation times of 10 min were also effective when samples were further diluted by a factor of two.) The filtrate, representing the FCI component, was analyzed by HPLC for ciprofloxacin content. The total amount of ciprofloxacin, representing both the encapsulated and free drug, was quantified by HPLC after diluting the CFI sample 20-fold into 80% methanol to solubilize the liposomes. The percent encapsulation was determined by comparing the free drug to the total drug in each sample.

In Vitro Release

The IVR assay measures the release of encapsulated ciprofloxacin when incubated at 37°C in 50% (v/v) bovine serum.[21] Briefly, the CFI samples were diluted to 50 μg/mL ciprofloxacin in HEPES-buffered saline (HBS: 20 mM HEPES, 145 mM NaCl, pH 7.4) and mixed one-to-one with chilled (2°C–8°C) bovine serum (Hyclone) and placed in a shaking water bath [Techne, TSBS40 (Staffordshire, UK)] at 37°C and 150 rpm. Duplicate samples were removed after incubation for 30, 60, 120, and 240 min, diluted 1:1 with chilled (2°C–8°C) HBS and placed in an ice-water bath to terminate any further release of encapsulated drug from the liposomes. The released ciprofloxacin was separated from the liposome-encapsulated ciprofloxacin by transferring 400 μL of each chilled sample to a Nanosep centrifugal device and centrifuging at 8100*g* for 18 min. The filtrate was removed for subsequent quantitation of the released ciprofloxacin by HPLC. This value was normalized by multiplying by 1.05, to correct for a small but reproducible loss of free drug in the filtration devices in the presence of serum.[21] The original CFI sample was diluted into 80% methanol to dissolve the liposomes and allow for quantitation of the total amount of ciprofloxacin by HPLC. The percent release at each time point was calculated by comparing the free drug to the total drug.

Cryo-TEM

To obtain visual images of the liposome formulations, cryo-TEM analysis was performed using a JEOL 2100 (Tokyo, Japan) instrument operated at 200 kV. The CFI samples were diluted to approximately 5 mg/mL (10 mg/mL liposomes) with water and 3 μL of each sample was applied to a glow discharge Quantifoil carbon grid (Jena, Germany) in a chamber controlled to 22°C and 100% relative humidity. Grids were blotted once with filter paper, at a blotting angle of 2 mm for 2 s, and vitrified by plunging into liquid ethane using a Vitrobot (F.E.I., Eindhoven, Netherlands). The vitrified samples were stored in liquid nitrogen prior to cryo-TEM analysis.

HPLC

The amount of ciprofloxacin in each sample was quantified using an HPLC method as described previously.[22] Briefly, HPLC analysis was performed using a Nucleosil C-18 column (5 μm, 4.6 × 150 mm^2; Canadian Life Science (Peterborough, Ontario)) protected with a Nucleosil C-18 guard column (4 × 3.0 mm^2; Phenomenex (Torrance, California)) both at 35°C. The mobile phase was a mixture of 0.5% TEA in water, pH 3.0, and 100% methanol (83:17, v/v), and the isocratic elution was performed at a flow rate of 0.9 mL/min. Ciprofloxacin was detected and quantified at a wavelength of 277 nm.

RESULTS

Liposomal Ciprofloxacin Encapsulation: Effect of Addition of Polysorbate 20

Polysorbate 20 is present in a number of approved pharmaceutical products and so was chosen for initial evaluation because of the general knowledge about its suitability in inhaled

formulations. When 50 mg/mL CFI was diluted with HB, and increasing concentrations of polysorbate 20, up to 2.0%, were added, there was a small concentration-dependent loss of encapsulated drug but the amount of free drug did not exceed 8% (Fig. 1a). The 30 and 10 mg/mL CFI samples decreased to approximately 95% and 92% drug encapsulation, respectively, at the highest polysorbate 20 concentration (i.e., 2%). As expected, intermediate CFI concentrations, that is, 12.5, 15, and 20 mg/mL, had encapsulation values which fell in between those two curves.

For any given surfactant concentration, there was an increase in drug release with decreasing CFI concentration, as would be expected because the ratio of surfactant to liposomes is greater. Therefore, one would expect more surfactant to be associated with each individual liposome for the lower CFI concentration preparations and thus to have the potential for a greater effect on drug release. At a constant ratio of surfactant to liposomes, if no other changes were introduced, it might be expected that the final encapsulation states would be comparable. Although the individual data points were not selected to keep the ratio of surfactant to liposomes constant, a comparison can be made by following the dotted black lines in Figure 1a for both a high and low ratio of surfactant to liposomes. In both cases, there was a slight decrease in encapsulation with decreasing CFI concentration over the concentration range of 30–10 mg/mL CFI, but the changes were modest (i.e., 1%–2%).

Liposomal Ciprofloxacin Encapsulation: Combination of Osmotic Swelling and Polysorbate 20

In the previous experiment, we were unable to produce meaningful increases in free drug, of more than 8%, even using a relatively large amount of polysorbate 20 (i.e., 2%). The next series of experiments were conducted to determine whether liposomes subjected to osmotic swelling would be more responsive to the addition of surfactant. In these experiments, CFI was diluted with water, instead of HB, to create a hypotonic (hypoosmotic) state. Polysorbate 20 was added immediately after dilution with water. In contrast to the previous studies for which there was a minimal loss of encapsulated drug (Fig. 1a), the addition of a surfactant in conjunction with osmotic swelling of the liposomes resulted in a much greater loss of encapsulated drug, especially for the lower CFI concentrations (Fig. 1b). The release of encapsulated drug happened very rapidly, well within 30 min, and no further release was observed thereafter. The degree of osmotic swelling would be expected to be the greatest for the lowest CFI concentration preparations (i.e., 10 mg/mL) because of the greater dilution of CFI and thus the largest reduction in tonicity. And indeed those samples showed the greatest loss of encapsulated drug. For the 10 mg/mL CFI preparation containing 2% polysorbate 20, the amount of released drug increased from approximately 8% to >50% when the liposomes were diluted with water versus HB suggesting that osmotic swelling acts synergistically with the addition of surfactant to modify the encapsulation state of the liposomes. The encapsulation state of the highest concentration CFI preparation (i.e., 30 mg/mL), across the range of polysorbate 20 concentrations, was no different when diluted with water or HB. This result suggests that there may not have been sufficient osmotic swelling of the 30 mg/mL CFI preparations to have any effect on release of encapsulated drug.

Congruous to the previous studies (Fig 1a), for any given concentration of polysorbate 20, there was a greater extent of release of encapsulated ciprofloxacin for more dilute CFI formulations than for more concentrated CFI formulations (Fig. 1b). In contrast to the previous studies, for which there was little variation in encapsulation state across a constant ratio of surfactant to liposomes (dotted lines in Fig. 1a), in these studies there was a dramatic difference in encapsulation states across a constant ratio of surfactant to liposomes (dotted lines in Fig. 1b). For the 30 mg/mL CFI formulation in 2% polysorbate 20, the encapsulation state was approximately 95%, whereas it decreased to approximately 56% for 10 mg/mL CFI in 0.67% polysorbate 20. Similarly, for the 30 mg/mL CFI formulation in 0.8% polysorbate 20, the encapsulation state was again approximately 95%, whereas it decreased to approximately 67% for 10 mg/mL CFI in 0.27% polysorbate 20. Although the ratio of surfactant to liposomes remained constant along the dotted lines in Figure 2B, the degree of tonicity, and hence the amount of osmotic swelling, did not remain constant. The lower concentration CFI preparations were more hypotonic and thus likely experienced more osmotic swelling. This may explain why the more dilute liposome solutions of CFI were more susceptible to surfactant-induced release of encapsulated drug than for the more concentrated solutions of CFI, at the same molar ratio of surfactant to liposomes.

Liposomal Ciprofloxacin Encapsulation: Effect of Dilution Order (Water and Polysorbate 20)

In a third experiment, the order of dilution was evaluated by adding the surfactant to the 50 mg/mL CFI immediately after dilution with water, as was performed in the previous experiments, or immediately before dilution with water. In this experiment there was no meaningful difference in the encapsulation state between the two preparations across the range of surfactant concentrations suggesting that order of dilution is not critical to the final encapsulation state, at least as long as the dilution steps are close together temporally (Fig. 1c).

Liposomal Ciprofloxacin Encapsulation: Effect of Equilibration with Polysorbate 20 prior to Dilution with Water

In a fourth experiment, the encapsulation states of 30 mg/mL CFI in a range of polysorbate 20 concentrations were measured after 30 min equilibration, and then were remeasured after dilution with water to 10 mg/mL CFI (Fig. 1d). In contrast to the previous experiment which suggested that the order of dilution did not have a significant impact on encapsulation state, in this experiment, the final encapsulation state was very dependent upon the dilution procedure. When the 10 mg/mL CFI formulation was prepared directly from the 50 mg/mL CFI by the addition of water and a surfactant, in either order (Fig. 1c), there was a much greater decrease in encapsulation state versus when the 30 mg/mL CFI was allowed to equilibrate with surfactant prior to dilution to 10 mg/mL (Fig. 1d). In the latter case, there was only a minimal change in the encapsulation state upon the threefold dilution (Fig. 1d). This result suggests that the final encapsulation state may be pathway dependent (hence also time dependent) and predominantly kinetically based, rather than equilibrium based. In other words, there may only be a transient time period for which the osmotically swollen liposomes are susceptible to surfactant modification and if the surfactant has already equilibrated with

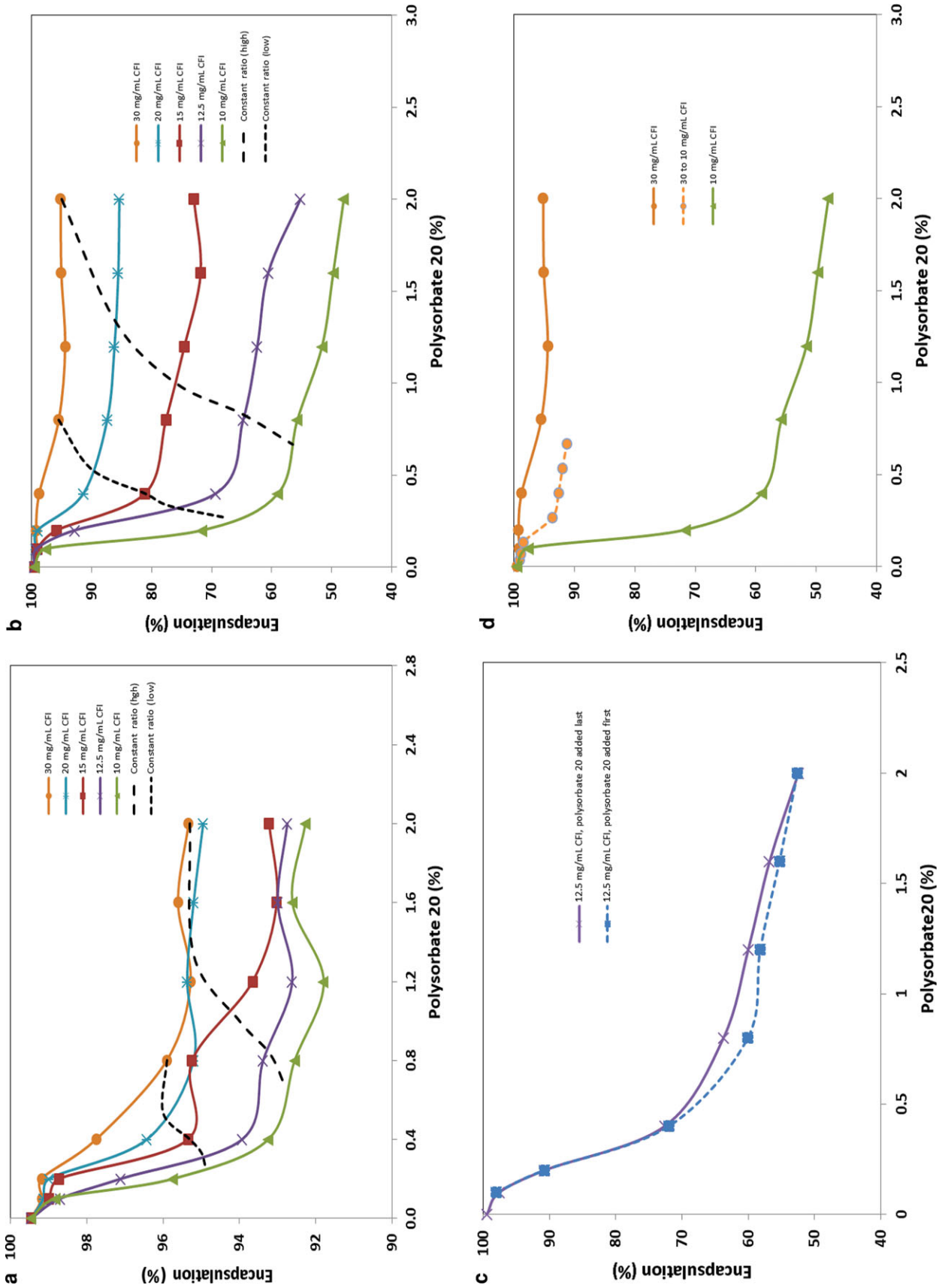

Figure 1. The effect of the addition of polysorbate 20 on the encapsulation state of ciprofloxacin in CFI. Each data point represents the mean ($n = 2$). CFI containing 50 mg/mL encapsulated ciprofloxacin was diluted to a final concentration of 30, 20, 15, 12.5, and 10 mg/mL ciprofloxacin with HB (a) or water (b) and an aliquot of 1% or 10% (w/v) polysorbate 20 to achieve a final surfactant concentration of 0.1%, 0.2%, 0.4%, 0.8%, 1.2%, 1.6%, or 2.0% (w/v). The dotted black lines represent a constant ratio of surfactant to liposomes. (c) CFI containing 50 mg/mL ciprofloxacin was diluted to a final concentration of 12.5 mg/mL ciprofloxacin with water and 1% or 10% (w/v) polysorbate 20 to achieve a final surfactant concentration of 0.1%, 0.2%, 0.4%, 0.8%, 1.2%, 1.6%, or 2.0% (w/v). In one experiment, the water was added to the CFI before the polysorbate 20 and in the other the polysorbate 20 was added to the CFI before the water. (d) CFI containing 30 mg/mL ciprofloxacin and 0.1%, 0.2%, 0.4%, 0.8%, 1.2%, 1.6%, or 2.0% (w/v) polysorbate 20 was diluted to a final concentration of 10 mg/mL ciprofloxacin with water to achieve a final concentration of 0.03%, 0.07%, 0.13%, 0.27%, 0.4%, 0.53%, and 0.67% (w/v) polysorbate 20.

the liposomes prior to osmotic swelling, as in this experiment, subsequent osmotic swelling does not appear to enable the surfactants to associate in the same way to produce larger losses of encapsulated drug.

Liposomal Ciprofloxacin Encapsulation: Applicable to Other Surfactants?

To determine whether the surfactant-induced release of encapsulated ciprofloxacin in a hypoosmotic environment was specific to polysorbate 20, or more broadly applicable to other closely related, nonionic surfactants, another series of experiments was performed with the following surfactants: polysorbate 80, Sp20, Sp80, poloxamer L44, and poloxamer L62. Upon the addition of a surfactant to CFI diluted with water to a final concentration of 12.5 mg/mL, all surfactants caused some release of encapsulated ciprofloxacin with the amount released increasing with increasing surfactant concentration (Fig. 2a). However, the amount of drug that was released was very dependent on the choice of surfactant, with polysorbate 80 resulting in substantial release of ciprofloxacin, whereas none of the other nonionic surfactants that were tested produced more than 1%–2% release of encapsulated drug over the surfactant concentration ranges that were evaluated (Fig. 2a). At the same surfactant concentrations, polysorbate 80 was more effective than polysorbate 20 (Fig. 2a vs. Fig. 1c) at causing greater release of encapsulated ciprofloxacin in the 12.5 mg/mL CFI preparations. More data points were not collected over the surfactant concentration range studied because the focus of this experiment was to understand the general behavior of the surfactant interactions with CFI, but not to fully map out the design space.

A follow-on experiment was conducted to more fully map out the design space for polysorbate 80, and to determine how reproducible this effect was for different manufactured batches of CFI. The response of each of the three batches of CFI to polysorbate 80 was comparable, with a large loss of 30%–35% encapsulated drug for 0.1% polysorbate 80, and 40%–45% loss of encapsulated drug for 0.2% polysorbate 20 (Fig. 2b). Higher concentrations of polysorbate 20, up to 0.6%, did not significantly alter the plateau value of approximately 45% released drug (55% encapsulated drug).

Vesicle Size

The vesicle size of the various preparations was measured to determine whether the addition of surfactant results in any measurable change in liposome size. The mean vesicle size of CFI was unchanged in the presence of either poloxamer L44 or poloxamer L62, increased by 1–3 nm for Sp20, Sp80, and polysorbate 20, and increased by up to 8 nm for polysorbate 80 (Table 1).

IVR Profile

We have shown that addition of polysorbate 20 and polysorbate 80 surfactants to CFI, in a hypotonic environment, leads to more extensive changes to the encapsulation state of the liposomes than in the absence of osmotic swelling. We were also interested whether the release properties of the liposomes could be modified by surfactant addition. The IVR assay measures the release of encapsulated ciprofloxacin from the liposomes after incubation with bovine serum at 37°C.[21] Consistent with the encapsulation assay data (Figs. 1b and 2a), the time zero release values in the IVR assay (Fig. 3a) represent the state of encapsulation before incubation with the release agent, that is, the amount of "burst." For the control CFI vial, the "burst" value is approximately 1%. For DRCFI, which is a mixture of approximately 70% CFI and 30% FCI solution, the "burst" value is approximately 30%, as would be expected. For the 12.5 mg/mL CFI containing various levels of surfactant, the amount of FCI, or "burst," increased with increasing polysorbate 20 from approximately 2% for 0.05% polysorbate 20 to approximately 27% for 0.4% polysorbate 20 in the IVR assay (Fig. 3a).

The last time point in the IVR assay, at 4 h, represents the complete extent of release of ciprofloxacin from the liposome preparations. By the 4 h time point, all of the formulations reached a plateau value in the IVR assay representing approximately 100% release of encapsulated ciprofloxacin.

The IVR profiles for the CFI formulations containing 0.05, and 0.1% polysorbate 20 were similar to that for CFI alone. However, the IVR profile for the CFI formulation containing 0.4% polysorbate 20 had marked differences to that for CFI as well as for DRCFI. The $T_{30\,\mathrm{min}}$ value is an approximate representation of the "midpoint" of release for CFI.[21] The $T_{30\,\mathrm{min}}$ values increased with increasing polysorbate 20, from approximately 44% for CFI alone to approximately 66% for CFI containing 0.4% polysorbate 20. However, one cannot directly compare the $T_{30\,\mathrm{min}}$ values of these formulations to assess changes to the release rates because the encapsulation states were not equivalent at the initial time point ($T_{0\,\mathrm{min}}$). To address this issue, one can "normalize" the $T_{30\,\mathrm{min}}$ values by subtracting the initial value, $T_{0\,\mathrm{min}}$, and dividing by the total available range for release ($T_{240\,\mathrm{min}} - T_{0\,\mathrm{min}}$) and converting to a percentage: $100 \times (T_{30\,\mathrm{min}} - T_{0\,\mathrm{min}})/(T_{240\,\mathrm{min}} - T_{0\,\mathrm{min}})$. The normalized $T_{30\,\mathrm{min}}$ values are 44.4% for CFI, and 43.4%, 46.8%, 47.6%, and 55.7% for CFI containing 0.05%, 0.1%, 0.2%, and 0.4% polysorbate 20, respectively. The encapsulated ciprofloxacin release rates were relatively comparable, except for the CFI formulation containing 0.4% polysorbate 20. In addition, although CFI containing 0.4% polysorbate 20 had a comparable initial release value, or "burst," to that for DRCFI, 27% versus 30%, respectively, its release rate was faster than DRCFI with a $T_{30\,\mathrm{min}}$ value (un-normalized, as the initial values are similar) of 66% versus 57%, respectively. The normalized $T_{30\,\mathrm{min}}$ values of 55.7% versus 41.6%, respectively, also demonstrate this difference in release rates.

Polysorbate 80 had an even greater effect on the IVR profile than polysorbate 20. CFI containing 0.2% polysorbate 80 had a faster IVR profile than for CFI containing twice as much (0.4%) polysorbate 20 (Figs. 3b vs. 3a). To investigate whether the surfactant has altered the membrane permeability of the liposomes and thus its release profile, or if the surfactant was simply operating as a release vehicle itself, control CFI was diluted into the release vehicle (serum) with or without polysorbate 80 and the IVR assay was repeated (Fig. 3b). The concentration of surfactant in the IVR assay in that experiment (0.0004%) was identical to that for 12.5 mg/mL CFI containing 0.2% polysorbate 80 after the 500-fold dilution into the IVR assay buffer. The presence of polysorbate 80 in the release buffer had no effect on the IVR profile of CFI (Fig. 3b).

Cryo-TEM

To better understand the effect of the addition of polysorbate 20 on the structure and state of the liposomal ciprofloxacin vesicles, 12.5 mg/mL CFI samples (diluted with water) with and

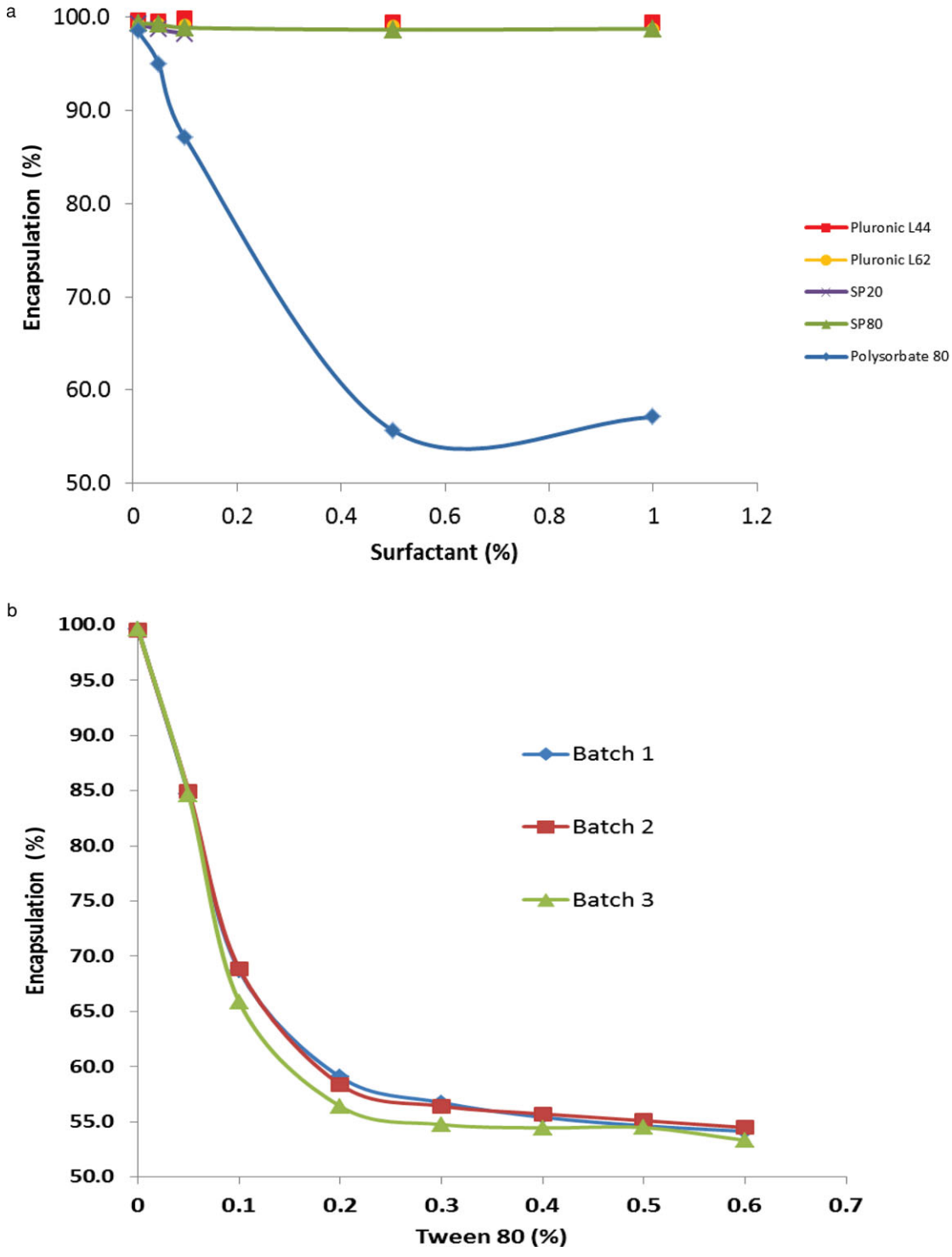

Figure 2. (a) The effect of the addition of various surfactants on the state of ciprofloxacin encapsulation. CFI containing 50 mg/mL of encapsulated ciprofloxacin was diluted to a final concentration of ~12.5 mg/mL ciprofloxacin with water and an aliquot of 1% or 10% (w/v) surfactant to achieve a final surfactant concentration of 0.01%, 0.05%, 0.1%, 0.5%, or 1.0% (w/v). The surfactants that were investigated included: poloxamer L44, poloxamer L62, Sp20, Sp80, and polysorbate 80. After vortexing and allowing each sample to equilibrate for at least 30 min, the ciprofloxacin encapsulation state was determined by centrifugal filtration in duplicate. Each data point represents the mean ($n = 2$). There are no data points using 0.5% or 1% (w/v) Sp20 because of the poor miscibility of the solution at those concentrations. (b) The effect of the addition of polysorbate 80 on the state of ciprofloxacin encapsulation for three different batches of CFI. CFI containing ~50 mg/mL of encapsulated ciprofloxacin was diluted to a final concentration of ~12.5 mg/mL ciprofloxacin with water and an aliquot of 1% (w/v) polysorbate 80 to achieve a final surfactant concentration of 0.05%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, or 0.6% (w/v). After vortexing and allowing each sample to equilibrate for at least 30 min, the ciprofloxacin encapsulation state was determined by centrifugal filtration.

Table 1. The Vesicle Size Distribution for 12.5 mg/mL CFI in the Presence of the Following Surfactants: Poloxamer L44, Poloxamer L62, Sp20, Sp80, Polysorbate 20, and Polysorbate 80

Surfactant (%)	SPAN 80	SPAN 20	Pluronic L62	Polysorbate 80	Pluronic L44	Polysorbate 20
0	93.5 [26.0]					
0.01	93.4 [26.0]	93.7 [27.6]	93.4 [27.6]	93.8 [29.0]	94.7 [34.8]	92.0 [30.6]
0.05	93.5 [26.5]	94.2 [28.9]	93.4 [26.5]	94.0 [24.9]	94.6 [30.4]	93.4 [30.8]
0.1	93.8 [28.2]	94.7 [29.9]	93.0 [25.2]	97.9 [29.2]	94.6 [30.3]	93.9 [30.7]
0.5	94.2 [34.0]	ND[a]	ND	101.3 [33.8]	94.7 [31.4]	95.2 [31.9]
1.0	96.7 [37.4]	ND[a]	93.8 [24.0]	101.6 [26.9]	94.3 [34.0]	94.6 [27.4]

[a]Preparation did not form a miscible solution.
Vesicle size data are reported as the mean (in nm) and [SD].
ND, not done.

without various levels of polysorbate 20 were imaged by cryo-TEM. The CFI formulation without surfactant was composed of spherical, unilamellar liposomes between approximately 50 and 100 nm in size (Fig. 4a). The CFI liposomes do not appear to form agglomerates in contrast to liposomes composed of cholesterol and distearoyl PC, which are nonspherical and interact attractively to form large clusters.[12,23] CFI samples containing 0.05% polysorbate 20 (Fig. 4b), 0.2% polysorbate 20 (Fig. 4c), and 0.4% polysorbate 20 (Fig. 4d) all appeared to be qualitatively similar to CFI with respect to liposome size and lamellarity. However, with increasing concentration of polysorbate 20, there were a greater proportion of liposomes that were lighter in shading suggesting a loss of encapsulated ciprofloxacin (Fig. 4d). There were also a very small fraction of disk-like fragments, which may represent pieces of ruptured liposomes (Fig. 4d). To determine whether the lighter density liposomes are consistent with loss of encapsulated drug, empty liposomes (Fig. 4e), and a 50:50 mixture of empty liposomes and CFI, were also imaged (Fig. 4f). The empty liposomes were comparable to CFI in terms of size and lamellarity, but had lighter density (Fig. 4e). The 1:1 mixture of the empty liposomes and CFI revealed a mosaic of liposomes with both light and dark shading in approximately equal proportion (Fig. 4f). These results suggest that the addition of increasing amounts of polysorbate 20, up to 0.4%, caused leakage of ciprofloxacin from a subset of liposomes which retained their physical integrity after loss of encapsulated drug.

DISCUSSION

We have investigated the interaction of surfactants with a liposomal ciprofloxacin formulation with the goal to develop novel formulations with modified encapsulation states and release properties. Our strategy was to use sublytic quantities of surfactant, below the level that solubilizes the liposomes, so that the liposome vesicles retain their integrity, but may have altered drug release rates.[15,16] The addition of polysorbate 20 resulted in a small loss of encapsulated drug when mixed with CFI using isotonic HB as the dilution vehicle; the amount of released drug increased with greater concentrations of surfactant, but even with 2% polysorbate 20 more than 92% of the ciprofloxacin remained encapsulated (Fig. 1a). These data are consistent with the established mechanism that sublytic concentrations of surfactant form transient pores in liposome bilayers allowing release of small quantities of the encapsulated drug before the membrane barrier is fully recovered.[15,16]

Liposomal ciprofloxacin preparations were also diluted with water to create a hypotonic environment that would be expected to result in osmotic swelling of the liposomes.[24–26] When polysorbate 20 was added just prior to or just after dilution with water, there was a much greater loss of encapsulated drug suggesting a synergistic effect of osmotic swelling and surfactant on release of encapsulated drug (Fig. 1c). The greater the dilution of CFI with water, and thus the greater osmotic imbalance between the interior and exterior of the liposomes, the larger the loss of encapsulated ciprofloxacin. The combination of osmotic swelling and 2% polysorbate 20 led to more than 50% free drug for 10 mg/mL CFI (Fig. 1b) in contrast to only 8% free drug in the absence of osmotic swelling (Fig. 1a).

The addition of polysorbate 20 (or polysorbate 80) causes enhanced leakage of drug in a hypotonic liposome environment. But does it also alter the release rate of the drug that remains encapsulated? For low concentrations of polysorbate 20, up to 0.2%, even in concert with osmotic swelling, there did not appear to be a meaningful effect on the IVR release rate for 12.5 mg/mL CFI (Fig. 3a). This result suggests that osmotic swelling of liposomes, when simultaneously exposed to low concentrations of surfactant, transiently allows enhanced release of drug, but without long-term effect on the bilayer properties that could alter drug release in the IVR assay. In contrast, for higher concentrations of surfactant, for example, 0.4% polysorbate 20 or 0.2% polysorbate 20, there was a significant increase in the IVR release rate for 12.5 mg/mL CFI (Figs. 3a and 3b). This result is consistent with the increased bilayer permeability model.[5,11]

In studies of surfactant interactions with liposomes and phospholipid vesicles, turbidity measurements of the system often increase with surfactant concentration as surfactant increasingly becomes associated with the bilayers, and this has been attributed to an increase in vesicle size.[11,27] Once the vesicles become saturated with surfactant, a decrease in turbidity is often observed because of the partial solubilization of the bilayers and formation of mixed micelles which scatter less light. Although no turbidity measurements were made in our studies, and our goal was to remain below the surfactant-solubilizing concentration, we did observe a general trend of modest increases of vesicle size with increasing surfactant for a number of surfactants tested, consistent with this model (Table 1). The increase in vesicle size was small for most surfactants but was the greatest for polysorbate 80 with an increase in mean vesicle size of up to 8 nm. This increase in vesicle size for polysorbate 80 is likely related to its structure and its propensity to be more disruptive to the membrane bilayer. Although there may be other explanations for this observation, the largest increase

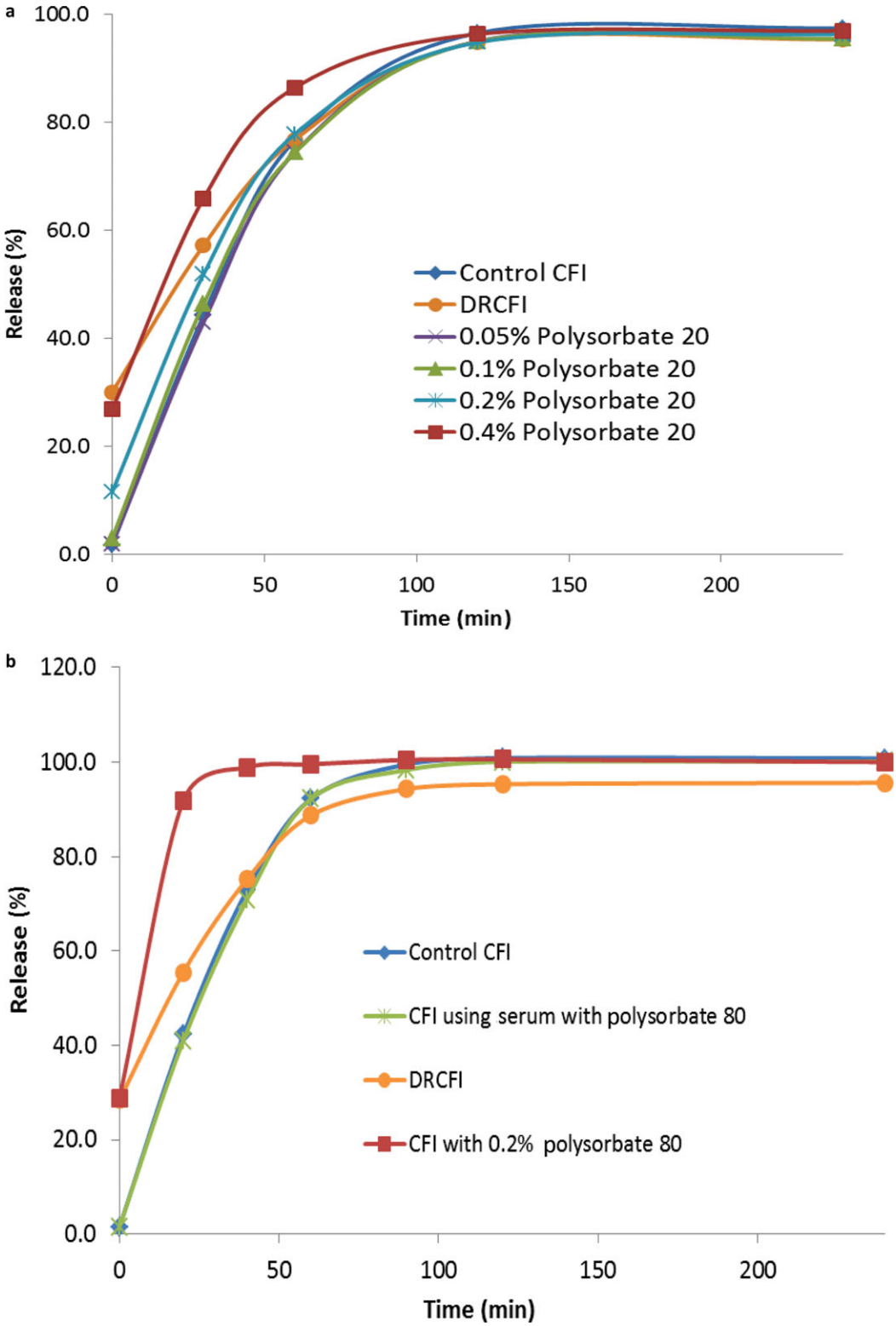

Figure 3. Evaluation of the effect of liposomal ciprofloxacin composition on the IVR assay. The *in vitro* release of 25 μg/mL ciprofloxacin in 50% (v/v) bovine serum and 10 mM HEPES buffered saline, pH 7.4 after incubation at 37°C for 4 h is reported. (a) IVR profiles for Control CFI (blue diamonds), DRCFI (orange circles), 12.5 mg/mL CFI in 0.05% (w/v) polysorbate 20 (purple crosses), 12.5 mg/mL CFI in 0.1% (w/v) polysorbate 20 (green triangles), 12.5 mg/mL CFI in 0.2% (w/v) polysorbate 20 (blue stars), and 12.5 mg/mL CFI in 0.4% (w/v) polysorbate 20 (red squares). Each value represents the mean ($n = 2$). (b) IVR profiles for Control CFI (blue diamonds), DRCFI (orange circles), 12.5 mg/mL CFI in 0.2% (w/v) polysorbate 80 (red squares), and 12.5 mg/mL CFI in serum containing 0.0004% (w/v) polysorbate 20 (green stars). Each value represents the mean ($n = 2$). Reprinted with permission from Ref. 21.

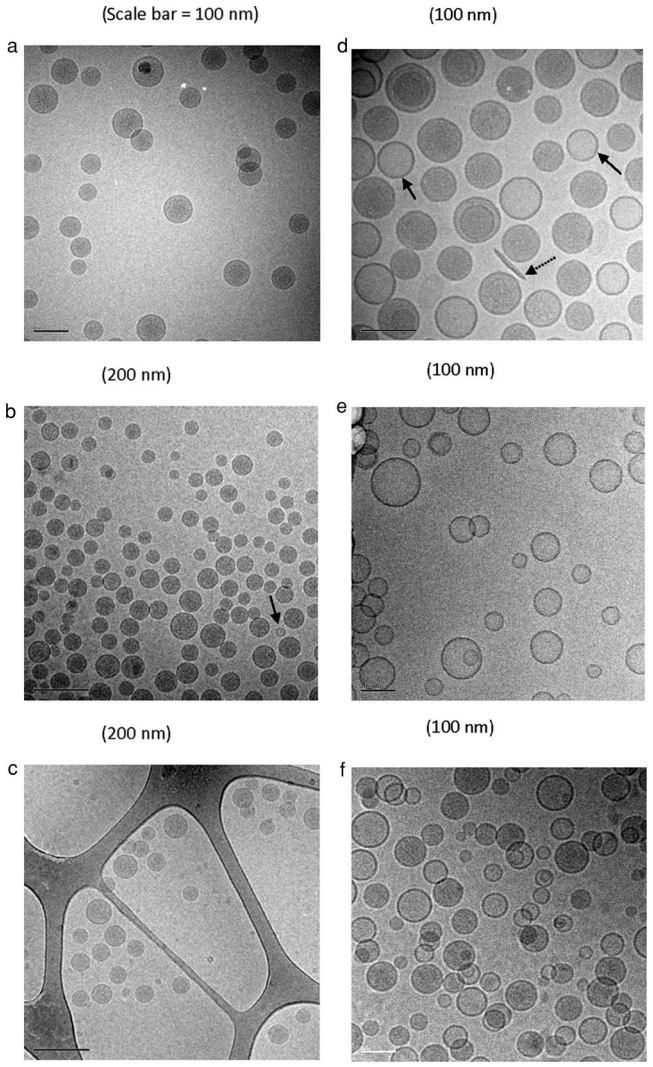

Figure 4. Cryo-TEM micrographs of various preparations of liposomal ciprofloxacin (CFI). The scale bar in the bottom left-hand corner of each micrograph is 100 nm for a, b, e, and f and 200 nm for b and c. All CFI samples were applied at a concentration of ~10 mg/mL liposomes. (a) 12.5 mg/mL ciprofloxacin; (b) 12.5 mg/mL ciprofloxacin in 0.05% (w/v) polysorbate 20; (c) 12.5 mg/mL ciprofloxacin in 0.2% (w/v) polysorbate 20; (d) 12.5 mg/mL ciprofloxacin in 0.4% (w/v) polysorbate 20; (e) empty liposomes; (f) 1:1 mixture of empty liposomes and CFI.

in vesicle size was also associated with the greatest loss in encapsulated drug (Table 1) and the greatest effect on IVR release profile (Fig. 3b), which may reflect upon the extent of the disruption and resulting size increase of the liposomes due to the association with surfactant. It is noteworthy that in the dynamic light scattering (DLS) studies, we did not observe a second population of smaller particles, which would have been consistent with the formation of surfactant micelles. It is possible that some micellar structures may have formed in these studies, as DLS may have been insensitive to the presence of a small percentage of micelles.

Polysorbate 80 had a more pronounced effect than polysorbate 20, both in terms of loss of drug encapsulation (Fig. 2a) and increase in IVR rate (Fig. 3b). Although the head groups are similar for both polysorbate surfactants, the hydrophobic tail for polysorbate 80 is almost twice as long as for polysorbate 20 (C16 vs. C10) and has a kink in the middle, unlike polysorbate 20 that has a saturated hydrophobic tail. Thus, the tail of the polysorbate 80 surfactant will be more disruptive upon insertion into the liposome bilayer than the polysorbate 20 tail, and explains the greater effect that polysorbate 80 has on the transient release of encapsulated drug prior to adjustment by the lipids and cholesterol in the membrane to a new, more-compact state. Once the membrane has accommodated the surfactant, there does not appear to be any further release of encapsulated drug (data not shown). For low concentrations of polysorbate 20, up to 0.2%, there did not appear to be much effect on the IVR release rate of 12.5 mg/mL CFI suggesting that the membrane permeability and packing were not too perturbed (Fig. 3a). However, the addition of 0.4% polysorbate 20 to 12.5 mg/mL CFI led to a more rapid release of encapsulated drug in the IVR assay (Fig. 3a). For this formulation, the ratio of surfactant to liposomes was 1:6.25 by weight, which suggests that the surfactant could represent up to approximately 14% of the liposome bilayer on average, if all of the surfactant was incorporated into the liposomes. One possible explanation for this transition is that once the surfactant reaches a critical level (between 0.2% and 0.4% for polysorbate 20 and less than 0.2% for polysorbate 80 in 12.5 mg/mL CFI), the cholesterol and lipids may be less able to accommodate the surfactant, and while the membrane retains its barrier properties to encapsulated drug, it may be more easily fluidized after mixing with serum in the IVR assay. The packing of cholesterol and lipids around the polysorbate 80 tail will be even less effective than for the polysorbate 20 tail, thus explaining the more pronounced disruption to drug encapsulation and IVR profile at lower surfactant concentrations for polysorbate 80.

To determine whether these observations are broadly applicable to other nonionic surfactants with similar chemistries, the encapsulation studies were repeated using poloxamer L44 and L62, and Sp20 and Sp80 surfactants. However, none of these surfactants resulted in meaningful changes in the amount of encapsulated drug. The Sp20 and Sp80 surfactants have identical hydrophobic tails to polysorbate 20 and polysorbate 80, respectively, but a different head group. These surfactants were much less disruptive than the polysorbates, indicating that the specific hydrophilic head group plays a key role in this process. For successful insertion of the surfactant tail into the bilayer, the surfactant head group apparently must successfully associate with the hydrophilic head group of the phospholipid. Both poloxamer surfactants, composed of a hydrophobic polyoxypropylene segment, flanked by two hydrophilic chains of polyoxyethylene, had very little effect on drug encapsulation (Fig. 2a), suggesting that they were also not effectively able to insert into the liposome bilayer.

Cryo-TEM studies were conducted to shed light on the consequences of the surfactant interactions with the liposomes. That so much more drug is released under hypotonic conditions raises the question of whether osmotic swelling is simply enhancing the transient release of encapsulated drug after exposure to surfactant, or if a large population of vesicles is being completely disrupted. In other words, are a large fraction of liposomes releasing all of their drug payload, or are all liposomes releasing some fraction of their payload, or is it a combination of the two. The cryo-TEM micrographs of mixtures of CFI containing low levels of polysorbate 20 are practically indistinguishable from CFI alone (Figs. 4a–4c). All three micrographs show predominantly spherical, unilamellar liposomes around

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50–100 nm in diameter. For these low levels of polysorbate 20 added to CFI in a hypotonic environment, there does not appear to be meaningful changes in liposome size, shape, or integrity. Micellar structures were not observed in any of the liposomal samples with added surfactant.

When 0.4% polysorbate 20 was added to 12.5 mg/mL CFI in a hypotonic environment, there was approximately 30% loss of encapsulated drug. By cryo-TEM imaging, the majority of liposomes appeared unaltered in size and shape and there was minimal liposomal debris which might be indicative of vesicle rupture (Fig. 4d). However, a second population of liposomes emerged, which were comparable in size, shape, and lamellarity to the unmodified CFI liposomes, but had lighter shading (Figs. 4d vs. 4a). These vesicles may have lost a substantial fraction of their encapsulated drug, or all of it, as the density of their interior space is not dissimilar to that for empty liposomes (Figs. 4d–4f). This result suggests that a second release mechanism may be occurring in the presence of higher concentrations of surfactant. Rather than simple leakage of a small fraction of the drug content from some of the liposomes, a subset of liposomes appear to have released all of their drug content and yet retained their vesicular shape. Very little liposomal debris or deformed liposomes were observed that might be indicative of liposome rupture. This is consistent with a mechanism that has been proposed that above a certain minimum surfactant concentration, but below the solubilizing concentration, the surfactant may cause an increase in the permeability of the membranes without having any effect on their structure.[5,11] However, that model does not explain why the majority of liposomes appeared unaltered (Fig. 4d).

Although the use of surfactants to produce therapeutic niosomes has received much attention,[28–30] there are few examples in the literature where surfactant was added to phospholipid vesicles or liposomes to intentionally modify their physicochemical properties or modulate drug release. In one study, polysorbate 20, 60, or 80 was added to soy PC to produce unilamellar vesicles or multilamellar aggregates containing caffeine as the model drug.[31] There was no sustained release of caffeine from the unilamellar liposomes, whether or not surfactant was present, as the release profiles were identical to that for caffeine in solution.[31] In contrast, in our study using unilamellar liposomes, although the presence of small amounts of polysorbate 20 or 80 had a minimal effect on the release rate, higher polysorbate concentrations, in combination with osmotic swelling, caused a faster release profile in the IVR assay. Another key difference is that the surfactant was added to intact liposomes in our study, whereas in the previous studies the surfactant was added prior to liposome manufacture.

Although these studies on modifying the release rate of a liposomal formulation speak to the promise of tailoring therapy to an individual's needs, there are many challenges that would need to be addressed before this approach could be realized in practice. The regulatory pathway for this new treatment paradigm is unclear and so would require discussion and agreement by the innovator with the Regulatory Agencies. As the personalization involves relatively minor qualitative changes in the formulation, and its primary purpose is to modulate the PK profile with the view to optimize the treatment efficacy and safety for each patient, this approach seems much easier to regulate than introduction of products with fundamentally different compositions or manufacturing processes. Another hurdle would be how to determine the ideal release profile for each patient. The answer clearly would depend upon the specific disease being addressed, the properties of the therapeutic being delivered, and the characteristics of the patient that demanded a personalized regimen. Finally, how would a clinical trial be designed to demonstrate safety and efficacy when patients may be receiving formulations with different release profiles? This may not be a totally foreign concept. The active drug is the same for each patient so it may be possible to apply a strategy similar to those for drugs that are dosed on a mg/kg basis. In those studies, the total amount of drug is individualized to the patient, based on their body mass, so why not modify the treatment based on other characteristics of a patient, like their need for a shorter or longer release profile? In the case of the liposomal ciprofloxacin formulation in this study, it is being developed as an aerosol therapy and has successfully completed Phase 2 trials in noncystic fibrosis bronchiectasis patients colonized with *Pseudomonas aeruginosa*.[32] One might imagine that if a sputum sample from a patient indicated colonization with a bacterial strain with a higher minimum inhibitory concentration, then the most effective treatment might be a faster release profile resulting in higher ciprofloxacin concentrations in the lung to better kill the more resistant pathogen. The clinical trial design and its safety and efficacy endpoints may not be much changed from the standard situation when the formulation is not individualized to the patient.

CONCLUSIONS

We have demonstrated that the encapsulation state and IVR properties of a liposomal ciprofloxacin formulation (CFI) could be modified by the addition of polysorbate surfactant to the formed liposomes. Moreover, the effect of surfactant was significantly enhanced if the liposomes were exposed to a hypotonic environment, to induce osmotic swelling, at the time of surfactant addition. Osmotic swelling alone, in the absence of surfactant addition, did not cause significant loss of encapsulated drug or a change in the IVR properties (data not shown). In the case for 12.5 mg/mL CFI containing 0.4% (w/v) polysorbate 20, or 0.2% (w/v) polysorbate 80, the encapsulation state was reduced from >99% to approximately 70%. The 12.5 mg/mL CFI preparation containing 0.4% (w/v) polysorbate 20, or 0.2% (w/v) polysorbate 80, also had altered IVR properties, with faster rates of drug release, suggesting that the permeability of the bilayer was also affected. These studies suggest that it may one day be possible to tailor therapy to an individual, by dialing in the desired encapsulation state and release rate of a liposomal formulation by simple addition of an aliquot of a selected surfactant, at a specific surfactant concentration, to the liposomal drug product. These studies may form the basis for a new paradigm of modifying the release profiles of a liposome formulation by the end user, to better meet their specific treatment needs.

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Conflict of interest: David Cipolla, Huiying Wu, and Igor Gonda are currently employed by Aradigm Corporation. Simon Eastman is currently employed by Northern Lipids, Inc. Hak-Kim Chan declared that no conflict of interest exists.

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