Phospholipids Critical Micellar Concentrations Trigger Different Mechanisms of Intrinsically Disorder Proteins Interaction with Model Membranes

Supporting Information

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Table of Contents

1 Computational Section

1.1 Simulations Setup

1.2 Unbiased Simulations

1.3 Umbrella Sampling-Based Free Energy Calculations

1.4 Simulation Results

2 Materials and Methods

2.1 Materials

2.2 Preparation of model membranes.

2.3 Preparation of free phospholipids solutions.

2.4 hIAPP preparation.

2.5 CD measurements.

2.6 ThT measurements.

2.7 Membrane leakage experiments.

2.8 Pyrene experiments.

3 Results

3.1 CD experiments: hIAPP secondary structure is perturbed by phospholipids.

3.2 CMC determination

Computational Section

1.1 Simulations Setup

The NMR structure (PDB:2KB8[1]) of the human Islet Amyloid Polypeptide (hIAPP), already equilibrated and simulated in water in our precedent work[2] has been used. The 276 lipids bilayer is made of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), structure and parameters are taken from the lipidbook website[3]. We first created two grids of lipids with a large area per lipid (APL) by using a home-made Python script.

A lateral pressure of 100 bar was used to equilibrate the bilayer in vacuum until a constant APL was reached.

Position restraints with a force constant of 10000 KJ mol^{-1} nm^{-2} were applied on the z-direction to the phosphate and the last tail atoms of the POPC molecules to avoid the folding of the lipids during the squeezing simulation in vacuum.

Subsequentially, the bilayer was hydrated and equilibrated for 50 ns with a salt concentration of NaCl 0.15 M.

1.2 ***Unbiased simulations***

The protein/lipid complexes were obtained by placing the protein in a water box with a different number of lipids: 1, 2, 3 and 10 (system C_1, C_2, C_3 and C_10, respectively). The initial minimum distance among lipids and protein was about 2,5 nm. In all simulations the formation of the complex was found to be irreversible. Furthermore, the systems P (only protein), C_1 and C_3 (protein/lipid complexes) were simulated together with the model membrane. The starting complex-membrane center of mass (com) distance was about 4,5 nm. All simulations were performed at 310K and NaCl 0,1M. Detailed set-up of the simulations are described in Table S1.

All simulations have been carried out with GROMACS 4.6.7 package[4] with gromos54a7 forcefield parameters[5] and water model SPC/E[6]. A time step of 2 fs was used. The temperature was kept constant by using the V-rescale[7] thermostat with a time constant of 2.0 ps. The pressure was handled by employing the Berendsen[8] barostat with 1bar for equilibration and production run. The electrostatic interaction was treated with particle mesh Ewald (PME) method[9] with real space cut-off of 1.2 nm and cubic interpolation.

Table S1. Unbiased Molecular Dynamics Simulations Details. The system naming follows the current method: M, the only membrane system; C_1, C_2, C_3, monomer of hIAPP in the presence of 1, 2 and 3 free POPC lipids respectively; P_M, monomer of hIAPP with a 276 POPC lipid bilayer; C_1_M, C_2_M, C_3_M, a 276 POPC lipid bilayer has been added to the free lipid-protein complexes formed during the C_1 and C_3 simulations.

System Name	**Nº hIAPP molecules**	**Nº POPC molecules**	**Nº Na^+**	**Nº Cl^-**	**Nº water molecules**	Time (ns)
M	0	276	67	67	25748	300
C_1	1	1	31	34	16762	500
C_2	1	2	31	34	16723	500
C_3	1	3	31	34	16687	500
C_10	1	10	31	34	16458	500
P_M	1	276	72	75	25882	500
C_1_M	1	1 + 276	72	75	30699	500

1.3 Umbrella Sampling-Based Free Energy Calculations

To improve the sampling and calculate the free energy at 310K required to bring the protein or protein-lipid complex in contact with the membrane, we employed the umbrella sampling technique[10] (biased simulations). This method enables us to evaluate also the free energy profile of the membrane insertion process, as shown in Figure 1 (main text). The energy profiles are shifted such that the point where the protein or protein/lipid complex is in at a non-interacting distance respect the membrane corresponds to a starting situation at 0 kcal mol^{-1}.

The umbrella sampling windows were initialized by pulling the protein (system P) or protein/lipid complexes (systems C_1 and C_3) along the normal to the membrane.

Thirty-forty 0.1 nm spaced windows (see Table 2 for umbrella sampling simulations details) were generated by using a pull rate of 0.0005 (nm/ps) with a force constant of 2000 (kJ mol^{-1} nm^{-2}).

The reconstruction of the potential of mean forces (PMF) as a function of the com of the POPC membrane and the protein along the bilayer normal are calculated by using the weighted histogram analysis method (WHAM)[11].

The statistical errors were estimated with the Bayesian block bootstrap approach using each independent simulation as a single block from 200 bootstrap samples. The error bar is estimated by calculating the standard deviation of the 200 bootstrapped profiles.

Table S2. Umbrella Sampling Simulations Details. The free energy calculations are made on the systems with the same set-up showed in the P_M, C_1_M and C_3_M systems. The system naming follows the method of the table S1, U means "umbrella sampling"

System Name	**Reference group 1**	**Reference group 2**	**Harmonic Force Constant** **($kJ*mol^{-1}*nm^{-2}$)**	**Nº windows**	**ns/windows**
P_M_U	Membrane COM	Protein COM	2000	31	100
C_1_M_U	Membrane COM	Protein COM	2000	41	100
C_3_M_U	Membrane COM	Protein COM	2000	44	100

1.4 Simulation Results

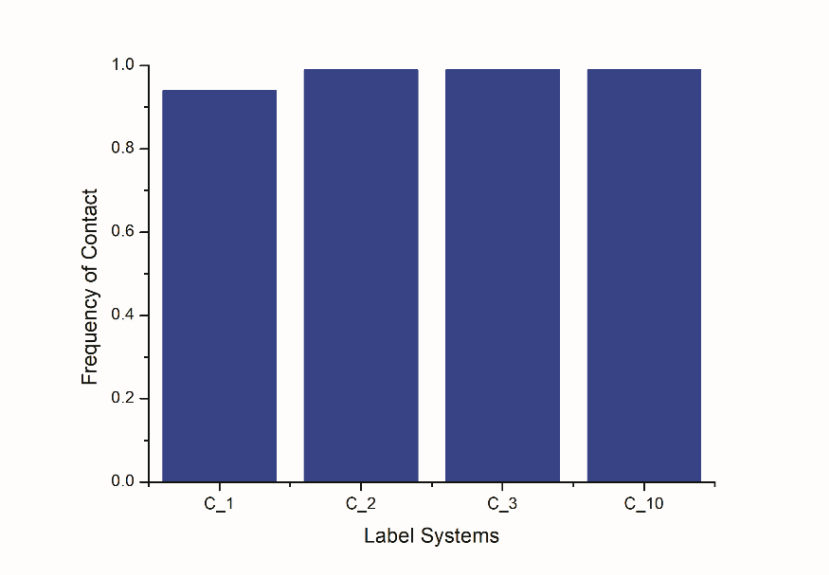

Figure S1. Contact frequency among lipids and protein for systems C_1, C_2, C_3 and C_10. Calculus was performed over 500 ns using a cutoff of 0.4 nm.

The Figure S1 shows the irreversibility of the interaction between the protein and free lipids in water bulk. So, the existence of these complex must be considered when protein/membrane interaction takes place, the results are showed and discussed in Figure 1 (main text). Furthermore, to rationalize the results showed in Figure 1, we investigated the effect of the lipid-protein complex (C_1_M and C_3_M systems) on the model membrane, performing MD simulations with the complex already adsorbed onto the lipid surface.

The presence of the lipid-protein complex reduces the local membrane thickness especially for the C_3_M system (see Figure S2 panel C and D). Similar behaviour is observable for the area per lipids reported in Figure S3: The 1:1 lipid-protein complex (C_1_M system) seems to do not perturb the membrane significantly. These analyses confirm the

trend showed in free energy profiles (Figure 1) and agree with the more favorable adsorption process explained for the C_1_M system.

The pictures showing thickness and area per lipid analysis in Figure S2 and S3 were generated by using the g_lomepro tool[12].

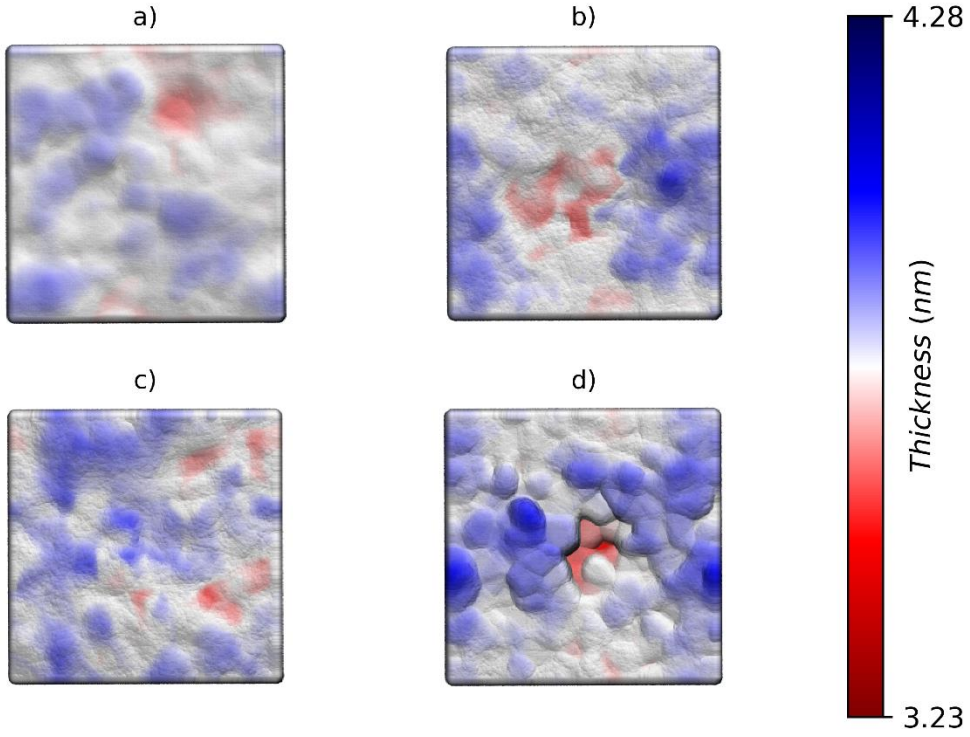

Figure S2. The membrane thickness of POPC membrane for different systems: a) M; b) P_M; c) C_1_M and d) C_3_M. The thickness is referred to as the distance between the phosphorus atoms of the upper and lower leaflets averaged over the last 200ns to the respective the unbiased simulations.

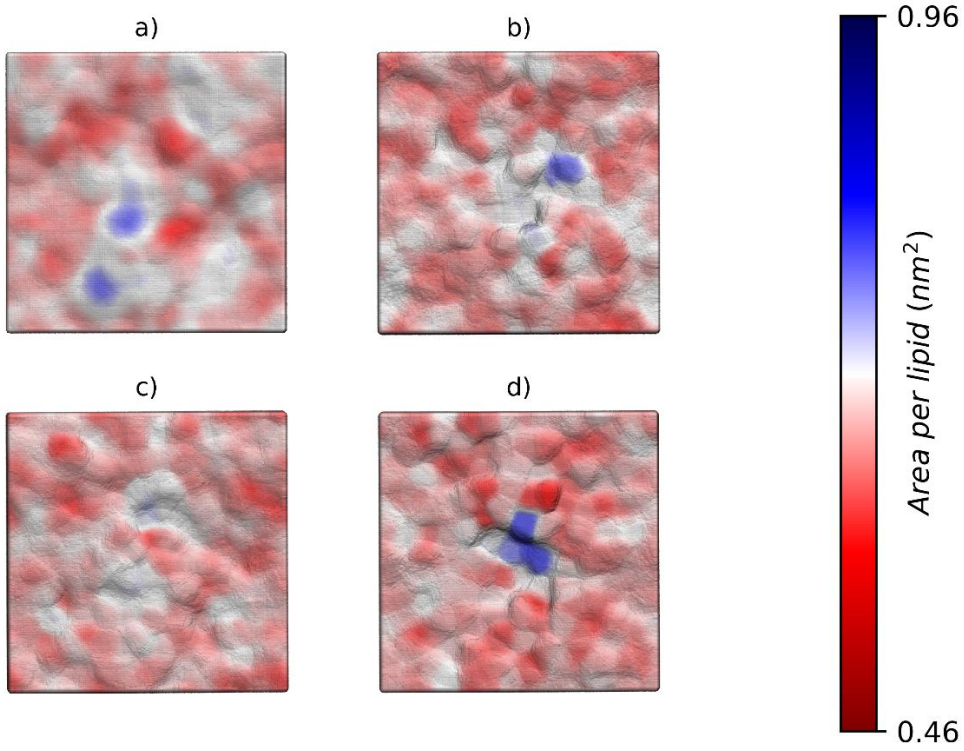

Figure S3. The Area per Lipid (APL) of POPC membrane for different systems: a) M; b) P_M; c) C_1_M and d) C_3_M. The APL calculated by averaging the last 200ns to the respective the unbiased simulations.

2. Materials and methods

2.1 Materials

Human amylin (hIAPP) was purchased from Synpeptide (Shanghai, China) with a purity >98%.
1,2 -dimyristoleoyl -sn- glycerol- 3- phosphocholine (PC14),

1,2−dipalmitoleoyl−sn−glycero−3−phosphocholine (PC16),

1,2−dioleoyl−sn−glycero−3−phosphocholine (PC18),

1,2−dieicosenoyl−sn−glycero−3−phosphocholine (PC20),

1,2−dierucoyl−sn−glycero−3−phosphocholine (PC22), were purchased from Avanti Polar lipids Inc. (Alabaster, AL, USA).
4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonic acid sodium salt (HEPES), (5)6-carboxyfluorescein, Thioflavin T (ThT), Sodium chloride (NaCl), Sodium fluoride (NaF), Sodium hydroxide (NaOH) and 1,1,1,3,3,3-hexa-fluoro-2-propanol (HFIP) were purchased from Sigma-Aldrich (St.Louis, MO) with a purity of 99%, Sodium hydrogen phosphate anhydrous (Na_2HPO_4) and Sodium dihydrogen phosphate ($NaH_2PO_4H_2O$) were purchased from CARLO ERBA with a purity of 99%;

2.2 Preparation of model membranes

In this study we used large unilamellar vesicles (LUVs) composed of five different types of phospholipids, with the same polar head (PC) a unique unsaturation, varying only the chain length. Model membranes were prepared as described elsewhere[13]. Briefly, phospholipidic films were prepared from $CHCl_3$ lipid stock solutions, a certain volume was taken and then dried under nitrogen flow and evaporated under high vacuum to dryness in a round-bottomed flask (at least 12 hours). Multilamellar vesicles (MLVs) were obtained by hydrating the lipidic films with an appropriate amount of aqueous solution (depending on the type of experiment, see below) and dispersing by vigorous stirring in a water bath. LUVs were obtained by extruding MLVs solutions through polycarbonate filters (pore size = 100 nm, Nuclepore, Pleasanton, CA) mounted in a mini-extruder (Avestin, Ottawa, ON, Canada) fitted with two 0.5 ml Hamilton gastight syringes (Hamilton, Reno, NV). Samples were typically subjected to 23 passes through two filters in tandem as recommended elsewhere[14].

2.3 Preparation of free phospholipids solutions

The method discussed above was used in order to obtain MLVs, the hydration step was led using PBS buffer solution (10 mM, NaCl 100 mM, pH=7.4). In this case, MLVs were centrifuged using EPPENDORF CENTRIFUGE 5810/5810R (30 min, 2000 rpm). Carefully the supernatant was poured off and used immediately for the experiments.

2.4 hIAPP preparation

To avoid the presence of any preformed aggregates, the protein was initially dissolved in HFIP at a concentration of 0.1 mg/ml and then lyophilized overnight. To be used for the experiments, the lyophilized hIAPP powders were dissolved in DMSO or in H_20 (pH=2.1) to obtain a stock solution with a final concentration of 250 μM. This solution was immediately used after its preparation.

2.5 CD measurements

Circular dichroism spectra were acquired by using a JASCO spectrophotometer J810. LUVs were prepared hydrating phospholipidic films with PBS buffer solution (10 mM, NaF 100 mM, pH= 7.4). Sample were prepared by adding aqueous hIAPP solution to buffer solution LUVs (200 μM) to obtain a final protein concentration of 10 μM.

2.6 ThT measurements

Kinetics of amyloid formation were measured using the well-known and widespread thioflavin T (ThT) assay. Samples were prepared by adding a specific quantity of peptide stock solutions to 100 μL of 200 μM model membranes in 10 mM PBS buffer solution (pH 7.4, 100 mM NaCl, containing 20 μM ThT) in order to obtain a final peptide concentration of 10 μM.

The same procedure was used for free phospholipids experiments. Peptide was added to a buffer solution (PBS 10 mM, pH 7.4, NaCl 100 mM, plus 20 μM ThT) of phospholipid, each one under his critical micellar concentration. Experiments were carried out in Corning 96 well non-binding surface plates. Kinetics profiles were obtained using a Varioskan (ThermoFisher, Walham, MA) plate reader, setting each read every 2 minutes and 30 s, at 37 °C, shaking the samples for 10 seconds before each read. The excitation and emission wavelengths were 440 nm 485 nm. Every curve is an average of three measurements.

2.7 Membrane leakage experiments

Membrane leakage experiments were performed by measuring the leakage of (5) 6-carboxyfluorescein dye from LUVs. Dye-filled LUVs were prepared by hydrating various dry phospholipid films with PBS buffer solution containing (5) 6-carboxyfluorescein (70 mM, pH=7.4) according to the procedure described above. Removal of residual non-encapsulated dye was realized by running the extruded LUVs through a Sephadex G50 gel exclusion column (Sigma-Aldrich, St. Louis, MO), the first colored band was collected.

The final concentration of lipid was checked by using the well-known Stewart assay[15].

Samples were prepared by diluting dye-filled vesicles with buffer solution (PBS 10 mM, NaCl 100 mM, pH=7.4) to a final concentration of 200 μM, and by adding to each solution an appropriate peptide volume in order to obtain a final concentration of 10 μM. Membrane damage was quantified by detecting the increase in fluorescence emission intensity of (5) 6-carboxyfluorescein due to its dilution (dequenching) in buffer as a consequence of the membrane leakage. Experiments were carried out in Corning 96 well non-binding surface plates, using a Varioskan (ThermoFisher, Walham, MA) plate reader, setting each read every 2 minutes and 30 s, at 37 °C, shaking the samples for 10 seconds before each read. The excitation and emission wavelengths were respectively 494 nm and 520 nm.

The fraction leaked was calculated as follows:

Fraction leaked = $\frac{(I_i - I_0)}{I_{100} - I_0}$

where I_i is the emission intensity of the sample, I_0 is the emission intensity in absence of peptide (baseline control). I_{100} represents the emission intensity linked to the maximum leakage (100%), caused by the addition of Triton X-100 (a detergent). All measurements were done in triplicate.

2.7 Pyrene experiments

Pyrene solutions were made by first dissolving pyrene in ethanol at 1 mg/ml followed by serial dilution down to 2 µM in 10 mM sodium phosphate buffer, 100 mM NaCl (pH 7.4). Pyrene emission spectra were recorded during titration with different lipids solutions using an excitation of 334 nm with excitation and emission slit widths of 2 nm. All measurements were performed in a temperature controlled (25°C), stirred cell.

3. Results

3.1 CD experiments: hIAPP secondary structure is perturbed by phospholipids

To study hIAPP secondary structure in presence of two phospholipid systems, CD measurements were performed. Among all phospholipids used in this work, the longer and shorter chain ones were chosen (PC14 and PC22), in order to compare two systems having CMC as different as possible.

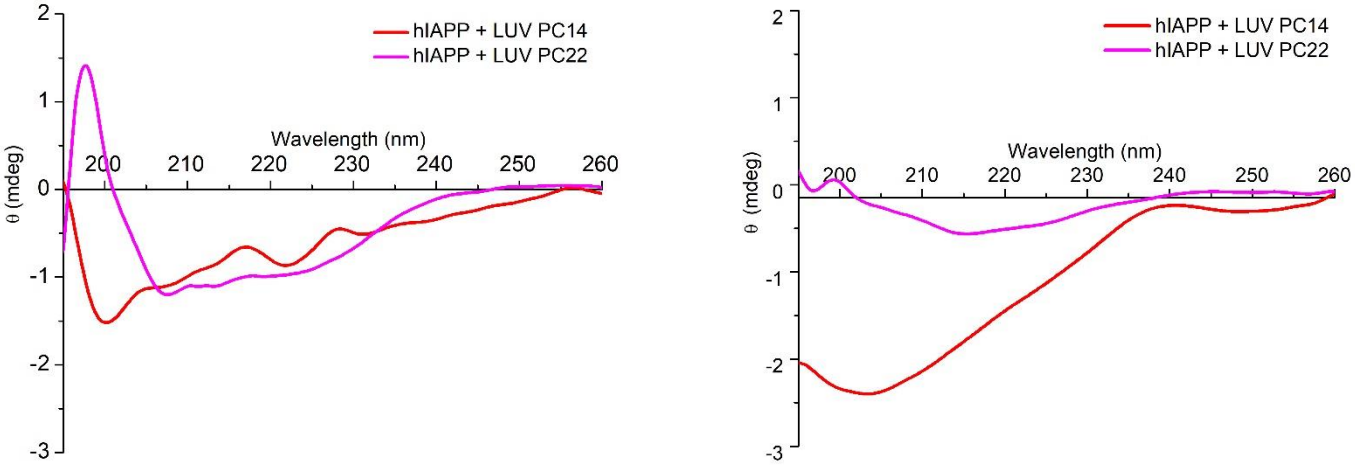

Figure S4. Effect of phospholipids on hIAPP secondary structure. Circular dichroism spectra of samples containing 10 µM hIAPP in presence of 200 µM PC14 LUVs (red curves) and 200 µM PC22 LUVs (magenta curves). All experiments were performed at 25°C in PBS buffer 10 mM, NaF 100 mM (pH= 7,4). Left panel shows the spectra obtained immediately after solution preparation, on the right after 18h of incubation.

As evidenced by the spectra (fig. S4), different phospholipids concentrations in solution influence the peptide secondary structure. Indeed, hIAPP in presence of PC14 phospholipid (higher CMC) shows a preeminent random-coil structure immediately after solution preparation (left panel), that is accentuated after 18h (right panel). On the other hand, the same peptide in a solution containing PC22 phospholipid (lower CMC) seems to assume mainly α-helix structure (t=0, left panel). After 18 h (right panel) the structure changes, increasing his β-sheet structure content.

3.2 CMC determination

This method is widely used in order to determine surfactants critical micellar concentration. Fluorescent properties of pyrene are extremely sensitive to chemical environment. Due to their high hydrophobicity, above the CMC, pyrene molecules solubilize in micellar phase. As the polarity environment decreases, the spectra changes [16].

Fluorescence emission spectrum of pyrene shows several vibronic bands, increased phospholipids concentration causes variation on intensity of the first (I_I) and the third one (I_{III}).

Particularly, intensity related to the first peak decreases, while the other one increases, that is associated to the formation of micelle-like structures [17].

Figure S5 represents pyrene spectra in presence of different concentration of the same phospholipid (PC 16). The same measurements were performed for the other four systems subjects of our study.

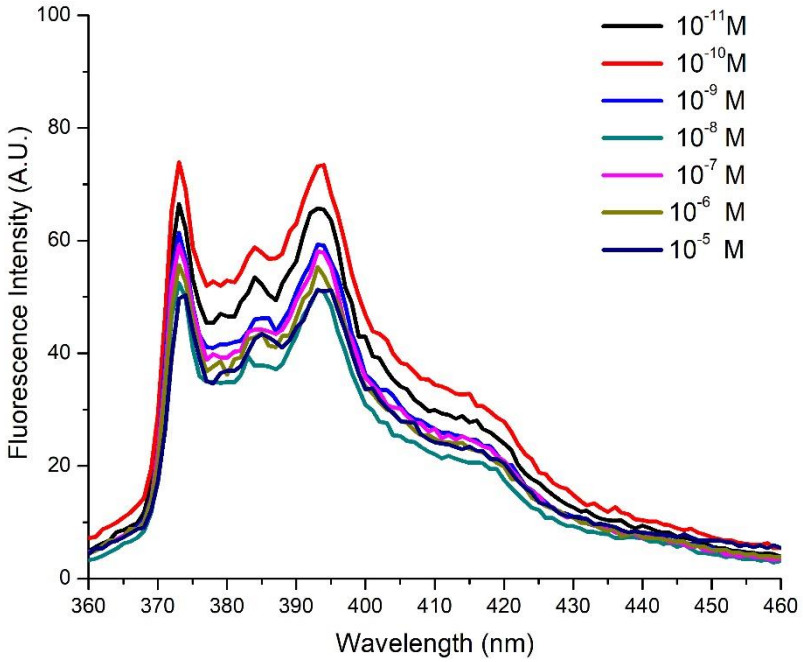

Figure S5. Fluorescence pyrene spectra in presence of PC16. Fluorescence emission spectra of samples containing 2 µM pyrene in presence of different concentration of PC16 phospholipid (ranging from 10^{-5}M to 10^{-11} M). All experiments were performed at 25°C in PBS buffer 10 mM, NaCl 100 mM (pH= 7.4).

Through a linear plot of I_{III}/I_I ratio as function of phospholipids concentration, two linear lines characterized by different slopes are obtained. It is possible to define an inflection point that is associated to CMC value[18].
Results obtained using this method are shown below (table 3).

Table 3. Critical micellar concentration of some diacyl (*n*) -phosphatidil-choline. In parenthesis the standard deviation.

PHOSPHOLIPID (*n*)	***log*[CMC]**
14:1	-7.0 (±0.35)
16:1	-7.2 (±0.36)
18:1	-7.7 (±0.46)
20:1	-8.2 (±0.49)
22:1	-9.1 (±0.64)

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