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Design and In Situ Characterization of Lipid Containers with Enhanced Drug Retention

*Benjamin Wong, Cecile Boyer, Christian Steinbeck, David Peters, Jason Schmidt, Ryan van Zanten, Bradley Chmelka, and Joseph A. Zasadzinski**

Drug delivery via liposomes has the potential to expand the therapeutic window by targeting and releasing drug at the disease site while minimizing drug concentrations elsewhere in the body.[1] In a liposome, a single bilayer defines the interior space, regulates release of the liposome contents, and protects the contents from the environment. However, finding a bilayer composition that provides the necessary physical integrity, drug retention, specific targeting and rapid contents release at the disease site has been problematic. Despite 40 years of research, only liposomal doxorubicin and amphotericin B are clinically available. Many drugs, including the antibiotic ciprofloxacin,[2,3] are retained in liposomes for weeks to months in buffer, but are released within minutes in serum.[1,4–9] Phospholipids and/or cholesterol can be removed from liposomes by high-density lipoproteins (HDL), leading to the formation of defects in the bilayer and release of small molecules.[5,9] Lipases and other enzymes can selectively break down lipids within the bilayer.[7] A possible solution is to protect the drug-encapsulating bilayer by surrounding it with a second, protective bilayer shell in a "vesosome".[7,10] The second bilayer increases the serum half-life of ciprofloxacin from <10 min in liposomes to 6 h in vesosomes. PEG-lipid coating prevents vesosome aggregation in blood, leading to a ~2 hour half-life in the mouse circulation. The biodistribution of vesosomes is similar to unilamellar liposomes as shown by near-infrared full body images. The vesosome structure may be a viable alternative for targeted delivery of weakly basic drugs, such as ciprofloxacin, that leak too rapidly from liposomes.

To open and close bilayer capsules so as to entrap other vesicles, we take advantage of the ethanol-induced interdigitated phase. When 100 nm dipalmitoylphosphatidylcholine (DPPC) liposomes formed by extrusion above 41 °C in the L_α phase are cooled to room temperature, the alkyl chains of the lipids "freeze" and tilt to accommodate the area mismatch between the phosphocholine headgroups and the frozen alkyl chains to form the $L_{\beta'}$ phase[10,11] (**Figure 1**). Adding 3 molar ethanol to the $L_{\beta'}$ phase swells the headgroup region, resulting in the formation of the interdigitated L_{bI} phase.[12] Interdigitation increases membrane rigidity and ruptures highly curved liposomes, leading to the formation of bilayer sheets[7,10,11] as shown by freeze-fracture transmission electron microscopy (FFTEM, Figure 1a).[13] X-ray diffraction (Figure 1b) shows a single reflection at $q \approx 15.3$ nm^{-1} indicating an untilted hexagonal lattice with $d \approx 0.41$ nm, consistent with the interdigitated $L_{\beta I}$ phase.[12] Figure 1c shows that the fracture pattern changes following removal of the ethanol from the bulk solution. The X-ray diffraction pattern also shows the changes in microstructure; the single reflection of the interdigitated phases separates into a sharp reflection at $q \approx 14.8$ nm^{-1} with a broad shoulder at $q \approx 15.25$ nm^{-1}. The tilted $L_{\beta'}$ bilayer phase that has a distorted hexagonal lattice with d-spacings of 0.41 nm and 0.42 nm has returned;[12] however, the bilayer sheets remain open after the ethanol is removed (Figure 1c), even though the bilayer is in the $L_{\beta'}$ phase (Figure 1d).

Heating the bilayer sheets above 41 °C into the L_α phase causes the bilayers to close; in the process, small vesicles and colloidal particles are encapsulated (see Supporting Information).[7,10] On cooling to room temperature, the bilayers re-enter the $L_{\beta'}$ phase, but the vesosomes remain closed. This metastable phase progression of $L_\alpha - L_{\beta'} - L_{\mathrm{bI}} - L_{\beta'} - L_\alpha - L_{\beta'}$ is responsible for vesosome production and the process can accommodate small fractions (~6 mol%) of fluorescently labeled lipids, cholesterol and/or polyethylene glycol-lipids with PEG molecular weights varying from 750 to 2000 Da, or larger fractions of saturated dipalmitoylphosphatidylglycerol.[10]

Pulse gradient stimulated echo (PGSE) NMR techniques[14] can distinguish between otherwise identical ciprofloxacin inside and outside liposomes without the need for labeling or extractions (Figure 2), which makes it ideal for release studies. The solution-state, isotropic ^{1}H and ^{19}F chemical shifts of specific moieties of ciprofloxacin (**Figure 2**) depend on the local concentration. For resolved signals in a PGSE NMR measurement, the measured echo-signal intensities, S, in the presence of a magnetic field gradient decay exponentially with the ciprofloxacin diffusivity, D: [14]

$$S = S(0)e^{-D\gamma^2 g^2 \delta^2 (\Delta - \delta/3)}$$

δ is the duration of a field gradient pulse of amplitude, g, γ is the gyromagnetic ratio of the ^{1}H or ^{19}F nuclei, and Δ is the time between the initial and reversed gradient pulses during which diffusion can occur. Ciprofloxacin inside liposomes diffuses

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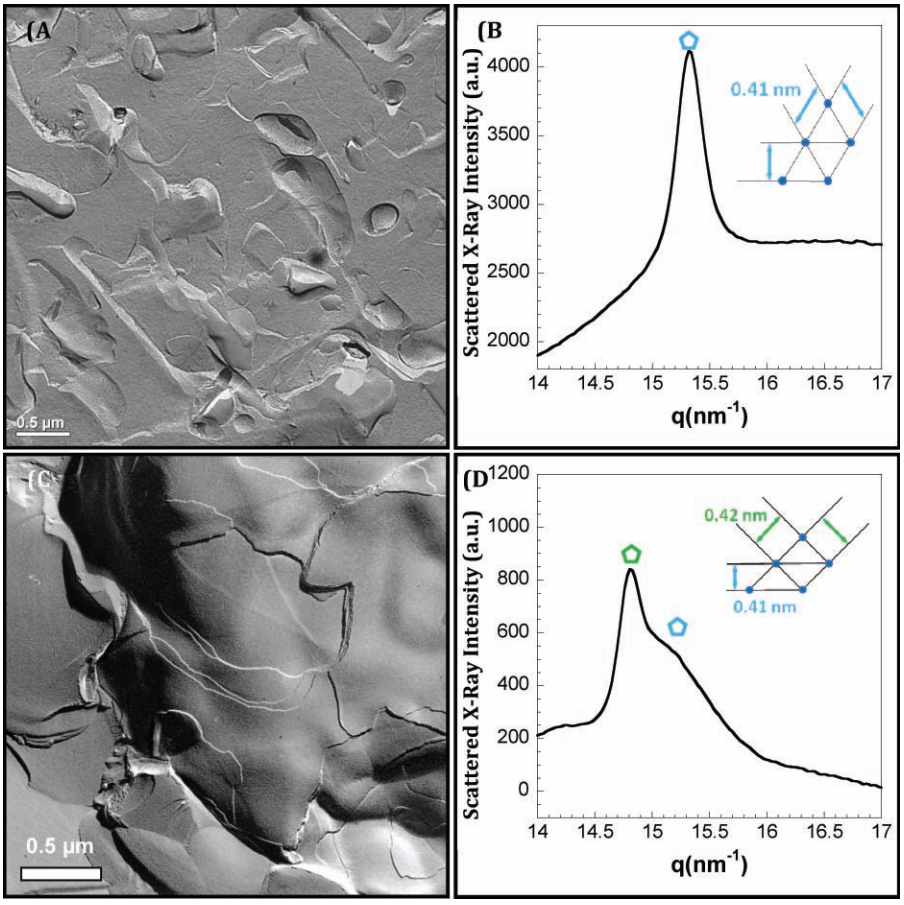

Figure 1. (a) FFTEM of interdigitated DPPC phase in 3M ethanol at room temperature. (b) X-ray diffraction of (a) shows a single reflection at $q \approx 15.3$ nm^{-1} indicating an untilted hexagonal lattice (inset) with $d \approx 0.41$ nm, consistent with the interdigitated $L_{\beta I}$ phase.[12] (c) FFTEM of L_{β}, phase at room temperature following removal of ethanol from (a). The sheet structure remains. (d) A sharp reflection at $q \approx 14.8$ nm^{-1} with a broad shoulder at $q \approx 15.25$ nm^{-1}, indicates a distorted hexagonal lattice with d-spacings of 0.41 nm and 0.42 nm, consistent with the tilted (inset) L_{β}, bilayer phase re-forming after ethanol removal.[12]

with the liposome, so that $D_{in} \sim 10^{-7}$ to 10^{-9} cm^2/s. By comparison, ciprofloxacin in solution has $D_{free} \sim 10^{-5}$ cm^2/s. By appropriate choice of Δ, the amplitude of the ^{1}H or ^{19}F signals corresponding to the fast diffusing, free ciprofloxacin are allowed to decay, while those of the slow diffusing, encapsulated ciprofloxacin are relatively unchanged (Figure 2b).[14,15]

Neutral ciprofloxacin (pK = 6.2), like many other weakly basic drugs, permeates within minutes through bilayers; however, if the liposome interior is acidified, ciprofloxacin is protonated, the bilayer permeability is negligible, and ciprofloxacin accumulates inside the liposomes.[16] To create a pH gradient, liposomes are hydrated with either a 150 or 300 mM ammonium sulfate, 10 mM histidine solution prior to extrusion.[16] Histidine is a NMR pH probe and ^{1}H PGSE NMR was used to follow the ciprofloxacin concentration and pH gradient (Figure 2b). Exchange of the external ammonium sulfate solution for phosphate buffered saline resulted in an liposome internal $pH_{in} \approx 4.5$ for 150 mM ammonium sulfate, and $pH_{in} \approx 4.0$ for the 300 mM ammonium sulfate; $pH_{ext} \approx 5.6$ in both cases. These pH gradients (1.1 and 1.7 pH units, respectively) were stable to 5% for a month. Ciprofloxacin was added to the external solution at a drug/lipid molar ratio of 0.4 and heated at 60 °C for 15 or 30 min. Ciprofloxacin concentrations inside and outside the liposomes were determined by averaging the peak areas from the ciprofloxacin H_5, H_8, and H_2 protons before and after PGSE diffusion filtering (Figure 2a,b). 85–95% of the available ciprofloxacin was concentrated in the liposomes containing 300 mM ammonium sulfate while 50% was encapsulated in liposomes containing 150 mM ammonium sulfate; there was little difference between heating 15 or 30 min at 60 °C. The fraction encapsulated was stable to within 5% for a month at room temperature, as was the pH gradient.[16]

To load vesosomes, ciprofloxacin was added to a vesosome suspension with the internal compartments containing 300 mM ammonium sulfate and heated at 60 °C for 30 min (the external vesosome compartment and the surrounding solution contained buffer at pH 5.5). ^{1}H NMR showed that 90% of the ciprofloxacin in solution was encapsulated. As was the case for unilamellar liposomes (Figure 2b), two peaks (at similar chemical shifts as for the liposomes in Figure 2a) were observed in the ^{1}H NMR spectrum and only one remained after diffusion-filtering via PGSE, corresponding to the entrapped ciprofloxacin.

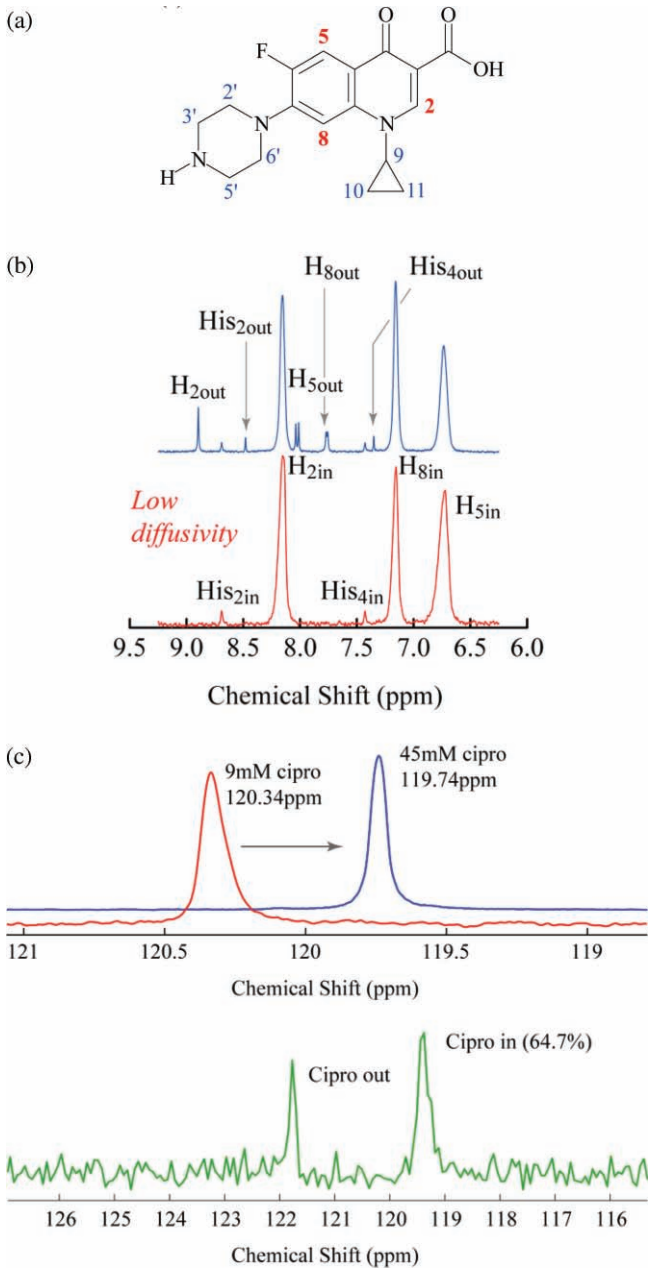

Figure 2. (a) Molecular structure of ciprofloxacin with protons labeled. (b) (top) Solution-state ^{1}H NMR spectrum showing signal from aromatic protons 2, 5 and 8 in a liposome suspension and from histidine used as a pH marker. (bottom) Diffusion-filtered ^{1}H PGSE NMR spectrum that shows only one signal from slow-diffusing ciprofloxacin within the liposomes. (c) Solution-state ^{19}F NMR spectra from ciprofloxacin (top) showing the concentration dependence of the ^{19}F isotropic chemical shift and (bottom) in a liposome suspension with signals corresponding to ciprofloxacin inside and outside of the liposomes as determined from a diffusion-filtered ^{19}F PGSE NMR spectrum (not shown).

Had a significant amount of ciprofloxacin accumulated in the space between the outer and inner vesosome bilayers, a third peak would appear at a chemical shift corresponding to that concentration. However, only two peaks were observed, confirming that ciprofloxacin accumulated in the internal ammonium

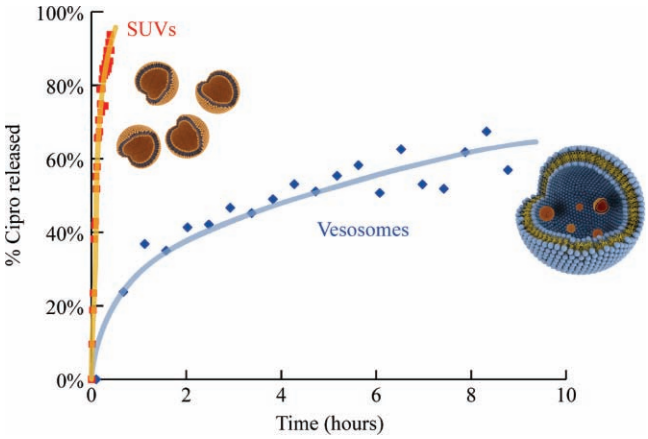

Figure 3. Fraction of ciprofloxacin released as determined from ^{19}F NMR (Figure 2C) at 37 °C in 50% calf serum from unilamellar DPPC/Chol liposomes and vesosomes (without PEG coating). Ciprofloxacin release from liposomes is complete within 30 min while the half-life in vesosomes is ~6 h. Lines are provided to guide the eye.

sulfate-containing compartments by permeating through two bilayers. The fraction encapsulated and pH were stable to within 5% for a month.

^{19}F PGSE NMR was used to determine the half-life of ciprofloxacin in serum to avoid the overlapping ^{1}H signals of the serum components. Liposomes or vesosomes were combined with freshly thawed calf serum in a 1:1 volume ratio, and NMR measurements were immediately initiated at 37 °C, yielding two ^{19}F signals, one at 119.75 ppm corresponding to high ciprofloxacin concentrations shown to be inside the liposomes or vesosomes by PGSE, and a low concentration signal that ranged from 120–122 ppm for free ciprofloxacin (Figure 2c). Within 30 min (**Figure 3**), the liposomes were empty, consistent with literature results for ciprofloxacin (and similar weakly basic drug) release from phospholipid/cholesterol liposomes.[3,4,6,17–19] In comparison, vesosomes released ~20% of their initial contents after exposure to 50% serum in the first 30 min. More than 40% of the encapsulated ciprofloxacin was retained after 10 h in serum at 37 °C; the half-life for release increased from <10 min in liposomes to ~6 h in vesosomes. Again, the absence of a third ^{19}F signal, intermediate in chemical shift between the inside and outside ciprofloxacin concentrations (Figure 2c), show that the external vesosome bilayer did not provide a barrier to ciprofloxacin release. During serum exposure, the pH of liposomes containing ciprofloxacin remained <5, while the ciprofloxacin exterior to the liposomes was at pH 7. No intermediate values of the liposome pH were seen. These results suggests that liposome release is due to failure of the bilayer barrier which led to rapid (about one minute between NMR measurements) equilibration of the liposome or vesosome contents and pH with the surrounding solution, rather than a gradual neutralization of the liposome interiors by permeation of the ammonium sulfate.

The external membrane of the vesosome increases retention by being a barrier, not to small molecules, but to entry of larger molecular weight enzymes, lipases, or complexes that disrupt the interior compartments. Phospholipase A_2 (PLA_2,

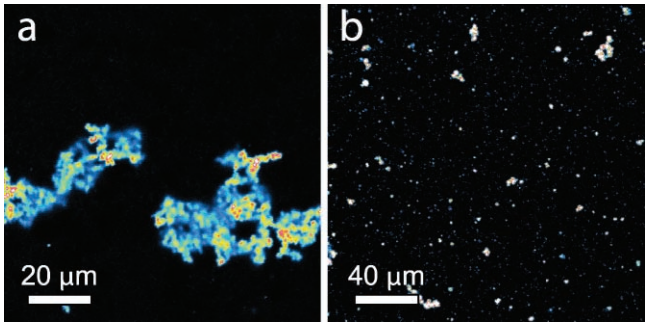

Figure 4. (a) Fluorescence image of aggregation of Texas-red DHPE labeled vesosomes after 30 min in human blood at 37 °C. (b) Fluorescence images of similar vesosomes as in (a) except for 4 mol% DPPE-PEG_{750} added to the outer bilayer shell. Aggregation is substantially reduced.

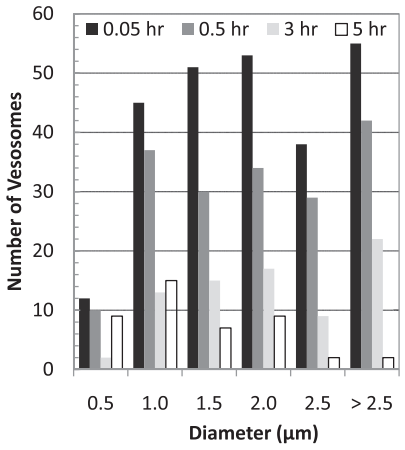

Figure 5. Size distribution of vesosomes with 4 mol% DPPE-PEG_{750} in the mouse circulation at different times after injection as measured from fluorescence images. The half-life of >2.5 μm diameter vesosomes is ~1 h compared to the >2 h half-life for vesosomes <2.5 μm.

14 kDa) releases carboxyfluorescein (and other small molecules) in hours from liposomes, but the same small molecules are retained in vesosomes for days.[7] In serum, HDL and other lipoproteins can form defects in the bilayer sufficiently large for small molecules to be released.[5,9] However, due to the vesosome structure, HDL or PLA_2 can generate defects in the interior compartments only if they can find a sufficiently large defect in the exterior bilayer that they can pass through. By this time, the external bilayer is sufficiently degraded that it would no longer serve as a barrier to small molecule release, consistent with the NMR results that show no ciprofloxacin accumulates in the space between the interior compartments and the external bilayer.

There is disagreement concerning whether one of the main functions of a PEG-lipid coating is to reduce liposome aggregation via steric repulsion, or to reduce plasma protein adsorption, which prevents opsonization and removal by the reticuloendothelial system (RES).[8] **Figure 4** shows fluorescently labeled vesosomes with and without 4 mol% DPPC–PEG_{750} in the exterior bilayer after 30 min in human blood at 37 °C. Without the PEG coating, the vesosomes form multimicron aggregates that are stable to vortexing (Figure 4a). With the PEG layer (Figure 4b), the vesosomes remain dispersed in blood. To determine the circulation lifetime of the PEG_{750} coated vesosomes, Texas-red DHPE-labeled vesosomes were injected into the tail vein of wild-type mice. Blood was drawn at regular intervals from the optic vein and images at uniform magnification were quantified for fluorescence intensity as a function of time post-injection. The overall fluorescence intensity measured from the images decayed exponentially with a half-life of 2 h. **Figure 5** shows the size distribution over time of vesosomes counted from a typical set of images. The initial distribution of the vesosome population was quite broad, with particles ranging from less than 0.5 μm to >5 μm. Some of the larger particles were likely aggregates as seen in Figure 4. Large particles were removed from the circulation faster than the smaller particles; after 5 h, the mean size of vesosomes in the blood decreased from 2.2 to 1.3 μm and almost all the particles with size >2.5 μm were removed.

To confirm the circulation lifetimes measured from the blood sampling, whole body, near-infrared fluorescence imaging was used to visualize the vesosome biodistribution in a living mouse (**Figure 6**). Empty vesosomes were labeled on the exterior of the membrane with Alexa Fluor 750 (AF_{750},$\lambda_{ex}/\lambda_{em}$ 749 nm/775 nm) covalently linked to DSPE-PEG. Tissue autofluorescence was

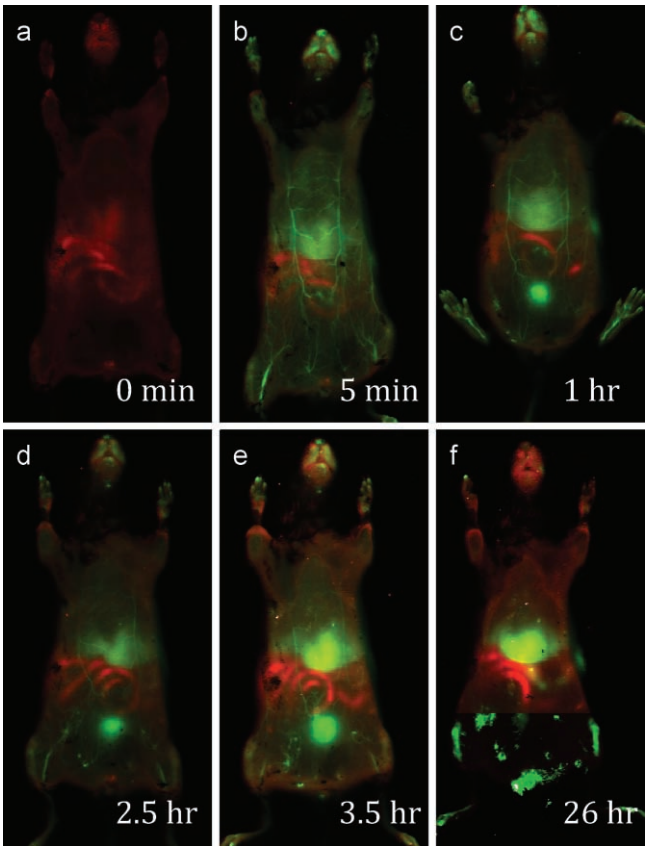

Figure 6. False-color near-infared fluorescence intensity maps of the ventral aspect of a mouse prior to injection with Alexa Fluor 750 labeled vesosomes and at 5 min, 1 h, 2.5 h and 3.5 h, following injection. The red is body autofluorescence detected at 700 nm and the green is fluorescence detected at 800 nm due to the labeled vesosomes.

detected at 700 nm (red/orange) as shown in the pre-injection image; the AF_{750} labeled vesosomes were detected at 800 nm (green) in the post-injection images. The vasculature can be seen immediately following injection, and the green fluorescence intensity decreases as the vesosomes are cleared from the circulation. Highly-vascularized regions such as the face, paws, liver and spleen show the highest green fluorescence intensity. This biodistribution is similar to that reported for small unilamellar liposomes.[1,4] There is an absence of vesosome accumulation in the lungs, heart, and gut. The bladder is also visible in several of the images as the circular green region near the bottom, implying processing of the vesosomes through the kidneys. However, even at 3.5 h, there is substantial green fluorescence visible throughout the larger vasculature, consistent with the ~2 h half-life measured by blood sampling.

In summary, X-ray diffraction and freeze-fracture microscopy show that vesosome assembly occurs via a metastable bilayer phase progression of $L_\alpha - L_{\beta'} - L_{bI} - L_{\beta'} - L_\alpha - L_{\beta'}$ as temperature and ethanol concentration are changed. In vesosomes, ciprofloxacin only accumulates in the interior compartments in response to a pH gradient. Both the encapsulated ciprofloxacin concentration and the pH gradient are stable for a month in buffer for both liposomes and vesosomes. In serum, the multilayered vesosome structure increased ciprofloxacin retention from <10 min to ~6 h compared to unilamellar liposomes of the same composition. Prior work on increasing ciprofloxacin (and similar weakly basic drugs) retention in liposomes has only achieved a maximum of ~1 h,[6] which shows the advantage of the vesosome structure. PEG-lipid coating prevents the aggregation of vesosomes in blood,[8] leading to a half-life in the mouse circulation of 2 h. Direct full body imaging of near infrared dye-labeled vesosomes show a similar circulation half-life and that the biodistribution of vesosomes is similar to that of conventional liposomes.

These results show a distinct advantage to adding a second bilayer to protect drug carrying compartments from blood components. The current generation of vesosomes would benefit from efforts to decrease the mean size, which would likely extend the in vivo circulation time. However, as ciprofloxacin retention (6 h half-life) in vesosomes is significantly greater that the circulation half-life (2 h), the vesosome might be useful as an actively targeted carrier. Non-specific vesosomes would have sufficient time to clear from the circulation and drug release would occur primarily from vesosomes retained at the target site.

Experimental Section

Materials and Methods: DPPC, DPPE-PEG_{750}, DPPE-PEG_{2000}, and cholesterol were purchased from Avanti Polar Lipids (Alabaster, Al). Lipid-conjugated fluorescent dyes NBD-HPC, Oregon Green DHPE, and Texas Red DHPE were purchased from Invitrogen. Alexa Fluor 750 was obtained from Invitrogen in its succinimidyl ester form, and conjugated to DSPE-PEG_{2000}-NH_2 followed by purification by HPLC. Ammonium sulfate and ciprofloxacin (98%) were purchased from Sigma-Aldrich (St. Louis). Newborn calf serum (Sigma #N4887) was thawed upon reception, divided into 10 mL aliquots and stored frozen at –20 °C.

Vesosome Preparation: DPPC and any necessary cholesterol, dyes or PEG-lipids were dissolved in chloroform in glass vials and the solvent removed by evaporation. The lipids were hydrated with the appropriate buffer at 55 °C, and 50 or 100 nm liposomes were prepared by standard extrusion methods using a Lipex Biomembranes Extruder (Vancouver, Canada).[16] DPPC or modified DPPC liposomes were transformed into interdigitated bilayer sheets by dropwise addition of ethanol (3 molar net ethanol concentration) to the liposome suspension at room temperature.[7,11] The interdigitated sheets were washed with buffer, followed by centrifugation. 55/45 DPPC/Chol liposomes to be encapsulated were added to the washed bilayer sheets and held at 55 °C for 20 min to induce encapsulation and form vesosomes. Vesosomes were separated from unencapsulated vesicles by size exclusion chromatography using Sephacryl S-1000 beads (GE Healthcare).

PGSE NMR: ^1H spectra were acquired at room temperature using a Varian Unity Inova 500 MHz NMR spectrometer. To eliminate the contribution from the water protons in the ^1H spectra, the W5 water suppression sequence was used.[20] For following ciprofloxacin release in serum, the samples were spun in the ^{19}F NMR probehead at ~20 Hz and allowed to equilibrate at 37 °C for 4 min before acquisition began. Each time point corresponded to 16 scans accumulated over 53 s for the liposome samples, and 128 scans accumulated over 7 min for the vesosome samples.

Fluorescence Microscopy: Whole human blood from healthy volunteers was mixed with EDTA before vesosomes were added to a final concentration of 1 mg/mL. The mixture was vortexed and left in a shaking water bath at 37 °C for 30 min. Aliquots were drawn, placed on glass slides and imaged under a fluorescence microscope.

Animal Studies: All animal studies were conducted under a protocol approved by the University of California, Santa Barbara Institutional Animal Use and Care Committee (Santa Barbara, CA). 100 µL of a 2.5 mg/mL dispersion of Texas-red DHPE labeled vesosomes filtered through a 5 µm filter was administered through the tail vein of wild-type mice. Blood was drawn from the optic vein under full anesthesia at various intervals, chelated with EDTA, and a 5–10 µL drop was placed on a microscope slide and sealed with a cover slip. Fluorescence images at fixed magnification were quantified by total fluorescence intensity and by counting individual vesosomes.

For full body imaging, wild-type mice were limited to a high-fat, no cellulose diet to minimize background fluorescence. Hair was removed with a commercial hair removal agent prior to vesosome administration through the tail vein. After being anesthetized, the mouse was placed on an Odyssey Infrared Imaging System at regular intervals post-injection.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

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Supporting Information

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Supplemental Materials

Figure S1: Freeze-fracture TEM image of as-prepared vesosomes. The short arrows point to small, 50 nm diameter liposomes of 55:45 DPPC:Chol that were encapsulated within a 94:4 DPPC:DPPE-PEG750 bilayer (large arrow) using the L_α - $L_{\beta'}$ - L_{bI} - $L_{\beta'}$ - L_α - $L_{\beta'}$ phase progression described in the text. The interior and exterior density of small liposomes (small arrows) is similar suggesting a passive encapsulation mechanism caused by the softening of the bilayer on heating. The untrapped liposomes can be removed by size exclusion chromatography to leave only the larger vesosomes. There are three distinct environments for ciprofloxacin – one exterior to the vesosome in which fast ciprofloxacin diffusion occurs, and two slow diffusing compartments, one inside the interior liposomes that have a low pH due to encapsulated ammonium sulfate and the space between the interior liposomes and the external bilayer that is at the same pH as the surrounding solution.

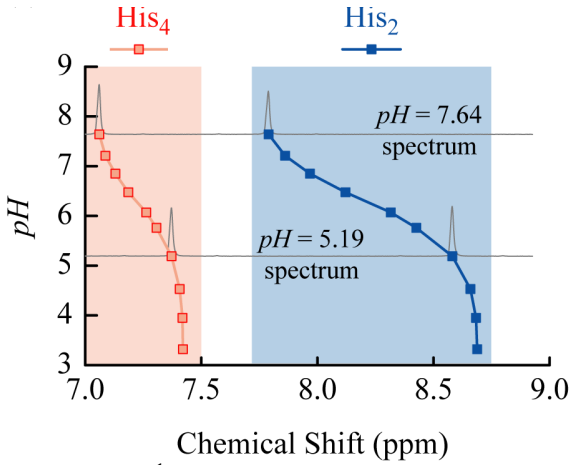

Figure S2. ^{1}H NMR spectra showing the chemical shift of histidine at pH 5.19 and 7.64. The calibration curves below for two histidine protons show the change in chemical shift with pH used to determine the vesosome and liposome internal and external pH in situ.

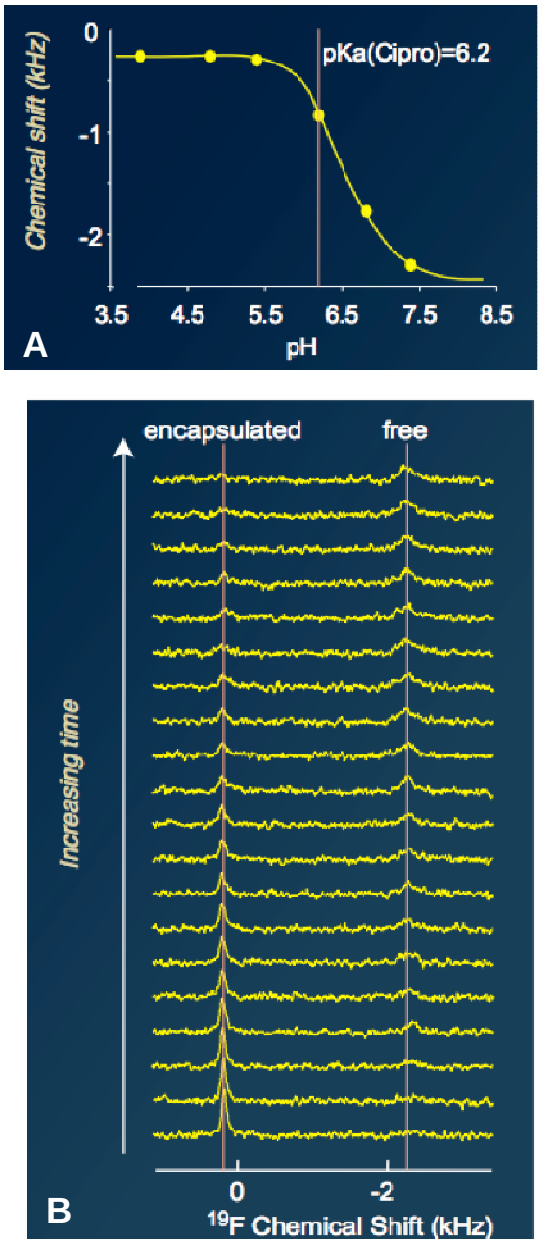

Figure S3. (A) The chemical shift of the ciprofloxacin fluorine in ^{19}F NMR also depends on the pH, similar to the proton spectra of histidine. (B) NMR spectra taken one minute apart of unilamellar liposomes containing cipro loaded with ammonium sulfate. The bottom spectra shows that all the encapsulated ciprofloxacin is in an environment with a pH of ~ 4.5. With time, the encapsulated ciprofloxacin peak decreases in amplitude, but the chemical shift does not change, suggesting that the internal liposome pH does not increase above ~ 5. The free ciprofloxacin in solution has a chemical shift corresponding to ~ pH 7, which also does not change. After ~ 30 minutes exposure to serum, the encapsulated ciprofloxacin peak has disappeared and the free ciprofloxacin peak has increased. From these spectra, the interior liposome pH is not gradually degraded over time by permeation of the ammonium sulfate pH gradient. Rather, it appears that the liposomes equilibrate rapidly, that is, within the time necessary for a single NMR spectra to be taken (~ 1 minute) with the surrounding fluid once the bilayer is compromised by serum components.

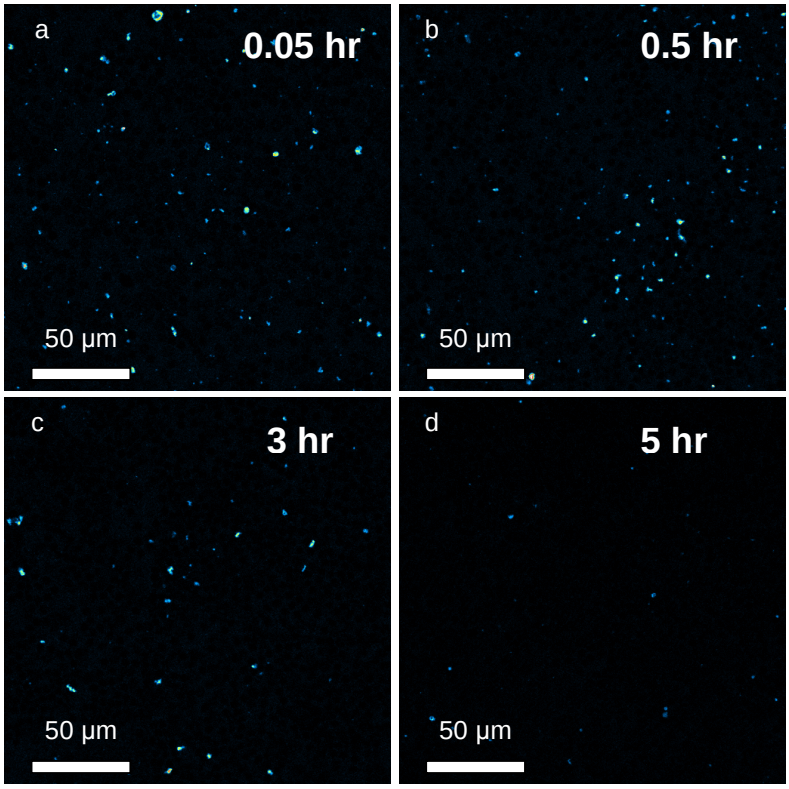

Figure S4. Fluorescence images of Texas red DHPE-labeled vesosomes taken from the optic vein of a mouse at various times after vesosome injection in the tail vein. From the images, the absolute fluorescence intensity and the size distribution and number of vesosomes could be quantified as shown in Fig. 5.

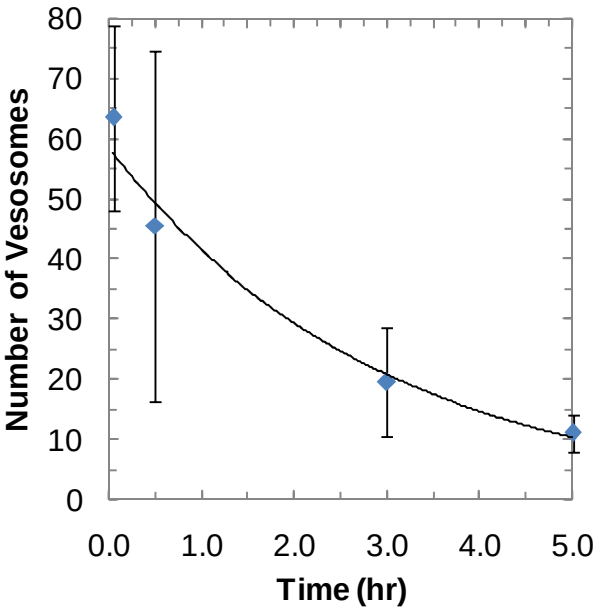

Figure S5. Vesosome half-life measured from average number of vesosomes counted per image as shown in Figure S4. The number decays exponentially with a half life of ~ 2 hours.

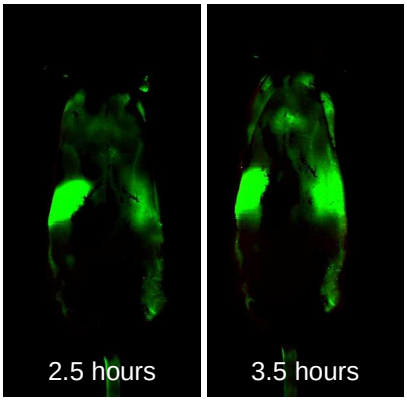

Figure S6. Dorsal view of mouse after injection with Alexa Fluor 750 labeled vesosomes. Bright green areas correspond to kidneys where the vesosomes are likely being filtered out of the circulation, similar to the biodistribution of liposomes in mice.