Supporting Information

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Chloroquine Liposome Preparation

For liposome preparation, DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine, Avanti Polar Lipids Inc.), Cholesterol(Avanti Polar Lipids Inc.), and Methoxy-PEG2kDa-DSPE (Laysan Bio Inc.) were mixed with chloroform at 10 mg/mL at a molar ratio of 3.8:2.6:1.0 (DSPC):(Cholesterol):(Methoxy-PEG_{2kDa}-DSPE) and then transferred into a 5-mL round-bottom flask. The chloroform was evaporated using a rotary evaporator to form a thin film of dried lipid mixture. Next, 500 µL of 1xPBS containing 40 mg/mL of chloroquine phosphate (Sigma-Aldrich) and adjusted to 60°C was added to the 5-mL round-bottom flask to cover the dried lipid film. The tip of the 5-mL round bottom flask was then immersed in a water bath at 60°C and agitated by hand via shaking in circular motions for 1 min. The tip of flask was then immersed in a sonicating bath (Branson Ultrasonic Cleaner) for 1 min. The steps were repeated for 2x times to full resuspend the dried lipid film in the PBS/chloroquine mixture forming polydisperse multilamellar lipid vesicles. This polydisperse mixture of lipid vesicles was then extruded using the Avanti Polar Lipids Mini Extruder set. The extruder block was set on the hotplate (70°C) to warm up, while the Mini Extruder itself was assembled following manufacturer's instructions with a polycarbonate membrane (100-nm, Avanti Polar Lipids Inc.) prewetted in 1xPBS. The mixture of multilamellar lipid vesicles was then transferred from the 5-mL round bottom flask into a 1-mL gas tight syringe. The syringe was inserted into one end of the Mini Extruder, with an empty syringe (1-mL) into the other end. The Mini Extruder was then placed into the pre-warmed heating block (70°C) and left for 10 min to equilibrate. The mixture of multilamellar lipid vesicles was then passed through the extruder into the other syringe. The extrusion process was completed after 21 passages. After the extrusion process, the solution was noticeably clearer than at the beginning due to the formation of monodisperse liposomes with an average diameter of 100 nm. The syringe

containing the extruded liposome solution was then removed from the extruder. The liposome solution was transferred into a 1.5 mL microcentrifuge tube. Below are the chemical structures of the lipids used for liposome synthesis as well as the structure of chloroquine.

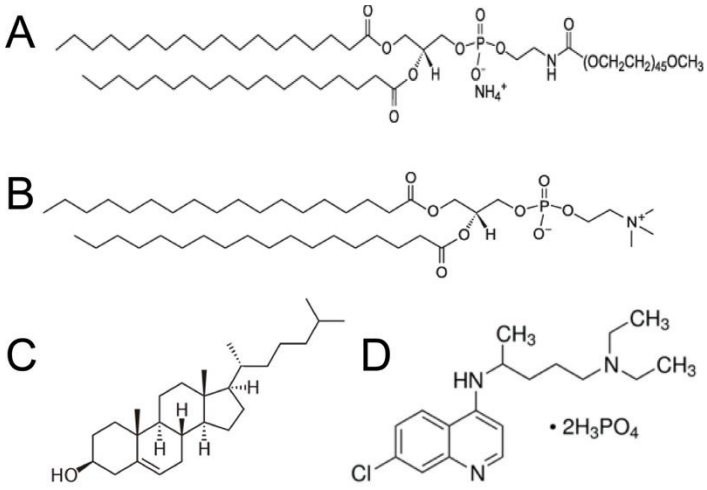

Figure S1: Molecular Structures of Chloroquine Liposome Components: A) Methoxy-PEG$_{2kDa}$-DSPE; B) DSPC; C) Cholesterol; D) Chloroquine diphosphate.

Chloroquine Liposome Purification

For chloroquine liposome purification the previously extruded liposome dispersion volume was measured and put into a 100 kDa centrifugal filter-0.5mL (Amicon Ultra) and spun at 14,000 xg for 20 minutes. The supernatant volume was measured and placed in a 1.5 mL tube. The wash's supernatant chloroquine concentration was measured using UV-Vis analysis (absorption peak at 343 nm). The volume of supernatant removed from the previous wash was added back to the

liposome solution using 1x PBS and then the washing and measuring steps were repeated until the concentration of chloroquine in the supernatant was around 2 mM (about 6 washes). Next, a size exclusion purification column (Illustra NAP-5, GE Healthcare) was set up on a support stand and prepared following the manufacturer's instructions. The previously filtered liposome solution was passed through the size exclusion column and collected in a 50 mL tube. The collected liposome solution volume was around 1.5 mL which required the liposomes to be isolated using another 100 kDa centrifugal filter-0.5mL (Amicon Ultra). The isolation process took 3 spins at 14,000 xg for 20 minutes. A final wash was completed by placing an amount of 1xPBS that returned the liposome solution to its original volume, and spinning it in the filter one more time. Lastly, 1xPBS was added to the filtered liposome dispersion, bringing its volume to the initial volume of ~1.5 mL.

Dynamic Light Scattering and Cryogenic Transmission Electron Microscopy (Cryo TEM)

Malvern ZetaSizer Nano ZS was used for dynamic light scattering and zeta potential measurements. For DLS standard 1.5 mL PMMA cuvettes were used to measure liposomes hydrodynamic diameter in 1x PBS. DLS data was collected in triplicates. For measuring zeta potential, disposable folded Malvern capillary cells (DTS 1070, Malvern) were used. Data was collected in triplicates.

To perform cryogenic transmission electron microscopy, C-Flat multihole copper grids were glow discharged using the GloQube Plus to hydrophilize the carbon film. The Leica EM GP Plunge Freezer was prepared by adding liquid nitrogen, liquid ethane, and water to the designated chambers until full. The system is then left alone until the liquid nitrogen chamber has reached -180°C and the humidity has reached 99%[1]. A grid is then attached to the plunge freezer using forceps, and 3 µL of the sample are applied to form a thin convex droplet. The droplet is blotted off by the Leica system once on the front and twice on

the back for one second each: the grid is blotted on the back to force the sample through the grid holes. Following the plunging of the sample into the liquid ethane chamber, the grid is transferred into the grid holder in preparation for imaging. The grid samples prepared using cryo plunge freezing were imaged using a JEOL TEM 2010-F Field Emission Microscope operated at 200kV. The images were captured with a Direct Electron DE-12 camera. Liposome diameters and bilayer thicknesses were found using ImageJ.

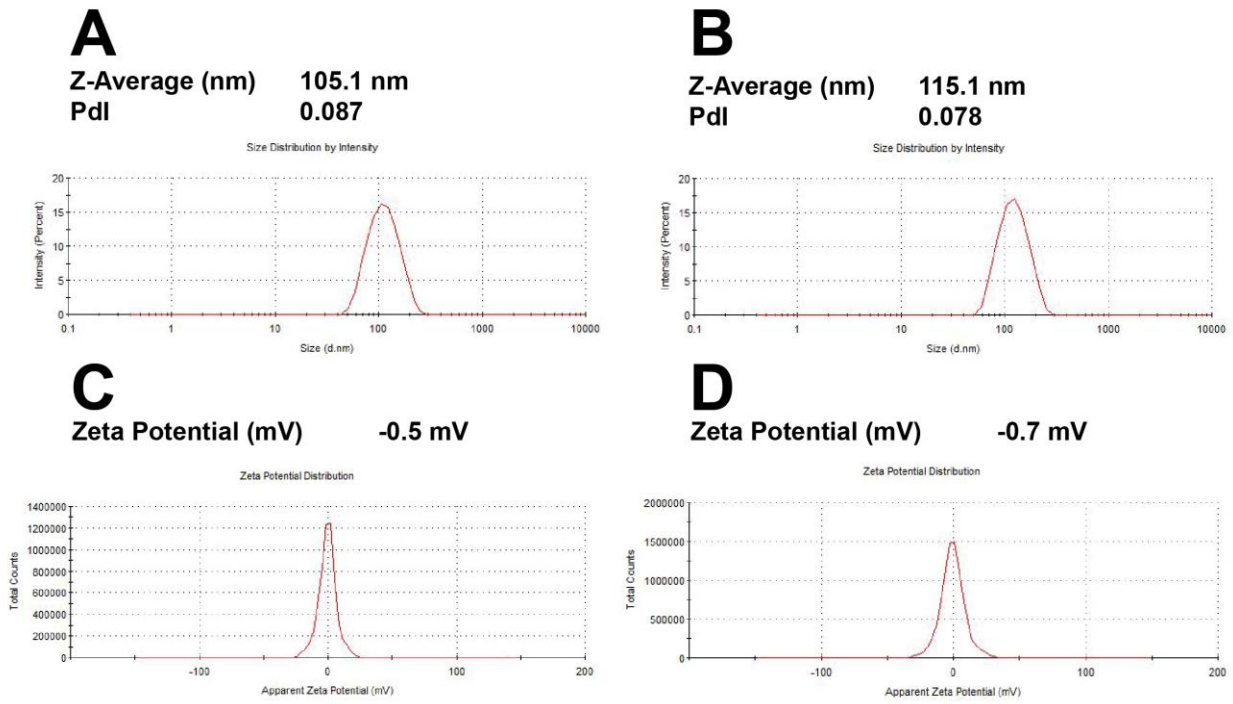

Figure S2: Dynamic light scattering characterization of liposomes with (A) and without (B) chloroquine encapsulation. Zeta potential measurements of liposomes with (C) and without (D) chloroquine encapsulation.

Chloroquine Liposomal Encapsulation via HPLC

All chloroquine samples were run on Alltima HP C18 Hi-Load analytical column 4.6x 250mm with an Agilent 1260 Infinity II HPLC system. The mobile phase consisted of HPLC-grade two phases: A) nanopure water with 0.01% triethylamine adjusted to pH 3.0 with phosphoric acid and B) acetonitrile. The flow rate was 2 mL/min. The detection wavelengths were set at 343 nm and 280 nm. The method that was used proceeded for the first 5 minutes with 100% aqueous phase. After 5 minutes, the device was program med to ramp up to 100% organic phase until 50 minutes had elapsed. 5 minutes later the aqueous phase was reestablished and allowed to equilibrate for an additional 5 minutes. Prior to HPLC analysis, chloroquine liposome dispersions were diluted 10-fold by taking an aliquot of 100 µL of chloroquine liposomes and adding that into 900 µL of 10% Triton X-100 to lyse the liposomes and release all chloroquine followed by brief sonication. From this diluted mixture, samples of 100 µL were injected in triplicate. Accounting for the dilution factor, we calculated the encapsulated chloroquine within liposomes based on the slope of a chloroquine standard curve in 10% Triton-X. At 13.6 minutes a sharp peak eluted at 343 nm which was determined to be chloroquine.

Chloroquine Liposomal Encapsulation via UV-Vis Spectrophotometry

Chloroquine encapsulation of the prepared and sized liposomes was determined by a lysing process using 10% Triton X-100 solution similar to the method used for HPLC analysis. Prior UV-Vis, chloroquine liposome mixtures were diluted by taking a 25 µL aliquot of the chloroquine liposome solution and adding that to 975 µL of 10% Triton X-100 in a 1.5 mL tube. The tube was then agitated for 5 minutes using sonication. From this diluted lysed mixture, a sample of 100 µL of the lysed liposomes was then taken and the chloroquine concentration encapsulated in the liposomes was measured using UV-Vis spectrophotometry analysis in a semi-micro quartz cuvette (absorbance peak of chloroquine at 343 nm) on an Agilent Cary 5000 UV-Vis-NIR spectrophotometer. A calibration curve based on various chloroquine concentrations was generated to obtain the molar decadic extinction coefficient of chloroquine which was applied to determine the concentrations of chloroquine in lysed liposomes accounting for the dilution factor and using Beer Lambert's law.

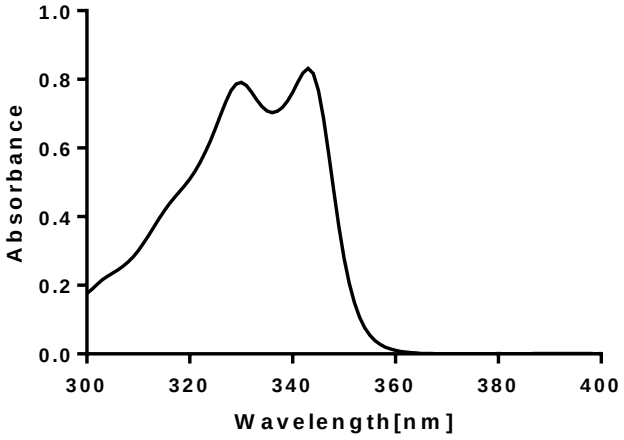

Figure S3: UV-Vis spectrum of 50-µM chloroquine from 400-300 nm where λ_{max} is 343nm.

Cytotoxicity XTT Assay on RAW264.7 Macrophages

RAW264.7 (from ATCC) cells were maintained in DMEM (10% FBS, 1% P/S) media and were seeded at a density of 30,000 cells per well into the wells of a 96 well plate and allowed to adhere overnight. The following day, the media was removed and replaced with fresh DMEM media containing varying dilutions of chloroquine, chloroquine liposomes, or blank liposomes. The cells were incubated at 37°C with the corresponding chloroquine concentrations. Next, the media containing the nanoparticles was removed and the cells were given fresh DMEM media containing the activated XTT reagent per the Sigma Aldrich's guidelines. The cells were incubated with XTT reagent and viability was measured by analyzing the absorbance at 450 nm and 650 nm. The total time the macrophages were exposed to chloroquine was 8 hours.

To determine the IC_{50} of free chloroquine, the following equation was used in GraphPad Prism:

Equation #2 $\mathrm{Y} = \mathrm{Bottom} + (\mathrm{Top} - \mathrm{Bottom})/(1 + 10\hat{}((\mathrm{LogIC50} - \mathrm{X}) * \mathrm{HillSlope}))$

Where the bottom and top values are the values for the plateaus in the curve, IC50 is the concentration of chloroquine that gives a response halfway between the bottom and top plateaus, and hillslope is the steepness of the family of curves. Using Equation #2 the following plot was generated:

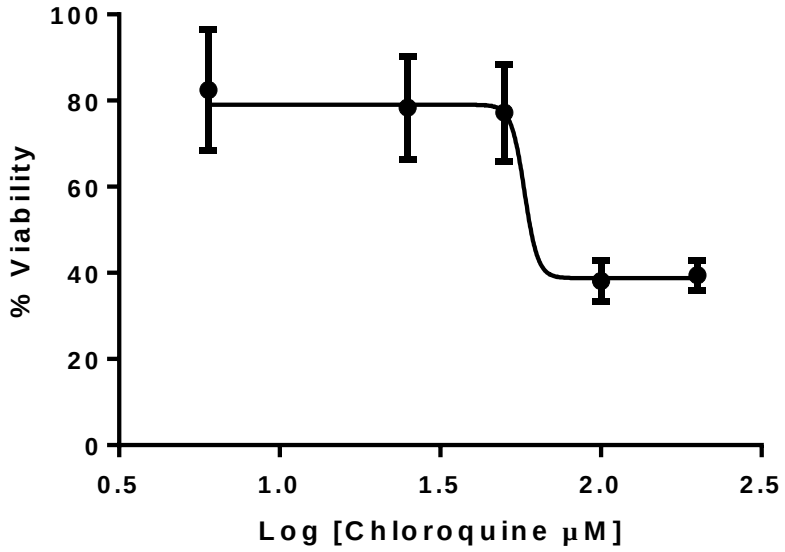

Figure S4: Cell viability in RAW264.7 macrophages plotted against the log of free chloroquine concentrations. The logIC_{50} was determined by GraphPad Prism to be ~ 1.756, yielding an IC_{50} of ~57.0 µM for free chloroquine. Data points represent mean +/- SD (n=5).

Fluorescence-based Quantification of Liposomal Release Kinetics of Chloroquine

Free chloroquine, liposomes without chloroquine, and liposomes encapsulating chloroquine were incubated with either in 10% FBS or 1x PBS in a Corning solid black flat bottom 96 well plate with a lid to prevent evaporation at 37°C for 8 hours. The final chloroquine concentration was 140 µM for each condition (i.e. free chloroquine and chloroquine liposomes) with a well volume was 150 µL to ensure optimal fluorescence signal. Chloroquine fluorescence measurements (ex. 320 nm / em. 400 nm) were taken every 2 hours on a Biotek Cytation 5 microplate reader set to 37°C. Based previous reports found in the main manuscript, and on an excitation and emission spectral scan, these wavelengths were chosen due to the high fluorescence signal and the large distance between the excitation and emission wavelengths with little interference from the solutions. Free chloroquine in solution is highly fluorescent over the course of 8 hours in both FBS and PBS. The background fluorescence signal from each medium was accounted for and subtracted from the measurements. The fluorescence intensity of blank liposomes is consistently below $1.0x10^5$ [a.u.] over the course of 8 hours indicating that the lipid components of liposomes are not fluorescent while chloroquine itself is highly fluorescent and stable in solution.

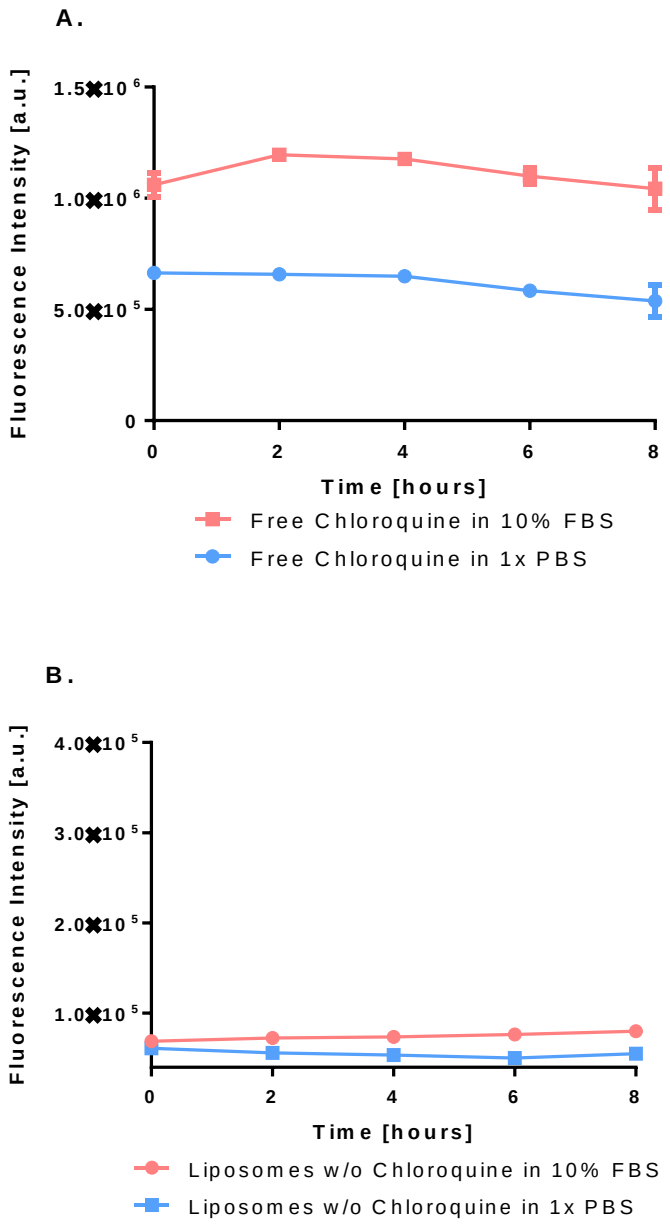

Figure S5: Fluorescence intensity signals from (A) free chloroquine incubated at 37°C; red: in 10% FBS; blue: in 1xPBS; (B) liposomes without chloroquine incubated at 37°C; red: in 10% FBS; blue as a function of time. Data points represent mean +/- SD (n=3).

To obtain the best fit and time constants(τ) for liposomes with chloroquine, the following pseudo first order kinetics Equation #3 was applied in GraphPad Prism:

Equation #1 $$Y = (X < X0,\ Y0, Y0\ +\ (P - Y0) * (1\ -\ \exp(-K * (X - X0))))$$

Where X_0 is the time at which fluorescence intensity signal begins to increase when $X > 2$ hours, Y_0 is the average fluorescence signal up to time X_0. *P* is plateau which was defined as the maximum fluorescence intensity observed for the chloroquine liposomes at 8 hours, and *K* is the rate constant and τ is the reciprocal of *K*.