Supplementary Information

Engineering bio-mimicking functional vesicles with multi-compartments for quantifying molecular transport

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Materials and Methods

Materials: *Escherichia coli* polar extract, 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(3-lysyl(1-glycerol))] (chloride salt) (Lysyl PG), 1′,3′-bis[1,2-dioleoyl-*sn*-glycero-3-phospho]-glycerol (sodium salt) (Cardiolipin), (1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (DOPG), 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) are from Avanti Polar Lipids, Atto-488 DOPE, Mowiol 28-99 (MW 145,000 Da fully hydrolysed Polyvinyl alcohol PVA), Antibiotics: Ciprofloxacin, Norfloxacin, KOH, NaCl, KCl. HEPES, Acetic Acid, Low Melting(LM) agarose (Melting T_m: <65˚C), and other chemicals used in this study are from Sigma-Aldrich Merck unless mentioned otherwise. Dermcidin peptide are purchased from Peptide Protein Research Ltd at >95% purity (HPLC) as lyophilized powders. Vacuum grease from Dow corning. Glass slides (26 x 76mm, 1mm thickness, labtech), Coverslips (22 mm x 0.13 mm thickness) from Blue star, O rings from Amazon.com, Recombinant Staphylococcus alpha-hemolysin protein from Abcam, Alexa Fluor 350 Hydrazide from Thermo-Fischer Invitrogen.

Methods:

Giant unilamellar vesicle formation

Cleaning of slides**:** Microscopic glass slides are sonicated for 30 minutes in 1M KOH, MilliQ water, and ethanol each. The slides are wiped thoroughly with Kim wipes and kept in UV for 15 minutes to prevent the dewetting, followed by nitrogen drying.

Gel-assisted methodology: Poly-vinyl alcohol (5% wt/v PVA) is prepared by dissolving in respective buffer at 90˚C (5mM acetate buffer for pH 5 and 10mM HEPES for pH 7 is used). For successful vesicle preparation, fully hydrolysed (99 - 99.8 % hydrolysis) PVA needs to be used to avoid its dissolution after buffer addition. 200 µL of PVA solution is coated uniformly with another glass slide, to make a film on the cleaned glass slide (covering ¾^{rth} of the slide) as shown in Fig. S1. The slides are kept for drying the PVA film in a hot air oven at 50° C for 1 hour or for 20 minutes on a hot plate at 40° C till a dry film is observed (Fig. S1). 20 µL of chloroform solubilised lipid at a concentration of 0.5 mg/mL to 1 mg/mL is coated uniformly using a clean glass slide/spatula till the chloroform evaporates (Fig. S1). The lipid solution is specifically spread in the O-ring marking as shown in the Fig. S1. The slide is kept under vacuum desiccator for 30 minutes to remove the residual organic solvent. Before addition of the buffer, a chamber is made using a greased O-ring surrounding the lipid film. 150 µL of buffer (137mM NaCl,)2.7 mM KCl in 5mM acetate (for pH 5), or 10mM HEPES (for pH 7) is added inside the O-ring and incubated in a water bath at 40° C for 30 minutes. In case of time dependence, the vesicle solutions are collected as mentioned in next section. The PVA-lipid film after hydration at different time points is shown in Fig.S1. The vesicle solution is pipetted using a cut tip (we usually recover close

to 100 µL of vesicle solution due to corrugated surface of PVA gel). After pipetting out all the solution, the PVA film is checked under the microscope for any remaining vesicles (Fig. S1) and another 50µL buffer is added on to the film to recover the sticking vesicles. This process can be repeated until the PVA film looks clean (Fig. S1). To note, second or third pipetted solution of vesicles tends to have more number aggregates as well.

Time dependent vesicle growth: In case of size control of vesicles, the same procedure is followed as described above to prepare vesicles. The vesicles are then collected at different time points using cut tips to obtain the different sizes. In case of 1.30 or 2 minutes sample, buffer is preheated and added to the lipid-PVA film and allowed to hydrate for 1.30 minutes before collecting. To achieve different sized liposomes, the lipid-PVA film is then hydrated for 5, 15, and 30 minutes for GP and GN vesicles at 40° C and at 15 and 30 minutes for DOPC at room temperature.

Encapsulation: The vesicles are prepared in the same manner as the above procedure. The molecule to be encapsulated in the lumen of vesicles is mixed with the hydration-buffer and then added to the lipid-PVA film. The vesicles are then collected in a time dependent manner. The imaging of vesicles is done by diluting the vesicles atleast 3 times in the respective buffer, this reduces the fluorescence intensity from the outside of the vesicle as compared to inside. The vesicles are allowed to settle on BSA-passivated glass slide before taking imaging.

Preparation of stage for GUV imaging and immobilization in agarose for transport experiments

The sonicated glass slides are wiped thoroughly using kim wipes, and the chamber is prepared with dimension 1.5 cm x 1.5 cm using invisible tape. The slides and coverslips are kept under UV for 15 minutes, and nitrogen dried after.

Low melting agarose (1% w/v, LM) is prepared in 137 mM NaCl, 2.7 mM KCl in 10mM HEPES for pH 7/ 5mM acetate for pH 5. The agarose solution is microwaved at 800 W for 30 seconds with mixing in 15 seconds for complete dissolving. LM agarose (15 µL, 1% w/v at 39° C) is mixed with GUV sample (15 µL) in an Eppendorf tube, mixed with a pipette and added to the tape chamber (Fig. S1). The coverslip is placed on the sample for uniform spreading of the sample inside the tape chamber and gelated on ice for 30 – 40 minutes as done previously.[51] The coverslip is removed, and the immobilized vesicles are observed under the microscope at 60X/40X objective in bright-field to spot the GUVs.

Imaging

The GUV's immobilized in agarose are imaged in Carl Zeiss fluorescence microscope or EVOS epifluorescence Microscope. One/few GUV's in one frame is spotted at a time, and focus is adjusted. The focus of the spotted GUV's is set correctly, and antibiotic solution (30 µL, 2mM) is added on top of the agarose gel, and a coverslip added on top of that. Stocks of Antibiotic (50 mM) norfloxacin and Ciprofloxacin are made in Milli Q water, concentrated HCl is added to the stocks to dissolve the

antibiotics completely. For measurements, 2 mM dilutions of the antibiotics are made in respective salt solutions at both pH 7 and 5, and their pH was checked before each measurement. Images are taken in a time-lapse manner at an interval of 10 seconds. In case of Dermcidin measurements, the peptide at concentration of 0.5 μM at pH 5 and 5- 200 μM at pH 7 is incubated with preformed GP GUV's at room temperature for 1 hour. 1mM of Alexa fluor dye with respective buffer is added on top of the vesicles immobilised in agarose. The coverslip is immediately placed and images taken at 1 minute intervals and after 10 minutes' images are taken every 5 minutes. For alpha-hemolysin measurements, the preformed GN GUV's incubated with alpha-hemolysin monomers (0.4-0.6 μM) for 45 minutes-1 hour at 30° C and then immobilised as described above. Buffer containing 1 mM Alexa fluor dye is added on top of agarose and images are snapped at 30 seconds interval. The corresponding blank measurements without a GUV is taken at the same focus in all cases and at the same time intervals as for the vesicles. For every measurement, the respective dark noise (by blocking the camera) is also taken at the same focus.

Optical setup used for various experiments

Main Fig. 2 A1 to E2 are taken using EVOS bright field mode with 40 X (N.A 0.65). Fig. 2 G1 and H1 are epi-fluorescence images from EVOS with 60X (N.A 0.75). G2 and H2 are snapped using apotome module of Carl Zeiss 40X (N.A 0.75). Encapsulation experiments in Fig. 2 K and L are snapped in dark-field/epi-fluorescence mode using EVOS at 60X (N.A 0.75). Fig. 3 are snapped in bright field mode with EVOS at 60X (N.A 0.75). Antibiotic measurements and alpha-hemolysin transport study for GN vesicles are performed with both EVOS (60X N.A 0.75) and Carl Zeiss (40X N.A 0.75) in epi-fluorescence. Dermcidin peptide measurements and antibiotic diffusion experiments in GP vesicles are performed in Carl Zeiss in epi-fluorescence mode with 40X (N.A 0.75).

Supplementary text

1. Analysis ImageJ/Fiji was used for the analysis of the data.

1.1 Image correction: For uneven illumination

To eliminate the uneven illumination, the correction is done using respective blank images without vesicles and black images(by blocking the camera) as described previously.[1,2] For image subtraction - the blanks are also taken together without vesicles in a time-lapse series. The corresponding GUV original images and corrected are represented in (**Fig. S11**). Image correction is done using equation (1).

$$\text{Corrected image} = \frac{(I - B)}{(S - B)} \times K \qquad (1)$$

I = original image, B = respective black noise image, S= Respective blank image, K= scaled factor <average intensity of the original image image>.

The images are corrected using ImageJ; the original image and blank image are subtracted individually from the black noise using the image calculator process of ImageJ. The resulting images are then further processed according to equation (1) using the Calculator Plus plugin of ImageJ. The image resulting from this process is the corrected image for uneven illumination.

1.2. Permeability coefficient calculation- Antibiotics

The permeability coefficient is determined using equation as described previously[3] (2)

$$P = -\frac{\ln(\Delta I_2 - \Delta I_1 + 1) \times R}{3 \times t} \quad (2)$$

Where

P = Permeability Coefficient, R = Radius of the vesicle, t = time between initial and final time point, t= $t_f - t_0$.

$$\Delta I_1 = \frac{I_{out} - I_1}{I_{out}}(t = 0) \quad (3)$$

$$\Delta I_2 = \frac{I_{out} - I_2}{I_{out}}(t = t_f) \quad (4)$$

I_1 and I_2 refer to the intensity at the initial time point t_0 and final timepoint t_f inside the vesicles, and I_{out} refers to the fluorescent intensity outside the vesicle. The last time point $tf = 3t0$, at t_0 we assume minimal diffusion process to have occurred. Hence a time, three times that of t_0, is chosen where a considerable diffusion takes place. The light intensity exterior to and interior of the vesicle at time point t_0 and t_f is determined using equation (3) and (4), respectively. The I_{out} and I_n (where n=1 or 2) are calculated on the uneven-illumination corrected images snapped at different time points. Average of six 18 x18 square pixels is created to determine the I_{out} and a 18 x18 box inside the vesicle to calculate the I_n, intensity inside the vesicle for respective times. Macros are created in ImageJ to do the above for all the time-dependent images for a single GUV to make the analysis semi-automated and accurate. For calculation of permeability coefficients, vesicles ranging from size 8 µm < R< 30 µm are only considered. A major/minor axis ratio of <1.2 was only taken into consideration.

Previous studies[3,4] used a microfluidic chip where the t_0 was almost immediately after the mixing of antibiotics. Here the process of antibiotic addition is manual, so the fastest we start recording data after antibiotics addition to agarose is close to 9 seconds (on average t_0 = 28s). We also observed that whatever the initial or final time point taken to calculate the permeability coefficients are, the coefficients are quite similar (at least until the time point where there is still contrast between the inside of vesicle and outside of vesicle data not shown).

2. Optical setup comparison: The Gram-negative model liposome experiments are done using both EVOS and Carl Zeiss microscope (statistics presented in Fig. 4 are combined from both instruments).

The Gram-positive model experiments are done only on Carl Zeiss microscope. Both EVOS and Carl Zeiss gave comparable results in-terms of permeability coefficients (**Fig. S15**)

Supplementary Table

Supplementary Table 1. Permeability values of Norfloxacin diffusion through DPhPC liposome

	Permeability Coefficient (cm/s)	Permeability coefficient reported in Literature (cm/s) [3]
pH 7	$4.5\pm 0.4 \times 10^{-7}$	$5.2 \pm 0.4 \times 10^{-7}$
pH 5	$0.9 \pm 0.1 \times 10^{-7}$	$0.5\text{-}1.5 \times 10^{-7}$

To validate the methodology and the instrument we used in this study, we calculated the permeability coefficient of Norfloxacin at pH 5 and pH 7 in DPhPC (1,2-diphytanoyl-*sn*-glycero-3-phosphocholine) vesicles under the same conditions (200mM sucrose in 10mM HEPES for pH 7 and 5mM acetate buffer for pH 5) reported previously using microfluidic chip and another microscope system[3]. The permeability coefficient values calculated using the agarose immobilization method and instruments used in this study were comparable with the previously reported values.

Supplementary Figures

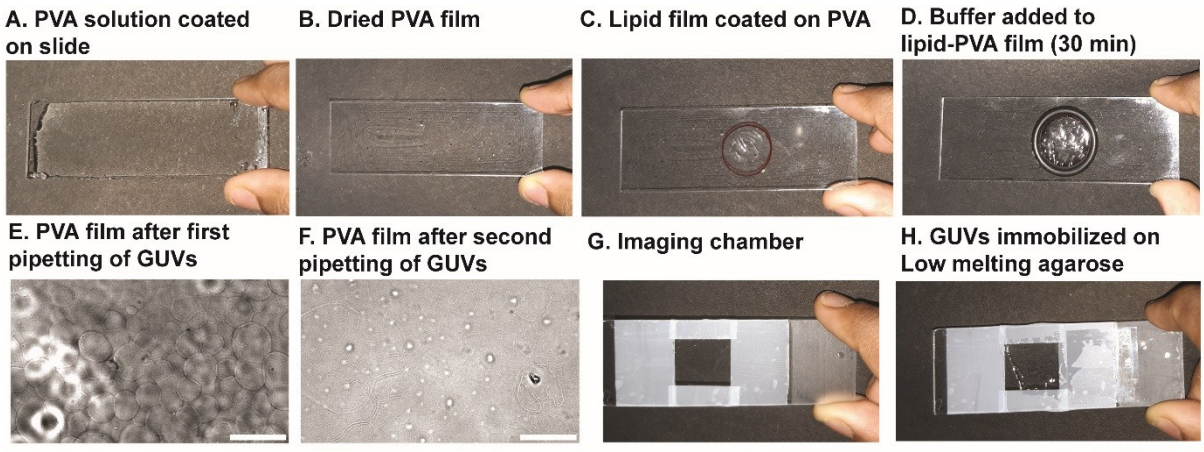

Fig. S1. A. 5 % Fully hydrolysed PVA solution coated on a clean glass slide with another glass slide, B. PVA film formed on glass slide after drying on a hot plate at 40 °C, C. 20 µL of lipid solution coated on dried PVA film either with a small size glass slide or with a spatula, D. 150 µL buffer added to the dried lipid-PVA film (after 30 min incubation), E. PVA film imaged on microscope after first pipetting and collection of GUVs, F. PVA film imaged on microscope after second pipetting and collection of GUVs. (scale bar: 20 µm) G. 1.5 cm*1.5 cm imaging chamber made with invisible tape on a glass slide, H. GUVs immobilized on 1% Low melting agarose (after removing coverslip)

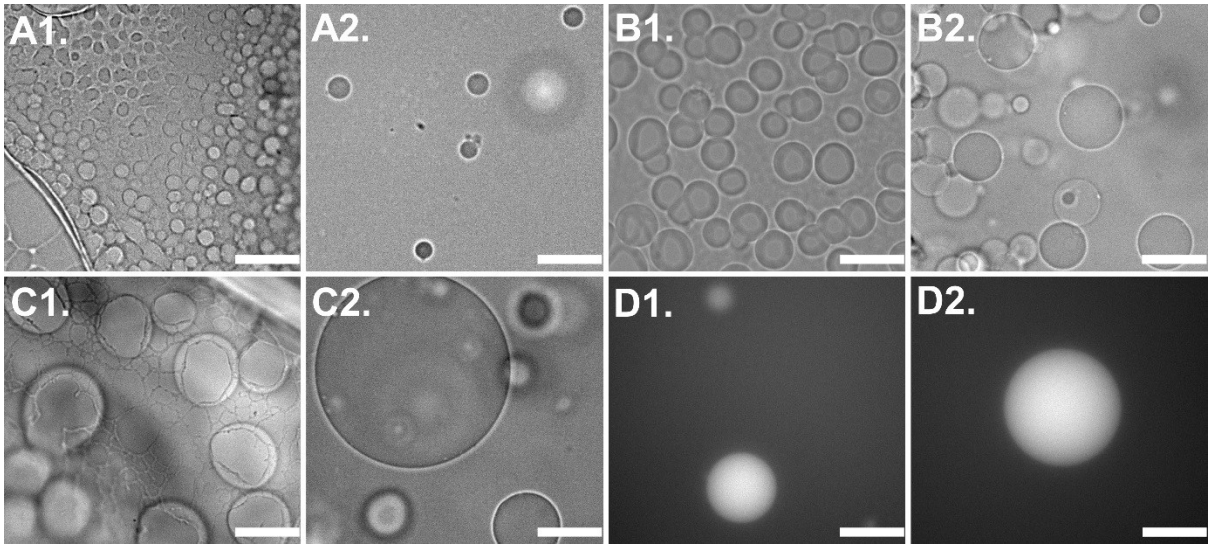

Fig. S2: Hydration time dependent growth of Gram negative giant vesicle A1. formation on PVA at 1.30 min, A2: collected in solution at 1.30 min, B1: formation on PVA at 5 min 7, B2: collected in solution at 5 min. C1 formation on PVA at 15 min, C2: collected in solution at 15 min. D1 and D2. encapsulation of Alexa Fluor in Gram negative vesicles collected at 30 minutes. conditions- 137 mM NaCl, 2.7 mM KCl 10mM HEPES pH 7. Scale bar: 20 μm

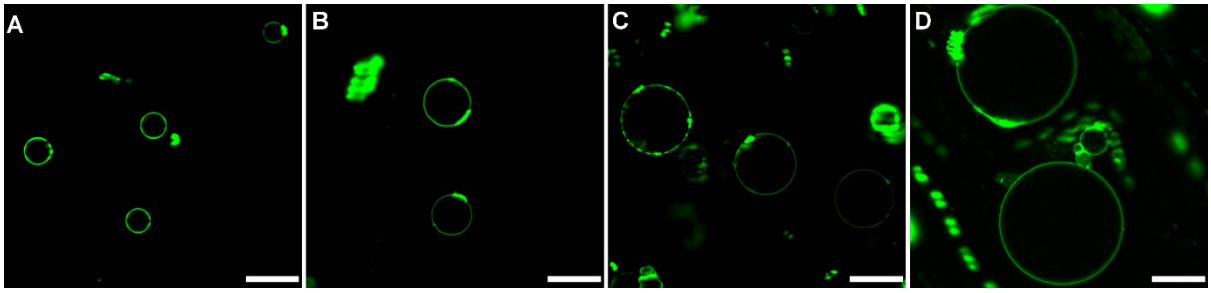

Fig. S3. Hydration time dependent growth of giant vesicle with 0.1 mol% Atto-DOPE mixed with lipid mixture A. GP vesicles collected in solution at 1 minute 30 seconds, B: GN vesicles collected in solution at 5 minutes 7, C: GP vesicles collected in solution at 15 minutes D. GN vesicles collected in solution at 30 minutes conditions- 137 mM NaCl, 2.7 mM KCl 10mM HEPES pH 7. Scale bar: 20 µm

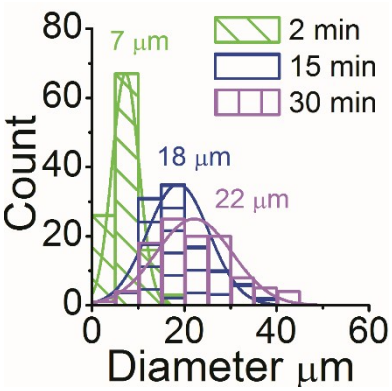

Fig. S4: Histogram of the distribution of size of DOPC vesicles collected at different hydration times.

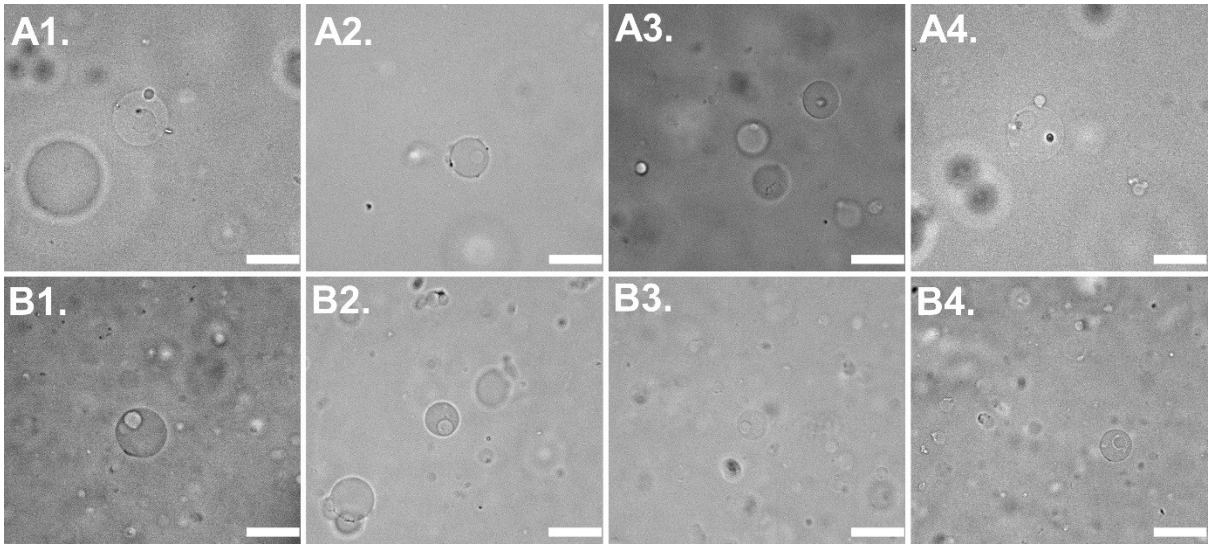

Fig. S5. Multicompartment or vesosomes formed using Inverse-phase precursor film in: A1, A2, A3, A4 Gram-negative vesicles, B1, B2, B3, B4 Gram-positive vesicles. Conditions: 137 mM NaCl, 2.7 mM KCl 10mM HEPES pH 7. Scale bar: 20 μm

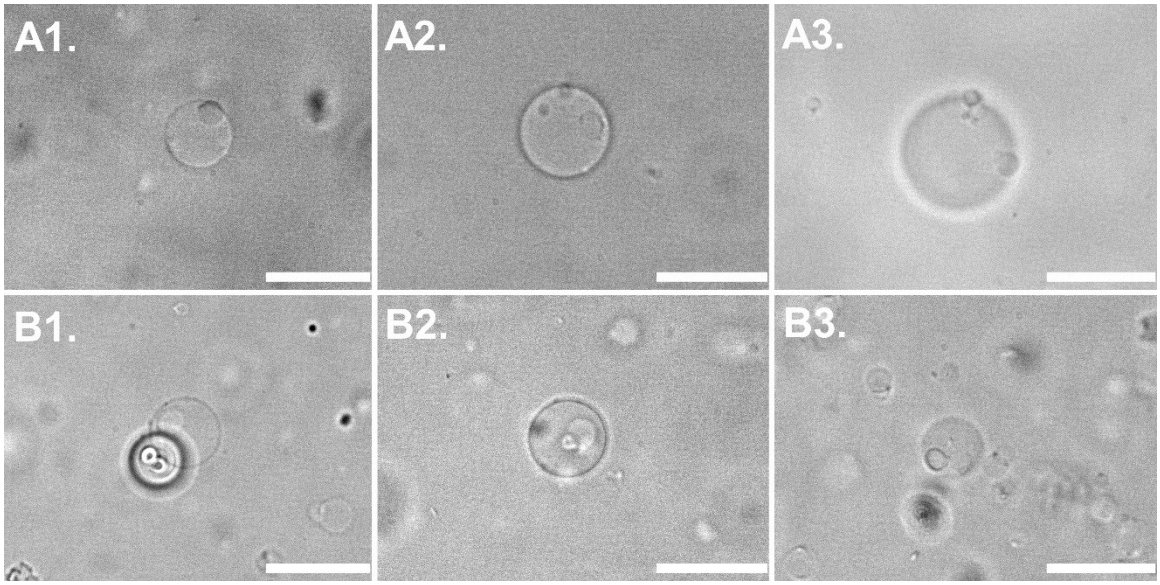

Fig. S6. Vesosomes or aggregates found inside vesicles using inverse-phase precursor film in: A1, A2, A3 Gram negative vesicles, B1, B2, B3 Gram positive vesicles. Conditions:137 mM NaCl, 2.7 mM KCl 10mM HEPES pH 7. Scale bar: 20 μm

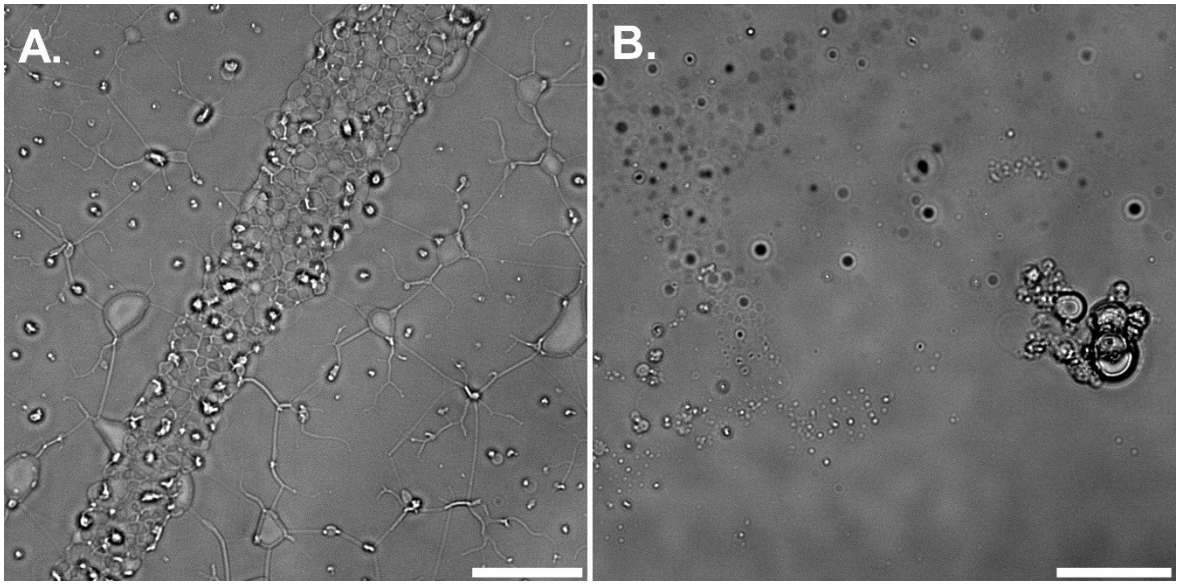

Fig. S7: PVA film coated with lipid film and hydrated with buffer unable to form vesicles of A. *E. coli* total lipid extract B. DOPG (55%), Lysyl-PG (40%) and Cardiolipin (5%). Scale bar- 50 µm

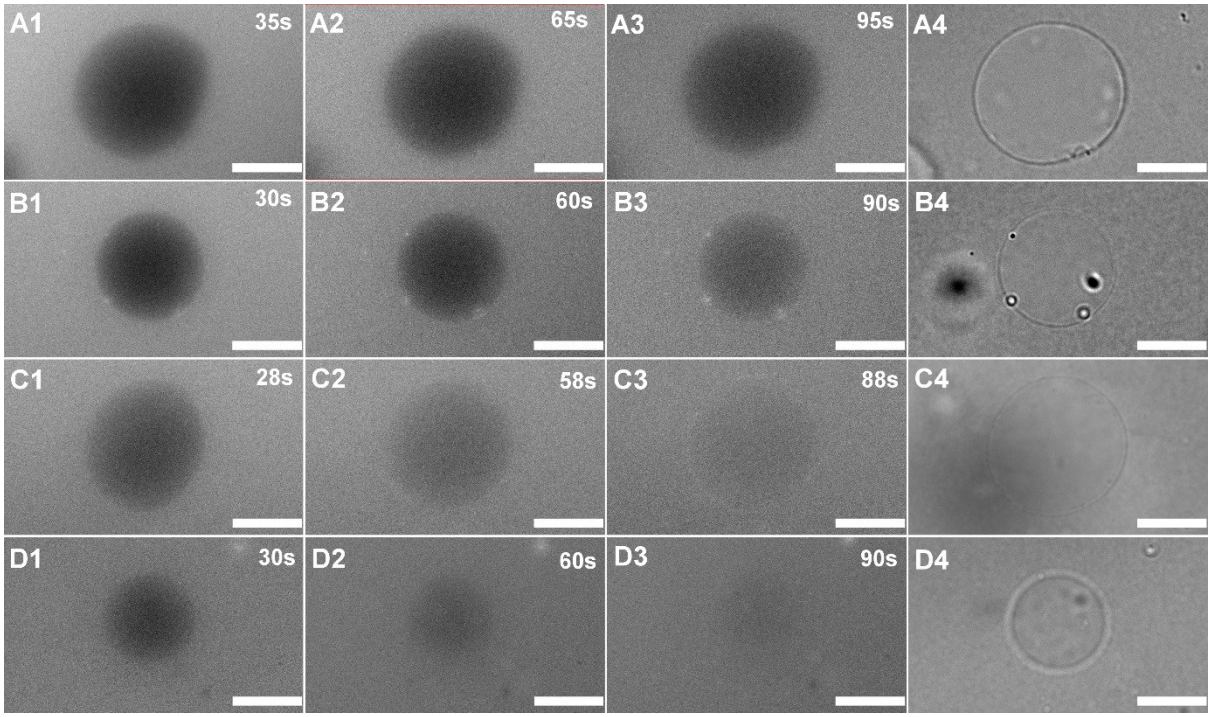

Fig. S8: Diffusion of antibiotic ciprofloxacin in a time dependent manner across- Gram-negative GUV model A1 to A3: at pH 7, B1 to B3: at pH 5 ; Gram-positive GUV model C1 to C3: at pH 7, D1 to D3: pH 5 in a time dependant manner. A4, B4, C4 and D4 depict the phase contrast images of the respective GUVs. **scale bar**: 20µm

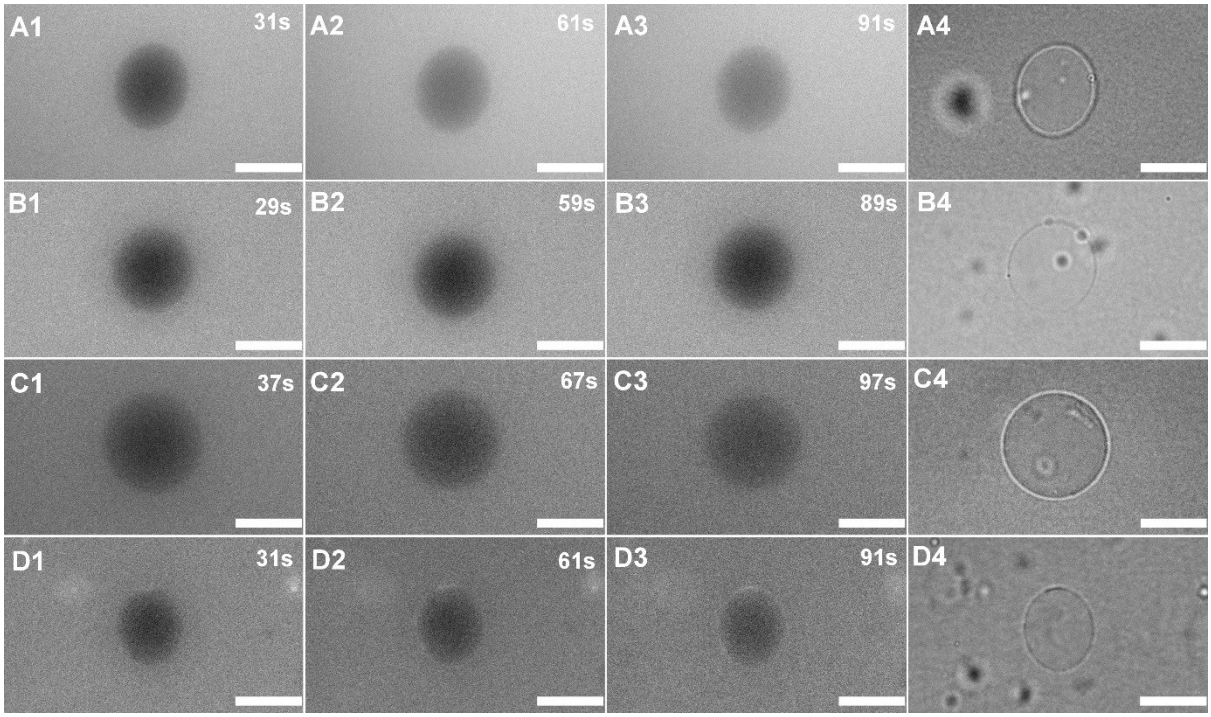

Fig. S9: Diffusion of antibiotic norfloxacin in a pH dependent manner across: Gram negative model GUVs. A. A1 to A3: at pH. B. B1 to B3: at pH 5; Gram-positive GUV model C1 to C3: at pH 7 D1 to D3: at pH 5. A4, B4, C4 and D4 depict the phase contrast images of the respective GUVs. **scale bar**: 20 μm

The diffusion of antibiotic Norfloxacin through the vesicles is depicted in the above figure. It can be seen that the fluorescence inside the vesicles is higher over time indicating diffusion of antibiotic to the interior of the vesicles in all cases.

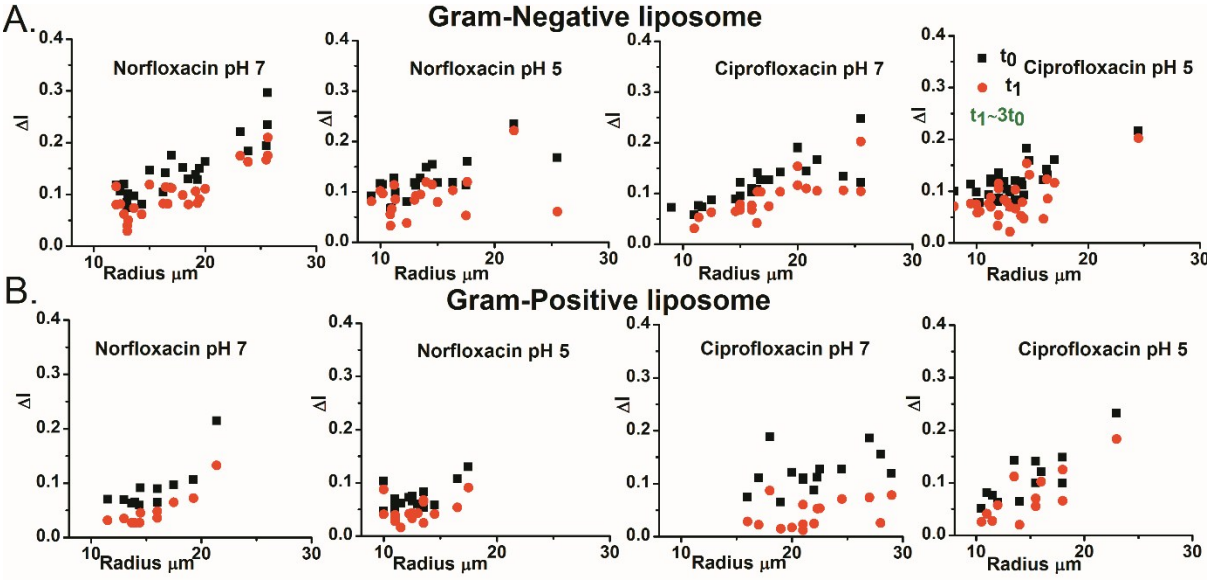

Fig. S10. Scatter plots, $\Delta I=(I_{out}-I_{in})/I_{out}$ versus the radius at t_0 (black squares) and t_1 (red circles) in A. Gram-negative mimicking giant vesicles, where t_0= 26± 10 s, t_1 $3t_0$ of antibiotics Norfloxacin pH 5 (N=16), Norfloxacin pH 7 (N=26), Ciprofloxacin pH 5 (N=17), Ciprofloxacin pH 7 (N=21) and in B. Gram-positive mimicking giant vesicles, where t_0= 30± 9 s and t_1 $3t_0$ for Norfloxacin pH 5 (N=17), Norfloxacin pH 7 (N=11), Ciprofloxacin pH 5 (N=12), Ciprofloxacin pH 7 (N=16).

I_{out} is the intensity of antibiotic outside the vesicle and is constant over time, but I_{in} increases over time due to diffusion of antibiotic inside vesicles. Consequently, the ΔI reduces over time (from t_0 to t_1) due to the influx of antibiotics represented by an increase in I_{in} (at t_1). The I_{in} and I_{out} are determined in ImageJ on uneven-illumination corrected images snapped in a time-dependent manner (Supplementary text 1). The ΔI is calculated in a time-dependent manner, where the ΔI is calculated for the initial image represented by t_0 (26± 10 seconds, the minimal time before time lapse images are captured and we assume minimal diffusion has occurred at this point) for individual GUV. The ΔI is also calculated for another time point t_1 (t_1= $3t_0$) for the same vesicle, where significant antibiotic diffusion should have occurred. The ΔI for these two time points for each GUV is plotted (S10 A Gram-negative and S10 B Gram-positive vesicle). The radial dependence of normalized intensity is similar to reports[3] as the measurements are done on an epi-fluorescence microscope.

In the case of both antibiotics at pH 7, the shift in the normalized intensity at t_1 is more prominent as compared to pH 5. The data confirms higher diffusion of both the antibiotics at pH 7 than pH 5 in respective bacterial membrane model.

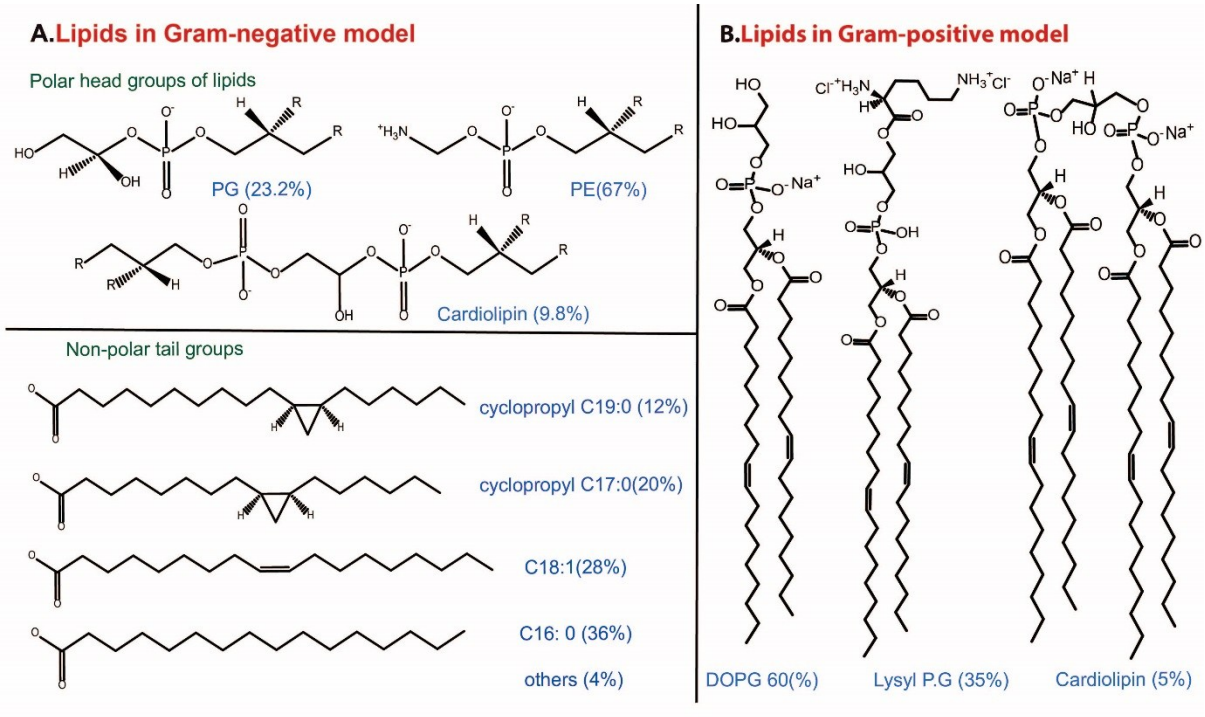

Fig. S11: Chemical structure of lipids used in GUV preparation. **A.** lipids present in *E. coli* polar extract used for preparing the Gram-Negative model. **B.** Lipids used to prepare the Gram-positive model

E. coli Polar extract used to prepare the Gram-negative model is a commercially available extract from Avanti polar lipids. It contains a heterogeneous mixture of lipids having phosphatidylethanolamine (PE)(67%), phosphatidylglycerol (PG)(23.2%), and cardiolipin(9.8%) as the composition of polar head groups, while the non-polar chain groups differ in length and structure as described previously.[5] Since the lipid mixture for Gram-positive bacteria is not commercially available, a mixture of dioleolyl phosphatidylglycerol (DOPG)(60%), Lysyl-Phosphatidylglycerol(35%), and cardiolipin(5%) was used to prepare the Gram-positive model of GUVs. DOPG has a negatively charged head group while Lysyl PG has a positively charged head group.

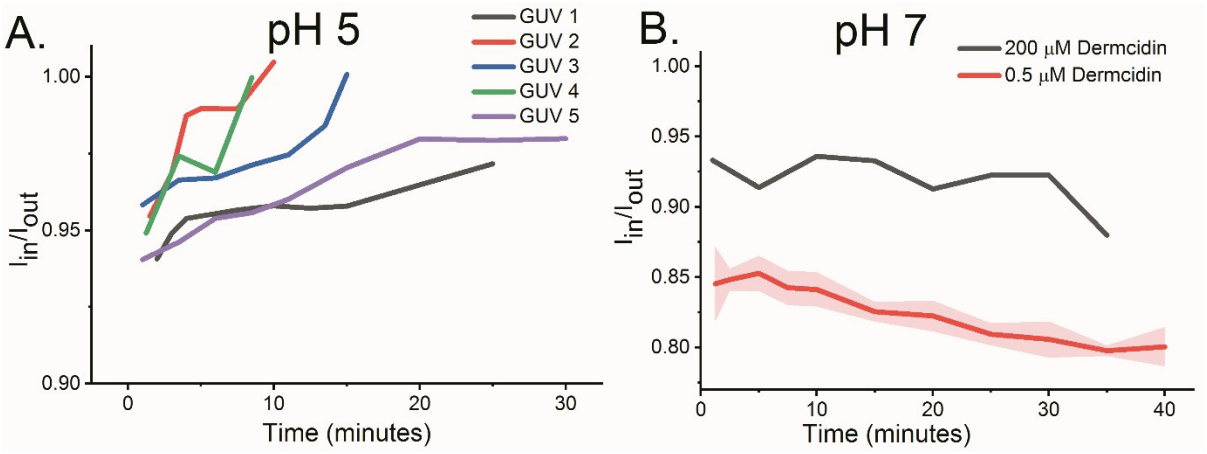

Fig. S12: I_{in}/I_{out} values for different Gram-positive mimicking liposomes incubated with Dermcidin at A. pH 5 with 0.5μM and B. pH 7 with different concentrations used.

We have tried direct addition of peptide to vesicles embedded in agarose. In case of pH 7, no change in intensity is observed even for concentration as high as 400μM over 40 minutes of experiment. In case of pH 5, we observed very high bursting of vesicles by addition of concentration like 10 μM to agarose-embedded vesicles, by addition of 0.5 to 1 μM we do not observe change within first 40 minutes likely due to the slow kinetic activity of Dermcidin peptide as observed *in-vivo*.

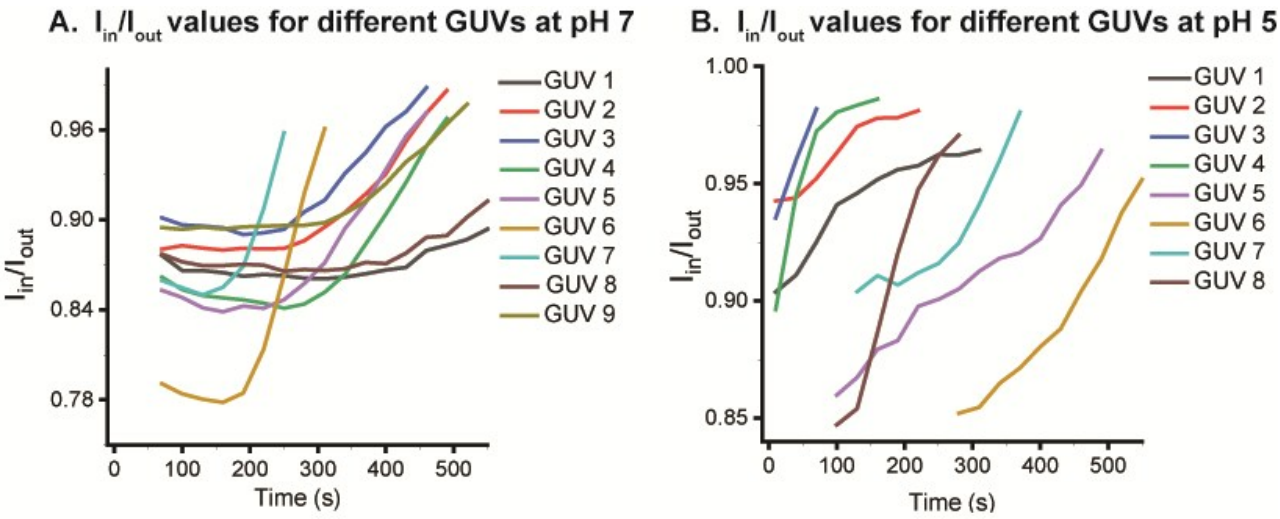

Fig. S13. I_{in}/I_{out} values for different Gram-negative mimicking liposomes incubated with alpha hemolysin at A. pH 7 and B. pH 5.

The figure represents individual proteo-vesicle and corresponding normalized intensity of Alexa Fluor 350 over time. Incubation of Gram-negative liposomes with Alpha hemolysin caused an influx of Alexa Fluor dye into the vesicle. The figure depicts the reconstitution efficiency of this membrane protein varies with different GUVs in the line graph. The main text figure Figure 5B and 5C the I_{in}/I_{out} values are average of N=3 GUV's in pH 7 and N=4 at pH 5 due to the issue of varying reconstitution efficiency.

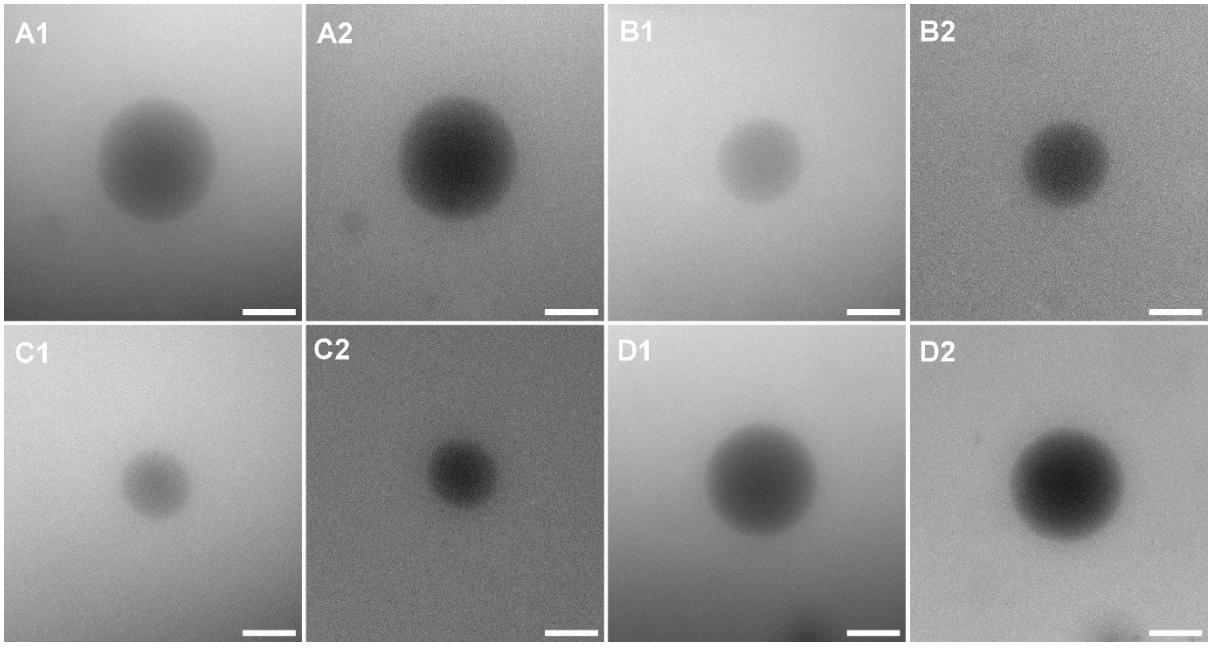

Fig. S14. Uneven Illumination correction of images using ImageJ analysis software of Gram-negative liposome A1. At pH 7 before correction, A2. At pH 7 after correction, B1. at pH 5 before correction, B2. at pH 5 after correction; Gram-positive liposome C1. at pH 7 before correction, C2. at pH 7 after correction, D1. at pH 5 before correction, D2. At pH 5 after correction. Scale bar: 20 μm

The overall fluorescent intensity of images was found to be non-uniform due to uneven illumination; hence, to solve this uneven illumination correction was done. The blank image was captured such that it doesn't contain any liposome and the black images by obstructing any light source to the sample.

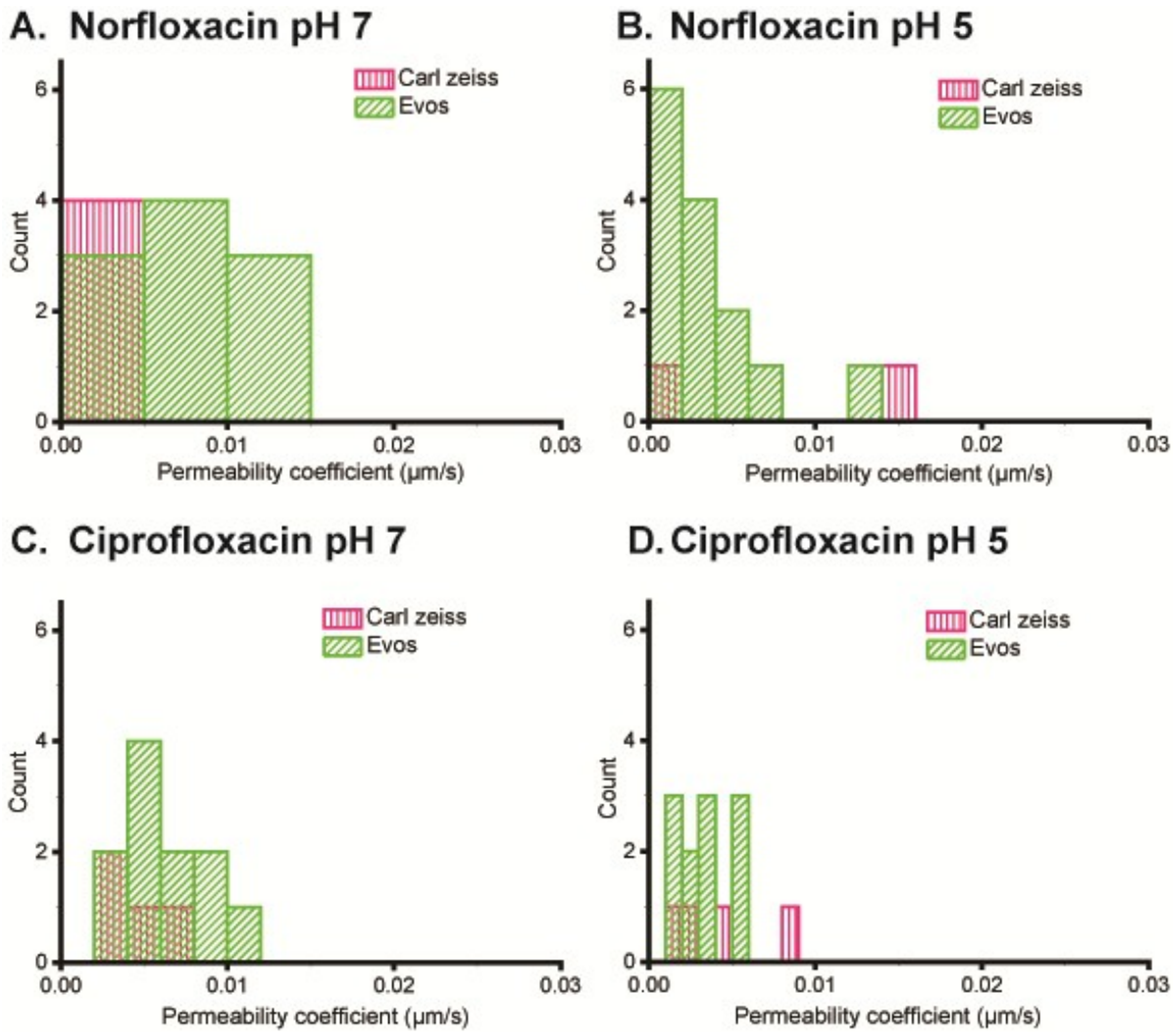

Fig. S15. Comparison of permeability coefficients measured using Carl Zeiss microscope and EVOS Epi-fluorescent microscope. Histograms of Permeability coefficients of A. Norfloxacin at pH 7. B. Norfloxacin at pH 5. C. Ciprofloxacin at pH 7. D. Ciprofloxacin at pH 5.

To revalidate the optical set, we repeated the same measurements under the same conditions in Carl Zeiss AxioImager2 upright microscope. The permeability coefficient values in both cases fall under the same range obtained from both the instruments. However, Carl Zeiss had better signal/noise ratio due to a better camera.

Supplementary Video legends

Supplementary Video S1: Multi compartment in Gram-negative vesicles by inverse phase method. Conditions: 137 mM NaCl, 2.7 mM KCl in HEPES, pH 7 Scale bar: 20 µm

Supplementary Video S2: Multicompartment in Gram-positive vesicles by Inverse phase method. Conditions: 137 mM NaCl, 2.7 mM KCl in HEPES, pH 7 Scale bar: 20 µm

Supplementary Video S3: Multicompartment in DOPC vesicles by Inverse phase method. Conditions: 50 mM KCl ,100 mM sucrose in HEPES pH 7, Scale bar: 50 µm

Supplementary Video S4: Stable DOPC-DOPG Microcompartment using Inverse method. Conditions: 5 mM NaCl, 50 mM sucrose and 5% PEG (MW: 8000). Scale bar: 20 µm

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