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TITLE:

Dynamic Light Scattering Applied to Probe-Free Characterization of Drug Induced Biophysical Changes in Lipid Membrane

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KEYWORDS:

Drug-membrane interaction; lipid phase transition; biophysical characterization; lipid bilayers; dynamic light scattering; lipid thin film hydration method

SUMMARY:

A simple yet reliable light scattering based protocol is described to monitor the phospholipid phase transitions. The simplicity of such a protocol can provide a new solution for routine evaluation of the interaction between chemical entities with phospholipid bilayers.

ABSTRACT:

Biological membranes are spherical boundaries formed by self-assembled lipid bilayers that enclose an internal aqueous core and are surrounded by an external aqueous environment. Biological membranes have strict biophysical properties that are essential for their function, such as defined thickness, structure, microviscosity, order, lateral pressure, surface charge and permeability. Considering the similarity between biological membranes and liposomes in their ability to mimic biophysical properties of the membrane, it is easy to understand why liposomes are simple but relevant cellular membrane models. In fact, liposomes are widely used to study the drug-induced effects on membrane biophysics, which can often be related to the drug's capacity to cross membranes and distribute to tissues or may be associated with membrane toxicity. In practical terms, the effects of drugs on membrane biophysics can be assessed by changes in the thermotropic behavior of lipid membranes which can be studied by monitoring parameters such as the main phase transition temperature (T_m) and the cooperativity (B) of the phase transition.

Herein we propose a probe-free protocol that uses dynamic light scattering (DLS) as the detection technique to determine T_m and B, taking advantage of the light scattering changes observed when the lipid components of a membrane transit from an ordered gel-phase (L_β) to a less ordered fluid phase (L_α). This protocol consists of a detailed explanation of a temperature trend standard operating procedure (SOP) based on the Dynamic Light Scattering technique as well as

an analysis of the data collected from the thermotropic study of liposomes in the presence and absence of drugs. For its simplicity, standardization and probe–free nature, this protocol has the potential to be used with a variety of drugs and other potential therapeutic agents to screen their effects on membrane biophysics and, more broadly, to relate these effects to drug biodistribution and membrane toxicity.

INTRODUCTION:

Liposomes are hollow spherical assemblies formed by single or concentric bilayers (unilamellar or multilamellar) enclosing an aqueous medium. Due to its spherical form and phospholipid composition, liposomes have been described as potential mimetic models of biological cell membranes, in which phospholipids (e.g., phosphatidylcholine) are the most abundant membrane components. Phospholipids are a family of amphiphiles consisting of a polar hydrophilic headgroup, a hydrophobic hydrocarbon tail (acyl chain) and an ester linkage attaching the head and tail groups. Considering liposomes as biomimetic models of cell membranes, their interesting thermodynamical properties are being widely studied towards a deeper understanding of the thermotropic behavior of cell membranes in conditions that are not compatible with live cells. Additionally, considering that these lipid bilayers are markedly influenced by their surroundings, the presence of exogenous molecules, such as therapeutic agents, can produce dramatic changes in the bilayer dynamics. Therefore, liposomes can be used to study the biophysical properties of membranes but can also be used to evaluate the effect of a chemical entity on these biophysical properties. Actually, changes in thermotropic phospholipid phase transitions in biological media may be correlated to impairment in biophysical properties of membranes with consequences in stability, fluidity or permeability of cell membranes[1].

Phospholipid bilayers can mainly assume three lamellar temperature-dependent phases (a crystalline phase (L_C), a solid-crystalline or gel phase ($L_{\beta'}$), and a liquid-crystalline or fluid phase (L_α)) and its existence or co-existence is regulated by the temperature. The temperatures at which the lipid phase transition occurs are called the sub-transition temperature, occurring from L_C to $L_{\beta'}$ (T_S) and the main transition temperature (T_m), occurring from $L_{\beta'}$ phase to L_α. It is also possible for an intermediated lipid phase – the ripple gel phase ($P_{\beta'}$) between $L_{\beta'}$ to L_α, and the temperature at which the $L_{\beta'} \rightarrow P_{\beta'}$ occurs is called the pretransition temperature (T_P). In the crystalline sub-gel phase (L_C), the hydrocarbon chains are in a completely stretched all-*trans* conformation and the polar headgroups are relatively static. After the sub-transition, at the gel phase ($L_{\beta'}$) the acyl chains remain preferentially in an all-*trans* conformation. Because the lateral chain-chain packing favors van der Waals attractive interactions in this lipid phase, the *all-trans* acyl chains are tightly packed together. The close packing loosens as the temperature reaches the T_m due to thermally mediated *trans-gauche* isomerization of C – C along the acyl chains. This isomerization results in an increased lipid molar volume, decreased bilayer width, and increased lateral chain-chain interaction gap within the bilayer, as well as increased intra- and intermolecular motion rates and amplitudes. The loose packing between the side chains also allows their average cross-sectional sum to almost perfectly match that of the headgroup, resulting in non-tilted acyl chains, nearly perpendicular to the surface of the bilayer. Regarding the characteristics of each lipid phase, it is easy to realize that the biophysical properties of cell membranes (stability, fluidity and permeability) are intimately connected to their phase transitions. Studies on the interactions of

various drugs with biomimetic models of cell membranes are extensively described in the literature, and it has been shown that the way drugs influence the conformational properties of phospholipids and/or their molecular order causes changes in the thermodynamic properties of the biomimetic system[2-7]. Consequently, the thermodynamic parameters of lipid membranes are being used as a tool in assessing drug biodistribution as part of the drug pharmacokinetic profiling[8].

The thermotropic behavior of lipid membranes can be studied by monitoring parameters such as T_m and the cooperativity (B) of the phase transition, which together allow prediction of the location of chemical entities interacting with the membrane. Actually, across the lipid bilayer of a membrane exists a fluidity gradient both above and below the transition temperature, in which the part of acyl chains near the center of bilayers (between C10 and C16) is more disordered than the part of the acyl chain closer to the headgroup region (C1 to C9)[9]. Regarding these assumptions, the cooperativity of the transition is largely regulated by the interaction in the region near C1 and C9, whereby changes in cooperativity induced by an external molecule can be taken as an indication of its location at a bilayer level. On the other hand, considering the increased disorder existent at bilayer core (C12 to C16), if an external molecule is placed at this level it would have subtle or no effect on the cooperativity of the transition[9].

Although the most widely used method to evaluate the lipid phase transition is Differential Scanning Calorimetry (DSC)[10-13], other methods for precisely determining the phase transition temperatures of lipids have been identified and include Nuclear Magnetic Resonance (NMR)[14,15], Electron Paramagnetic Resonance (EPR)[16,17], Fourier Transform Infrared spectroscopy (FT-IR)[18,19], fluorescence polarization or anisotropy studies[6], and Small and Wide-Angle X-ray Scattering (SAXS/WAXS)[20]. DSC measurements take into account the changes of enthalpy associated with the changes of the heat capacity of phospholipids during the phase transition from the gel to the fluid phase[8]. The determination of lipid phase transitions by FT-IR is focused on the temperature-dependent changes in the fundamental vibrations of CH_2 groups of hydrocarbon chains due to the *trans-gauche* C – C bond rotational isomerism that occurs at the hydrocarbon chain-melting phase transition[14,21,22]. However, some difficulties can be found in this analysis, since absorption bands from these vibrationally active groups are often very broad and there is the possibility of being in the middle, overlapped or obscured by adjacent or overlapping bands arising from the solvent. In addition, when lipid bilayers are in the hydrated form, the infrared activities of groups located on the polar region of headgroups will be dominated by interaction with the solvent, including when the lipids are forming quasi-crystalline lipid phases[14]. On the other hand, experiments of NMR studying the relaxation times of the protons of ammonium methyl groups ($N^+(CH_3)_3$) can be conducted to study lipid membrane dynamics, since NMR relaxation times associated with spin-lattice and spin-spin are both sensitive to the mobility of the NMR active species. Thus, as a molecule undergoes random thermal motion, fluctuating magnetic fields arise from interactions with the lattice. From the NMR spectra it is possible to obtain the lipid phase transitions by monitoring the variation of either the amplitude or the area of peaks originated by such protons[14]. Other methods require the incorporation of paramagnetic spin or fluorescence probes (EPR and fluorescence anisotropy, respectively). Both methods allow the phase transition temperatures determination by monitoring the rotational motion of extrinsic probes. The main

drawback of both methods relies precisely on the requirements of extrinsic molecules, that although are interesting to study the biophysical properties of cell membranes, become a hindrance on the evaluation of drug-induced effects, since the probe constitutes a secondary external element capable of disturbing the biomimetic system. In SAXS/WAXS studies, it is possible to distinguish the different lipid phases on fully hydrated lipids by monitoring the temperature-dependent changes in molecular order and packing [20]. Although all these methods allow observation of changes that phospholipids undergo with temperature with good agreement in the obtained results, the highly specific and complex instrumentation and skills required make them far from the ideal solution for routine study of phase transitions as a critical step toward elucidating drug-membrane interactions[1]. With demand for a simple method to determine the phase transitions of phospholipids as well as the effect provoked by a chemical entity aiming for a molecular understanding of its biodistribution, a method based on the application of the dynamic light scattering (DLS) will be described in this protocol. This protocol is based on the liposomal optical properties' dependence on the temperature, in which at critical temperatures, such as those temperatures at which phase transitions occur, the changes in conformational structure, lateral diffusion and expansibility as well as bilayer thickness, lead to modification on the light that is scattered. Basically, at lower temperatures ($T<T_m$), the rigid structure of liposomes causes a higher scattering of the incident beam light and with the temperature increasing ($T>T_m$) as the lipid membranes are transitioning to a less organized and more fluid conformation, the ability to promote the scattering of the incident beam light is decreased[1,3,4]. Therefore, by monitoring the intensity of the light scattered as a function of the temperature, it is possible to analyze the phases' transitions. The average number of photons detected per second are reflected by the mean count rate (MCR) measured by DLS. This value is a representation of an emerging macroscopic phenomenon and not directly size dependent and reflects the changes in optical properties of the different phase transition state of the liposomes. The validity and reproducibility of this method was first proved by Michel et al.[1] and in this protocol, this method will be validated as a routine step in the pharmacokinetic profiling of chemical entities, such drugs. Thus, representative results of one very-well characterized lipid – dimyristoylphosphatidylcholine (DMPC) – was analyzed by the proposed method and compared with literature. Also, the same lipid systems were analyzed in the presence of several model drugs – caffeine (CF), diclofenac (DCF), resveratrol (RSV) and acyclovir (ACV) – and the results were compared to what were previously published in literature.

PROTOCOL:

1. Preparation of the lipid systems

1.1. In a volumetric flask of 25.00 mL, dissolve 338.97 mg of DMPC powder in an organic solvent (e.g., ethanol absolute HPLC grade) to achieve a final concentration of 2.00 x 10^{-2} M.

NOTE: Chloroform can be also used instead of ethanol absolute.

1.2. Transfer an adequate portion of the stock solution obtained in the step 1.1 to a round bottom glass flask. For example, to obtain a LUVs final concentration of 1.00 x 10^{-2} M in 5 mL of

buffer solution, transfer 2.5 mL to the round glass flask.

1.3. Evaporate the organic solvent under a nitrogen flow and constant flask rotation to promote the formation of a thin-lipid film on the bottom of a glass flask.

1.4. Hydrate the dried lipid films with an adequate quantity of aqueous solution (Tris Buffer, 100 mM, pH 7.4) pre-heated at a temperature above the main transition temperature of the lipid (for DMPC T> 23 °C) to achieve a final lipid concentration of 1.00 x 10^{-2} M.

1.5. Perform ten cycles of 1 min of vortex and 1 min of heated bath at a temperature above the main phase transition, to promote the formation of multilamellar vesicles (MLVs).

1.6. Extrude the MLV suspension (Step 1.4) at controlled temperature ($T > T_m$) five times through polycarbonate filters with pore diameters of 400 nm, ten times through polycarbonate filters with pore diameters of 200 nm, and ten times through polycarbonate filters with pore diameters of 100 nm to obtain Large Unilamellar Vesicles (LUVs).

2. Sample preparation

2.1. Prepare a drug stock solution by dissolving the drug in an aqueous solution.

2.2. Prepare the samples by mixing adequate portions of LUVs suspension, Tris buffer solution (100 mM, pH 7.4) and drug solution accordingly to the desired final concentrations.

NOTE: Herein, a final concentration of liposomes of 5.00 x 10^{-4} M was used in the presence and absence of pre-established drug concentrations of 100 µM CF and DCF, 150 µM of ACV, and 200 µM of RSV.

2.3. Incubate the samples for 30 min at 37 °C (a temperature chosen because it is above the main transition temperature of the lipid and to mimic physiological conditions).

2.4. Keep the samples at low temperatures (in the refrigerator at T = 4° C) for least 24 h to promote the phospholipid organization in the crystalline sub-gel phase (if it is intended to monitor the transition temperatures below the T_m).

3. Adjust measurement configurations for the sample

3.1. Transfer 1 mL of the buffered suspension containing liposomes (in the presence or absence of a drug) to a disposable polystyrene cell.

3.2. Place the cell on the sample holder of the DLS equipment.

3.3. Perform a ‘Size’ measurement to allow the equipment to automatically optimize the measurement setting parameters: ‘Cell position’ and ‘Attenuator Value’.

3.4. Take note of the optimal values for such parameter established be the equipment.

4. Setting up the temperature trend standard operating procedure (SOP)

4.1. Configure a New SOP in the DLS equipment software (menu **File → New → SOP**).

4.2. Choose the Size Temperature Trend as 'Measurement Type' (**Trend → Temperature → Size**).

4.3. Fill the 'Sample' configurations ('Material', 'Dispersant' and 'Cell configurations') according to the experiment. Herein, polystyrene latex, water, and a disposable cuvette, were respectively filled.

4.4. Change configurations of the 'Trend' section according with the temperature range chosen. This choice depends on the lipid used and on its main phase transition temperature. For example, in the case of DMPC, to be able to evaluate pre-transition and main transition temperatures, the range of temperatures should vary from 10 °C to 40 °C with temperature increments of 1 °C.

4.5. Define the 'Size measurement' section, 300 seconds of equilibration time for each of the 5 measurements.

4.6. Change the attenuator position of the cell in the **Advanced → Measurement Settings** section, from 'automatic' to 'Fixed position' and introduce the values annotated in step 3.4.

4.7. Start the measurement.

5. Data treatment

5.1. Represent the MCR (Kcps) as a function of the temperature (T in °C) to obtain a sigmoidal profile.

5.2. Apply two independent linear fittings to both plateaus of the sigmoid trend obtained in step 5.1.

5.3. Use the modified Boltzmann regression curve presented below to fit the sigmoidal profile:

$$MCR = b_1 + m_1 \cdot T + \frac{b_2 - b_1 + m_2 \cdot T - m_1 \cdot T}{1 + 10^{B\left(1/T - 1/T_m\right)}}$$

where m_1 and m_2 are the slopes resultant from the two independent linear fittings applied to the plateaus before and after T_m, respectively, and b_1 and b_2 are the corresponding *y*-intercepts.

5.4. Fix m_1, m_2, b_1 and b_2 values to determine T_m and B.

REPRESENTATIVE RESULTS:

The main purpose of this protocol is to prove the reliability of a simple method to evaluate the lipid phase transition of liposomal systems as well as to monitor the biophysical changes provoked by exogenous molecules. Following the protocol, sigmoidal profiles are obtained by plotting the average of photons scattered by the liposomal systems as a function of the temperature. The thermodynamic parameters T_m and B can be calculated from the sigmoidal profile using a non-linear fitting method based on the Boltzmann regression, as described in the protocol (5.3).

Figure 1A presents the full profile of the DMPC transition from 10 °C to 40 °C, with insets for each of the observable transitions in **Figure 1B**, **1C** and **1D**. Moreover, **Table 1** presents the values for each phase transition observed by DLS in comparison with the temperatures observed using other common techniques. From the Boltzmann regression fitting to the data points, it was possible to determine the pre-transition of DMPC from the $L_{\beta'} \rightarrow P_{\beta'}$ phase occurring at 14.66 ± 0.13 °C. This transition was also observed by DSC and fluorescence anisotropy[23]. However, Pentak *et al.* were unsuccessful to observe this transition by DSC, FTIR, NMR and EPR[14]. From **Figure 1C** it was possible to determine T_m and B of the transition from the $P_{\beta'} \rightarrow L_{\alpha}$ phase as 24.03 ± 0.05 °C and 591.3, respectively. The value of T_m agreed with the value described in the literature. Interestingly, a less marked transition was also found at 33.10 ± 0.63 °C. This transition was firstly observed by Pentak *et al.*[14] by DSC and FTIR; however, the authors were not able to observe this latter transition by the other tested techniques (EPR and NMR). This transition at higher temperatures may be due to the spontaneous formation of some MLVs from extruded LUVs[24] during the storage time, as the electrical neutral charge of DMPC liposomes makes them more prone to aggregation.

In order to test the applicability of this protocol to unveil possible biophysical perturbations produced by interaction of cell membrane biomimetic models with chemical entities with biological interest, the thermotropic study of the DMPC system was also performed in the presence of CF, ACV, DCF and RSV. Thus, **Figure 2** shows the sigmoidal profiles of the DMPC phase transition from $L_{\beta'} \rightarrow L_{\alpha}$ in the absence (black dots and lines) and in the presence of each of these compounds (grey dots and lines). From the sigmoidal profiles obtained, it was possible to determine the T_m and B by a modified Boltzmann regression fitting curve and the results are presented in **Table 2**. With these studies, it is intended to show that the technique presented in this protocol can be applied to a varied range of chemical structures.

CF is a small amphiphilic molecule that has been widely investigated due to its potential antioxidant activity[25] and in previous studies with lipid membranes it was found that it has the ability to incorporate in the lipid bilayers at the headgroup-acyl chain interface, promoting a decrease in the membrane fluidity through formation of local water pockets at this interface[25,26]. In the present study, CF does not significantly change the T_m (24.12 °C); however, the addition of this compound to the lipid system increased the cooperativity of the transition (870.4) as can be

seen in **Figure 2A**, which is in line with the studies presented in the literature[25,26]. Since the cooperativity reflects the lipid organization, increased values of cooperativity are correlated with a faster lipid transition between phases, which in turn suggests an increased lipid organization on the bilayer with decreased fluidity[27]. This decreased fluidity can be correlated with the preferred location of the CF on the bilayers (on the interface of headgroups region and the acyl chains) as previously documented[26].

ACV is an antiviral drug used for the treatment of *herpes simplex*[28] and little is known about its interaction with biomimetic models of cell membranes. Herein, it is possible to observe that its addition to the DMPC system causes no significant changes in T_m but provokes a significant increase in the cooperativity of the system. Once again, as the cooperativity is intimately connected with the mean size of the ordered or fluid regions existing at different stages of the transition, this means that a high cooperative process is characterized as a transition where the fluid regions increase rapidly simultaneously with a fast decrease of ordered regions[29]. Thus, it is possible to conclude that the addition of ACV to the lipid system decreases the phospholipid mobility. Even for temperatures above the T_m, it is possible to see that the normalized average number of photons scattered by the liposomal system increases in the presence of ACV, which corroborates the lower lipid fluidity and higher lipid organization promoted by the presence of ACV that acts as a stiffening agent at the bilayer.

Figure 3C presents the sigmoidal profile resultant from the addition of DCF to the DMPC system as a comparison with the pure DMPC system. DCF belongs to the class of non-steroidal anti-inflammatory drugs (NSAIDs) and it is one of the most widely used NSAID worldwide[3]. Several studies have been performed regarding the molecular understanding of the interaction of such drug with PC bilayers[3,30,31]. It is well documented that the anionic form of DCF at pH 7.4 preferentially locates in a shallower region of the bilayer[3,30]. This is in accordance with the significant decrease observed in the T_m value (20.66 °C) of the DMPC upon DCF addition. In general, a decrease in T_m is associated with alterations in the lipid headgroups hydration and thus with headgroup-headgroup interactions, which leads to changes in the packing density at this region. In fact, these changes in packing density can be intimately related to the decrease in cooperativity of the transition observed upon addition of DCF to the DMPC system (**Table 2**). Thus, from the reduction of T_m and B value it is possible to conclude that DCF has a significant membrane disordering effect.

RSV is a polyphenolic molecule present in grapes and red wine that has been studied due to its therapeutical potential in several diseases[32]. Regarding its therapeutic potential, numerous studies have been published documenting its interaction with lipid bilayers[32-34]. From the **Figure 2D** it is possible to understand that RSV largely affected the phase transition profile of DMPC. The decrease in T_m and B is in accordance with the results documented in the literature[32-34]. From the sigmoidal profile, it is possible to highlight the fluidizing effect of RSV since a phase transition occurs at lower temperatures than the pure DMPC. However, an interesting fact is that although RSV have this fluidizing effect, which leads to a phase transition at lower temperatures, for temperatures above the T_m, a stiffening effect is clear, since the number of photons scattered increased. This ambivalent behavior of RSV is in agreement with the literature[32-34]. Additionally,

the values obtained suggest an interaction of RSV with the polar headgroups of the bilayers and regarding the high decrease in cooperativity it is possible to predict a preferential location of RSV at the interface region between the polar headgroups and the C1 – C9 region[9].

In the figures shown, the phase transitions were clearly shown as the measurements were expressed as MCR as a function of temperature. It is important to highlight that although size changes also occur during phase transitions, it is not advised to use size alterations to infer about phase transitions, as the differences can be negligible. **Figure 3** shows a comparison of the transition temperature profiles of DMPC liposomes obtained by measuring MCR or size as a function of temperature. There is a clear variation in liposomal size with increasing temperature, but this variation is not as clear as the variation in average count rate of light scattered intensity. Because all the conclusions drawn were consistent with those reported in the literature and obtained using other techniques such as DSC, EPR, SAXS, WAXS, and NMR, the results presented are indicative of the reliability of the DLS-based protocol as a probe-free characterization method of drug-induced biophysical changes in lipid membranes.

FIGURE AND TABLE LEGENDS:

Figure 1 – Evolution of the normalized mean count rate as function of temperature for DMPC liposomes displaying the whole curve obtained by DLS (A) and insets of three phase transitions: B) the pre-transition (T_p 14.66 ± 0.13 °C), C) the main phase transition (T_m 24.03 ± 0.05 °C) and D) a latter transition at 33.10 ± 0.63 °C. The whole curve experiment had the duration of 9 hours. All experiments were performed at pH 7.4 (Tris Buffer, 100 mM).

Figure 2 – Sigmoidal profiles of normalized mean count rate as function of temperature for DMPC liposomes in the presence of 100 µM of Caffeine (A), 150 µM Acyclovir (B), 100 µM Diclofenac (C) and 200 µM Resveratrol (D), including the non-linear fitting by Boltzmann regression. All experiments were performed at pH 7.4 (Tris Buffer, 100 mM) and each experiment had the duration of 90 minutes.

Figure 3 – Profiles of mean count rate (Kcps) (red) and of liposomal size (blue) both quantified as function of temperature for DMPC liposomes. All experiments were performed at pH 7.4 (Tris Buffer, 100 mM).

Table 1 – Comparison of the values obtained by DLS for the different phase transition temperatures with the values described in the literature using the main techniques for this determination [14,23].

Table 2 – Thermodynamic parameters (T_m and B) calculated from the Boltzmann regression fitting applied to the data obtained for DMPC in the absence and in the presence of caffeine, acyclovir, diclofenac and resveratrol.

DISCUSSION:

This DLS-based protocol has proven to be a simple, probe-free alternative method for evaluating drug-induced biophysical changes in lipid membranes, specifically how thermodynamic

parameters like T_m and B are altered. When the purpose is to evaluate the lipid phase transition of liposomal systems *per se*, it is advisable to test several different concentrations, although the method has been proven to be independent of the liposomal concentration. However, when the main objective of the study is to evaluate the effect of chemical exogenous molecules on the bilayer dynamics, the pure liposomal system serves as the control, and the results must be a comparison between the absence and the presence of the external molecules. Furthermore, the comparison should be always done after a normalization of the data obtained. Different scenarios can be obtained; however, it is worth highlighting that changes in T_m should be always evaluated considering the changes in B. Additionally, although this protocol is very useful in routine evaluation of lipid phase transitions, it is important to be complemented with other techniques, such as drug location at bilayer level, order and packing evaluation, and others, to support the discussion of results. Although herein not presented, the influence of the external molecules on pre-transition temperature can be also monitored. Another important point is that although the main phase transition results in size changes, it is not advisable to use size alterations to infer about phase transitions, as the differences can be negligible.

In comparison to other techniques used to determine similar biophysical techniques, it is important to note that, while this protocol based on DLS analysis may take longer periods of time, there is no operator intervention required during the measurement of MCR with the temperature range, as this can be done automatically. Furthermore, the data treatment is expedited. Finally, the reliance and applicability to analysis of bioactive compounds/membrane interactions was demonstrated.

In conclusion, because this protocol is simple, reliable, and requires minimal operator intervention, it has the potential to be used as a screening procedure in routine assays to evaluate drug-membrane interactions for *in vitro* drug distribution evaluation or, in the case of the development of liposome-based nanocarriers, to evaluate the effect of drugs loaded in the lipid carrier.

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DISCLOSURES:

The author reports no conflicts of interest in this work.

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Figure 1

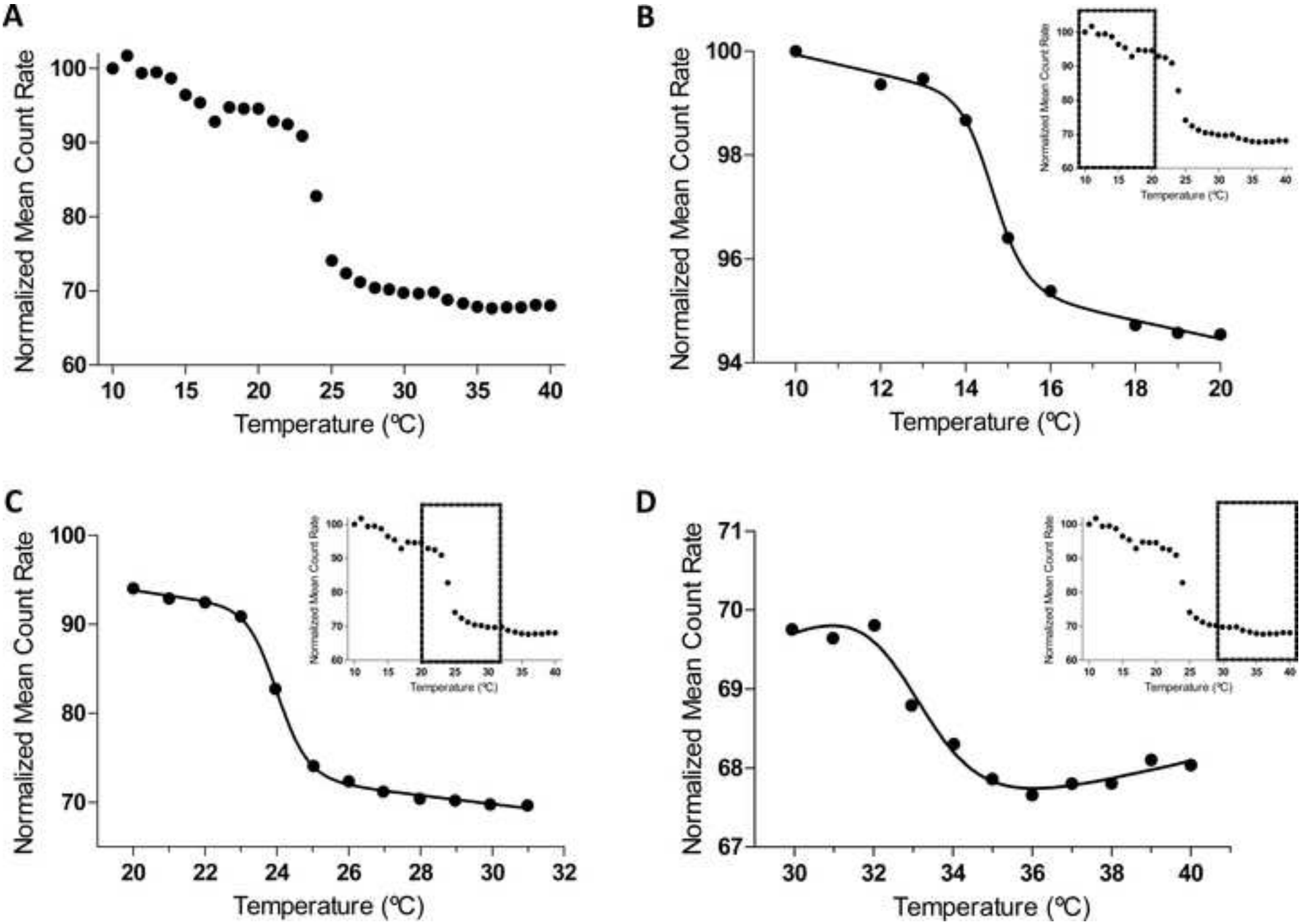

Figure 2

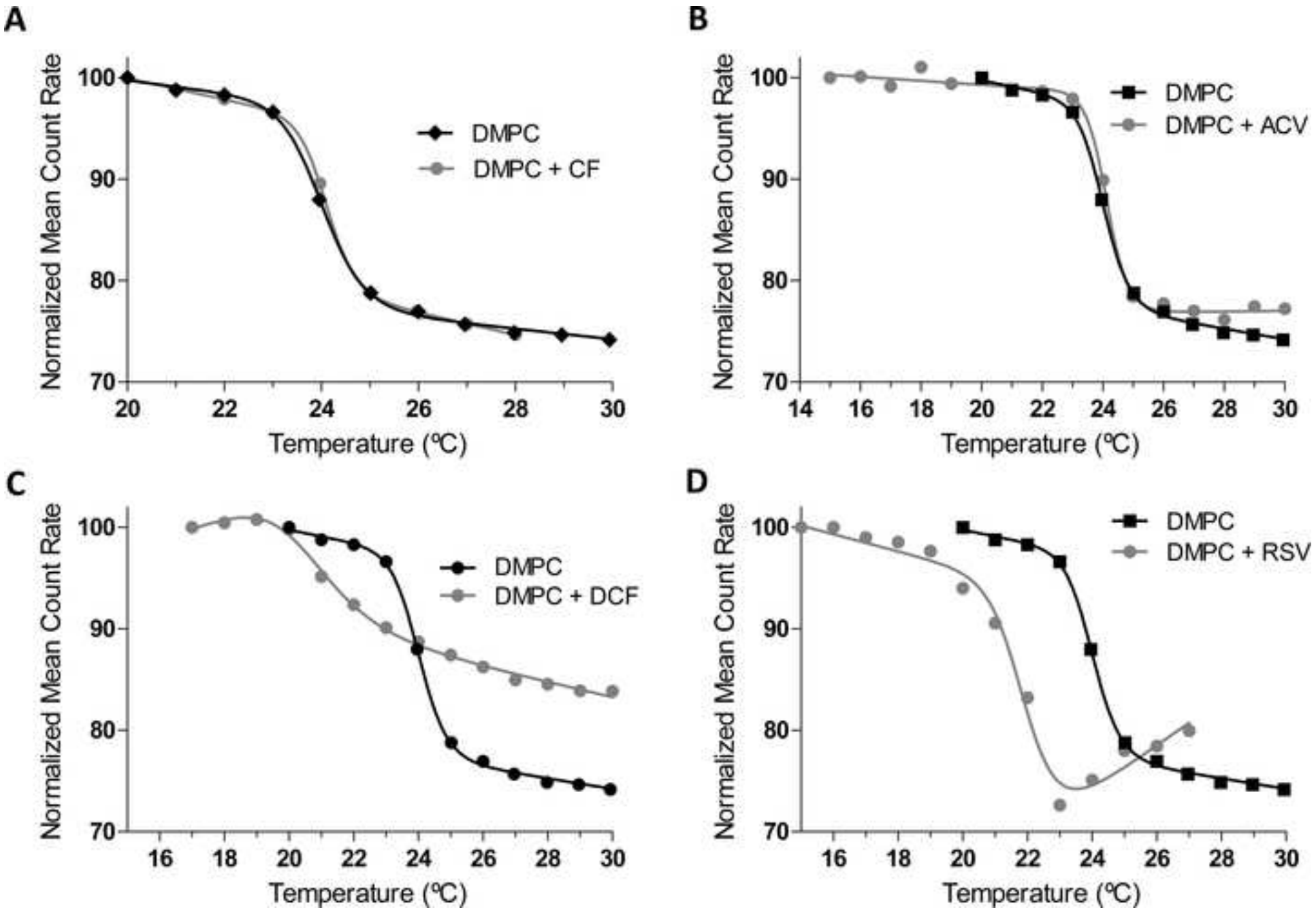

Figure 3

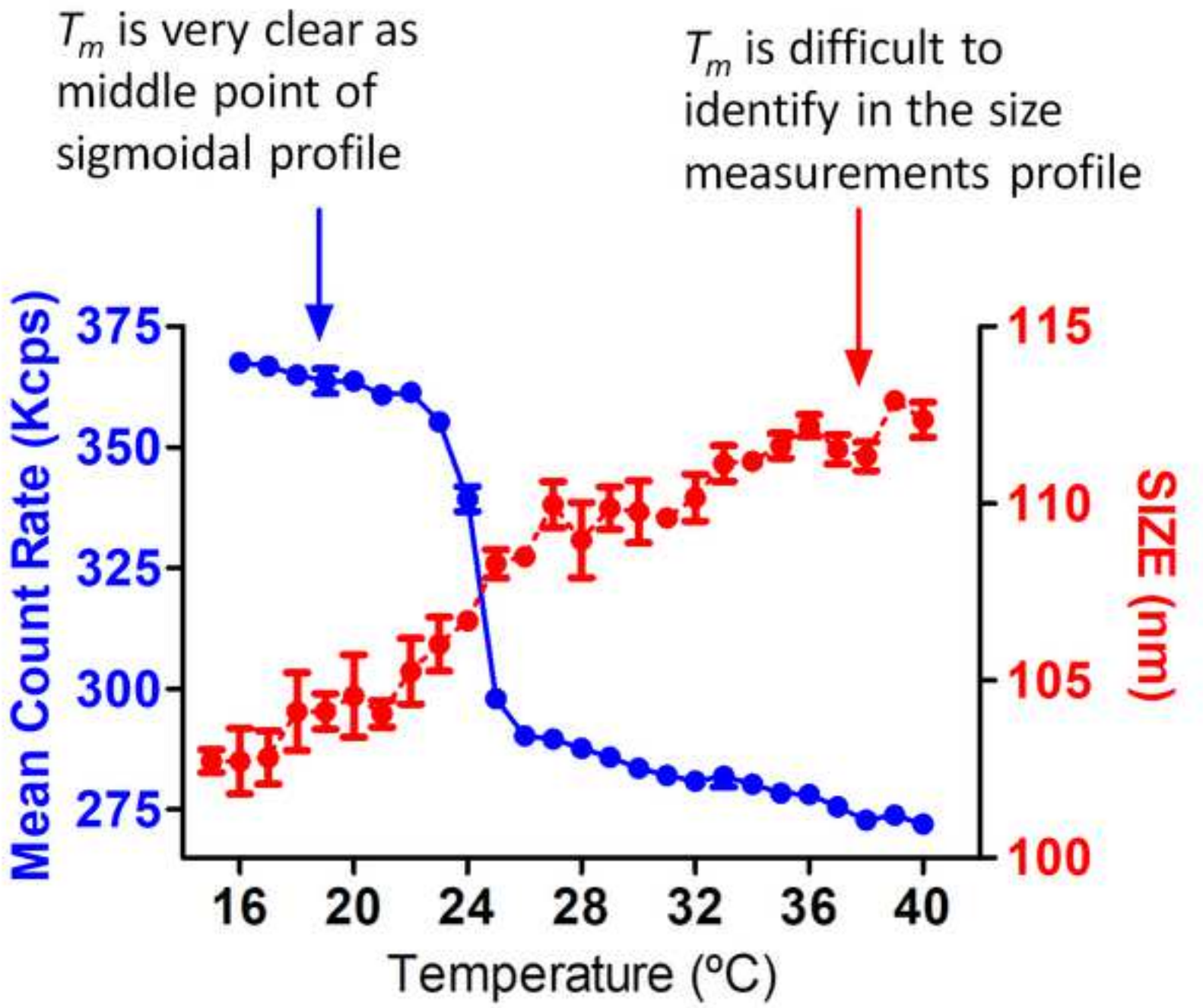

Table 1

	DMPC		
	Tp (°C)	Tm (°C)	Other transition (°C)
Literature Values	15 (DSC)	23.58 (DSC) 23.65 (FTIR) 23.72 (EPR) 24.13 (NMR)	32 - 34 (DSC and FTIR)
DLS results	14.82	24.07	33.86

Table 2

Lipid System	Tm (°C)	B
DMPC	24.03 ± 0.05	591.3 ± 64.15
DMPC+CF	24.12 ± 0.01	870.4 ± 39.18
DMPC+ACV	24.14 ± 0.05	724.4 ± 112.1
DMPC+DCF	20.66 ± 0.29	209.4 ± 44.87
DMPC+RSV	21.99 ± 0.20	389.9 ± 114.0

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1,2-dimyristoyl-*sn* -glycero-3-phosphocholine (DMPC)	Avanti Polar Lipids	850345	
Acyclovir			Kindly offered by Labesfal Genéricos (Lisbon, Portugal)
Caffeine	Sigma-Aldrich	C0750	
Diclofenac Sodium	Sigma-Aldrich	D6899	
Disposable Polystyrene Cuvette	Malvern Panalytical	DTS0012	
Ethanol Absolute	Enzymatic	E/0650DF/C17	Brand - Fisher Chemical
Membrane Nucleopore 25 mm 0.1 µm	Enzymatic	110605	Brand - Whatman
Membrane Nucleopore 25 mm 0.2 µm	Enzymatic	110606	Brand - Whatman
Membrane Nucleopore 25 mm 0.4 µm	Enzymatic	110607	Brand - Whatman
Resveratrol	Sigma-Aldrich	R5010	
Tris buffer Molecular Biology Grade	Sigma-Aldrich	648315-100ML	
Zetasizer nano ZS	Malvern Instruments Ldt		

RESPONSE TO EDITOR

Manuscript Number: JoVE62360R1

Title: Dynamic Light Scattering applied to probe-free characterization of drug induced biophysical changes in lipid membrane

Dear Editor of JOVE,

We appreciate your interest in our work and your willingness to reconsider a revised version of the manuscript. After careful reading, the paper was revised to address all of the editorial and reviewer concerns. We truly believe that the corrections address all of your comments and suggestions while also improving the overall quality of the manuscript to meet JOVE requirements. Following that, all changes made to the manuscript are highlighted with word revision tools in the revised version, and a more detailed explanation and answers are provided.

In addition, we would like to take this opportunity to apologize for the lack of video submissions to date. In fact, in this pandemic scenario, all laboratories and universities in Portugal remain closed, making it impossible to submit the video protocol for consideration for publication alongside the manuscript. However, we would like to inform you that as soon as the government grants permission to open universities and laboratories, we will record and edit the video that will be submitted.

Once again, we thank you for the opportunity to consider our work for publication in JOVE.

Yours sincerely,

Marlene Lúcio*, PharmD, PhD

*corresponding author

EDITORIAL CORRECTIONS:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. .e.g line 193

Following the suggestion of the editor, all the spelling or grammar was carefully revised on entire manuscript as it is possible to confirm by the changes highlighted in the new document.

2. Please revise the following lines to avoid previously published work: 82-85

To avoid excessive repetition in the text of other published work, not only the indicated lines, but also the entire introduction text, were revised.

3. Please provide an email address for each author.

The affiliations and contact information were updated to include each author's email address. Instead of a number representing one institution, each number is assigned to one of the authors, and all author information is provided below.

BEFORE CHANGES

'Eduarda Fernandes[1] and Marlene Lúcio[1,2]

1 – CF-UM-UP – Centro de Física das Universidades do Minho e Porto, Campus de Gualtar, Braga, Portugal

2- CBMA, Centro de Biologia Molecular e Ambiental, Departamento de Biologia, Universidade do Minho, Campus de Gualtar, Braga, Portugal'

AFTER CHANGES:

'Eduarda Fernandes[1] and Marlene Lúcio[2]

1 – CF-UM-UP – Centro de Física das Universidades do Minho e Porto, Campus de Gualtar, Braga, Portugal, eduardabfer@gmail.com

2- CF-UM-UP and CBMA –, Centro de Física das Universidades do Minho e Porto and Centro de Biologia Molecular e Ambiental, Departamento de Biologia, Universidade do Minho, Campus de Gualtar, Braga, Portugal, mlucio@fisica.uminho.pt'

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

We sincerely apologize for the authors' misinterpretation of the JOVE template. The protocol's numbering was changed in response to revision indications. In addition, bullets and dashes were removed from the protocol.

5. Line 168: Please specify the quantity of DPMC dissolved. Also, specify the grade/concentration of solvent used.

Although this type of information is dependent on the assayed desired final concentrations, we agree with the editorial comments about the lack of information. Following that, the amount of DMPC used and the grade of ethanol used were added to the protocol text. A DMPC mass of 338.97 mg is dissolved in ethanol absolute of HPLC grade to produce a stock solution with a DMPC concentration of 2.0×10^{-2} M.

6. Line 199: How much suspension is used?

This is an important question, and while the suspension quantity varies depending on the equipment and cuvette used, the used quantity was added to the protocol text. In the case of our equipment and sample cuvette requirements, we performed the measurement with 1 mL of suspension.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that

cannot be written in the imperative tense may be added as a "Note." E.g.: "Place samples in…" instead of "Samples are placed in..", etc.

We deeply understand the concerns expressed in the editorial comments, and as a result, all of the protocol text has been revised and all of the editorial comments have been taken into account. The word revision tool highlights all changes made in the revised manuscript, allowing you to double-check them.

8. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. E.g. ZetaSizer, Excel, Origin, etc.

We agree with the editorial comment, so all commercial language was removed to meet JOVE requirements, and the products are referenced in the Table Materials.

9. Please include a figure or a table in the Representative Results showing the effectiveness of your technique backed up with data and describe the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title.

The JOVE template was misinterpreted, and the representative results were included in the discussion. As a result of reorganizing the text, we believe we now have a correct representative results section emphasizing how the results confirm the protocol's success and how to interpret the data. We've also included a third figure to show an example of both a positive (MCR measurement) and a negative result (size measurement).

10. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I.,

LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate journal names.

We appreciate the observation and have revised and corrected all references in accordance with the Editorial comments. All of the abbreviated journals were also corrected to the full title.

Reviewers' comments:

Reviewer #1:

General comment:

This paper reports a protocol where the effect of drugs on the main phase transition temperature and cooperative of lipid membranes is evaluated by dynamic light scattering. This is an important topic once those membranes are used to mimetize the biological membranes. Besides the use of this new technique allows to provide a simple and fast way for membranes' characterization. The paper is well written, the protocol is detailed and clearly described and the discussion is a logical outcome of the data. Thus, I truly recommend this paper for publication as is.

Response to reviewer's general comment:

We were grateful for the reviewer's recognition of our work as an important topic, as well as all of the compliments on the significance of such protocol. In addition, we appreciate the time spent reviewing our manuscript as well as the publication recommendation.

Reviewer #2:

Manuscript Summary:

This manuscript reports a method that uses dynamic light scattering to rapidly assess phase transitions and, biophysical changes created by exogenous molecules in the phospholipid bilayer of liposomal systems. This simple approach is a very important in the context of drug-induce

effects on biomembranes, therefore key to drug discovery and formulation.

The title proposed well describes the method presented.

The introduction is well organised and give a good overview on the method theory and approach. I therefore recommend this method-manuscript for publication in JOVE.

Response to reviewer's general comment:

We deeply acknowledge the interest demonstrated in our work and the availability to review our manuscript, as well as the positive feedback regarding the publication.

Major Concerns:

None

Minor Concerns:

Line 25: The sentence of the Abstract sounds confusing, I would suggest to re-word it.

We agree with the reviewer's observation. Indeed, the sentence sounded odd. However, the sentence was reworded, and we believe that it sounds clearer in its current format.

BEFORE CHANGES

'*Biological membranes are self-sealing spherical boundaries that enclose an internal aqueous core, protect it from an external aqueous environment and consist of self-assembled lipid bilayers*.'

AFTER CHANGES

'*Biological membranes are spherical boundaries formed by self-assembled lipid bilayers that enclose an internal aqueous core and are surrounded by an external aqueous environment.*'

Line 97: The authors use the word "along" maybe they mean "across".

We acknowledge the reviewer suggestion and actually, 'across' fits in better in this context. Thus, we have changed the phrase:

BEFORE CHANGES:

*'Actually**, along with** the lipid bilayer in a membrane exists a fluidity gradient…'*

AFTER CHANGES

*'Actually, **across the** lipid bilayer in a membrane exists a fluidity gradient..'*

Line 185: It says ".....liposomes (LUVs) in aqueous solution is diluted in buffer ...." I would suggest to specify which buffer the authors are referring to.

The buffer solution used in the protocol was specified as suggested. Additionally, some changes in the phrase were made by suggestion by Editor. Thus, the final text form is '*2.2. Prepare the samples by mixing adequate portions of LUVs suspension, **Tris buffer solution** (100 mM) and drug solution accordingly to the desired final concentrations. NOTE: Herein, a final concentration of liposomes of $5.00x10^{-4}$ was used in the presence and absence of pre-established drug concentrations of 100 µM CF and DCF, 150 µM of ACV, and 200 µM of RSV.'*

Line 192: A space is missing between the number 37 and the sign °C

We acknowledge the reviewer's observations, and a space between the number and the sign of unity has been added.

Line 193: A space is missing between the number 4 and the sign °C

Once again, we acknowledge the reviewer's observations , and a space between the number and the sign of unity has been added.