Supplementary Information:

Manufacturing

ATRA was encapsulated in different delivery systems produced with different manufacturing techniques. Neutral and positive charged liposomes were prepared with Thin Layer Evaporation (TLE) technique. Liposome ATRA 1 (neutral liposomes) was prepared by dissolving ATRA:PC: Cholesterol: DSPE-PEG2000 (8:70:30:4 molar ratio)[1] in ethanol and dried at vacuum rotary evaporator. When the lipid film was formed, it was hydrated with PBS pH 7.4. Liposome ATRA 2 was prepared with the same process conditions, by mixing ATRA:PC:Cholesterol:DOTAP (10:70:20:20 molar ratio) to have positive charged liposomes. Both liposome formulations were extruded at 800nm with a handheld extruder to reach a desired particle size dimension and homogeneity.

ATRA 1.25mg/ml was always incorporated into lipids emulsion phase. In brief, NP2 was prepared by mixing ATRA with a melted mass (at 85°C) of with Compritol888ATO®, Polysorbate 80 and Cholesterol as lipid phase. Then, a PEG 0.1% (w/v) and EDTA 0.01% (w/v) pre-heated solution was nanoprecipitated in lipids emulsion under Ultraturrax T-25 homogenizer (Ika Labortechnik, Germany) for 10 minutes at 85°C. After cooling, the nanoparticles were formed by solidification. Finally, NP1 was prepared with Precirol 5 ATO®, Labrafac Lipophile WL1349® as the oily phase and heated at 85°C. The aqueous phase with Polysorbate 80 5% (v/v) was heated and nanoprecipitated in lipids phase under Ultraturrax for 10 minutes at 85°C.

In all the formulations prepared, ATRA was encapsulated 100% and no crystal presence was detected. To conduct measurements on membrane-bound ATRA, we centrifuged 1 mL liposomal (membrane made of phosphatidylcholine and cholesterol) solutions at 8000 rpm and then dried them under a hood for 6 hours.

SAXS

Small Angle X-Ray Scattering (SAXS) and data analysis

SAXS measurements were performed at Chiesi Farmaceutici S.p.A., Parma, Italy. We used an Empyrean (Malvern PANalytical) X-ray diffraction/scattering instrument with an evacuated beam path (ScatterX78). We used a sealed high-resolution X-ray tube (Cu anode) operating at 40 kV and 40 mA. The Cu Kα radiation (λCu Kα = 1.5418 Å) was line-collimated, and the spectra were recorded for 1 h by a PIXcel3D-Medipix3 1x1 detector; resolution 55 μm. The scattering vector $q = (4\pi/\lambda)\sin\Theta$, with 2Θ being the scattering angle, was calibrated before the analysis. Data were collected using the PANalytical Data Collector software, on a q-interval of 0.006-0.3 Å-1. The measurements were performed at 25°C. After the acquisition, the spectra were reduced and analyzed using custom-made codes written in the Wolfram Language (Mathematica, Version 13.0, Wolfram Research, Inc., Champaign, IL). Data reduction included background subtraction (PBS buffer), and the data was edited to remove the noisy q-range and select the q-range of 0.06-2.5 nm-1. Subsequently, the reduced data were analyzed according to Brzustowicz et al. [2] to estimate the electron density profile of the bilayer of the liposome. The center of the profile is set as the center of the bilayer, which is considered asymmetric. The same procedure has been applied to both samples to compare the unloaded liposome and the loaded liposome (ATRA). The estimation is based on the parametric nonlinear regression of the sum of three Gaussian function, by minimizing residual least squares. To regress the model presented by Brzustowicz et al.[2], we used the logarithms of the data pairs.

Nano DSC

Differential scanning calorimetry experiments were carried out using a Nano- Differential Scanning Calorimetry (nDSC) by Ta-Instruments(New Castle, Delaware, USA). Samples and references were loaded into the autosampler and then injected into the capillary cells. They were heated from 0°C to 95°C with

a scan rate of 2°C/min and were given 600 seconds of equilibration at the initial temperature. Before and after each sample, a blank scan was performed using buffer in both cells.

Data were collected using DSC Run Software and analyzed with NanoAnalyze Software by TA-Instruments. Data analysis consider mostly three parameters: transition temperature (T_m), enthalpy variation (ΔH) and half-width of peak ($\Delta T_{m1/2}$). From the parameters obtained, we also computed the cooperative number (CN) using **eq.(1)**, following the method proposed by Kasian et al.[3].

CN = $4RT_m^2$ / $\Delta T_{m1/2}$ ΔH	**(1)**

FLIM

Fluorescence Lifetime Imaging Microscopy (FLIM) was carried out using a Leica TCS SP5 confocal microscope from Leica Microsystems (Mannheim, Germany). The samples were stimulated with a pulsed diode laser with a frequency of 80 MHz and an excitation wavelength of 400 nm, with an average power of 10-20 µW at the sample. Emission was detected in the range of 470 to 570 nm by a photomultiplier tube, connected to a PicoHarp 300 Time Correlated Single Photon Counting (TCSPC) card from PicoQuant in Berlin.

The Phasor Analysis of the FLIM data was done using custom-made Python 3.6 routines. Specifically, for every pixel in the image, the fluorescence decay measured in the time domain was transposed to the phasor plot. In this plot, a phasor is represented by two coordinates: the real and imaginary parts of the Fourier Transform of the fluorescence lifetime decay, which are calculated at the measurement's angular repetition frequency.

In frequency domain for each pixel one can exploit modulation $m_{i,j}$ and phase shift $\phi_{i,j}$ of the signal to map the phasor plot as reported in **eq. (2.a)-(2.b).**

$g_{\{i,j\}} = m_{i,j} \cdot \cos(\phi_{i,j})$	**(2.a)**
$s_{\{i,j\}} = m_{i,j} \cdot \cos(\phi_{i,j})$	**(2.b)**

For a single exponential decay, the intensity can be described using equation **(3.a)**. In contrast, when the decay is multi-exponential, the intensity can be expressed using equation **(3.b)**. In these equations, the subscripts "f", "b", and "p" indicate the intensity of the drug in its free form, its form associated with a membrane, and its form in precipitates, respectively.

$I_{mono}(t) = A_f e^{-t/\tau}$	**(3.a)**
$I_{multi}(t) = A_f e^{-t/\tau_f} + A_b e^{-t/\tau_b} + A_p e^{-t/\tau_p}$	**(3.b)**

When two distinct molecular species are present in the same pixel, a combination of the two molecular species' weightings can be used to produce phasors distributed along a straight line connecting the unique phasors of the two pure species.

Phasors are typically found within a semi-circle, known as the universal semi-circle, which is centered at (½, 0) with a radius of ½ and a positive x-axis. The point where the lifetime is zero is located at (1, 0) and the point where the lifetime is infinite is located at (0, 0). Mono-exponentially decaying species lie on the semi-circle itself, while signals found outside the semi-circle are less common and likely to be caused by delays in excited state reactions [4].

We established the phasor positions by aggregating cumulative phasors [5,6]. This process involved assigning weights to each data point in accordance with the pixels' intensity values. To provide clarity, it's important to note that a cumulative phasor is formed by consolidating the signals emanating from the pixels of each replica, thereby creating a composite phasor. We estimated the positional error by taking into account the average coordinates g and s, considering all the points starting from the 75th

percentile. This was feasible because we consistently ensured that the variability in the phasor distribution's primary component of PCA remained below 0.6.

When three different molecular species are present, the possible phasors generated by the system will be distributed across a triangle. The vertices of this triangle represent the unique phasors of the pure species. As a result, through utilizing a graphical method, described previously by Tentori et al. [7], it is possible to determine the combination of two or more species that have contributed to a given experimental phasor based on the phasors of the isolated pure components.

Formulations	Tm [°C]	ΔH [kJ/mol]	CN	ΔTm ½
PC	51,23 ± 0,130	0,7089	616	8
PC + ATRA	56,6 ± 0,126	0,6796	529	10
Placebo	44,65 ± 0,038	0,7200	773	6
LipoATRA 1	61,39 ± 0,132	0,6763	731	7,5

Table S1 NanoDSC extrapolated data from DSC thermograms of various nanoformulations. in terms of melting temperature (Tm), enthalpy change (ΔH), cooperative number (CN) and Full Width Half Maximum ΔTm ½).

Nanoformulation	ATRA concentration [mg/mL]	Type	Excipients
Lipo ATRA 1	1	Liposome	PC Cholesterol DSPE-PEG2000
Lipo ATRA 2	4	Liposome	Pc Cholesterol DOTAP
NP ATRA 1	1.25	Nanoparticle	Precirol Labrafac Tween80
NP ATRA 2	2	Nanoparticle	Cholesterol Tween 80 Compritol PEG EDTA

Table S2 Investigated nanoformulations. *Liposomal ATRA 1 is investigated in the whole paper and simply referred to as liposomal ATRA, while Liposomal ATRA 2 is included to evaluate the impact of distinct excipient formulation*

Sample	free	Precipitated	bound
LipoATRA1	23.91±0.54	65.58±0.57	10.51±0.03
LipoATRA2	32.41±0.49	16.40±2.93	51.68±2.44
ATRA PC DOTAP	26.94±1.99	44.54±2.47	28.52±0.48
ATRA PC Cholesterol	12.87±5.54	58.54±4.32	28.29±1.22

Table S3 Fractional intensities of LipoATRA nanoformulations. *Liposomal ATRA is mainly encapsulated precipitated ATRA, whereas LipoATRA 2 is mainly encapsulating membrane-bound ATRA as a consequence of the liposomal composition.*
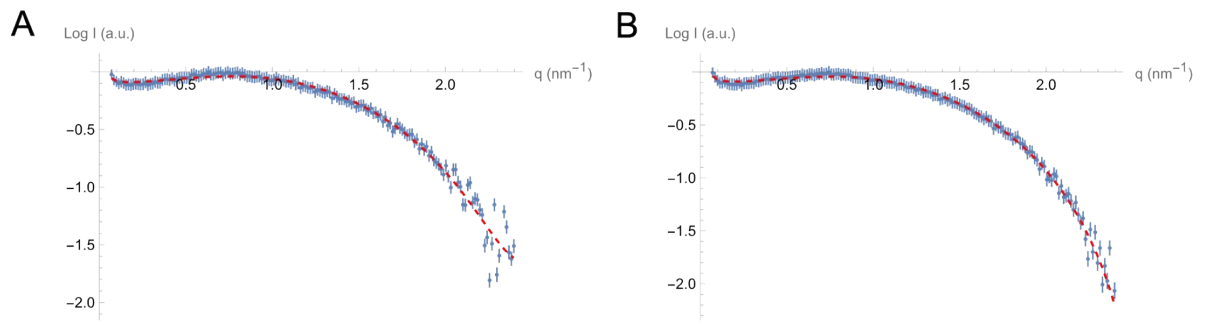
Figure S1. Raw fitting of the SAXS model *A) Fitting of the liposome placebo SAXS pattern B) Fitting of the liposome loaded with ATRA SAXS pattern.*
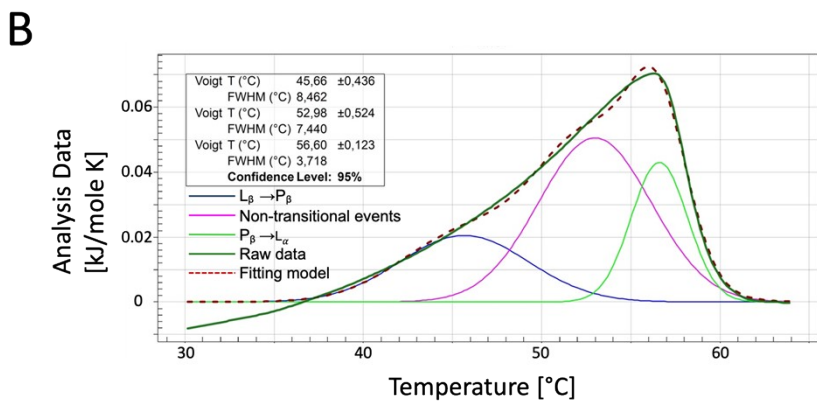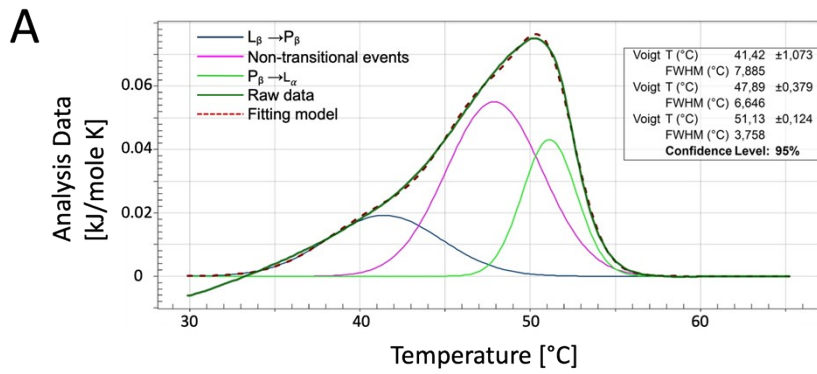
Figure S2 PC formulations thermograms with deconvolution analyzed samples. *A) PC thermogram with voigt parameters of B) PC+ATRA thermograms with Voigt fitting parameters shows a clear shift towards higher melting temperarures.*
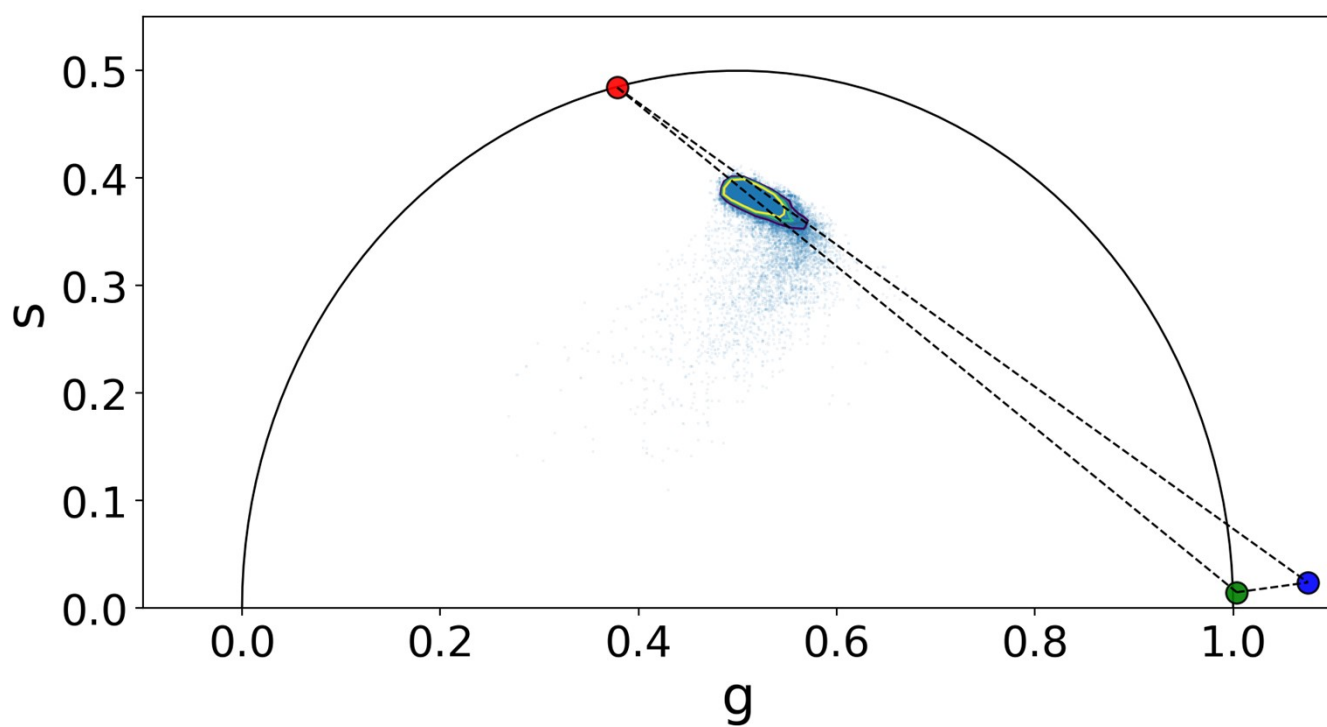
Figure S 3 Phasor-FLIM raw data of isolated membrane interacting with ATRA molecules. *Cumulative phasor extracted by replica (N=3) of a basic liposomal formulation made of Phosphatidylcholine and Cholesterol. The fractional intensity of membrane-bound ATRA is approximately 80%.*
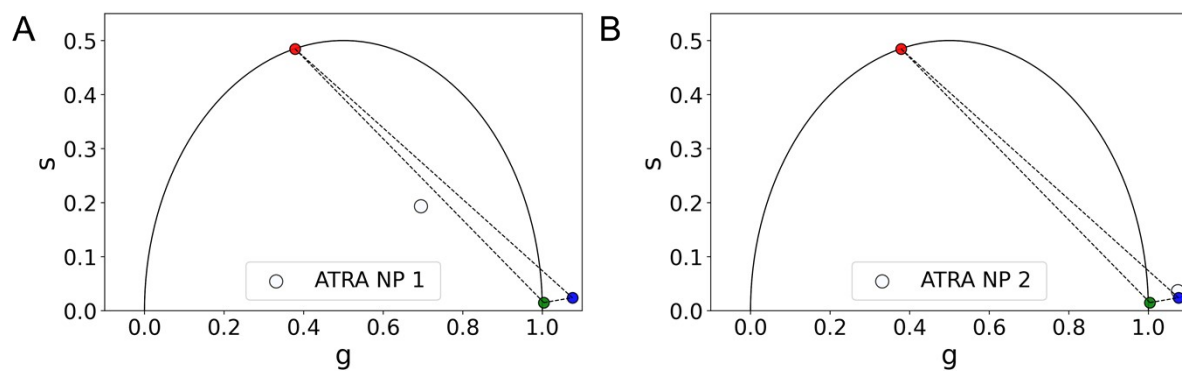
Figure S 4 FLIM testing of non-lipidic nanoformulations. *A) NP ATRA 1 of **Table S2** reveals non-lipidic interactions in fact the phasor is lying outside of the free-, precipitated and membrane bound description B) NP ATRA 2 of **Table S2** reveals that ATRA is fully precipitated in this nanofromulation.*

References

1 D. C. Berry and N. Noy, *Mol Cell Biol*, 2009, 29, 3286–3296.

2 M. R. Brzustowicz and A. T. Brunger, *J Appl Cryst*, 2005, 38, 126–131.

3 N. Kasian, O. Vashchenko, L. Budianska, R. Brodskii and L. Lisetski, *J Therm Anal Calorim*, 2019, 136, 795–801.

4 L. Malacrida, S. Ranjit, D. Jameson and E. Gratton, *Annual Review of Biophysics*, 2021, 50, 575–593.

5 M. A. Digman, V. R. Caiolfa, M. Zamai and E. Gratton, *Biophysical Journal*, 2008, 94, L14–L16.

6 A. Vallmitjana, B. Torrado, A. Dvornikov, S. Ranjit and E. Gratton, *J Phys Chem B*, 2020, 124, 10126–10137.

7 P. Tentori, G. Signore, A. Camposeo, A. Carretta, G. Ferri, P. Pingue, S. Luin, D. Pozzi, E. Gratton, F. Beltram, G. Caracciolo and F. Cardarelli, *Nanoscale*, 2022, 14, 8901–8905.