Deciphering Melatonin-Stabilized Phase Separation in Phospholipid Bilayers

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Supporting Information

ABSTRACT: Lipid bilayers are fundamental building blocks of cell membranes, which contain the machinery needed to perform a range of biological functions, including cell–cell recognition, signal transduction, receptor trafficking, viral budding, and cell fusion. Importantly, many of these functions are thought to take place in the laterally phase-separated regions of the membrane, commonly known as lipid rafts. Here, we provide experimental evidence for the "stabilizing" effect of melatonin, a naturally occurring hormone produced by the brain’s pineal gland, on phase-separated model membranes mimicking the outer leaflet of plasma membranes. Specifically, we show that melatonin stabilizes the liquid-ordered/liquid-disordered phase coexistence over an extended range of temperatures. The melatonin-mediated stabilization effect is observed in both nanometer- and micrometer-sized liposomes using small angle neutron scattering (SANS), confocal fluorescence microscopy, and differential scanning calorimetry. To experimentally detect nanoscopic domains in 50 nm diameter phospholipid vesicles, we developed a model using the Landau–Brazovskii approach that may serve as a platform for detecting the existence of nanoscopic lateral heterogeneities in soft matter and biological materials with spherical and planar geometries.

INTRODUCTION

Phase separation is an important feature for a range of physical and biological processes,1–9 including the compartmentalization of a solution in immiscible liquid components.10–12 It can be induced through changes in pressure, temperature, concentration, and other thermodynamic and physicochemical parameters. In biological systems, this liquid–liquid coexistence has been experimentally observed in different systems.13–15 For example, biological membranes compartmentalize chemical reactions in order to facilitate the spatiotemporal regulation of cellular functions. Moreover, many of the biological condensates in the cytoplasm and nucleoplasm resulting from phase separation are liquids16 and are associated with functions such as restructuring of the genome17 and repairing DNA.18 Certain organelles and nuclear bodies also form through liquid–liquid phase separation.19 Liquid–liquid phase separation may also describe the aggregation of intrinsically disordered proteins, which are relevant for the formation of structures like P granules16,20 and the development of diseases such as Alzheimer’s. In the case of Alzheimer’s, it is thought that Tau proteins undergo liquid–liquid phase separation, forming liquid protein droplets that trigger the disease.21 Biological membranes consist of a liquid bilayer that is responsible for membrane structure and dynamics.22–24 Importantly, biological membranes are laterally inhomogeneous structures and, in model systems, their heterogeneity depends on pH, temperature, pressure, ionic strength, and so on. Phase separation in biological membranes has been implicated in biological processes such as viral budding, cell fusion, and cell–cell communication.25–27 With regard to technological applications, targeted phase-separated liposomes with an ability to easily fuse with other membranes have been used to deliver their payloads with greater efficacy.28 As the...

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direct observation of lipid rafts or domains in living cellular membranes remains a challenging and elusive task, model lipid membranes have been used extensively to study phase separation in biomimetic membranes.

Here, we studied the effect that melatonin, a hormone produced by the brain’s pineal gland, has on phase separation in model membranes at length scales ranging from the nanometer to the micrometer and at different temperatures. Melatonin is of current scientific interest as it is believed to act as an antioxidant, responsible for regulating the human sleep cycle, and is proposed to protect against a variety of different diseases, including cardiovascular, Alzheimer’s, and certain types of cancers. Despite its biological importance and the fact that it is intimately associated with membranes, there are only a few studies that looked into the effects of melatonin on membrane lateral heterogeneity.

For the current study we used fluorescence microscopy, small angle neutron scattering (SANS), and differential scanning calorimetry (DSC) to study the effect(s) that melatonin has on phase separation in nanoscopic unilamellar vesicles (ULVs) and macroscopic giant unilamellar vesicles (GUVs). We found that, irrespective of size, melatonin stabilizes and preserves lipid phase separation over a range of temperatures. This effect was observed regardless of experimental technique and vesicle size, suggesting that melatonin is capable of modulating phase separation with possible and significant physiological consequences.

In order to interpret the newly acquired SANS data from laterally heterogeneous lipid vesicles, we developed a scattering model using a phenomenological free energy approach. This approach is based on general symmetry principles and is able to address different phase-separated states. Unlike previous SANS analyses that assume a particular geometry for the separated phases, such as a single disc-like domain or an assortment of circular domains on the vesicle surface, our model is based on the most probable thermodynamic state of the membrane and does not assume any specific domain geometry. This so-called Landau–Brazovskii approach for interpreting scattering data from laterally heterogeneous lipid vesicles, may be applicable in identifying many different domain configurations, including patterned membranes and disordered phases with transient structures.

**Materials and Methods**

**Confocal Microscopy of GUVs. Sample Preparation.** GUVs of a four-component lipid mixture composed of 1,2-distearoyl--sn-glycero-3-phosphocholine (DSPC, 39 mol %), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, 33 mol %), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, 6 mol %), and cholesterol (Chol, 22 mol %) were prepared by a modified electroformation procedure as described previously. Briefly, lipid mixtures in chloroform and melatonin in 2,2,2-trifluoroethanol (TFA, Sigma) are mixed at the appropriate mole fractions and placed onto a conductive indium–tin-oxide (ITO)-coated slide (Delta Technologies). Following transfer, the film-ITO slide was placed in a vacuum for 1 h to remove residual solvent. GUVs were swelled at 55 °C in sucrose for 2 h and then cooled to room temperature. Electroformation was performed at 1 V peak-to-peak and at a frequency of 5 Hz. The GUV solution was then transferred to a PCR tube and GUVs were given 1 h to settle prior to confocal microscopy imaging. Solutions were used within 24–48 h of preparation.

**Imaging.** GUVs were imaged using a raster scanning, confocal microscope (Zeiss LSM T-PMT). A GUV solution was transferred to the center of a silicon gasket placed on a glass microscope slide. A glass coverslip was then placed over the solution and the silicon glass coverslip was then placed over the solution and the silicon.

gasket. GUV images were collected using a 561 nm filter for exciting the Dil-C12 probe added to the film solution at a concentration of 0.05 mol %. A 405 nm filter was used to excite naphthopyrene that was added to the film solution at a concentration of 10 mol %. Importantly, the fluorescence spectrum of melatonin has maximum at ~340 nm, significantly below the lower value cutoff filter. All Dil-C12 and naphthopyrene were chosen because they are known to selectively partition into the Ld and Ld phases, respectively, of GUVs. All GUVs were imaged using the red (Dil-C12) and blue (naphthopyrene) channels to check for partitioning of the dye into the incorrect phase. For red channel images, black regions of the membrane indicated the presence of Ld phase-separated regions and vice versa for the blue channel. In doing so, the integrity of the vesicles exposed to light was determined. GUV images were collected at 25 and 45 °C. Samples were equilibrated for 15 min after a change in temperature.

**SANS Measurements of ULVs. Sample Preparation.** Unilamellar vesicles (ULVs) were prepared by extrusion, as previously described. To study the low-q behavior, we contrast-matched the average SLD of the solvent, headgroup, and acyl chains using 34.6% D2O water and 65.4% of chain perdeuterated DSPC-D2O measured as a percentage of total DSPC. As mentioned, lipids were dissolved in chloroform and melatonin in 2,2,2-trifluoroethanol (TFA, Sigma). Samples were prepared by transferring desired volumes of solutions to a glass tube. Chloroform/trifluoroethanol were then removed under a stream of N2 and moderate heat. Samples were then placed under vacuum for a minimum of 12 h to remove any traces of solvent. Dry lipid films were hydrated with a predetermed D2O/H2O mixture preheated to 50 °C, followed by vortexing. The multilamellar vesicles (MLVs) were then incubated at 50 °C for 1 h, followed by five freeze–thaw cycles between −80 and 50 °C. ULVs were prepared using a minietruder and polycarbonate filters with 50 or 100 nm pore diameters, also preheated to 50 °C. The MLV solution was passed through the appropriate diameter filter 31 times. The extruded samples were measured using SANS at concentrations of 10−20 mg/ml within 24 h of extrusion.

**Measurements.** SANS measurements were carried out at the BL-6 Extended Q-range small angle neutron scattering (EQ-SANS) instrument located at the Spallation Neutron Source (SNS), Oak Ridge National Laboratory (ORNL). ULVs were loaded into 1 mm path-length quartz banjo cells (Hellma USA, Plainview, NY) and mounted in a temperature-controlled cell holder with ~1 °C accuracy. EQ-SANS data were collected at a sample-to-detector distance of 2.0 m using 2.5−5.5 Å wavelength neutrons for a total scattering vector of 0.01 < q < 0.3 Å−1. The 2D SANS data were reduced using the ORNL Mantid software. Data were corrected for detector pixel sensitivity, dark current, sample transmission, and background scattering from water.

**Results and Discussion**

**Micrometer Length Scale Lipid Phase Separation.** A four-component lipid mixture can exist in the plane of the membrane as an isotropically liquid-disordered Ld phase (i.e., entropy exceeds the heat of mixing) or as coexisting liquid-ordered Lα and liquid-disordered Ld phases (i.e., heat overcomes entropy of mixing); GUVs are commonly used to study lipid phase separation on micrometer length scales. DSC/PPOP/DOPC/cholesterol mixtures are extensively used because they are capable of forming coexisting Lα−Ld phase bilayers that are well characterized. For example, at 25 °C it has been observed that Lα−Ld bilayers have mismatched bilayer thicknesses and very different bending moduli, features that contribute to a line tension at domain interfaces.

At 45 °C, bilayers without melatonin are in the isotropically liquid disordered, Ld phase. Lipid lateral diffusion, which is moderately restricted at 25 °C, increases at 45 °C, and the...
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conditions. SANS, however, is an inherently nanoscopic technique,
nanoscopic biomaterials under physiologically relevant con-
is because there are few techniques capable of studying
among the few techniques capable of providing both nanoscale
structural information without the use of extrinsic probes
Previous studies show that, at intermediate and elevated
concentrations, melatonin strongly interacts with the phos-
pholipid membrane at the membrane/water interface. Specifically, melatonin molecules act as a surfactant, forming a highly uniform structure aligned parallel to the membrane.
This may be a possible mechanism for the domain stabilization, whereby the ordered melatonin structure prevents domain melting, especially if the melatonin preferentially partitions into either the L₀ or L₄ phase of the membrane.
Another possibility, is that melatonin is a linactant, binding at the L₀−L₄ boundary interface, decreasing the line tension and preserving the domain structure. This is, for example, a known mechanism for micro- and nanodomain formation in lipids and is plausible given melatonin’s propensity to associate with the membrane.

Zooming in on Nanoscopic Lipid Phase Separation.
Vesicle finite size effects and their role in nanoscopic lipid phase separation have yet to be explored thoroughly. This is because there are few techniques capable of studying nanoscopic biomaterials under physiologically relevant conditions. SANS, however, is an inherently nanoscopic technique, and contrast matching approaches have been used to highlight different aspects of the lateral membrane structure. SANS is among the few techniques capable of providing both nanoscale structural information without the use of extrinsic probes (e.g., fluorescent labels) that are sometimes known to alter the interactions responsible for domain formation.

The coherent neutron scattering differential cross section for polydisperse ULVs with a uniform number density n is fully specified by the vesicle form factor, F(q), and structure factor, S(q). It can be written as

$$ F(q) = \int d\mathbf{r} \rho(\mathbf{r}) \rho(\mathbf{r} + q) e^{-i\mathbf{q}\cdot\mathbf{r}} $$

where ρ is the scattering length density, SLD, of the solvent and ρ(\mathbf{r}) is the vesicle SLD. For a dilute solution of vesicles (i.e., 10−20 mg/mL) used for experimentation, we can approximate S(q) ≈ 1 by ignoring vesicle-vesicle interactions that contribute to the scattering. Thus, we are only concerned with the spherically averaged intensity, I(q), that depends on the magnitude of the wave vector, q = |q|, which is related to the neutron wavelength, λₙ, and the scattering angle, θ, according to q = (4π/λₙ)sin(θ/2). The scattered intensity is an orientationally averaged cross section. For a dilute solution of vesicles I(q) can be written as

$$ I(q) = \frac{I_0}{4\pi} \int |F(q)|^2 d\Omega_q + I_{inc} $$

where I_{inc} is the q-independent incoherent scattering contribution. Since for dilute samples the vesicles all scatter independently, the overall amplitude is proportional to the vesicle number density: I₀ ∝ n. Note that the SANS experiment averages over all directions Ωₚ of the wave-vector \( \mathbf{q} \). Here we have the convenient notation dΩₚ = sin θ dθ dφ, where (θ, φ) are the spherical coordinate angles for the vector \( \mathbf{q} \) in the integration.

The ensemble average in \( \langle |F(q)|^2 \rangle \) includes an average over a thermal distribution of ULVs, that is, scattering experiments are carried out at fixed temperatures, T. Moreover, the extrusion process used to generate ULVs results in vesicles with a distribution of radii, R. Thus, averaging over all different size ULVs, we can rewrite the averaged scattered intensity as

$$ \langle |F(q)|^2 \rangle = \int_0^\infty P(R) \langle |F(q)|^2 \rangle dR $$

where P(R) is a probability distribution of ULVs with radius R, and \( F(q) \) is the form factor for scattering from vesicles of given R. Previously, experiments using extruded ULVs have shown that the Schultz distribution is a physically reasonable descriptor of ULV polydispersity, that is,

$$ P(R) = \frac{R^{z+1}}{\Gamma(z+1)} \exp \left[ -\frac{R}{\bar{R}} \right] $$

where \( \bar{R} \) is the average ULV radius and z parametrizes the distribution variance: \( \sigma_R^2 = \bar{R}^2/(z + 1) \). At this point, all that remains is to develop a physically justified scattering model for the form factor, \( F(q) \). Moreover, we will also distinguish between our high- and low-q SANS data.

As an initial test of our scattering model with high-q SANS data, we considered vesicles with \( \bar{R} = 50 \) nm radii (on average) composed of ternary lipid mixtures of DSPC-DOPC-POPC, along with cholesterol (the same mixture used for the optical experiments), placed in 100% D₂O solution. We then used a simple spherical shell model with a Gaussian SLD contrast along the radial direction to estimate the lipid bilayer size of the vesicles. (For this composition, we did not have any data, we considered vesicles with"

$$ \frac{d\Sigma}{d\Omega}(q) = n|F(q)|^2 \left[ 1 + \frac{|F(q)|^2}{|F(q)|^2} (S(q) - 1) \right] $$

where the averages \( \langle \cdot \rangle \) are ensemble averages over many vesicles, which can vary in size (i.e., polydisperse). The form factor is given by

$$ F(q) = -\int d\mathbf{r} \rho(\mathbf{r}) \rho(\mathbf{r} + q) e^{-i\mathbf{q}\cdot\mathbf{r}} $$

$$ I(q) = \frac{I_0}{4\pi} \int |F(q)|^2 d\Omega_q + I_{inc} $$

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modeling and contrast-matching approach that resolves lateral heterogeneities.

**Low-q SANS Scattering Model.** We studied \( R = 25 \) nm ULVs with the same composition as the GUVs used in the optical measurements, that is, DSPC (39 mol %), POPC (33 mol %), DOPC (6 mol %), and Chol (22 mol %). However, in order to make the ULVs “invisible” to neutrons at 45 °C, a fraction of the DSPC lipids were deuterated (DSPC-protiated, 34.6%; DSPC-d40, 65.4%; see Materials and Methods for more details). In other words, at high T, when the lipids are uniformly mixed (i.e., in the L2 phase), this composition leads to the absence of the coherent scattering signal and \( I(q) = I_{\text{inc}} \). However, at low temperatures (i.e., 25 °C), the lipid phase separates (i.e., domains) and \( \rho(r) \neq \rho_c \neq 1 \), resulting in a coherent scattering signal. We can approximate these spatial variations as follows:

\[
\rho(r) \equiv \psi(\theta, \phi) | \Theta(r - R + t/2) - \Theta(r - R - t/2) | \tag{6}
\]

where \( \Theta(x) = 0 \) for \( x \leq 0 \) and \( \Theta(x) = 1 \) for \( x > 0 \) is the Heaviside step function, and \( \psi(\theta, \phi) \) is the lateral SLD variation, and use a “top-hat” function to represent the SLD contrast in the radial direction. This particular choice, however, does not filter the data in any meaningful way, as the low-q features are dominated by \( \psi(\theta, \phi) \). Here, \( t \) represents the lipid bilayer thickness and, as such, it is convenient to expand \( \psi(\theta, \phi) \) using spherical harmonics:

\[
\psi(\theta, \phi) = \sum_{l,m} \psi_{l,m} Y_l^m(\theta, \phi) \tag{7}
\]

where \( Y_l^m(\theta, \phi) \) is the spherical harmonic with index \( l = 0, 1, 2, \ldots \) and azimuthal index \( m = -l, -l+1, \ldots, l \). Note that the spherical harmonics form a complete basis for (square-integrable) functions on the sphere. We can thus represent any configuration \( \psi(\theta, \phi) \) using this spherical harmonic expansion. To calculate the form factor, we expand the Fourier component \( e^{-i\mathbf{q}\cdot\mathbf{r}} \) as follows:

\[
e^{-i \mathbf{q} \cdot \mathbf{r}} = 4\pi \sum_{l=0}^\infty \sum_{m=-l}^l (-i)^l j_l(qr) Y_l^m(\theta_r, \phi_r) Y_l^m(\theta, \phi) \tag{8}
\]

where \( j_l(x) \) is the spherical Bessel function and \( (\theta_r, \phi_r) \) are the spherical coordinate angles for the vector \( \mathbf{q} \). The form factor now reads as

\[
F(q) = 4\pi \sum_{l,m} \left( \int_{R-1/2}^{R+1/2} dr r^2 j_l(qr) \right) \psi_{l,m}^*(i)^l Y_l^m(\theta_r, \phi_r) \psi_{l,m} Y_l^m(\theta, \phi) \tag{9}
\]

and the scattering intensity takes the form of

\[
I = 4\pi l_0 \sum_{l,m} \left\{ \int_0^{2\pi} P(R)|\Xi_l^m| \, dR \right\} \langle |\psi_m|^2 \rangle + I_{\text{inc}} \tag{10}
\]

where \( l_0 \propto n \) is the overall vesicle concentration-dependent amplitude. We also obtain the radial integration result in the closed form:

\[
\Xi_l^m(q) \equiv \int_{R-1/2}^{R+1/2} dr r^2 j_l(qr) \tag{11}
\]

\[
= \frac{\sqrt{\pi} q}{2^{l+1} (l + 1/2)(l + 3/2) 4^{l+5/2}} \left\{ \left( R + \frac{t}{2} \right)^{l+3} I_{2l+1} - \left( R - \frac{t}{2} \right)^{l+3} I_{2l+1} \right\} \tag{12}
\]

where \( \Gamma(x) \) is the gamma function and \( I_{2l+1}(a;b,c) \) is the generalized hypergeometric function.

At this point, it is important to develop an appropriate model for \( \langle |\psi_m|^2 \rangle \). When ULVs are at 45 °C, all domains have melted, \( \rho_{\text{uc}} = \rho_0 \) where \( \rho_0 \) is the SLD of hydrocarbon acyl chains. As the temperature is lowered, the lipid phase separates, but as no new material is added or removed from the lipid membrane, we expect that the SLD integrated over the entire ULV remains the same. Therefore, regardless of temperature, we expect the \( l = 0 \) mode to vanish:

\[
\int d\Omega_4 |\psi(\mathbf{r}) - \rho_0| = \psi_0^2 = 0 \tag{13}
\]

\( l = 0 \) is absent in our expansion, and any lateral phase separation will result in coherent scattering contributions from modes \( l \neq 0 \), for our lipid mixture domains have previously been observed by SANS using similar size ULVs. When domains form, there will be some SLD contrast variation at a length scale \( \lambda_0 \) on the surface of ULVs, which will correspond to a mode \( l_0 \approx 2\pi R/\lambda_0 \). We can thus propose a model to describe lateral heterogeneities through \( \psi(\theta, \phi) \), representing a phase-separating field with a Landau–Brazovskii free energy, which has minima exhibiting spatial modulations at the scale \( \lambda_0 \). This approach describes spatially modulated phases, which are ubiquitous in soft matter systems and are thought to arise when the lipid membrane shape is coupled with a phase-separating lipid composition. In addition, depending on the parameters of the free energy, the system may form a disordered microemulsion, a fully phase-separated phase, or a fully mixed, disordered phase. This mathematical formalism is general and has been successfully used to describe pollen grain pattern formation, lipid domains on spherical vesicles, viral capsid formation, and block copolymer assembly on a sphere. Recent coarse-grained computer simulations of lipid bilayers have also suggested that domain formation can be thought of as a curvature-induced microemulsion. Thus, based on this prior evidence, we thought it appropriate to incorporate the Landau–Brazovskii approach into the framework of our scattering model.

In our scattering model, the correlations of the spherical harmonic modes are given by

\[
\langle |\psi_m|^2 \rangle = \frac{\alpha}{(l^2 - l_0^2) + R^2 / \xi^2} \tag{14}
\]

where \( \alpha \) is the overall amplitude and \( \xi \) is the temperature-dependent correlation length. This form is what one would expect in the disordered region of the modulated phase diagram, that is, a microemulsion phase. On the other hand, in the case of an ordered modulated phase, there would be a large component of the scattering at a single \( l = l_0 \) representing a single domain size. However, as we shall see later, our results
are in better agreement with a range of domain sizes and, possibly, a microemulsion phase. It should also be pointed out that when $\lambda_0$ is comparable to the vesicle radius, our proposed model becomes essentially indistinguishable from an Ising-like model of phase separation, which may also be relevant in describing phase-separated lipid systems. In other words, the membrane could be phase-separated, but as the liquid-ordered and liquid-disordered phases coarsen, eventually they will merge and form a single domain. As we will see, our SANS results are consistent with both scenarios, that is, that of a microemulsion with a large $\lambda_0$ or a simple phase-separated vesicle; more detailed studies are needed to differentiate between them experimentally. Substituting eq 13 into eq 10, we obtain a scattering model that describes the overall scattering density, which we write as follows:

$$I(q) = I_{inc} + A \sum_{l=1}^{\infty} \int_{0}^{\infty} \frac{4\pi(2l+1)P(R)\Xi^{R_{fi}}(q) }{(l^2 - 4\pi^2 R^2/\lambda_0^2) + R^2/\xi^2} dR$$

(14)

where $A$ is the overall scattering amplitude that needs to be fitted and is proportional to the vesicle concentration, $n$. For this model there are seven parameters, namely, $A$, $\lambda_0$, $\xi$, $I_{inc}$, $R$, $z$, and $t$. However, integrating over the radius distribution, $P(R)$, cannot be carried out analytically and needs to be approximated. We do this by using the trapezoidal rule (with 10 segments) and integrating from $\sigma_R$ to $R + 2\sigma_R$, where $\sigma_R = R/\sqrt{2} + 1$. That is, for any function $f(R)$, and for the current case, the function multiplying $P(R)$ in the integration gives rise to

$$\int_{0}^{\infty} P(R)f(R) dR \approx \frac{R}{2} \sum_{k=0}^{10} \frac{P(R + \Delta R)f(R + \Delta R) + P(R)f(R)}{2} \Delta R$$

(15)

with $\Delta R = 2\sigma_R/10$. We also examined this solution using more segments or by extending the integration interval with similar results.

Some of our fit parameters are known from independent experiments. For example, ULV diameters are known from the pore size of the polycarbonate filters used to extrude them ($R = 25 \text{ nm}$), and the lipid bilayer thickness, $t$, is known to be approximately $4 \text{ nm}$ for the lipid mixtures considered here. We note that at low-$q$, ULV polydispersity can significantly modify the scattering curve, and a good estimate of $R$ and $z$ are important as the fitting procedure is particularly sensitive to the values of these parameters, unlike the case of the high-$q$ data, where the scattering curve is much less affected by $z$ and $R$. Knowing this, we allowed $z$ and $R$ to vary in the fits and then used the fixed estimates of $z = 15$ and $R = 25 \text{ nm}$ to fit all the data, since the extrusion process was the same for all samples, that is, $z$ and $R$ should be the same for all samples at low-$q$. Finally, $I_{inc}$ can be estimated from the high temperature scattering data, where the SLDs of the solvent and ULVs are matched, resulting only in incoherent scattering. From the high temperature data, we estimate $I_{inc} \approx 0.14$. For the final fits of the low-$q$ scattering data (Figure 2), we only used three parameters, that is, $A$, $\lambda_0$, and $\xi$. However, we found that the fit was not sensitive to $\lambda_0$ as long as $\lambda_0$ was sufficiently large and will be discussed in more detail below. As a result, we fixed $\lambda_0$ to a large value and fitted $A$ and $\xi$. Since it is not trivial to analytically sum over all $I = 1, ..., \infty$, we defined a cutoff value of $I_{max} = 14$ and tested increasing cutoff values to detect any appreciably changes to the results, which we did not. We tabulate the fitted results from our two lowest temperature scattering data of ULV samples with lipid domains in Table 1. For the highest two temperature intensities we used: $I(q) \approx I_{inc} \approx 0.14$.

![Figure 2](image.png)

**Figure 2.** SANS intensities of 50 nm diameter ULVs for $\phi_{int} = 0 \text{ mol } \%$. $T = 45$ and $54 \text{ °C}$. Scattering curves are, for the most part, featureless, consistent with pure incoherent scattering. $I_{inc} = 0.14$ was used to fit the two lower temperature data. For those data, we estimated polydispersity parameters $z = 15$ and $R = 250 \text{ Å}$, which we estimated from the pore size of the filters used to extrude the ULVs and consistent with previously published data. Finally, we performed nonlinear least-squares fits to the data by varying the parameters $A$ and $\xi$. Our fits were not sensitive to $\lambda_0$ as long as $\lambda_0$ was sufficiently large. We therefore fixed $\lambda_0 = 200 \text{ nm}$, and the best fit values are given in Table 1.

<table>
<thead>
<tr>
<th>$T$ (°C)</th>
<th>$A$ (arb. units)</th>
<th>$\lambda_0$ (Å, f.)</th>
<th>$\xi$ (Å)</th>
<th>$I_{inc}$ (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>$(2.4 \pm 0.2) \times 10^{-11}$</td>
<td>$10^4$</td>
<td>$15.1 \pm 0.9$</td>
<td>$0.14$</td>
</tr>
<tr>
<td>37</td>
<td>$(10 \pm 3) \times 10^{-12}$</td>
<td>$10^4$</td>
<td>$11 \pm 2$</td>
<td>$0.14$</td>
</tr>
</tbody>
</table>

*Certain parameters were fixed ($Å$) based on independent measurements or other physical considerations as discussed in the main text. The following parameters we fixed as follows: $z = 15$, $R = 250 \text{ Å}$, and $t = 40 \text{ Å}$.

Our results in Figure 2 indicate that our vesicles phase separate at low temperatures and that the domain sizes of the phase-separated vesicles are comparable to the vesicle radius, that is, $I(q)$ is peaked at $q$ values consistent with domain sizes on the order of $10 \text{ nm}$. In particular, we found that $\lambda_0 \gg \xi$, $R_0$ (see Table 1). The fitting procedure was largely insensitive to the value of $\lambda_0$ provided that it was large compared to $\xi$. We therefore fixed the value of this parameter to $1 \mu\text{m}$, as shown in Table 1. For these parameters it is difficult to distinguish between different bilayer phases (microemulsion, modulated, or ordered, phase-separated). It is also possible that the bilayers are somewhere in the vicinity of the Lifshitz point, where these different phases are in close proximity on a phase diagram. However, because of the size of the ULVs used, we may not be able to detect detect, for example, a large characteristic domain size. Nevertheless, our free energy approach captures the features of the scattering profile and expect it to be a valuable tool for better understanding phase separation in lipid vesicles. As expected, the correlation length $\xi$ decreases as we increase $T$ and move away from the phase transition temperature. This decrease in $\xi$ corresponds to a
widening of the scattering spectrum (Figure 4), consistent with a more disordered system. The inset to Figure 4 shows examples of microemulsions that are consistent with the measured scattering profiles.

**Low-q Behavior in the Presence of Melatonin.** In the presence of melatonin, the lipid mixture SLD contrast is no longer matched as melatonin causes $\rho_{\text{mel}} \neq \rho_{\text{fl}}$. Melatonin molecules are small compared to the lipids and localize below the lipid headgroup, creating SLD contrast. To model this, we assumed that the melatonin localizes equally in both membrane leaflets of ULVs, $R \pm \delta_{\text{mel}}$ at a distance $\delta_{\text{mel}}$ from the bilayer center. Melatonin thus generates a Gaussian-shaped SLD contrast with a width $\sigma_{\text{mel}}$. The scattering model then contains a homogeneous contribution from melatonin and an inhomogeneous contribution from the lipid domains and, possibly, of melatonin preferentially partitioning in the domains:

\[
I = 4\pi A \int_0^{q_{\text{max}}} \left\{ \frac{2\rho_{\text{mel}}^2 e^{-q^2\delta_{\text{mel}}^2}}{q^2} \cos^2(\xi \delta_{\text{mel}}) \right. \\
\left. \times \left( (q\delta_{\text{mel}} + \delta_{\text{mel}} \tan(q\delta_{\text{mel}})/\cos(q\delta_{\text{mel}}) + R \sin(q\delta_{\text{mel}}))^2 \\
+ \sum_{l=1}^{\infty} \frac{(2l + 1)\pi^l}{(U^2 - 4\pi^2 R^2/l^2)^2 + R^2/\xi^2} \right) \right\} 
\]

where $\rho_{\text{fl}}$ is the SLD contrast between melatonin and the solvent and which depends on melatonin concentration. Importantly, this scattering model introduces three additional melatonin-related parameters: $\rho_{\text{mel}}$, $\delta_{\text{mel}}$, and $\sigma_{\text{mel}}$. As in the case of the low $T$ results with no melatonin, we find that $\lambda_0 \gg \xi R$ for these scattering curves, implying that it is not possible to resolve a microemulsion phase or a completely phase-separating mixture from our data. Because the fitting procedure is not particularly sensitive to the choice of $\lambda_0$ (as long as it is large compared to $\xi$ and $R$), we set $\lambda_0 = 10^4 \text{ Å}$ to fit the data. $I_{\text{fl}}$ was estimated independently by examining the large $q$ scattering behavior, where the coherent scattering signal from the membrane is suppressed. Furthermore, we assumed that melatonin resides somewhere below the lipid head groups \(^{56,57}\) and set $\delta_{\text{mel}} = 15 \text{ Å}$. We may also constrain $\sigma_{\text{mel}}$ because we know that melatonin is small compared to the lipid molecules. We set $\sigma_{\text{mel}} = 5 \text{ Å}$ in our fits, but verified that the fits did not change substantially when this number was varied by a few angstroms. We were thus left with three fitting parameters: $A$, $\xi$, and $\rho_{\text{fl}}$.

As shown in Figure 3, our fitting results are consistent with lateral lipid phase separation at low temperatures in melatonin-containing ULVs. This is evidenced by the scattering excess (a “shoulder” in the scattering curve) for $q$ values between approximately 0.01 and 0.03 Å\(^{-1}\). This signal persists even at high temperatures, although it becomes less pronounced due to the signal from melatonin, the sample is not perfectly contrast matched. Our fits to the data (dashed lines in Figure 3) are also consistent with the presence of lipid domains: we find a non-negligible contribution to the fit from lateral inhomogeneities. However, a more definite observation of lateral heterogeneity will require better contrast matching between melatonin and the lipids.

Despite the less than ideal contrast matching conditions in samples with melatonin, our data is consistent with the presence of lipids domains, even at high $T$. To further verify this claim, we plotted the fitted intensity curves with $\rho_{\text{fl}} = 0$ so that only the scattering signal originating from the lipids is shown (dotted lines in Figure 3). We see from the dotted lines in Figure 3 that, for all temperatures, the scattered intensity curves have some contribution from lateral phase separation and are, at a minimum, consistent with a phase-separated lipid bilayer. The various membrane parameters for the $\phi_{\text{mel}} = 10$ mol % case are presented in Table 2. Note that the fitting parameters have large estimated errors, indicative of the difficulty in disentangling the melatonin and lateral heterogeneity contributions.

![Figure 3](image)

**Figure 3.** SANS intensities of 50 nm diameter ULVs for $\phi_{\text{mel}} = 10$ mol %. The curves are offset vertically for better visualization. The shoulder in the scattering data at around $q \approx 0.01$ Å\(^{-1}\) is consistent with lipid-phase separation. However, this signal is overwhelmed at low-$q$ due to increased scattering from melatonin. As in previous studies,\(^7\) we estimated a polydispersity parameter $z = 15$ and $R = 250$ Å. Due to the additional melatonin-related scattering parameters needed to describe the data, we fixed $\lambda_0 = 10^4 \text{ Å}$, as was determined from experiments with no melatonin. However, because of its magnitude, this parameter does not much influence the fitting results. We then performed a nonlinear least-squares fit by varying $A$, $\xi$, and $\rho_{\text{fl}}$. The best-fit values are listed in Table 2. Importantly, the dotted line, due to lateral lipid-phase separation, has a non-negligible contribution for $q$ and differs from what one would expect from pure incoherent scattering. Our results are therefore consistent with the presence of domains at all temperatures studied. This claim would, however, be even more definitive if we had better control over the contrast between melatonin and the aqueous solution.
meltin in lipid bilayers noticeably affects melting of lateral heterogeneities at intermediate and elevated temperatures.

scans of 0 mol % melatonin are consistent with previous studies. Note that lipid mixtures melt over a wide range of temperatures, in contrast to single component systems. The melting in multicomponent lipid membranes takes place over consecutive stages, that is, premelting, main transition melting, and postmelting. Importantly, both premelting and postmelting stages can be characterized by fast and slow kinetics, respectively. The thermotropic phase behavior of both hydrated MLV lipid dispersions (0 mol % and 20 mol %) exhibit slow kinetics toward their main melting transition centered around \( T_m = 40 \, ^\circ C \), see Figure 5. Both thermograms in Figure 5 are noticeably broad, and their effective melting \((T_{\text{completion}} = T_m + \frac{F \text{WHM}}{2})\), \( F \text{WHM} \) is the melting transition width, \( F \) is the full width at half-maximum, completes at \( T_m \approx 45 \, ^\circ C \) (0 mol %) and \( T_m \approx 48 \, ^\circ C \) (20 mol %), respectively. This implies that the 0 mol % melatonin–lipid mixture at 45 °C is in the liquid disordered state, while the 20 mol % melatonin–lipid mixture is not completely melted (i.e., premelted) due to the presence of phase-separated lateral heterogeneities. In this regard, the DSC data is consistent with our fluorescence microscopy observations (see Figure 1). Also, the 0 mol % melatonin–lipid mixture exhibits a steep (fast kinetics) DSC slope toward 45 °C (see Figure 5), and it is also consistent with a zero signal in SANS data, implying that the 0 mol % melatonin–lipid system is well mixed and domains are completely melted, see Figure 2. The DSC profile of the 20 mol % melatonin–lipid mixture undergoes a thermodynamic crossover at around 48 °C, followed by “shoulder-like” slow kinetics toward \( T_m \approx 60 \, ^\circ C \). This implies that the lipids with melatonin are still in the phase-separated state, even at \( T_m = 54 \, ^\circ C \) (see Figure 5), consistent with our ULV SANS measurements (see Figure 3).

## CONCLUSIONS

In this study we presented experimental data revealing melatonin’s ability to stabilize liquid–solid phase separation in a biomimetic model system of lipid membranes composed of saturated DSPC, unsaturated POPC and DOPC, and cholesterol. We used confocal fluorescence microscopy to image micrometer GUVs and SANS to explore nanometer ULVs with the same lipid composition. We developed a scattering model to explain the lipid phase behavior, where lateral lipid heterogeneities were treated using the Landau–Brazovskii approach.

The molecular basis of melatonin’s domain stabilizing ability relies on the competitive forces between melatonin–lipid and lipid–lipid interactions. In phospholipids, van der Waals forces are a substantial component of the intermolecular interactions, and weaken near physiological temperatures. As a result, the mobility of the lipids changes and gives rise to lipid behavior seen with increasing temperature. However, melatonin–lipid interactions are noticeably stronger than the lipid–lipid interactions. Specifically, melatonin’s strong interactions with lipid headgroups are most likely responsible for melatonin’s ability to stabilize lipid domains over a wide range of temperatures, as observed in this work using optical microscopy and SANS. In this regard, the thermodynamically stabilized lipid phase separation across different length scales is
of particular interest, since phase-separated lipid domains are implicated in a myriad of physiological functions.

Finally, our newly developed scattering model may serve as a platform in deciphering nanoscopic lateral heterogeneities in soft materials with spherical and planar geometries in the presence of proteins, hormones, peptides, pathogens, and other biomolecules. Moreover, the use of the Landau–Brazovskii approach enables our scattering model to identify spatially modulated phases at the molecular level utilizing X-ray or neutron scattering techniques.

**REFERENCES**

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