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SUPPORTING INFORMATION

Sensing Cholesterol-Induced Rigidity in Model Membranes With Time-Resolved Fluorescence Spectroscopy and Microscopy

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Materials and Methods

Biological Materials and Dyes. All lipids and cholesterol used in this study were purchased from Avanti Polar Lipids. DI-8-ANEPPS was purchased from Biotium. 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoylsn-glycero-3-phospho-(1'-rac-glycerol) sodium (POPG), Cholesterol (plant-derived) and DI-8-ANEPPS were dissolved in chloroform for the lipids and ethanol for DI-8-ANEPPS and mixed in a glass vial in molar ratios as written in table 1. Note that the overall POPC and POPG amount decreases as cholesterol increases, but the POPC:POPG ratio is maintained at 3:1 so as not to drastically change the overall charge of the liposomes. The lipids and dyes in a solvent were dried under a gentle stream of argon in a fume hood, and then the lipids were further dried in a vacuum oven set to 100C for 30 minutes to obtain a thin lipid film. To prepare liposomes, the dried film was rehydrated by adding 1X PBS buffer (phosphate-buffered saline, GIBCO) to a final lipid concentration of 3.125mg/mL and vortexed for 1 min. Then, using an Avanti Mini Extruder, the liposomes were extruded using a 200 nm membrane first and then a 100 nm membrane at room temperature 21 times, which were stored at 4C until further use. Note that the lipid film was rehydrated in 0.1X PBS for Z-potential measurements and then extruded as described.

Bulk Steady State and Time-Resolved Spectroscopy. All the absorption spectra reported here were measured using a Cary 8454 Uv-Vis (Agilent, Santa Clara, CA) and the fluorescence spectra were collected using a photoluminescence spectrometer (FLS-1000) from Edinburgh Instruments. All measurements were carried out at STP by diluting 100 uL of the stock solution created as described above by 20 in 1900 uL of PBS All photoluminescence lifetimes were taken using the same FLS-1000 from Edinburgh Instruments used from steady-state characterization. In all cases, the lifetimes were measured at the λ_{max} of the emission spectra (Table S1) using an

excitation wavelength of 456 nm. In all cases, the repetition rate of the diodes for collecting the data was 5 MHz. That is, a 200 ns collection window was used.

Fluorescence anisotropy measurements. The fluorescence anisotropy was obtained using an ISS PC1 spectrofluorometer, which includes polarizer features for efficiently conducting fluorescence anisotropy measurements. In all cases, the excitation wavelength used for characterization was the same used for fluorescence lifetime characterization (456 nm), and polarized fluorescence was detected at each sample λ_{MAX} (Table S1). In synthesis, absorption and emission events occur in a polarized fashion among a fixed axis, and by controlling the orientation of the optical excitation and detection (polarized), one can inform about restrictive events in the medium. To that end, one needs to track the changes in the orientation of a chromophore in a medium, focusing on the time between the absorption and emission events. In an isotropic medium, where the anisotropy value tends to be ~0, the chromophores are fluid and have less motion restriction. In an anisotropic medium, in which the anisotropy value tends to be ~0.4, the chromophores are motion-restricted. In the context of the experiments carried out here, an increment in fluorescence anisotropy represents a higher degree of structural order or low membrane fluidity.[1] Calculating the fluorescence anisotropy (*a* - ***Equation S1***) and polarization (*P* - ***Equation S2***) can be obtained by:

$$a = \frac{I_{\parallel} + I_{\perp}}{I_{\parallel} + 2I_{\perp}} \qquad \textbf{Eq. S1}$$

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \qquad \textbf{Eq. S2}$$

Where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensity of the vertically and horizontally polarized emission, when the sample is excited with vertically polarized light.[2]

Fluorescence Lifetime Imaging Microscopy (FLIM). Fluorescence Lifetime Imaging Microscopy (FLIM). Fluorescence Lifetime Imaging Microscopy (FLIM). Microscopy was done

using a Leica Stellaris SP8 point scanning confocal microscope equipped with a white light laser excitation source and an Acusto Optical Beam Splitter. Images were acquired using the intensity and FLIM modes concurrently with the excitation set at 460nm and detected using the Power HyDX detector from 525-700nm using an HC PL APO 63x/1,20 W motCORR CS2 water immersion objective. Fluorescence lifetime decays of each image were fit in LAS X FLIM software with a threshold per pixel of >10 photons to remove background noise and fit to n-Exponential Tail Fit. Images were taken at 1024x1024 pixels at a 1000 Hz scan rate.

Z-Potential Measurements. Liposomes were made following the same protocol described above but resuspended in 0.1X PBS to minimize the influence of salts in the measurement. The Z-Potential measurements were taken on a Malvern Zetasizer Nano-S. Samples were measured using a Folded Capillary Zeta Cell at 25 °C for all samples. New cuvettes were used for each measurement. Three separate measurements with 20 runs each were done for each sample. Note that the liposomes in 0.1X PBS were used only for Z-potential characterization. For all of the fluorescence characterizations, liposomes diluted in 1X PBS were used.

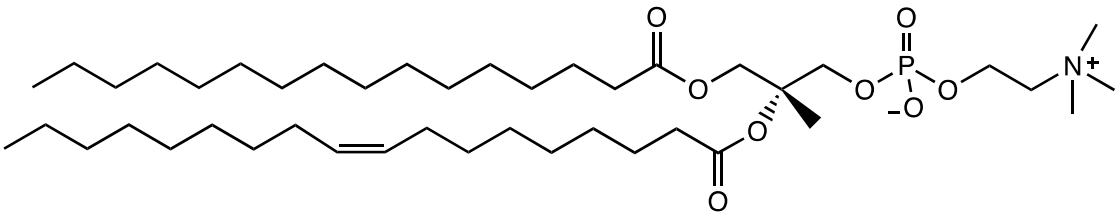
Transmembrane potential (Ψ_d) determination with R.

Evaluating the fluorescence intensity of DI-8-ANEPPS when detected at the edge of its fluorescence spectra (λ_{670nm}) upon excitation at the blue edge (λ_{420nm}) and red edge (λ_{510nm}) of its absorption spectrum can inform about Ψ_d. The fluorescence intensity at excited at λ_{420nm} and detected at λ_{670nm} is divided by the fluorescence intensity at excited at λ_{510nm} and detected at λ_{670nm}, which gives you the variable **R**, see **Equation S3**.[3]

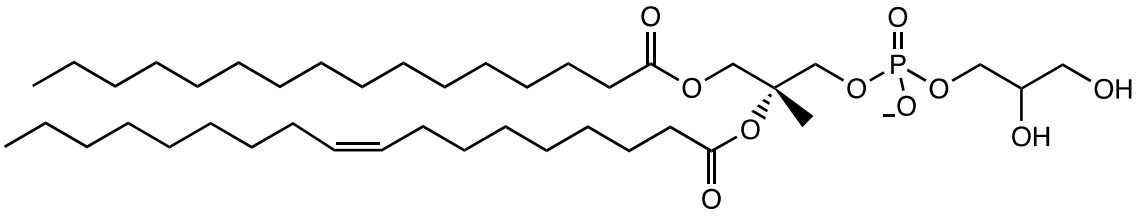
$$\Psi_d = \frac{(R+0.3)}{4.3\ x\ 10^{-3}} \qquad \textbf{Eq. S3}$$

With this approach, tt is believed that selecting these wavelengths minimizes membrane fluidity effects on $\boldsymbol{R}$.[4] After determining R, simple arithmetic will give you the $\boldsymbol{\Psi_d}$ value. The fluorescence spectras for $\boldsymbol{R}$ determination can be found in ***Figure S6***.

POPC



POPG



Schematic S1. Molecular structure of the lipides used in this study: 2-oleoyl-1-pamlitoyl-sn-glyecro-3-phosphocholine (POPC) and 2-oleoyl-1-pamlitoyl-sn-glyecro-3-glycerol (POPG).

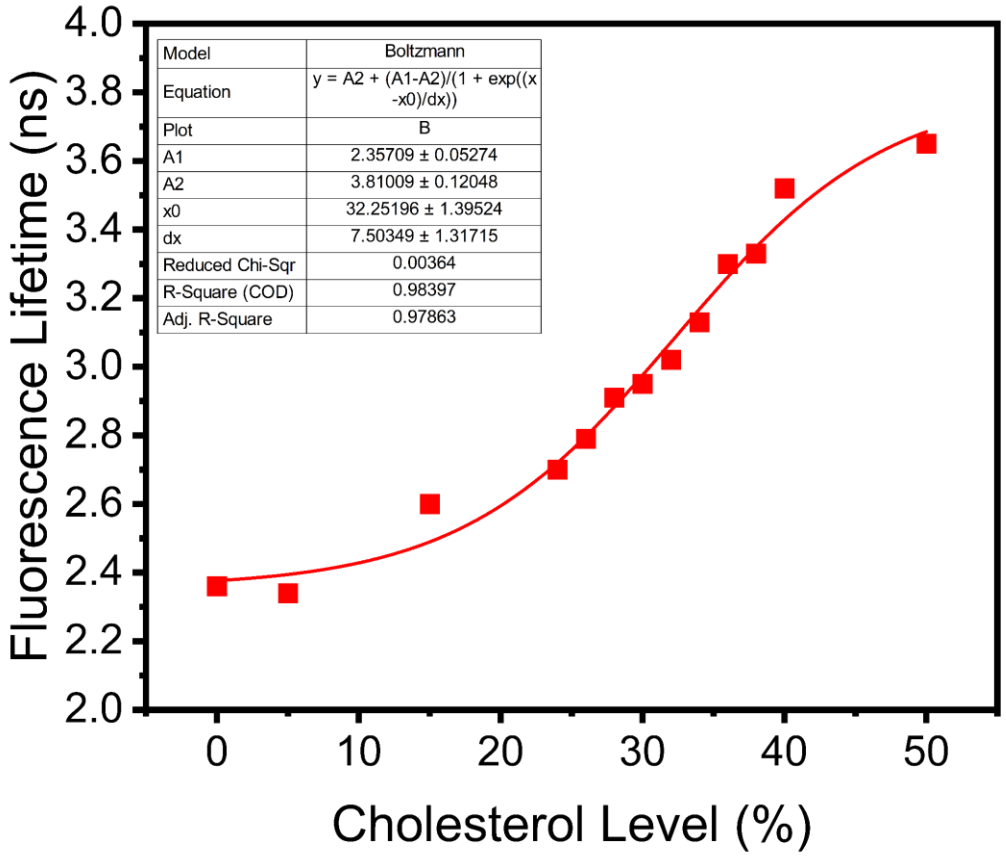


Figure S1. Fluorescence lifetime correlation equation following a Boltzmann sigmoidal fit curve.

Table S1. Photophysical data. The fluorescence lifetime detected at λ_{MAX} was fitted to a bi-exponential decay, while the fluorescence lifetime detected at λ_{670nm} was fitted to a mono-exponential decay.

Cholesterol Level (%)	Fluo Lifetime (ns) λ_{MAX}	Absorption λ_{MAX}	Emission λ_{MAX}	Stoke Shift	R/Ψ_d
Liposomes with Different Cholesterol Levels					
0	1.03/2.36	470 nm	596 nm	126 nm	1.44/405 mV
5	0.98/2.34	468 nm	590 nm	122 nm	1.52/423 mV
15	1.05/2.60	466 nm	584 nm	118 nm	1.72/468 mV
24	0.96/2.70	464 nm	580 nm	116 nm	n/a
26	0.97/2.79	464 nm	578 nm	114 nm	n/a
28	1.16/2.91	464 nm	576 nm	112 nm	n/a
30	1.10/2.95	463 nm	574 nm	111 nm	2.30/604 mV
32	1.22/3.02	463 nm	574 nm	111 nm	n/a
34	1.24/3.13	462 nm	572 nm	110 nm	n/a
36	1.41/3.30	461 nm	572 nm	110 nm	n/a
38	1.40/3.33	461 nm	572 nm	111 nm	n/a
40	1.56/3.52	460 nm	570 nm	110 nm	n/a
50	1.34/3.65	458 nm	568 nm	110 nm	2.55/663 mV
PC:PG Ratios – 15% Cholesterol					
1:7	1.06/2.52	467 nm	586 nm	119 nm	1.56/432
1:1	0.97/2.47	465 nm	584 nm	119 nm	1.75/476
3:1	1.05/2.60	465 nm	584 nm	119 nm	1.72/468
7:1	1.12/2.60	464 nm	583 nm	119 nm	1.83/495

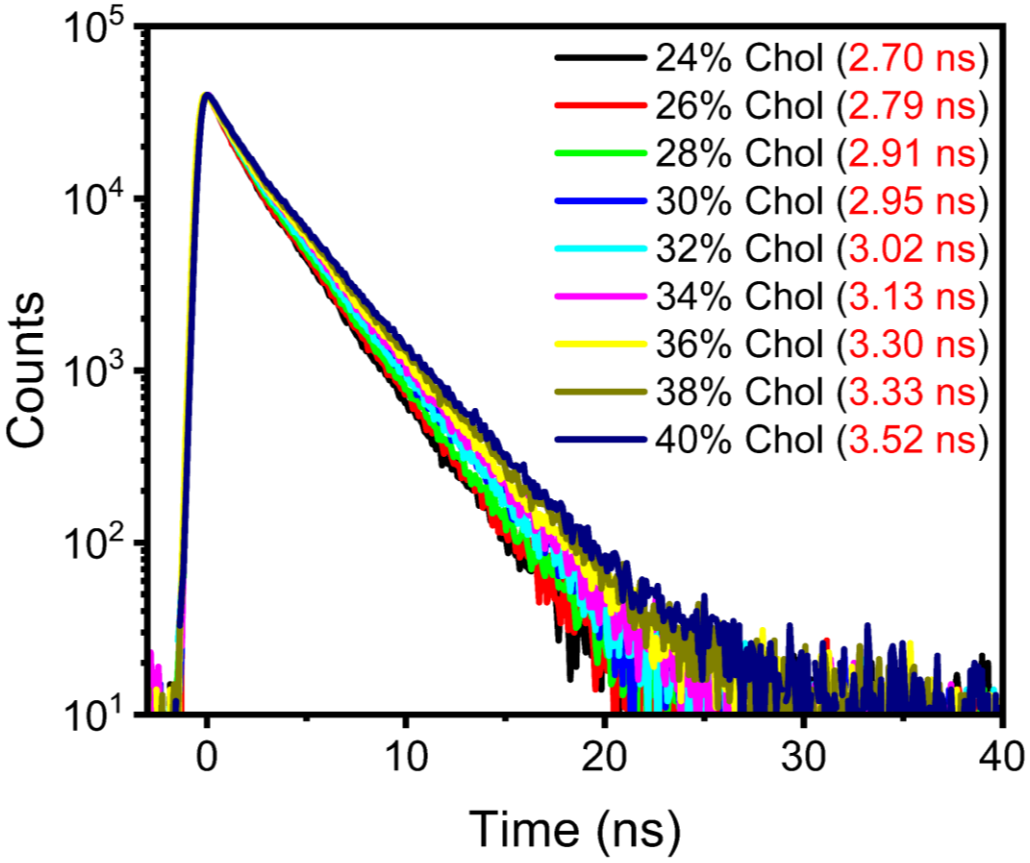


Figure S2. Fluorescence lifetime of DI-8-ANEPPS-intercalated liposomes with cholesterol levels within physiological relevance (**A**) and their respective fluorescence spectra (**B**).

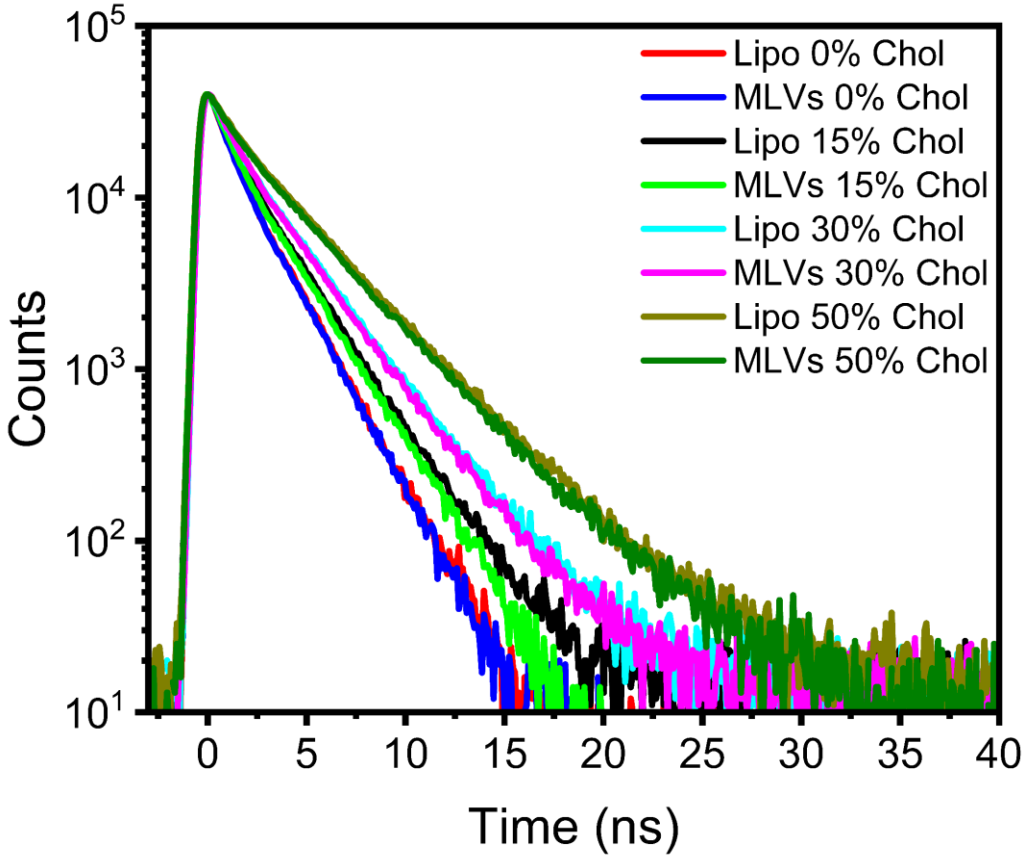


Figure S3. Representative FLIM images for DI-8-ANEPPS-intercalated into GMLVs with different cholesterol levels.

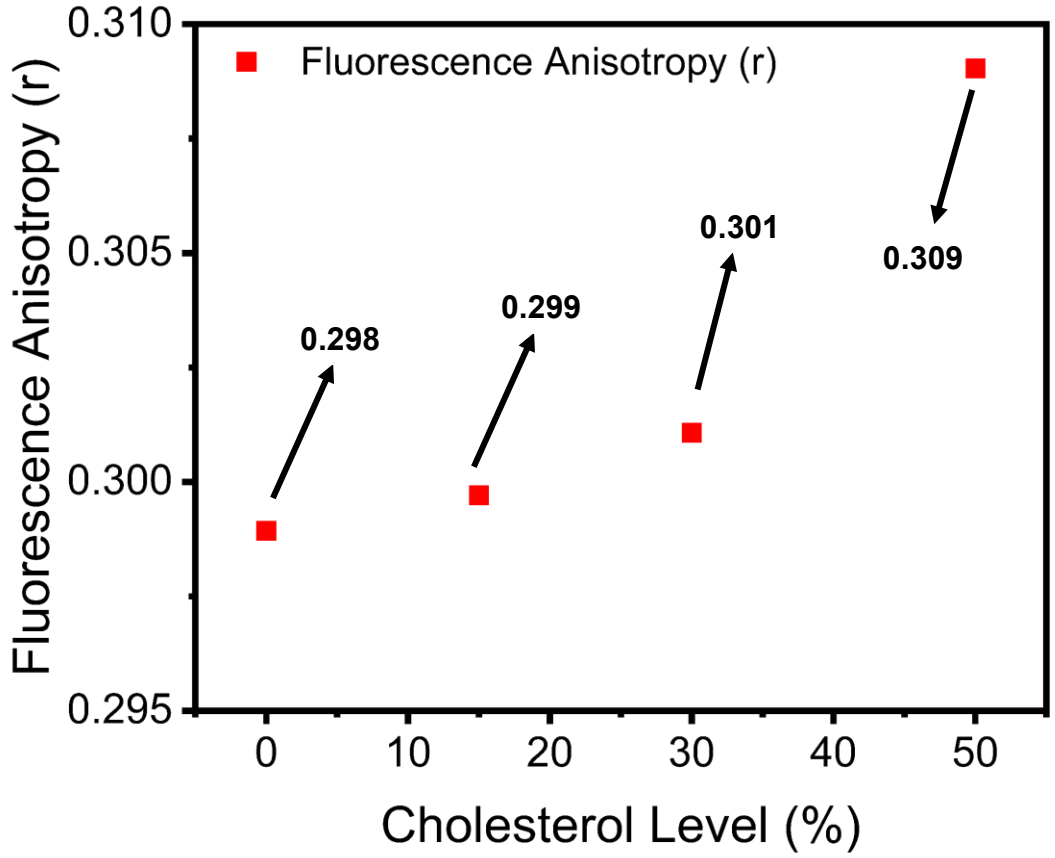


Figure S4. Fluorescence Anisotropy (r) for liposomes with different cholesterol levels. In all cases, the excitation was 456nm and the detection was at their respective $\lambda_{MAX.}$

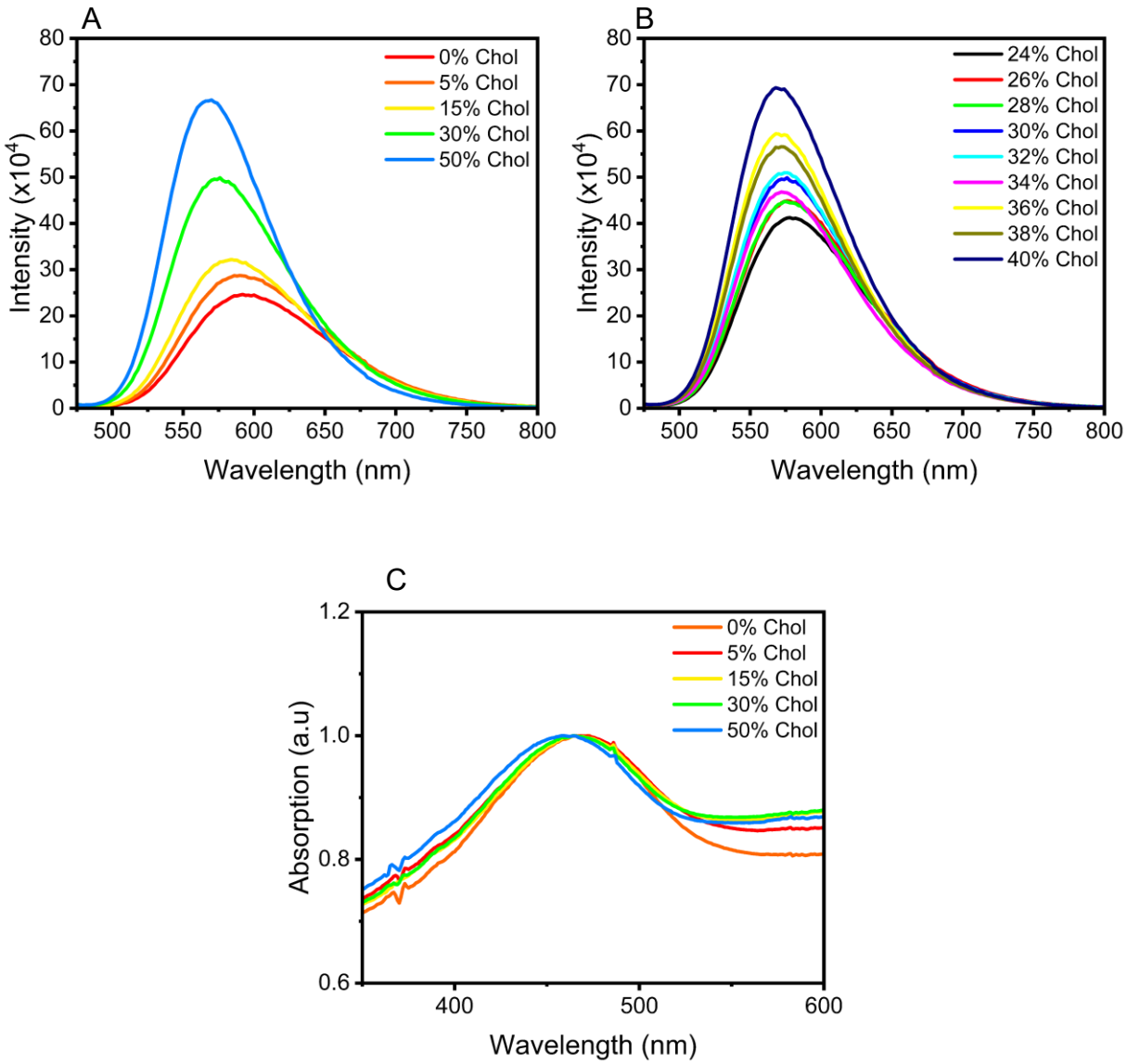


Figure S5. Fluorescence (**A** + **B**) and Absorption (**C**) spectra of the DI-8-ANEPPS-intercalated liposomes with different cholesterol levels.

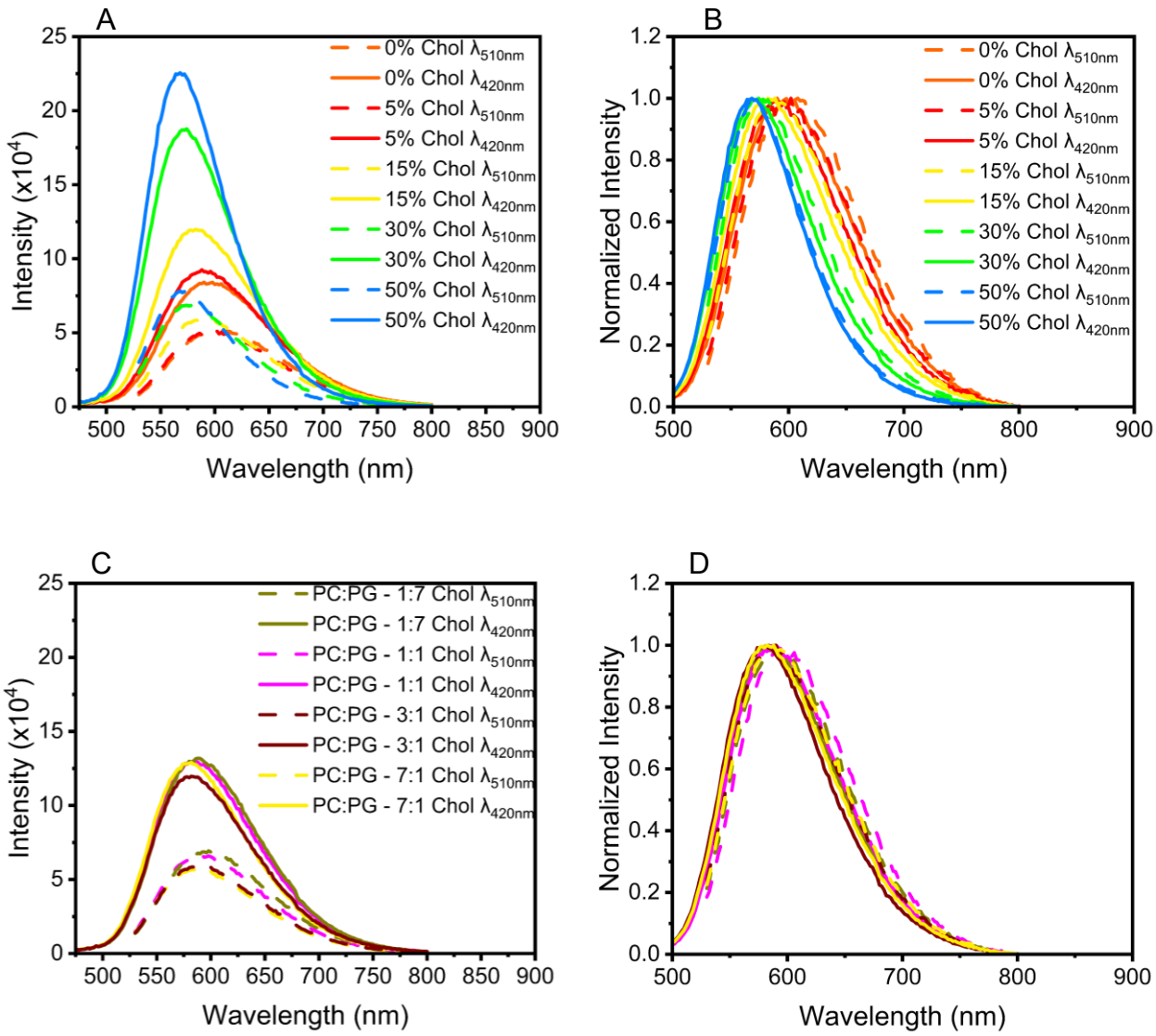


Figure S6. Raw (**A**) and normalized (**B**) fluorescence spectra of the DI-8-ANEPPS-intercalated liposomes with different cholesterol levels. Raw (**C**) and normalized (**D**) fluorescence spectra of the DI-8-ANEPPS-intercalated liposomes with 15% cholesterol levels but different PC:PG ratios. Figures C and D have the same legend.These spectra were used to determine the R and Ψ_d values. These spectra were used to determine the R and Ψ_d values.

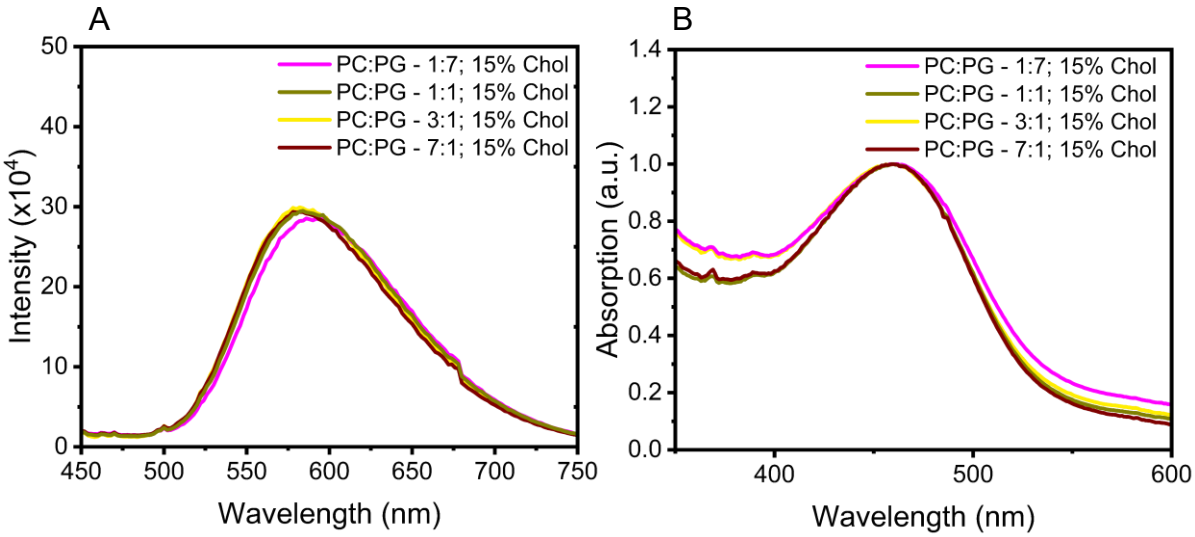


Figure S7. Fluorescence (**A**) and Absorption (**B**) spectra of the DI-8-ANEPPS-intercalated liposomes with 15% cholesterol levels but different PC:PG ratios.

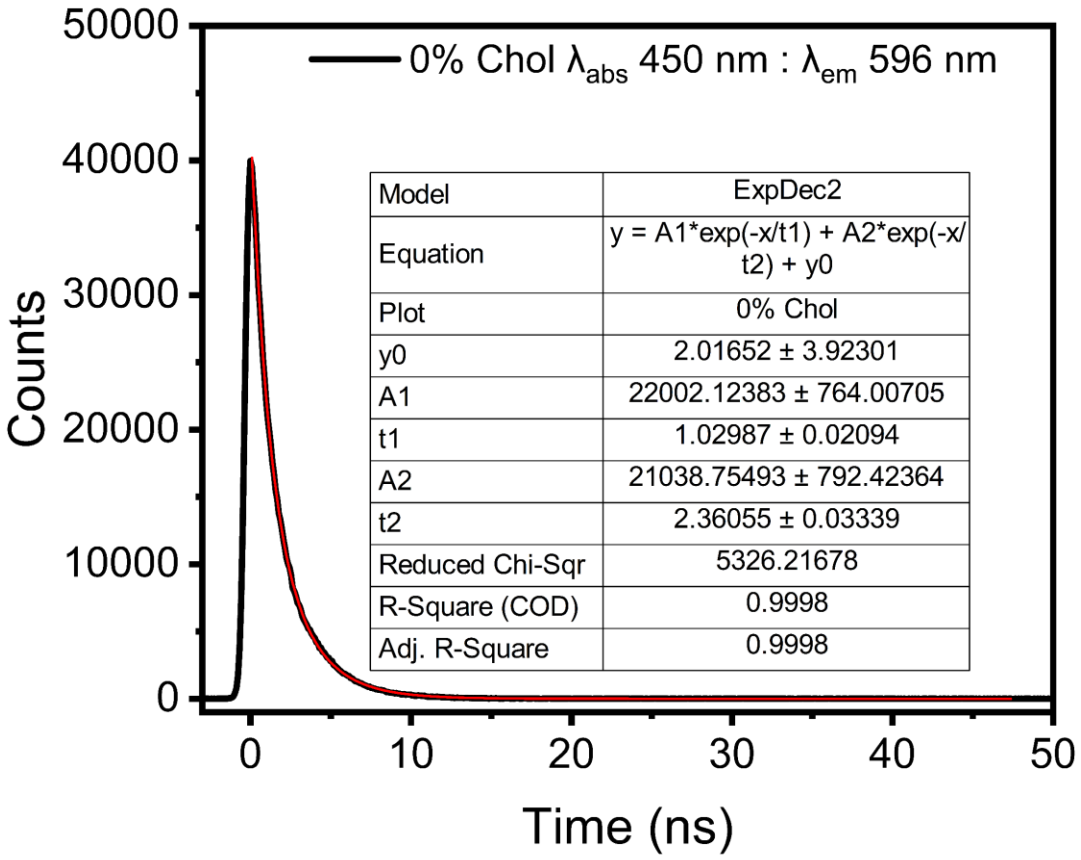


Figure S8. Fitting for the fluorescence lifetime of DI-8-ANEPPS in liposomes with 0% cholesterol

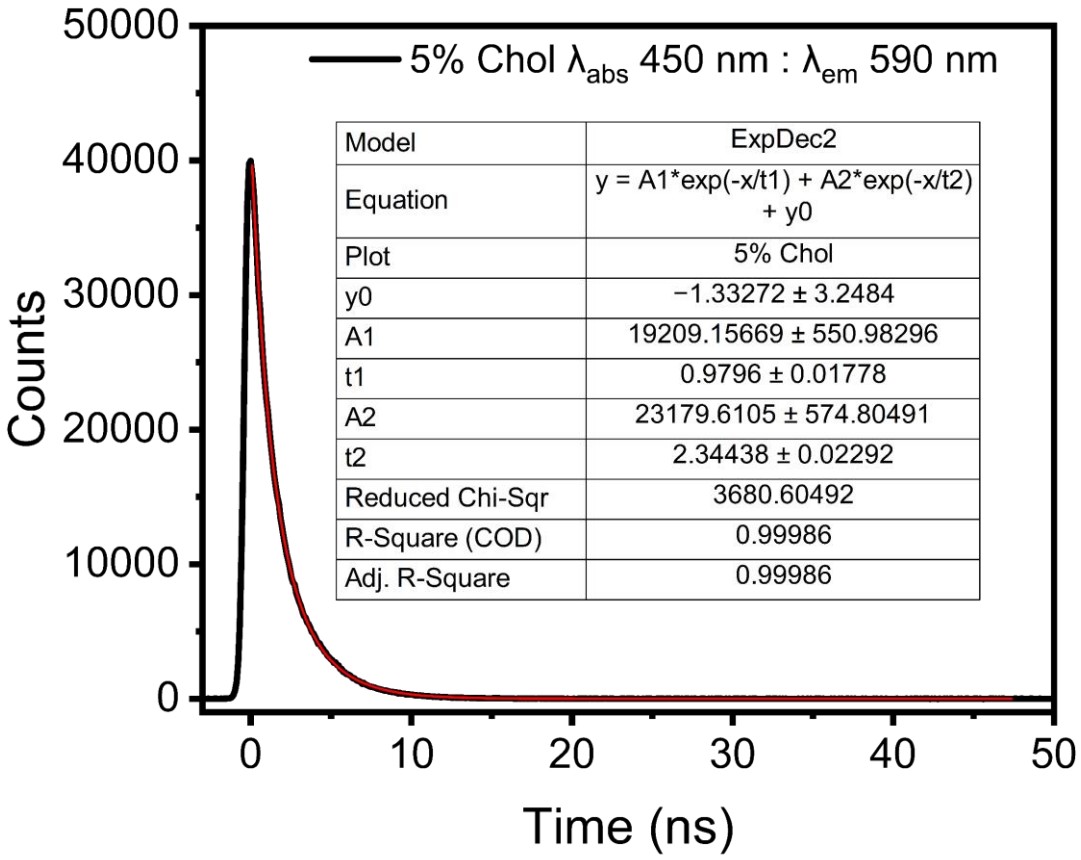
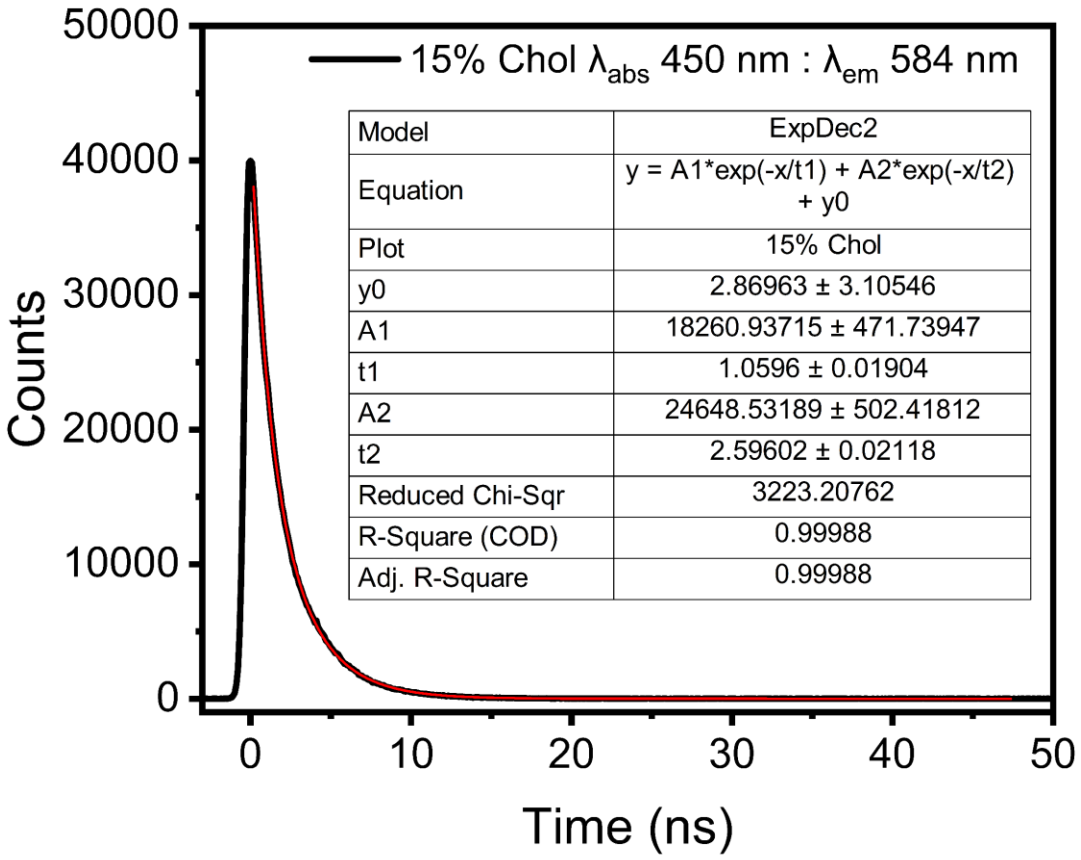
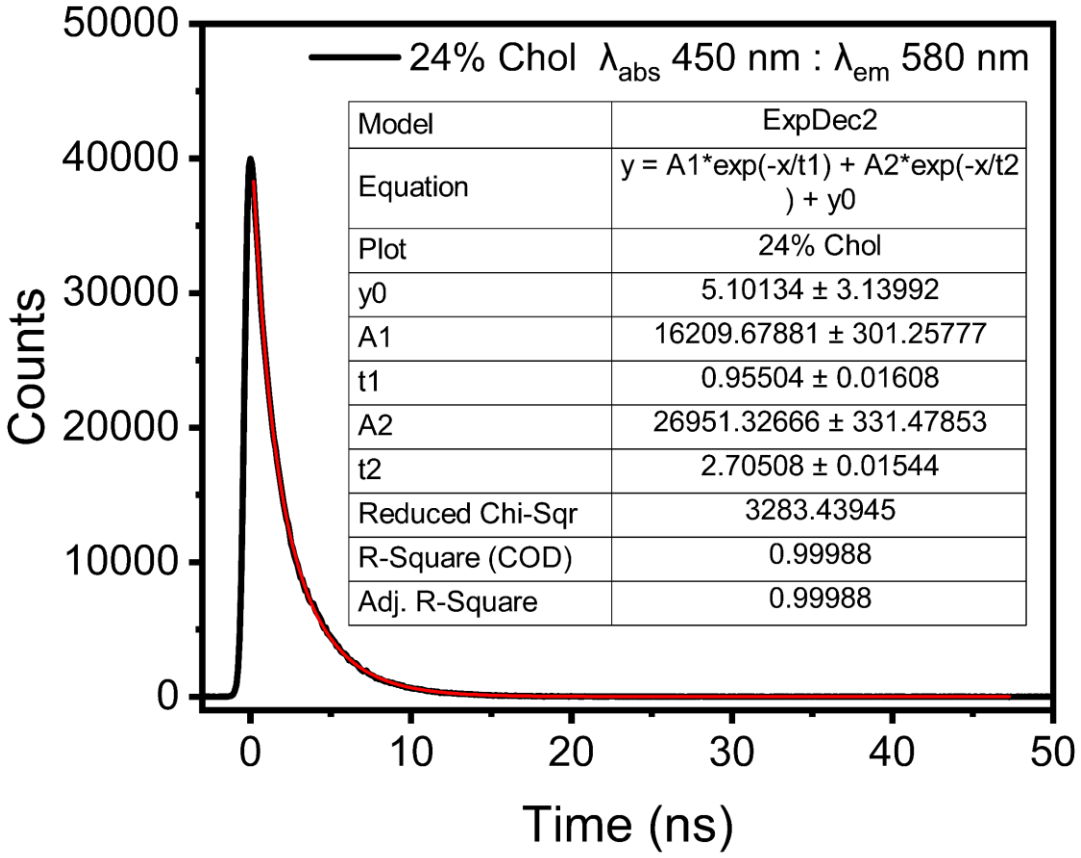


Figure S9. Fitting for the fluorescence lifetime of DI-8-ANEPPS in liposomes with 5% cholesterol



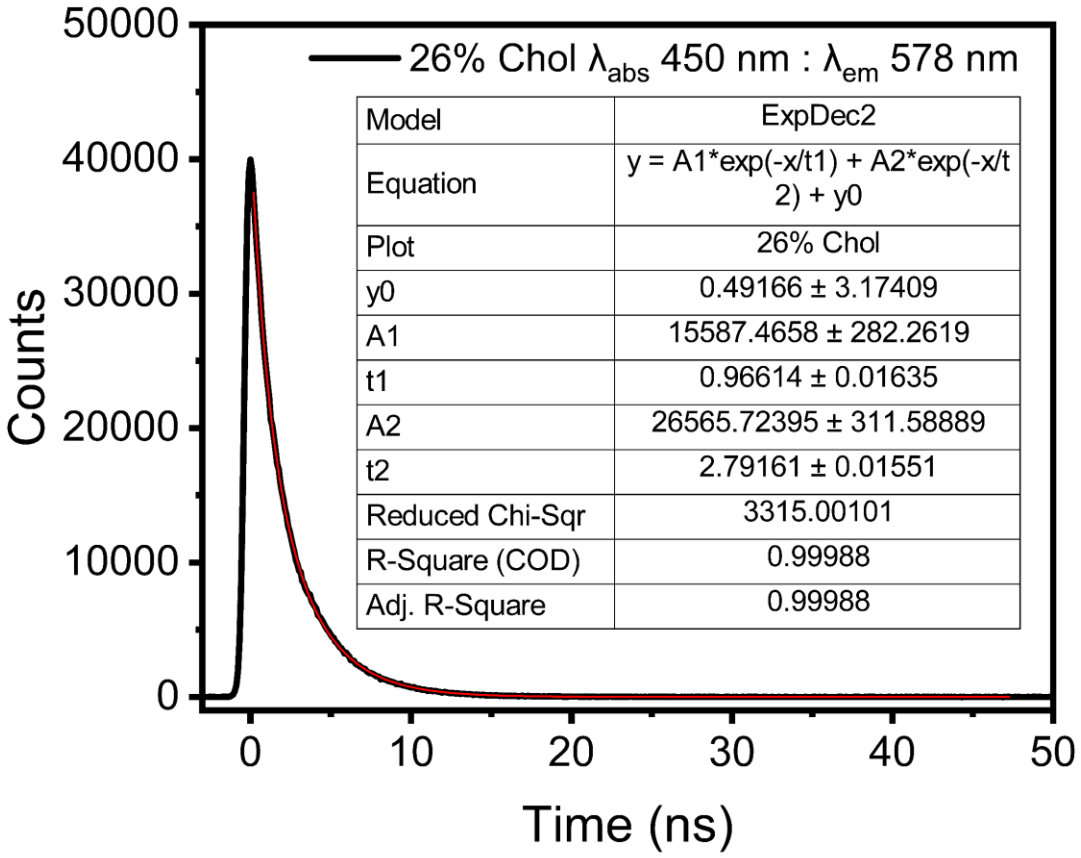
Model	ExpDec2
Equation	y = A1*exp(-x/t1) + A2*exp(-x/t2) + y0
Plot	15% Chol
y0	2.86963 ± 3.10546
A1	18260.93715 ± 471.73947
t1	1.0596 ± 0.01904
A2	24648.53189 ± 502.41812
t2	2.59602 ± 0.02118
Reduced Chi-Sqr	3223.20762
R-Square (COD)	0.99988
Adj. R-Square	0.99988

Figure S10. Fitting for the fluorescence lifetime of DI-8-ANEPPS in liposomes with 15% cholesterol



Model	ExpDec2
Equation	y = A1*exp(-x/t1) + A2*exp(-x/t2) + y0
Plot	24% Chol
y0	5.10134 ± 3.13992
A1	16209.67881 ± 301.25777
t1	0.95504 ± 0.01608
A2	26951.32666 ± 331.47853
t2	2.70508 ± 0.01544
Reduced Chi-Sqr	3283.43945
R-Square (COD)	0.99988
Adj. R-Square	0.99988

Figure S11. Fitting for the fluorescence lifetime of DI-8-ANEPPS in liposomes with 24% cholesterol



Model	ExpDec2
Equation	y = A1*exp(-x/t1) + A2*exp(-x/t2) + y0
Plot	26% Chol
y0	0.49166 ± 3.17409
A1	15587.4658 ± 282.2619
t1	0.96614 ± 0.01635
A2	26565.72395 ± 311.58889
t2	2.79161 ± 0.01551
Reduced Chi-Sqr	3315.00101
R-Square (COD)	0.99988
Adj. R-Square	0.99988

Figure S12. Fitting for the fluorescence lifetime of DI-8-ANEPPS in liposomes with 26% cholesterol

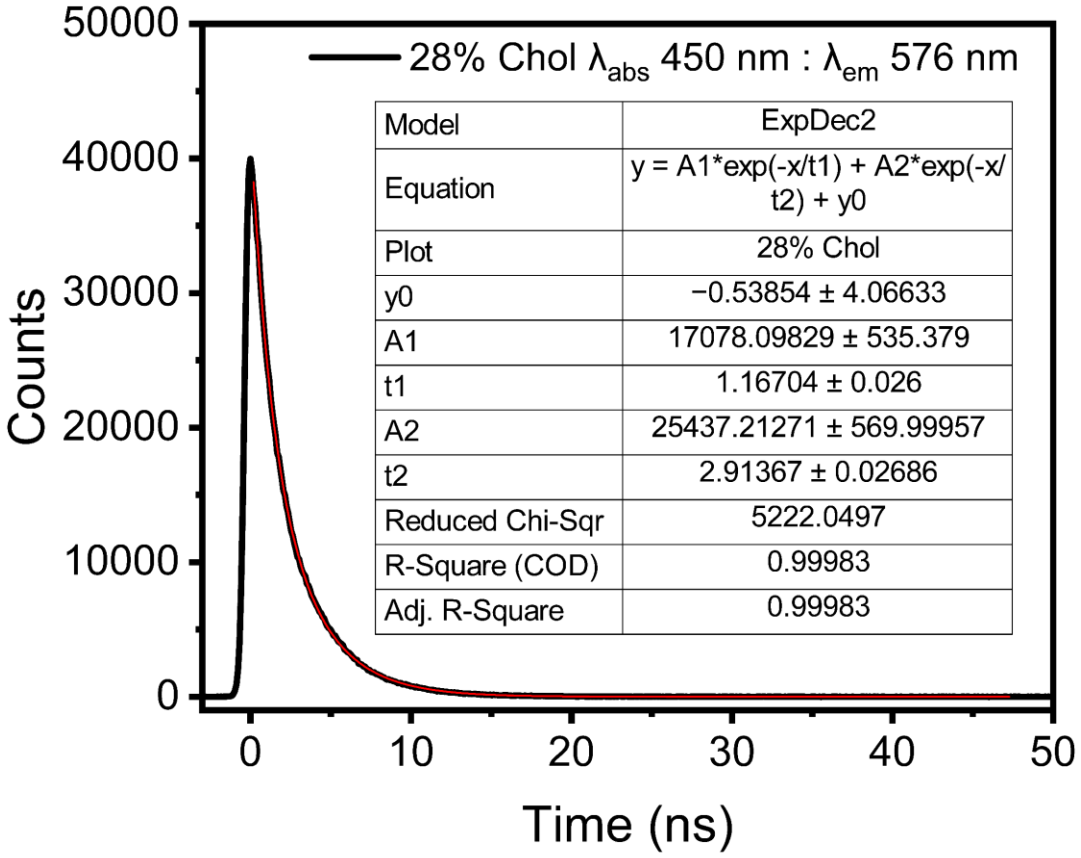
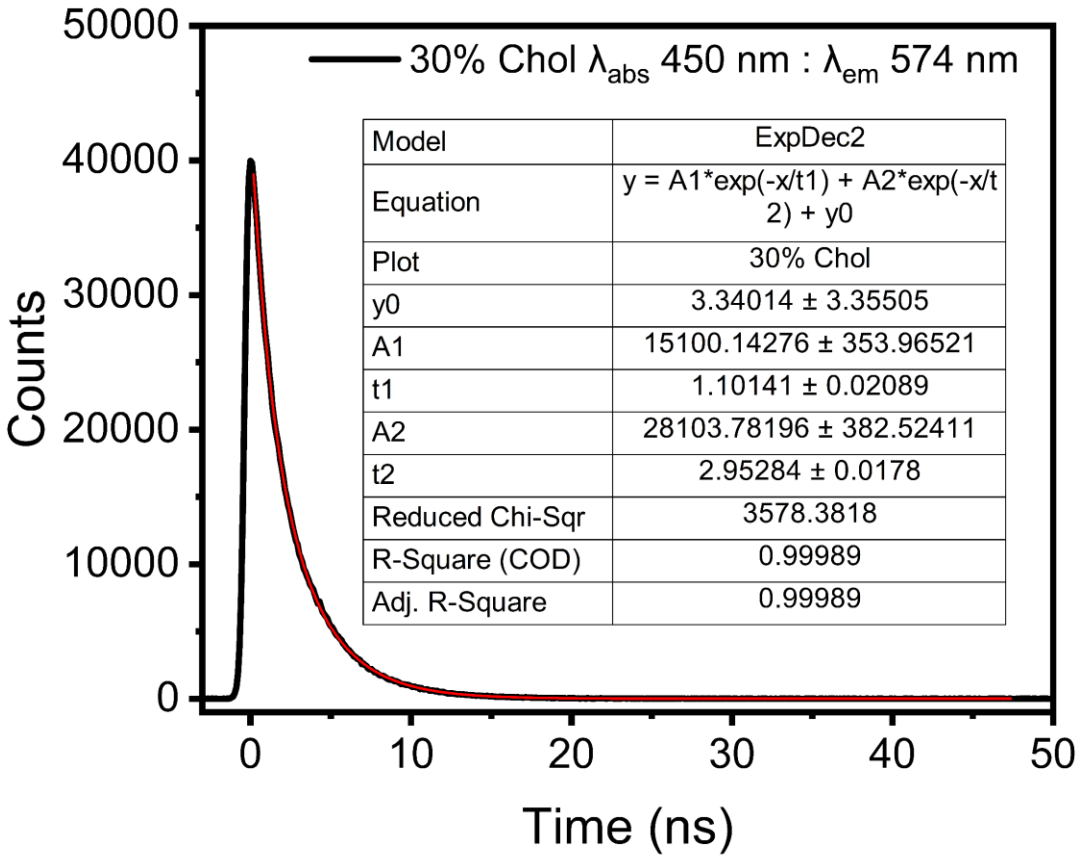


Figure S13. Fitting for the fluorescence lifetime of DI-8-ANEPPS in liposomes with 28% cholesterol



Model	ExpDec2
Equation	y = A1*exp(-x/t1) + A2*exp(-x/t2) + y0
Plot	30% Chol
y0	3.34014 ± 3.35505
A1	15100.14276 ± 353.96521
t1	1.10141 ± 0.02089
A2	28103.78196 ± 382.52411
t2	2.95284 ± 0.0178
Reduced Chi-Sqr	3578.3818
R-Square (COD)	0.99989
Adj. R-Square	0.99989

Figure S14. Fitting for the fluorescence lifetime of DI-8-ANEPPS in liposomes with 30% cholesterol

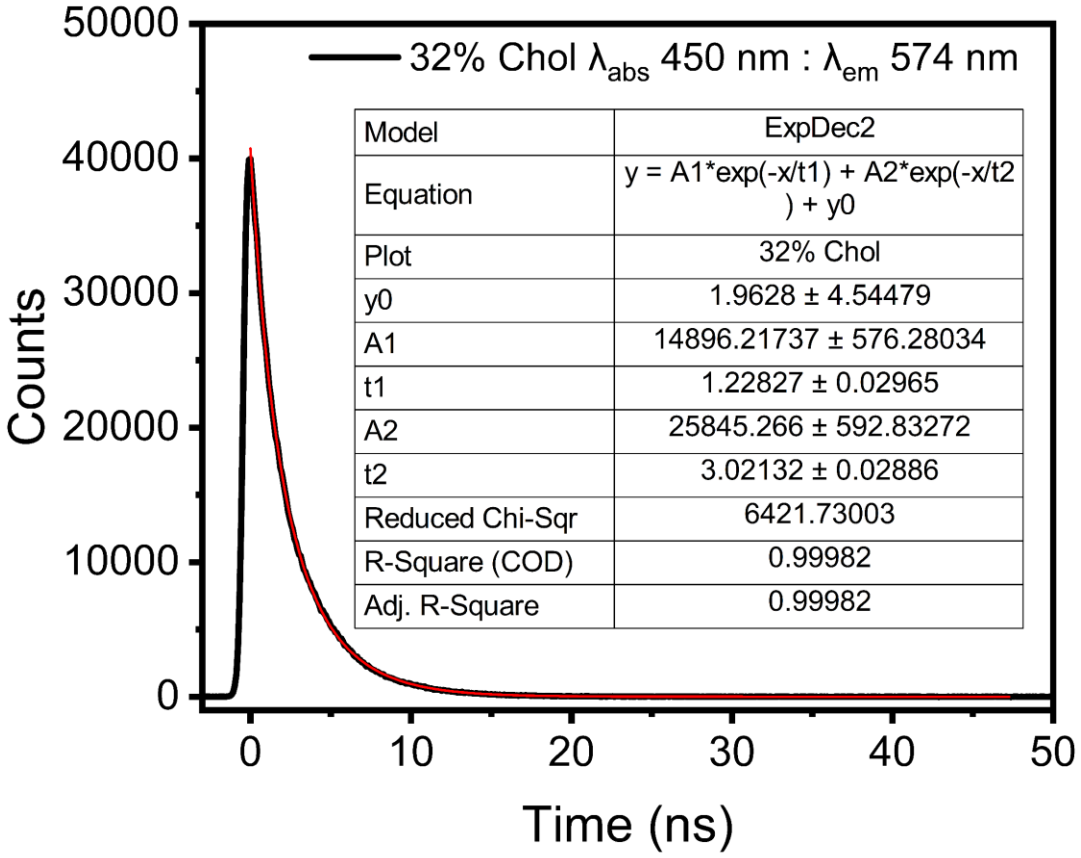


Figure S15. Fitting for the fluorescence lifetime of DI-8-ANEPPS in liposomes with 32% cholesterol

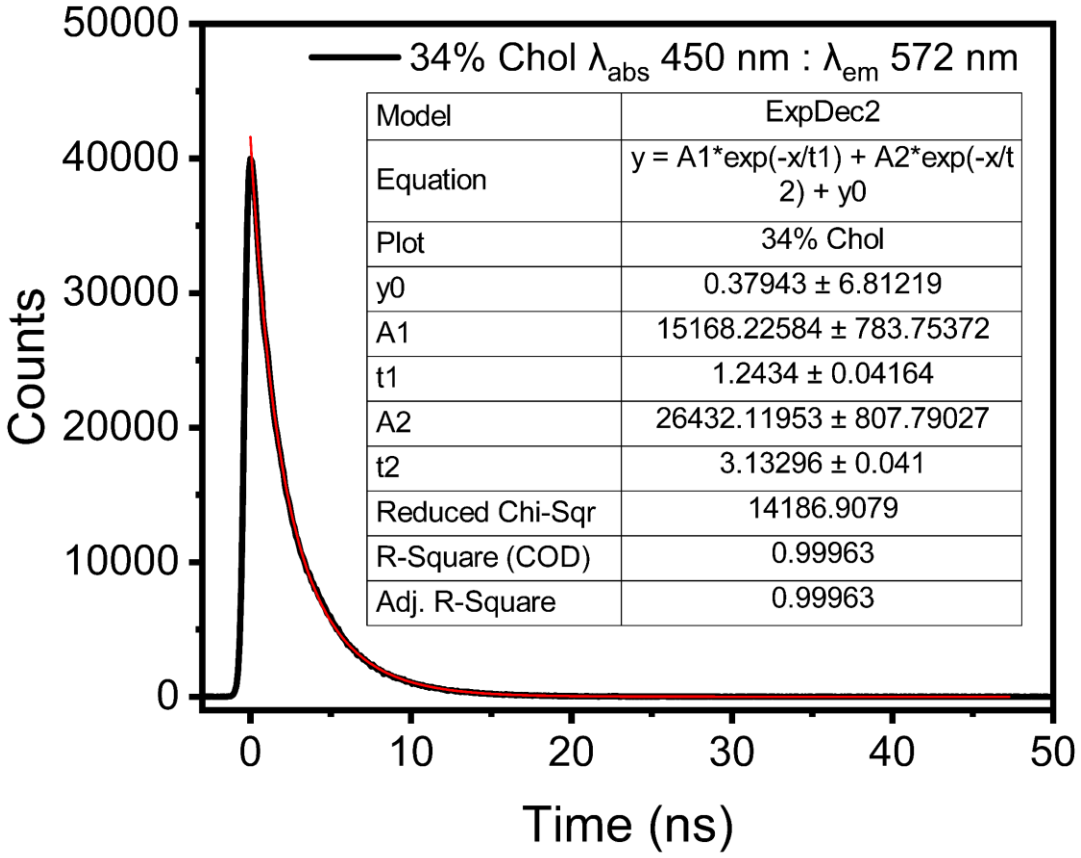
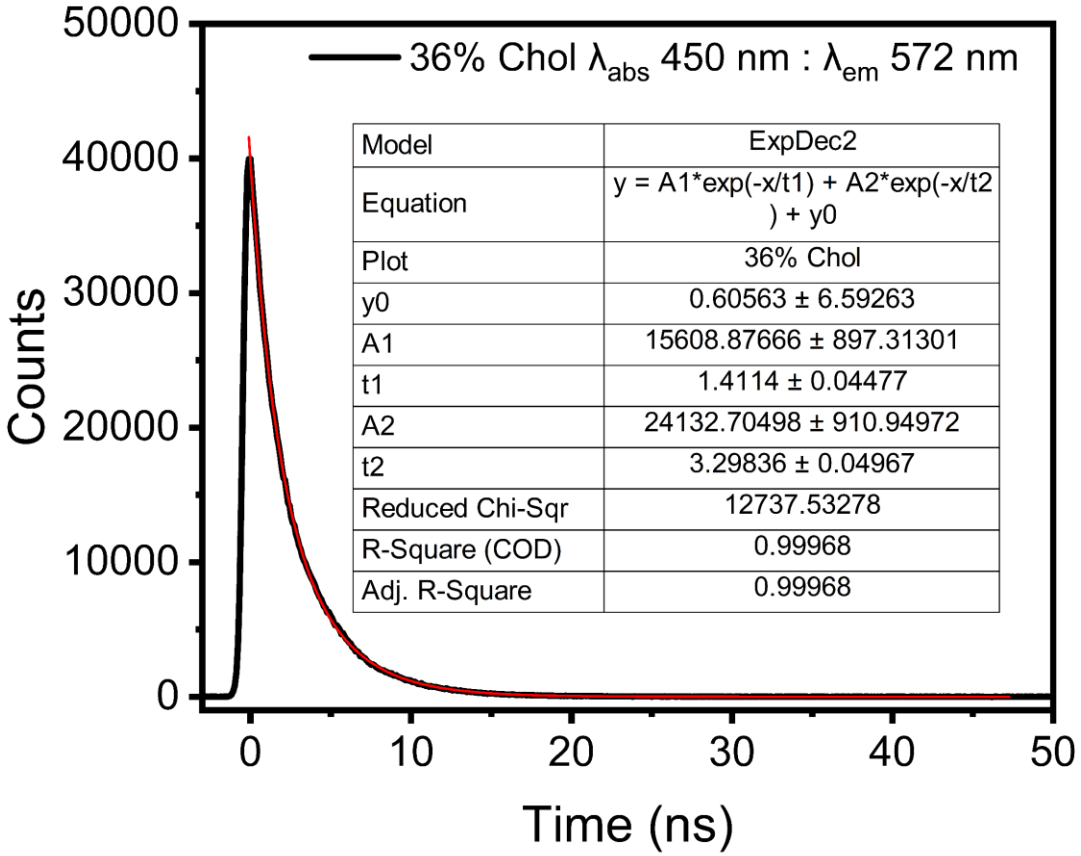


Figure S16. Fitting for the fluorescence lifetime of DI-8-ANEPPS in liposomes with 34% cholesterol



Model	ExpDec2
Equation	y = A1*exp(-x/t1) + A2*exp(-x/t2) + y0
Plot	36% Chol
y0	0.60563 ± 6.59263
A1	15608.87666 ± 897.31301
t1	1.4114 ± 0.04477
A2	24132.70498 ± 910.94972
t2	3.29836 ± 0.04967
Reduced Chi-Sqr	12737.53278
R-Square (COD)	0.99968
Adj. R-Square	0.99968

Figure S17. Fitting for the fluorescence lifetime of DI-8-ANEPPS in liposomes with 36% cholesterol

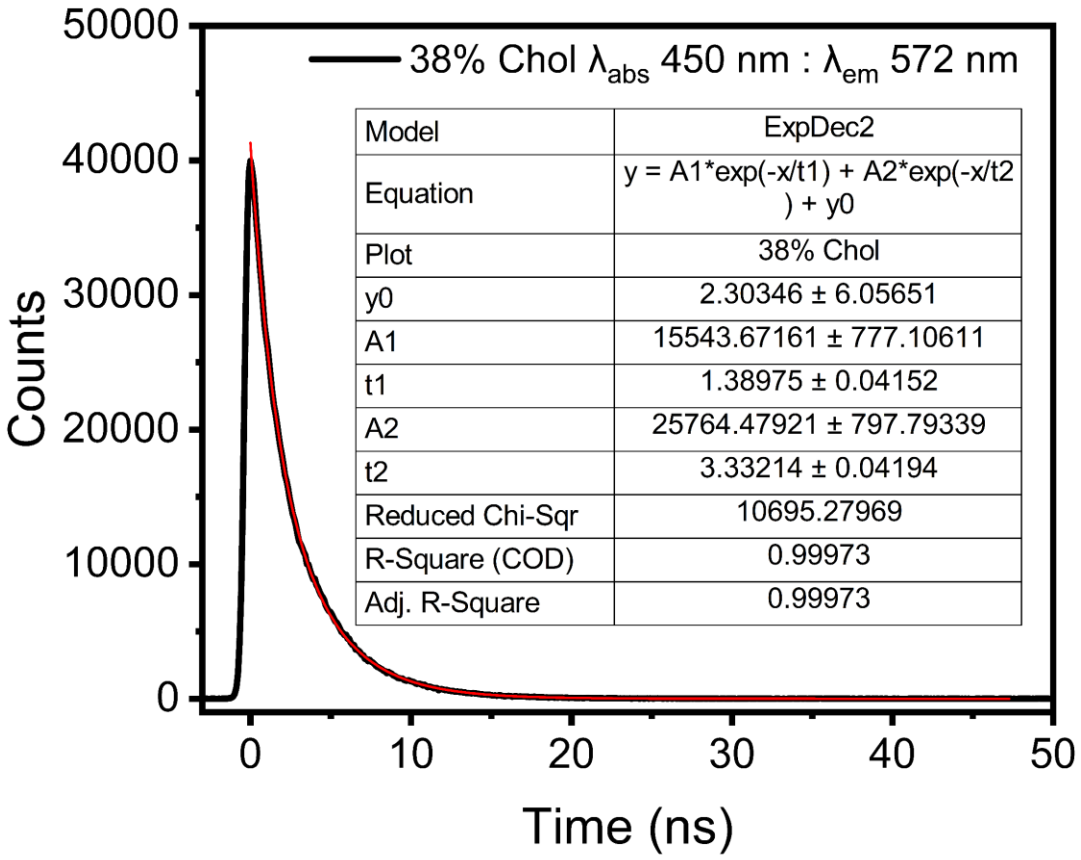


Figure S18. Fitting for the fluorescence lifetime of DI-8-ANEPPS in liposomes with 38% cholesterol

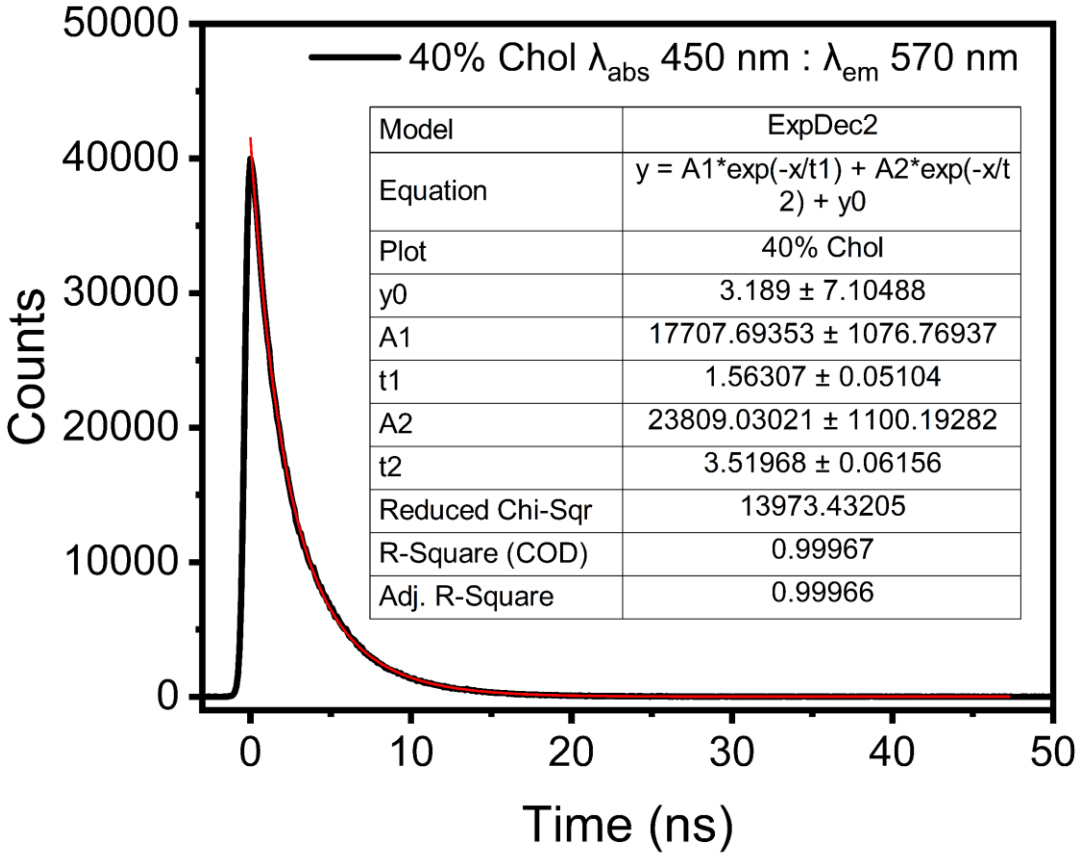


Figure S19. Fitting for the fluorescence lifetime of DI-8-ANEPPS in liposomes with 40% cholesterol

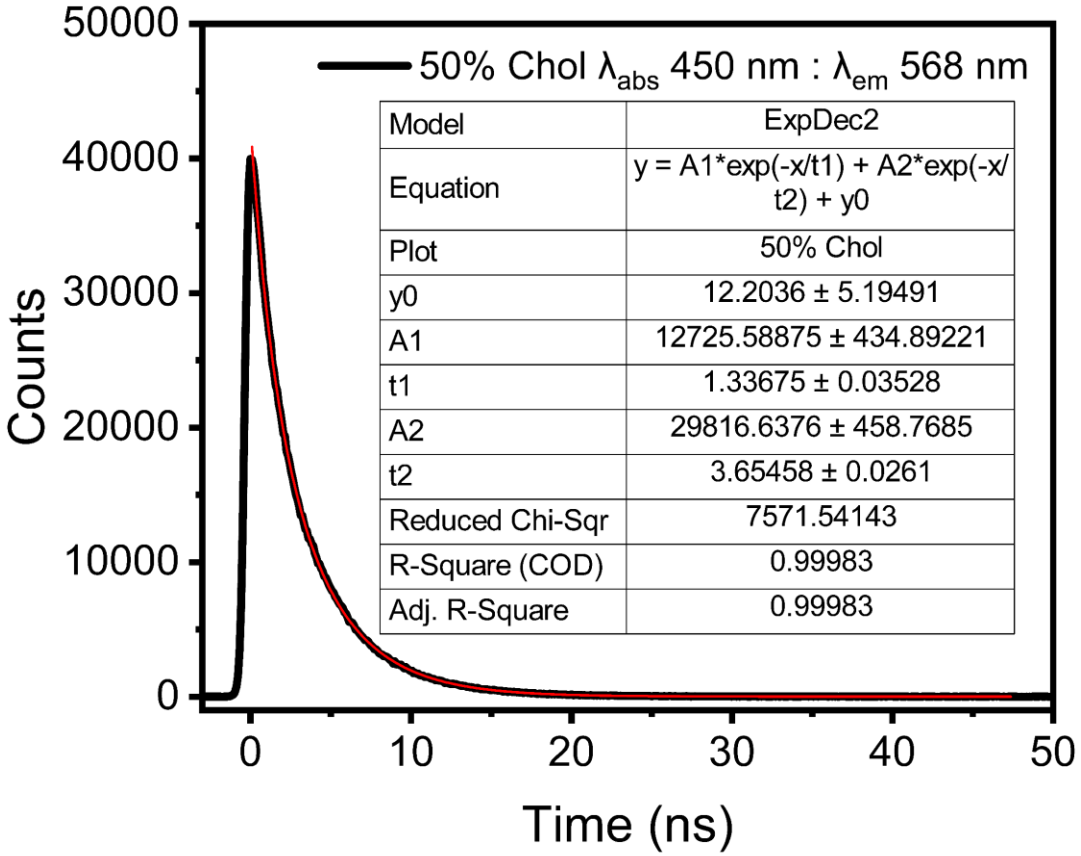
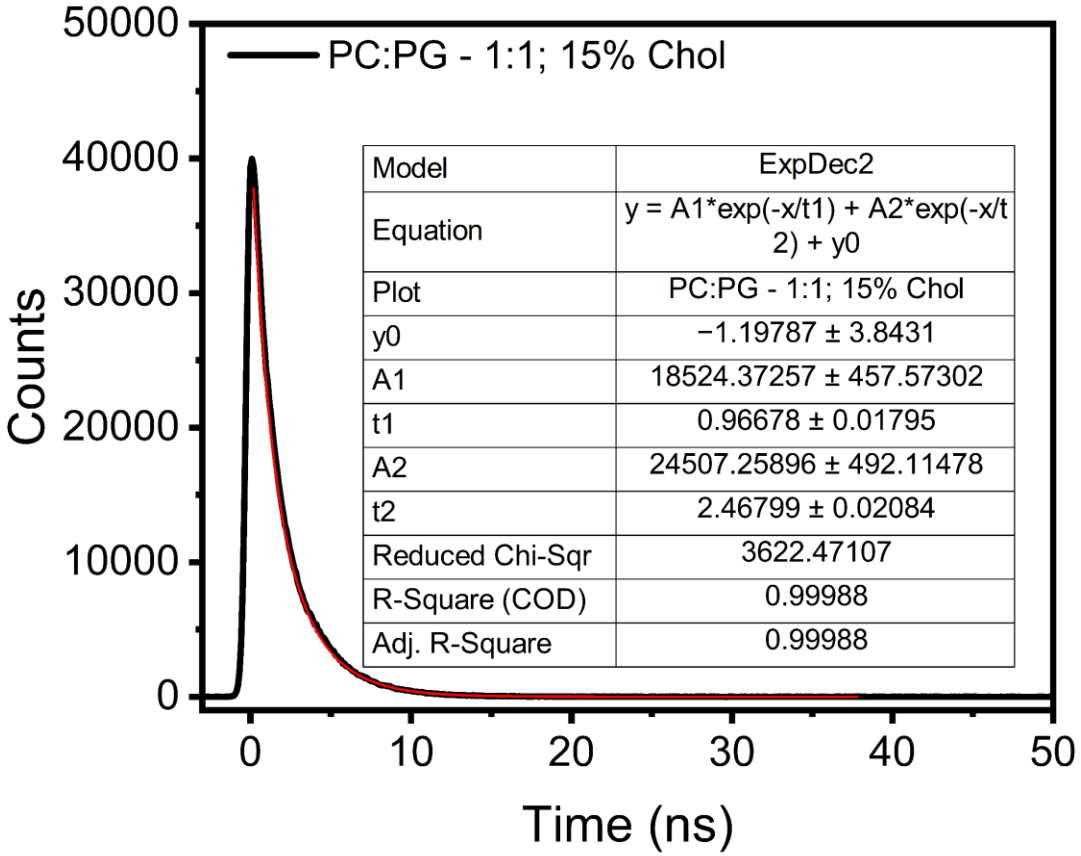


Figure S20. Fitting for the fluorescence lifetime of DI-8-ANEPPS in liposomes with 50% cholesterol



Model	ExpDec2
Equation	y = A1*exp(-x/t1) + A2*exp(-x/t2) + y0
Plot	PC:PG - 1:1; 15% Chol
y0	−1.19787 ± 3.8431
A1	18524.37257 ± 457.57302
t1	0.96678 ± 0.01795
A2	24507.25896 ± 492.11478
t2	2.46799 ± 0.02084
Reduced Chi-Sqr	3622.47107
R-Square (COD)	0.99988
Adj. R-Square	0.99988

Figure S21. Fitting for the fluorescence lifetime of DI-8-ANEPPS in liposomes with 15% cholesterol

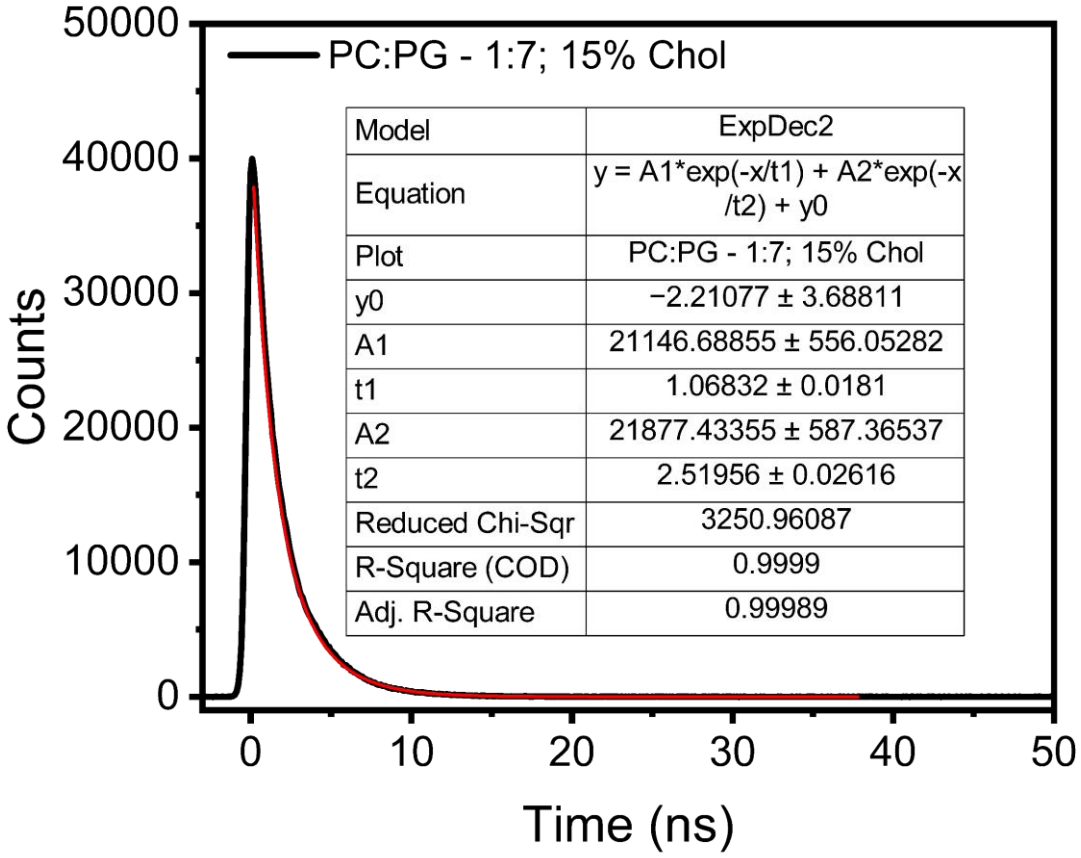


Figure S22. Fitting for the fluorescence lifetime of DI-8-ANEPPS in liposomes with 15% cholesterol

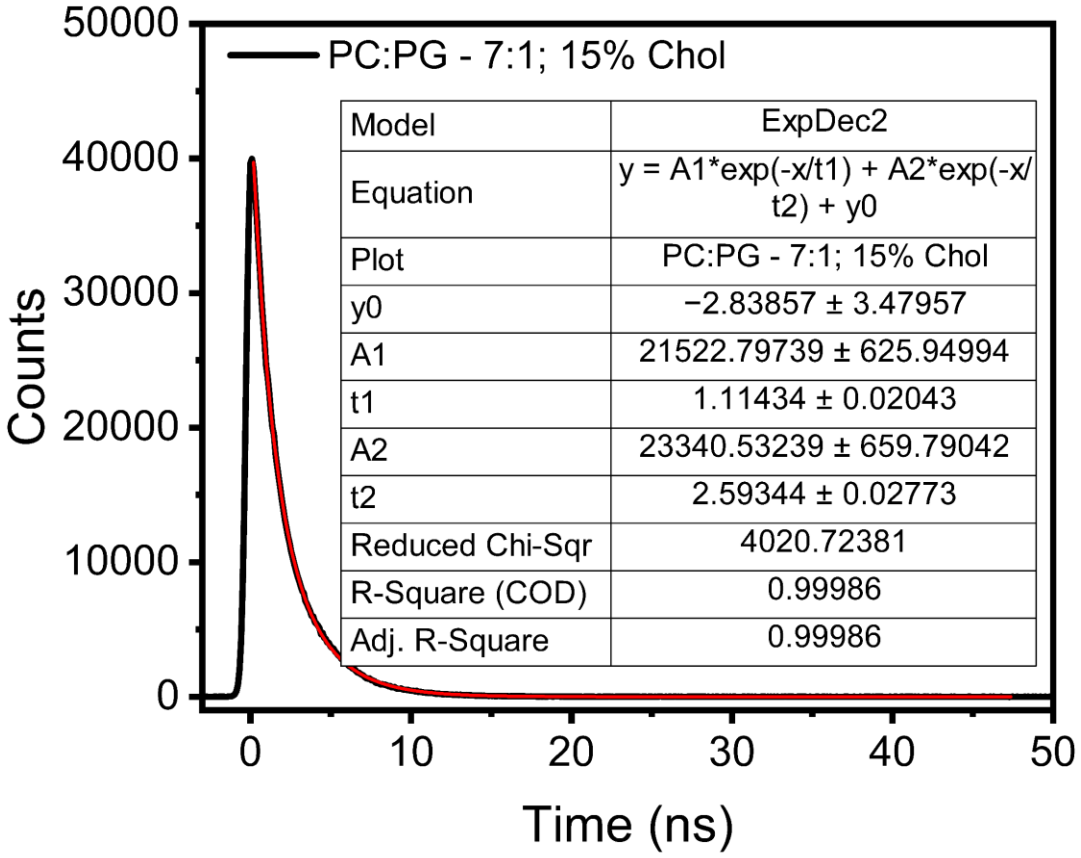


Figure S23. Fitting for the fluorescence lifetime of DI-8-ANEPPS in liposomes with 15% cholesterol

Reference:

1 A. Marczak, *Bioelectrochemistry*, 2009, **74**, 236–239.

2 C. Albrecht, *Anal Bioanal Chem*, 2008, **390**, 1223–1224.

3 T. Starke-Peterkovic, N. Turner, P. L. Else and R. J. Clarke, *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 2005, **288**, R663–R670.

4 R. J. Clarke and D. J. Kane, *Optical detection of membrane dipole potential: avoidance of fluidity and dye-induced effects*, 1997, vol. 1323.