Electronic Supplementary Material (ESI) for Biomaterials Science.

Laser-responsive sequential delivery of multiple antimicrobials using nanocomposite hydrogels

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SECTION 1: Materials and Methods

Liposome formation and characterization. To create liposomes, established protocol of thin film hydration method was used with few modifications.[1] Lipid films were prepared by dissolving (in separate vials) dipalmitoylphosphatidylcholine (DPPC, phase transition temperature (T_m) of 41 °C, Avanti Lipids, USA) and distearoylphosphatidylcholine (DSPC, T_m of 55 °C) in chloroform and subsequently transferred to a glass vial to have a final weight of 1 mg. The lipids were then dried using a stream of nitrogen gas for 1-2 minutes, followed by overnight lyophilization. The vials with the dried lipid films are stored at -20 °C until use.

To form rifampicin-loaded DSPC multilamellar vesicles (MLVs), rifampicin (7.5 mM, 1ml) was dissolved in citrate buffer (10 mM, pH 4, with 3.75% v/v ethanol) and added to the DSPC lipid film. The solution was then heated and vortexed at 65 °C (10 °C higher than the T_m of DSPC) for 30 minutes resulting in rifampicin-loaded DSPC MLVs. For EDTA-loaded DPPC liposomes, 1 mL of 0.25 M EDTA was first dissolved in 1 M NaOH and added to the DPPC lipid film. For calcein-loaded liposomes, 1 mL of 112 mM calcein was dissolved in 0.27 M NaOH and was added to the DPPC lipid film. The solution was then heated and vortexed at 51 °C (10 °C higher than the $T_m$of DPPC). The multilamellar EDTA/calcein loaded DPPC liposomes were then extruded (through a 100-nm polycarbonate filter over 15×) to form unilamellar vesicles (ULVs).

To separate the drug-loaded liposomes from the unloaded drugs, the resulting solution was passed through a 28-cm size exclusion column made of Sephadex G-50 medium beads that was equilibrated with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer at pH 8.5. The drug-loaded liposomes, due to their bigger size, would pass through the column first, before the unloaded drugs (Figure S1A). These fractions containing the liposomes were collected and centrifuged at 16,000 g (4 °C) for 1 hour to pellet down the liposomes. The resulting supernatant was re-subjected to centrifugation to further pellet the remaining liposomes. The liposome pellets were then combined and diluted to a final volume of 100 µL in HEPES buffer (Figure S1B).

The amount of calcein and rifampicin loaded was quantified by measuring the absorbance using a UV/Vis spectrophotometer (Cary 60 UV-Vis, Agilent Technologies). For imaging using transmission electron microscopy (TEM; Hitachi TEM H7500 MegaView III, Olympus, USA), 1 % uranyl acetate (UA) stain was used to visualize the liposomes. Here, the liposomes were first drop casted onto a formvar coated copper grid for ~2 minutes and excess solution was then wicked off. Subsequently, 5 µL of the 1% UA stain was added, and the excess was wicked off after one minute. The hydrodynamic diameter of the EDTA- and calcein-loaded DPPC ULVs was measured using Dynamic Light Scattering (DLS; NanoBrook Omni Brookhaven Instruments).

Preparation of nanocomposite hybrid hydrogel for testing sequential cargo release. For testing the temperature-dependent sequential release, hydrogels embedded with liposome were formed in a cuvette. Here, 2.5% of a low gelling temperature (26-30 °C) agarose gel (Sigma Aldrich, USA) was first prepared. 5 mg agarose gel powder was melted with 100 µL HEPES (10 mM, pH 8.5) in a cuvette at 70 °C for 2 minutes in a water bath, allowed to cool down for one minute in a 35 °C water bath, and 100 µl of

the liposome solution was subsequently added to this gel and mixed thoroughly. The resulting gel in the cuvette was then immediately placed at 4 °C, for at least 30 minutes before testing drug release.

For testing the cargo release, HEPES buffer (10mM, pH 8.5) was first added to the cuvette. (Figure S5). The temperature of the system was raised using the heating system (single cell holder, Peltier thermostated) of the UV/Vis spectrophotometer. For calcein-loaded DPPC ULVs, drug release was tested at 25 °C (room temperature) and 41 °C (T_m of DPPC), whereas for rifampicin-loaded DSPC MLVs, drug release was tested at 41 °C and 55 °C ($T_m$of DSPC). The drug release in the buffer was measured every 2.5 minutes for 20 minutes at each temperature, and the cuvette was inverted before each measurement to ensure the drug is thoroughly mixed in the buffer solution. Similarly, for testing the sequential drug release, both calcein-loaded DPPC and rifampicin-loaded DSPC liposomes were added in the same gel in a 1:10 ratio. Since calcein has a higher molar absorptivity than rifampicin, a lower amount of calcein was added to clearly visualize the absorbance by both drugs. The absorbance for the drug released was measured using a UV/Vis spectrophotometer at 25 °C, 41 °C, and 55 °C, every 2.5 minutes for 15 minutes at each temperature.

Bacterial Growth Inhibition using Sequential Delivery of EDTA and Rifampicin. To test the efficiency of our sequential delivery system in inhibiting bacterial growth, Gram-negative *Escherichia coli* (strain AR3110, Tetracycline resistant)[2] was used. *E. coli* was grown for 18 hours in LB media liquid culture with 5 µg/ml tetracycline. Bacterial pre-culture was then transferred to new LB media and was harvested after 4 hours by centrifuging at 2000 g for 5 minutes, followed by washing with M9 minimal media. The M9 minimal media used contains 0.05 mM Ca^{2+} and 0.5 mM Mg^{2+} ions. In the sequential delivery assay, the 2.5% low gelling agarose hydrogels were made as mentioned above but in Eppendorf tubes, with a final volume of 50 µl. Four different types of hydrogels were made: hydrogels containing no liposomes, only EDTA-loaded liposomes, only Rifampicin-loaded liposomes, and both EDTA- and Rifampicin- loaded liposomes. After forming the hydrogels, 50 µl of the *E. coli* bacterial solution (OD 0.1) was placed on top of the hydrogel and subjected to heat treatment using water baths. They were treated for 2 minutes at 41 °C, followed by a 10-minute break at room temperature, another 2 minutes at 55 °C, and another 10-minute break at room temperature. After the treatment, the *E. coli* solution was mixed with M9 media in a 1:1 ratio, and the growth was monitored for 20 hours at 37 °C at 244 rpm using a microplate reader (Infinite M Nano, Tecan, Männedorf, Switzerland).

Laser-induced Sequential Drug Release. To raise the temperature of the hydrogel to the liposomes' phase transition temperatures for sequential drug release, we leveraged on the photothermal conversion ability of polydopamine nanoparticles (PDNPs). Here, a low gelling temperature agarose gel (2.5%) with a final volume of 50 µL was prepared in an Eppendorf tube as mentioned above with a final PDNP concentration of 1 mg/ml. PDNPs were prepared as previously reported.[3] To test the laser-induced sequential delivery of calcein and rifampicin, PDNPs were added to the drug-loaded liposomes (1:10 ratio of calcein-loaded DPPC ULVs and rifampicin-loaded DSPC MLVs were mixed). The resulting PDNP-liposome-agarose hydrogel was then immediately placed at 4 °C to solidify the gel, for at least 30 minutes before testing drug release.

For testing the laser-induced drug release, a 400mW CW 808 nm laser with ~3mm diameter (MDL-III-808, CNI Laser, China) was used. HEPES buffer was added on top of the PDNP-liposomes-agarose hydrogel inside an eppendorf tube, and the laser power was cranked up to raise the temperature of the system to approximately 25 °C, 41 °C, and 53 °C. Once the temperature was reached, the laser power was adjusted as needed to maintain the temperature for 5 minutes. The HEPES buffer was then collected and replaced with new buffer between each temperature. The amount of drug released at each temperature was determined by measuring the absorbance using NanoDrop 2000c Spectrophotometer (Thermo Scientific™).

To test the efficacy of the laser induced sequential release of EDTA and rifampicin on *E. coli* growth, the PDNP containing hydrogels were prepared as mentioned above with either no liposomes or embedded with both EDTA- and Rifampicin-loaded liposomes. *E. coli* solution grown as mentioned above with OD 0.1 was added on top of the hydrogel and subjected to laser treatment. The laser power was adjusted to maintain the hydrogel temperature at 41 °C for 2 min, followed by a 10-minute equilibration at RT. Afterwards, the hydrogel was subjected to another set of laser illumination to maintain the temperature at 53 °C for 2 min, followed by a 10-minute equilibration at RT. The treated *E. coli* solution was collected and mixed with fresh minimal M9 media in a 1:1 ratio and growth was monitored for 20 hrs, using a microplate reader reader (Infinite M Nano, Tecan, Männedorf, Switzerland). The number of colony forming units (CFU), after this 20-hour growth, was obtained by CFU counting on LB agar plate (with 5 µg/ml tetracycline).

SECTION 2: Purification and Characterization of Drug Loaded Liposomes

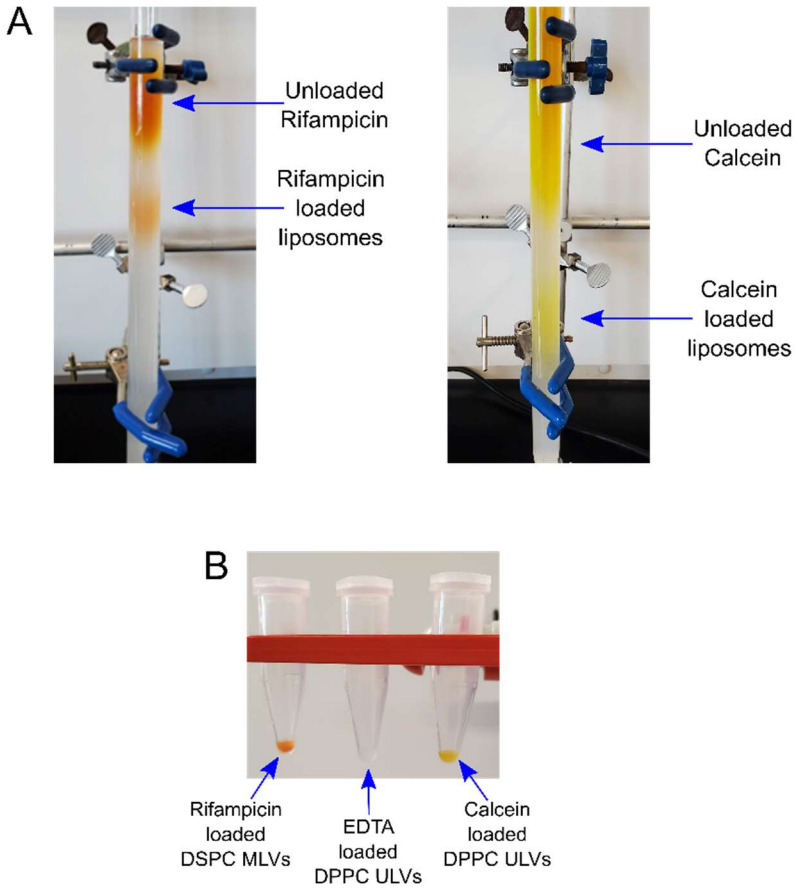

Figure S1. Purification of Drug-Loaded Liposomes. (A) Sephadex G-50 column was used to separate rifampicin-loaded DSPC MLVs from unloaded rifampicin (left) and calcein-loaded DPPC ULVs from unloaded calcein (right). Since the drug loaded liposomes are bigger than the unloaded drug, they pass through the column first and can be collected. (B) Pellets of rifampicin-loaded DSPC MLVs, EDTA-loaded DPPC ULVs, and calcein-loaded DPPC ULVs.

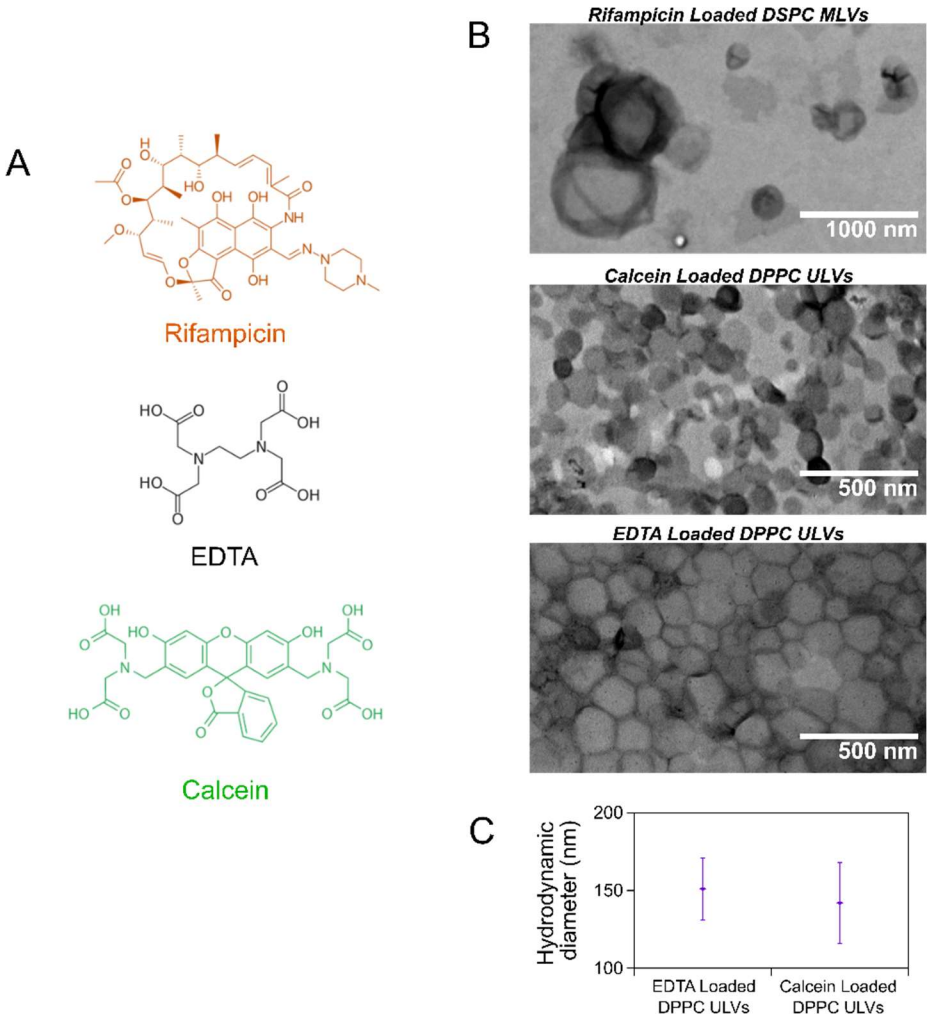

Figure S2. Characterization of drug loaded liposomes. (A) Structures of rifampicin, EDTA, and calcein. (B) Transmission electron microscopy (TEM) images for rifampicin-loaded DSPC MLVs, EDTA-loaded DPPC ULVs, and calcein-loaded DPPC ULVs. The multi-lamellarity of rifampicin-loaded DSPC MLVs is evident, showing that rifampicin-loaded MLVs can be formed, isolated, and purified, which could then be further incorporated into the hybrid hydrogel drug delivery platform. The diameter of EDTA- and calcein- loaded DPPC ULVs measured using the TEM images was found to be 103 ± 26 nm and 124 ± 35, respectively. (C) Hydrodynamic diameters of calcein-loaded DPPC ULVs and EDTA-loaded DPPC ULVs measured using Dynamic Light Scattering (DLS). The hydrodynamic diameters measured via DLS for EDTA- and calcein-loaded DPPC ULVs were found to be 151 ± 20 nm and 142 ± 26 nm, respectively. Both these liposomes were monodispersed with polydispersity indices (PDI) of 0.062 ± 0.03 and 0.0764 ± 0.064, respectively. As rifampicin-loaded MLVs are not monodisperse (TEM images and polydispersity index (PDI) = 0.240 ± 0.02 from DLS measurement), its hydrodynamic size cannot be reliably obtained using DLS.

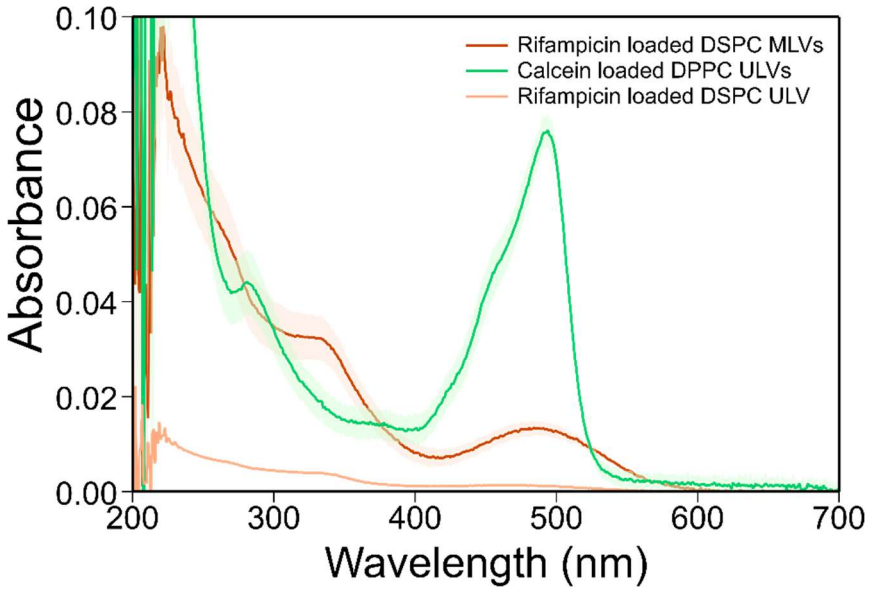

Figure S3. Loading capacity of rifampicin-loaded DSPC MLVs (orange) and calcein-loaded DPPC ULVs (green).

Rifampicin DSPC ULVs:
Absorbance of Rifampicin loaded DSPC ULVs was measured. Background correction was performed using a polynomial (x^2) fitting.
Concentration of Rifampicin Loaded = 0.061 mM
Mass of Rifampicin Loaded = 0.005 mg and Mass of lipids used = 1 mg
Loading Capacity % (w/w) = $\frac{\text{Mass of Rifampicin Loaded (mg)}}{\text{Mass of lipid (mg)}} \times 100$ = 0.5 %

Rifampicin DSPC MLVs:
Absorbance of Rifampicin loaded DSPC MLVs was measured. Background correction was performed using a polynomial (x^2) fitting.
Concentration of Rifampicin Loaded = 2.27±0.2 mM
Mass of Rifampicin Loaded = 0.0373±0.004 mg and Mass of lipids used = 1 mg
Loading Capacity % (w/w) = $\frac{\text{Mass of Rifampicin Loaded (mg)}}{\text{Mass of lipid (mg)}} \times 100$ = 3.7±0.3 %

Calcein:
Absorbance of Calcein loaded DSPC MLVs was measured.
Concentration of Calcein Loaded = 3.0±0.1 mM
Mass of Calcein Loaded = 0.0190±0.001 g and Mass of lipids used = 1 mg
Loading Capacity % (w/w) = $\frac{\text{Mass of Calcein Loaded (mg)}}{\text{Mass of lipid (mg)}} \times 100$ = 1.9±0.1 %

While forming rifampicin-loaded DSPC liposomes, the lamellarity of the liposomes was found to be a crucial factor that could dictate drug loading capacity. Figure S3 shows that the rifampicin loading capacity is drastically higher for MLVs than for ULVs. Similar inconsistencies have been reported for the encapsulation efficiency of rifampicin in liposomes. For instance, Justo et al. reported a 0% rifampicin encapsulation, whereas Manca et al. reported a 74% encapsulation efficiency.[4, 5] For both studies, the same lipid 1,2- distearoyl-sn-glycero-3-phosphocholine and cholesterol were used, along with a similar

thin film hydration method for drug loading. However, one key difference in these groups was the lamellarity of the liposomes formed. Since MLVs have a higher lipid content due to their multiple number of lipid bilayers, they can encapsulate hydrophobic drugs more efficiently than ULVs which only has a single bilayer.[6] As such, rifampicin due to its hydrophobic nature, loads efficiently in MLVs as compared to ULVs.

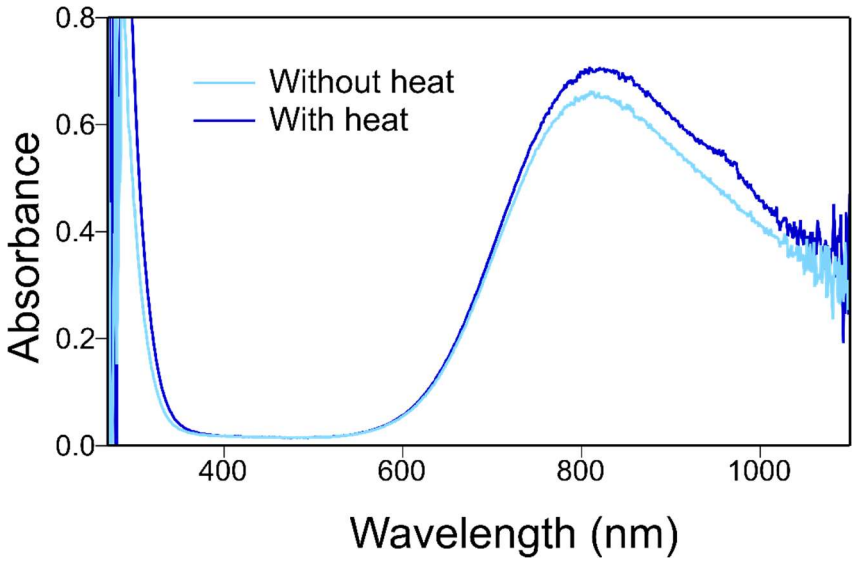

Figure S4. Loading of EDTA in DPPC unilamellar liposomes. To confirm the successful loading of EDTA, EDTA-loaded DPPC ULVs were added in 2.5% low gelling agarose hydrogel with 0.05 M $CuCl_2$ solution added on top of the gel. Upon heating the gel at the T_m of DPPC (41 °C) for 5 minutes, a shift in the absorbance of $CuCl_2$ solution was observed compared to the absorbance without heating. Being a metal ion chelator, this shift in absorbance suggests release of EDTA and chelation of the Cu^{2+} ions in the solution, further suggesting successful loading of EDTA into the liposomes.

SECTION S3 Temperature-dependent release of cargo from liposomes.

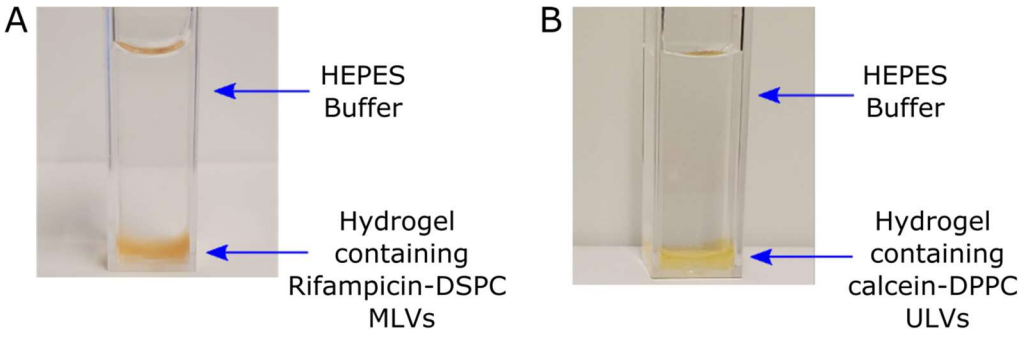

Figure S5. A 2.5% agarose gel containing (A) Rifampicin loaded DSPC MLVs and (B) Calcein loaded DPPC ULVs. The gel is prepared in a cuvette, and once fully formed, HEPES buffer is added on top of the gel. The cargo containing liposomes would remain inside the gel, whereas the cargo (Rifampicin or calcein) released would migrate to the buffer on the top of the gel. The amount released was then calculated using UV/Vis spectrophotometer to measure the absorbance of the drug released.

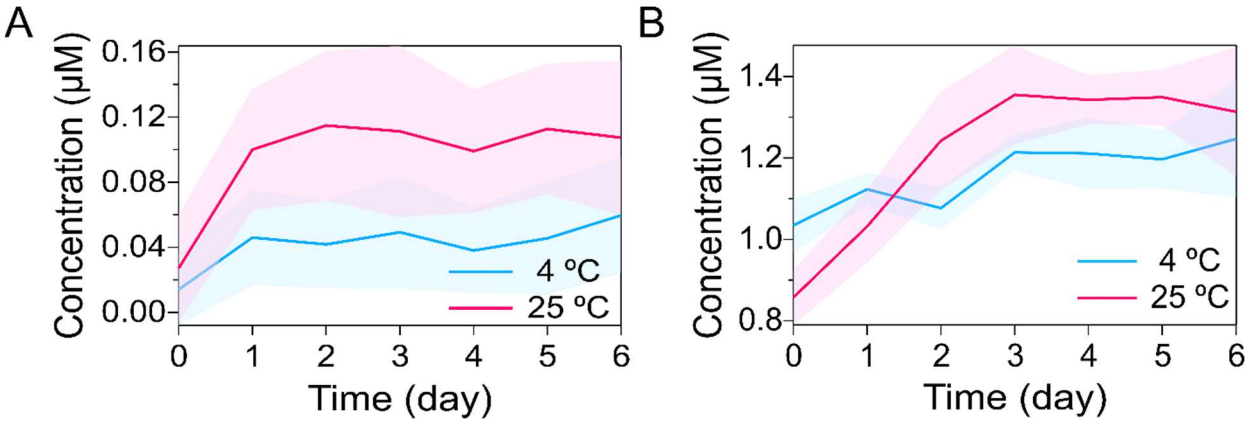

Figure S6. Stability tests for (A) calcein-loaded DPPC ULVs and (B) rifampicin-loaded DSPC MLVs at 4 ℃ and 25 ℃ over 6 days.

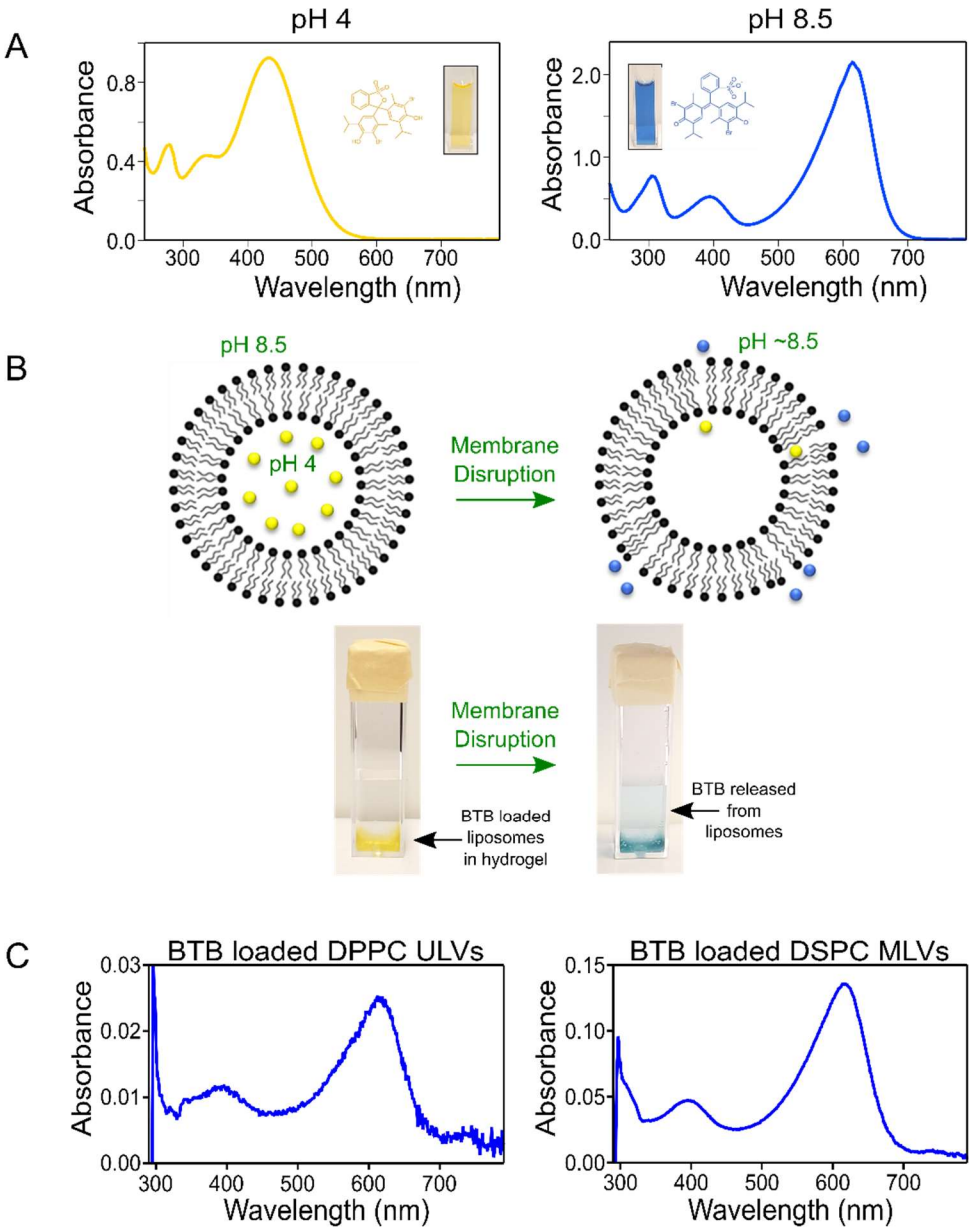

Figure S7. Bromothymol blue assay (BTB) to confirm the temperature dependent leakage of cargo from liposomes. (A) BTB is yellow under acidic conditions (pH <6) and blue under basic conditions (pH >7.6). (B) When liposomes were formed at pH 4, it resulted in yellow liposomes which were added to an agarose hydrogel, with pH 8.5 buffer added on top of the gel. As such, if the liposomes leaked out of the agarose gel, absorbance spectrum corresponding to yellow BTB would be observed. However, if the BTB was released from the liposomes into the basic buffer, absorbance spectrum corresponding to blue BTB right would be observed. (C) For BTB containing DPPC ULVs (left) and DSPC MLVs (right), upon heating the agarose gels at their respective phase transition temperatures for 10 minutes, an absorbance spectrum corresponding to blue BTB is seen, confirming that the loaded cargo is indeed being released from the liposome.

SECTION S4 External heating and *Escherichia coli* growth

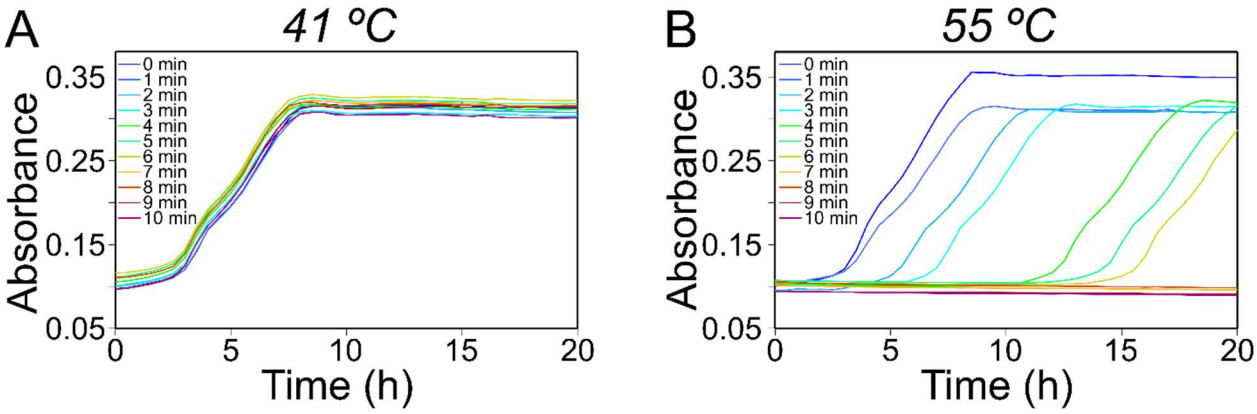

Figure S8. Effect of external heating on *E. coli* growth at (A) 41 ℃and (B) 55 ℃. When bacteria were heated at 41 ℃ for up to 10 minutes, little to no effect was observed on their growth. However, when heated at 55 ℃, the lag phase of *E. coli* growth drastically increased with longer exposure.

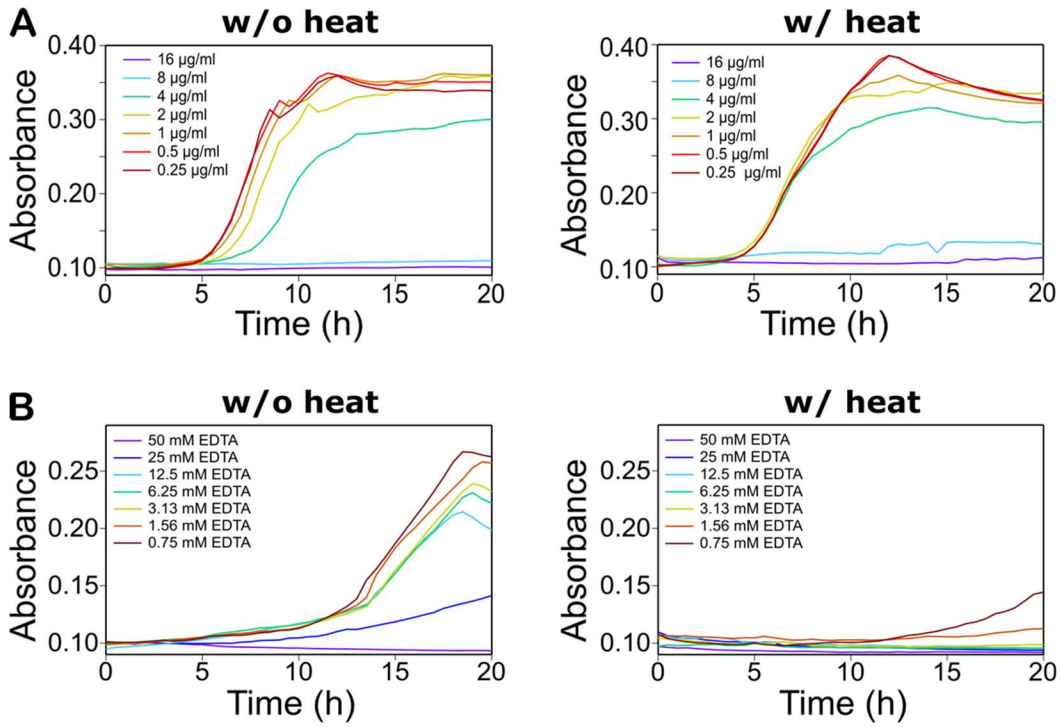

Figure S9. Effect of heat on *E. coli* growth with (A) rifampicin and (B) EDTA. In the presence of EDTA, the bacterial growth is inhibited more when heated (2 minutes at 41 ℃ followed by 2 minutes at 55 ℃), as compared to without heating. However, in the presence of Rifampicin, the bacterial growth inhibition is similar for both with and without heat treatment.

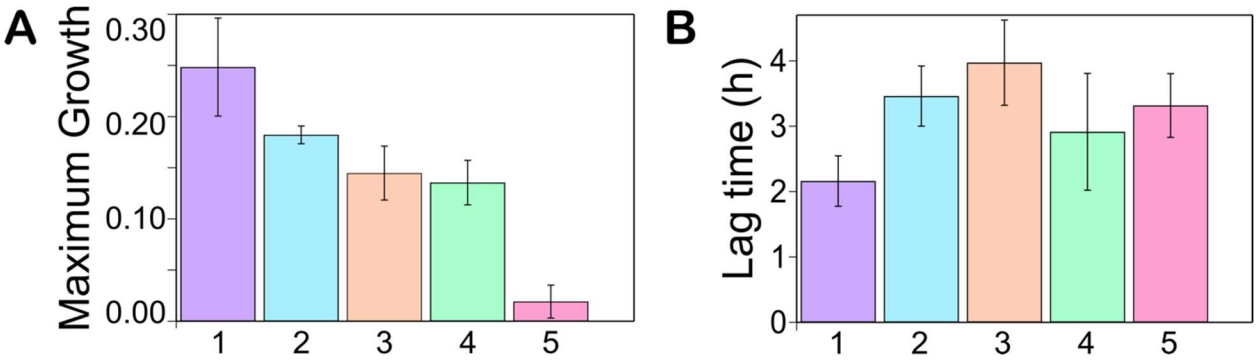

Figure S10. Effect of external heating induced sequential release of EDTA and rifampicin using external heating on *E. coli* growth. (A) Maximum Growth and (B) Lag time for (1) no treatment control, (2) treatment with no liposomes, (3) only rifampicin-loaded liposomes, (4) only EDTA-loaded liposomes, and (5) both EDTA- and rifampicin-loaded liposomes. The maximum growth is drastically inhibited when both EDTA- and rifampicin- loaded liposomes are present in the hydrogel than with no liposomes or either only EDTA- or rifampicin-loaded liposomes are present. For lag time, an increase was observed when both EDTA- and rifampicin- loaded liposomes were present compared to no liposomes condition.

SECTION S5 Laser induced heating of Gram-negative bacteria

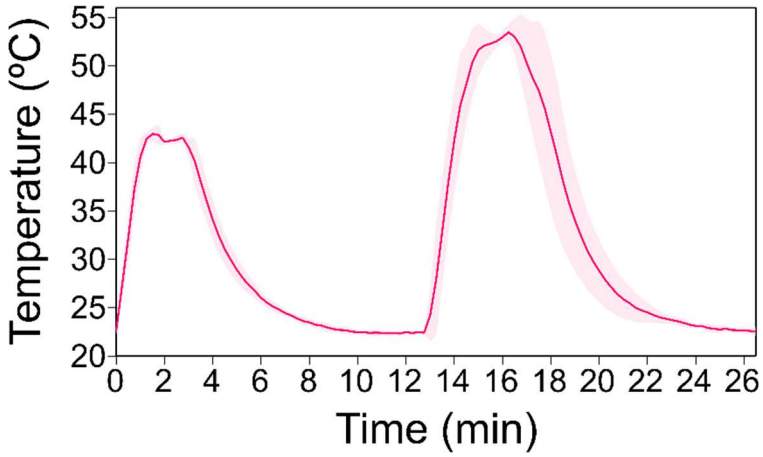

Figure S11. Temperature profile for sequential release of EDTA and rifampicin with laser induced heating. To achieve the sequential release, laser power was adjusted to raise them temperature for 2 minutes at 41 ℃, followed by a 10-minute break, 2 minutes at 55 ℃, followed by another 10-minute break.

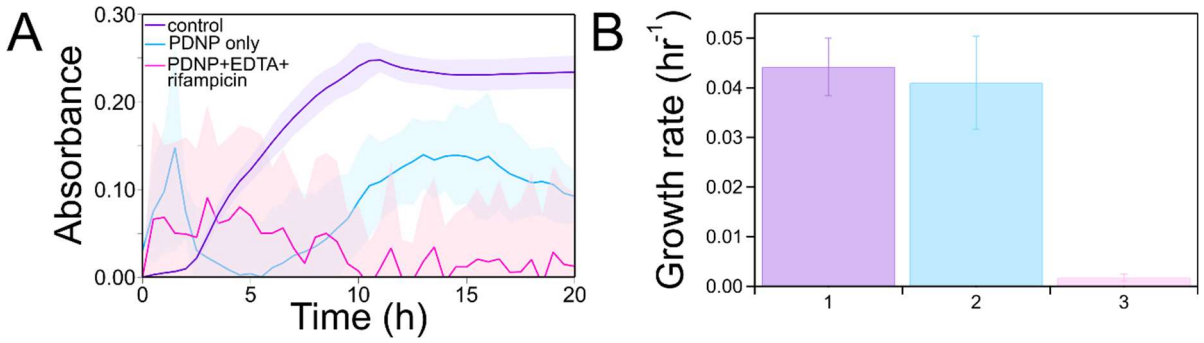

Figure S12. Effect of sequential release of EDTA and Rifampicin using laser induced heating on *E. coli* growth. (A) Growth curves for *E. coli* with no treatment (control, purple) and treatment with no liposomes (PDNP only, blue), and both EDTA- and rifampicin-loaded liposomes (pink) in a hydrogel. (B) Growth rate for (1) no treatment control, (2) treatment with no liposomes, (3) both EDTA- and Rifampicin-loaded liposomes in a hydrogel. The growth rate is drastically inhibited when both EDTA- and rifampicin- loaded liposomes are present in the hydrogel than with no liposomes.

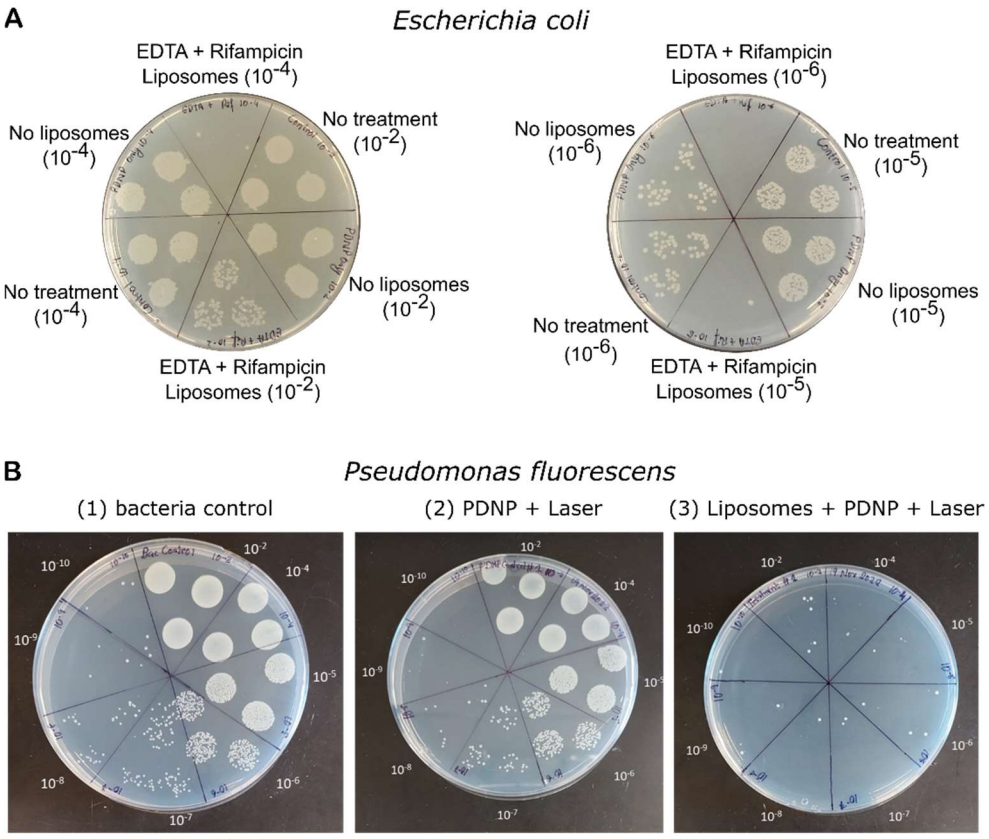

Figure S13. Corresponding agar plate images at different dilutions for the CFU/mL reported for *E. coli* (Figure 3C) and *P. fluorescens* (Figure 3D) at three different conditions: (1) no treatment/no laser (control), (2) no liposomes (PDNP only, with laser), and (3) with both liposomes and PDNP present in the hydrogel and irradiated with laser.

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