Effect of the Incorporation of Nano-sized Titanium Dioxide on the Interfacial Properties of DPPC Langmuir Monolayers

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Abstract

The effect of the incorporation of hydrophilic titanium dioxide (TiO_2) nanoparticles on the interfacial properties of Langmuir monolayers of 1,2-Dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC) has been evaluated combining interfacial thermodynamic studies, dilatational rheology and Brewster Angle Microscopy (BAM). The results show that the TiO_2 nanoparticles are able to penetrate into DPPC layers, modifying the organization of the molecules and, consequently, the phase behaviour and viscoelastic properties of the systems. Measurements of dilational viscoelasticity against the frequency have been performed, using the Oscillatory Barrier method, at different values of the surface pressure corresponding to different degrees of compression of the monolayer. The presence of TiO_2 nanoparticles also affects the dynamic response of the monolayer modifying both the quasi – equilibrium dilatational elasticity and the high frequency limit of the viscoelastic modulus. Principal aim of this work is to understand the fundamental physico-chemical bases related to the incorporation of specific nanoparticles of technological interest into interfacial layer with biological relevance such as phospholipid layers. This can provide information on potential adverse effects of nanoparticles for health and environment.

Introduction

Titanium dioxide (TiO_2, titania) is accounted among the most frequently used materials in technology, especially due to their extraordinary optical properties, e.g. photocatalytic properties and UV-resistance.[1] That has led to the use of this TiO_2 in many fields including paints, cosmetics, health care products and energy generation devices.[2] Moreover, the use of TiO_2 in the aforementioned fields has been favored for long time because it is considered a cheap, innocuous, chemically stable, environmentally friendly and biocompatible material.[3]

However, the recent developments on nanoscience and nanotechnology, and the increasing interest on the fabrication of technological devices with reduced dimensions have raised new questions associated with the potential toxicity and hazards of micro- and nano-sized materials.[4-8] Among them, particulate materials with size in the sub-micron scale present a central importance because they can be easily spread into the atmosphere or the water-bearing, interacting with flora and fauna.[9-10] The potential adverse effects for human health and environment,[11-13] related to these interactions, have stimulated the discussion on the potential toxicity of nanoparticles.[14-15] This is a controversial issue as evidenced the fact that nanoparticles are commonly used for the development of drug delivery platforms, while other studies are advising against the use of nanoparticles because their interaction with life tissues and bio-fluid is a possible source for several diseases such as cancer, pulmonary diseases, etc.[16-20] Therefore, the increasingly environmental exposure to TiO_2 particles makes necessary to deepen on their potential toxicity, especially because several studies on mammals have evidenced effects associated with the development of diseases, e.g. cell proliferation, inflammation, oxidation stress and histopathological changes. This has fostered the research on the understanding of the relationship existing between the toxicity of TiO_2 and its physico-chemical properties.[21] It is worth to mention that there are

studies that claims that the crystalline structure of TiO_2 determine its toxicity because of the different interaction of the biomolecules with rutile and anatase forms.[22]

An elegant method to test the effect of different agents, such as nanoparticles, on the structure and properties of biological relevant systems is investigating their interaction with Langmuir monolayers of fatty amphiphiles at the water/vapor interface. These systems are good models for understanding many fundamental aspects related to the physico-chemical behavior of biological relevant systems and their interactions with external components.[23-26]

Moreover, it is worth mentioning that several studies on surfactant systems have clearly evidenced that nanoparticles are able to modify the thermodynamic and kinetic behavior of the adsorption layer, influencing also the interfacial structure and composition.[27-29] This is because the study of the interaction between nano-sized materials and lipid layers is a central issue for understanding the potential adverse effects associated with nanoparticles incorporation. In many cases, in fact, the normal physiological functionality of the biological systems is related to the structure, composition and mechanical properties of the lipid interfaces.[30-32]

This work is aimed to understand the effect of nano-sized titanium dioxide particles (TiO_2) on the interfacial properties of Langmuir monolayers of 1,2-Dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC). This lipid, a phosphocholine with two saturated fatty chains, has been chosen because it is accounted among the most common lipid in biological membranes and bio-fluids such as lung surfactant. Its importance is associated with their ability to form condensed phases at physiological temperature.[33-38] Thus, it is possible to assume that the understanding of the behaviour of DPPC monolayers may provide the bases for developing new studies on more complex systems.

In recent years, several studies have been devoted on the effect of nanoparticles of different nature on the physico-chemical properties and structure of lipid monolayers.[26, 39-48] These studies have shown the strong influence of the specific properties of the particles on their effects.[49] Despite the extensive development of this research, the understanding of the effect of nanoparticles on the dynamic response has been neglected for long time, even if it is probably a central aspect for exploring the adverse effects on the physiological response,[30, 49-51] providing important information to complement previous studies on the interaction between TiO_2 and biological relevant systems.[52-54] For the purpose of this study, we combine Langmuir trough experiments, equilibrium isotherms and oscillatory barriers experiments, with the morphological information obtained by Brewster Angle Microscopy (BAM). Thus, it is possible to establish correlations between the changes of the equilibrium and dynamic mechanical properties of the layer due to the nanoparticles incorporation, with the changes on the molecular ordering and layer structure. This study can be useful to improve the understanding of the most fundamental physico-chemical bases associated with the potential toxicological effects of nanoparticles in their interaction with biological relevant systems.

Materials and Methods

Materials

1,2-Dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC) was purchased from Sigma (Germany) at 99 % purity and used without further purification. The molecular weight of this lipid is 734.1 g/mol. Solutions of lipids for the spreading were prepared using chloroform for HPLC from Sigma (Germany).

TiO_2 aqueous dispersions at different solid concentration, in the 0.1 – 10 mg/mL range, were obtained by diluting a 30 wt % commercial dispersion (VP Disp. W 2730X from Degussa, Germany). Note that both concentrated TiO_2 nanoparticles dispersions and diluted ones were sonicated before their use to avoid undesired effect associated with particles aggregation in bulk dispersions. The commercial sample is formed mainly by TiO_2 nanoparticles in the anatase form, presenting a specific surface area and a density of 96.5 m^2/g and 3.88 g/cm^3, respectively. This nanoparticles are negatively charged in Milli-Q water (pH ~ 6.5) as evidenced the negative value of their ζ-potential (-47 ± 5 mV) obtained using a Malvern Zetasizer Nano ZS instrument (Malvern Instrument, Ltd.) and present a hydrodynamic diameter around 38 ± 10 nm as was obtained by Dynamic Light Scattering. The dispersions do not contain any stabilizing agent, which could false the data interpretation, being non surface active materials as was pointed out by independent surface tension measurements.

Water for all the reported measurements was deionised and purified by a multi-cartridge system (Elix plus Milli-Q, Millipore) providing a resistivity greater than 18 MΩ·cm. and a surface tension of 72.5 mN/m without any appreciable kinetics over several hours. The pH of the water used was around 6.5 and no salt was added to fix the ionic strength.

Methods

Lipid monolayers were spread on the aqueous subphase contained in a Teflon trough (Langmuir balance), by dropping controlled volumes of their chloroform solution with a precision syringe (Hamilton). From this volume and the solution concentration (~ 1 mg/mL) was possible to control the number of molecules remaining at the water/vapor interface after evaporation of the solvent. For all the experiments, the temperature was at a controlled value of 22.0 ± 0.1 °C. Note that the studies have been carried out at a

temperature far from the physiological one (~ 37 ºC). However, assuming that DPPC presents its phase transition temperature above the physiological temperature,[55] it can be considered that most of the results could be extrapolated to physiological conditions.

A Langmuir balance (KSV minitrough, Finland) equipped with two Delrin® barriers allowing for symmetric compression / expansion of the free liquid surface. The total free surface area of the trough is 243 cm^2 (length 32.4 cm, width 7.5 cm and depth 0.4 cm). The surface tension, γ, was measured using a paper Willhelmy plate (Whatman CHR1 chromatography paper, effective perimeter 20.6 mm, supplied by KSV), ensuring a zero-angle contact angle. Surface pressure is then obtained as $\Pi = \gamma_w - \gamma$, where γ_w is the surface tension of pure water.

Using the Langmuir balance the Π - A isotherms of the monolayers were obtained by compressing the free area, at a rate of 2 cm^2/min. This compression rate was found to be slow enough to avoid undesired non-equilibrium effects in the isotherms obtained.[33, 56] Compression was started after one hour given time from the solution deposition. This time was checked to be long enough to ensure the complete evaporation of the solvent and, in case of nanoparticle dispersions, the achievement of the equilibrium of the composite system, driven by the nanoparticle - lipid interaction. The Langmuir balance also enables for dilatational rheological measurements by the Oscillatory Barrier Method in the 0.001 Hz to 0.150 Hz frequency range.[57-58] Thus, these experiments allows studying the system upon dilatational deformations with frequencies similar to that occurring during the respiratory cycle on healthy human adults, which are reported to be in the range 0.050 – 0.090 mHz.[59] For the purpose of this experiments, after equilibrating the spread monolayer, a slow compression (2 cm^2/min) is imposed until the desired values of surface pressure is reached, and then the complex modulus of dilatational viscoelasticity is obtained from the

response of the surface pressure to an imposed sinusoidal variation of the surface area, according to the procedure described in our previous publication.[60] Being evaluated through the measurement of the γ response. E is affected by an error ~ 2 mN/m. For the oscillatory barrier experiments, the amplitude for the area deformation was fixed in $\Delta u = 0.01$, which was checked to be an appropriate value to ensure results within the linear regime of the layer response.

In addition to the rheological studies carried out within the linear regime, and to characterize the viscoelastic response of the systems upon a dilatational deformation similar to that occurring during the respiratory cycle. Additional experiments of dilatational rheology fixed frequency (0.050 Hz) were performed at different values of Δu in the range 0.01 – 0.4. These studies were carried out at a fixed value of surface pressure (~ 40 mN/m) that is considered a reference state for the study of bio-systems such as lung surfactant or biomembranes.[61-63] The increase of the amplitude for the area deformation is expected to induce non-linearity in the response. This makes impossible to use the classical approach based on the fitting of the response to a sinusoidal function for the analysis of the experimental results, being necessary to use a description based on a complex sum of trigonometrical functions which makes difficult the data analysis.[64] This can be solved in part using a Fast Fourier Transform algorithm, implemented in the software package Origin® (Origin Lab Corporation, U.S.A.) as was described elsewhere.[30, 58, 60, 65-67] From the analysis of the surface pressure using the FFT algorithm, it is possible to obtain information of the response as a spectrum in the frequency domain, which provides information about the nature of the signal. When the response to the deformation remains in the linear regime, the resulting FFT spectrum shows only a peak centred at the characteristic relaxation frequency, whereas for those cases in which non-linear effects domains the response, several peaks appears on the FFT spectrum. These are associated

with characteristic relaxation frequency and the the integer multiples of such frequency, which corresponds to the harmonics (further details are presented in our previous publication[60]). The evaluation of the non-linearity of the response can be carried out on the bases of the values of Total Harmonic Distortion, THD, defined as[68]

$$THD = \frac{\sqrt{\sum_{k>1} \Delta\sigma_k^{\,2}}}{\Delta\sigma_1} \qquad (1)$$

where $\Delta\sigma_k$ are the k-Fourier coefficient, or the amplitude of the k-order harmonics, when the signal is represented as a Fourier series, and $\Delta\sigma_1$ is the amplitude of the fundamental harmonic. According to its definition, systems assuming vanishing values of THD are considered linear system, whereas those with larger values of THD are considered non-linear ones.

The Langmuir trough is coupled to a Brewster Angle Microscope (Multiskop, Optrel, Germany) allowing for the acquisition of information on the layer morphology simultaneously to the study of their interfacial properties.

Results and Discussion

Thermodynamics of DPPC + TiO_2 mixed layers

The incorporation of TiO_2 into DPPC monolayers has been analysed from the differences observed between pristine DPPC monolayers and DPPC monolayers spread onto nanoparticles (NPs) dispersions containing different NPs concentration, in the range 0.1 mg/mL - 10 mg/mL. The nanoparticle concentration was chosen to match with previous studies in the literature.[69] As TiO_2 NPs are negatively charged and highly hydrophilic,

their incorporation into DPPC monolayers is expected to be mediated through the electrostatic interaction between the charged groups on NPs surface and the ammonium head group of the lipid molecules, oriented toward the water phase. Similar incorporation mechanism has been previously reported for SiO_2 nanoparticles.[50-51] Figure 1 shows the $\Pi - A/A_0$ isotherms, where A_0 represents a normalization factor referred to the area available per spread DPPC molecule at the initial free interface with area 243 cm^2, for the monolayers of DPPC spread at the pristine water/vapour interface and onto TiO_2 dispersions with different concentration of solid NPs.

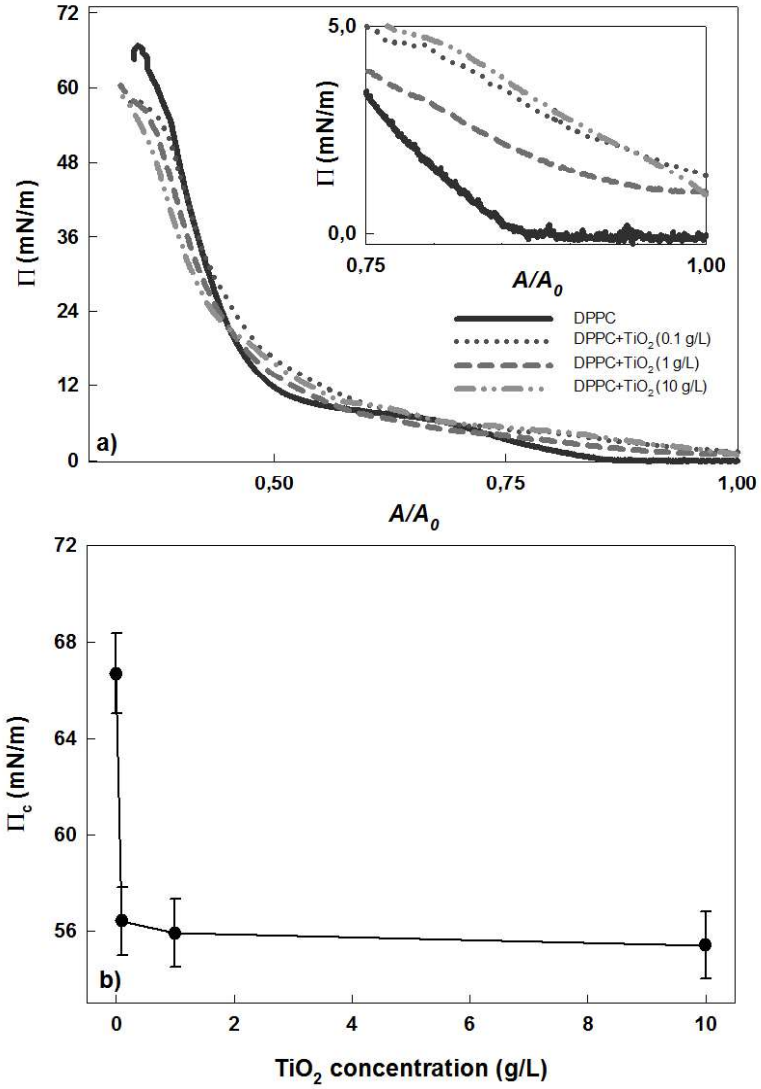

Figure 1. (a) Π-A/A_0 isotherms of Langmuir monolayer for DPPC spread at the water/vapour interface and onto TiO_2 dispersions with different concentrations. The inserted Figure is a spam of low compression degree region. Note that the isotherms are the average of three measurements with reproducibility better than 2 mN/m. (b) Π_c dependence on the TiO_2 concentration. The solid line is a guide for the eyes.

It is worth mentioning that the amount of spread DPPC molecules at the initial state was the same for all the TiO_2 concentration investigated and was chosen in order to ensure the formation of DPPC monolayers in a highly expanded phase. The isotherm for pristine DPPC monolayer obtained in this study agrees well with those previously discussed in the literature.[24, 56-57, 70-71] The main peculiarity of this well-known isotherm is the presence of two plateau in the Π-A/A_0 diagram, one at the low compression region (highest values of A/A_0) and the second one at intermediate compression which correspond to the coexistence of two phases of DPPC, the gas - liquid expanded and the liquid condensed - liquid expanded, respectively. This latter, as usually evidenced by BAM analysis, is characterized by the presence of typical elongated domains of condensed phase. The incorporation of TiO_2 into the DPPC monolayers shifts the isotherms to higher values of A/A_0, leading to an excluded area like effect.[72] This effect is slighter than that previously reported for SiO_2 NPs.[71] The shifting of the DPPC isotherm in presence of TiO_2 is clearer from the inserted Figure in Figure 1. These results show the absence of clear dependences of the shifting of the isotherm on the nanoparticles concentration.

Furthermore, the significant change of the isotherm shape confirms the TiO_2 penetration into the DPPC monolayer, driven by the aforementioned electrostatic interaction, which leads to the trapping of the particles into the lipid layer, already when it is in an expanded phase. The first modification of the compression isotherm of the DPPC monolayers, observed in presence of TiO_2 nanoparticles in the subphase, is the disappearance of the gas-liquid expanded coexistence region at the lowest compression degree (highest values

of A/A_0). In fact, the isotherms of DPPC in presence of TiO_2 NPs show a monotonic increase of the surface pressure with the compression degree from the initial stage of the compression. Therefore, the penetration of NPs leads the DPPC monolayers to a state that can be considered reminiscent of a liquid expanded phase, presenting a lateral cohesion higher than that of DPPC spread on pure water, at the same compression degree. The penetration of NPs reduces the complexity of the phase behavior of DPPC monolayers, being noticeable the hindering of the liquid expanded –liquid condensed coexistence, i.e. the complete disappearance of the coexistence plateau characteristic of pristine DPPC monolayers. Thus, it is possible to assume that the penetration of TiO_2 into the monolayer introduces an important restriction to the monolayer packing, becoming the phase behavior of DPPC similar to that expected for a disordered lipid monolayer. The absence of coexistence regions is also evidence by the almost complete disappearance of condensed domains in the monolayer in presence of NPs as found by BAM imaging (Figure 2). This is explained considering that the driving force for the domains nucleation and growth is the re-orientation of the DPPC dipole at the interface. This observation supports the hypotheses that the incorporation of charged particles in the monolayer alters the electrostatic interaction between lipids modifying, as a consequence, the morphology of the layer. From the BAM images is possible to assume that titania is incorporated mainly as single particles or as aggregates presenting sizes below the wavelength of the laser used for these experiments.

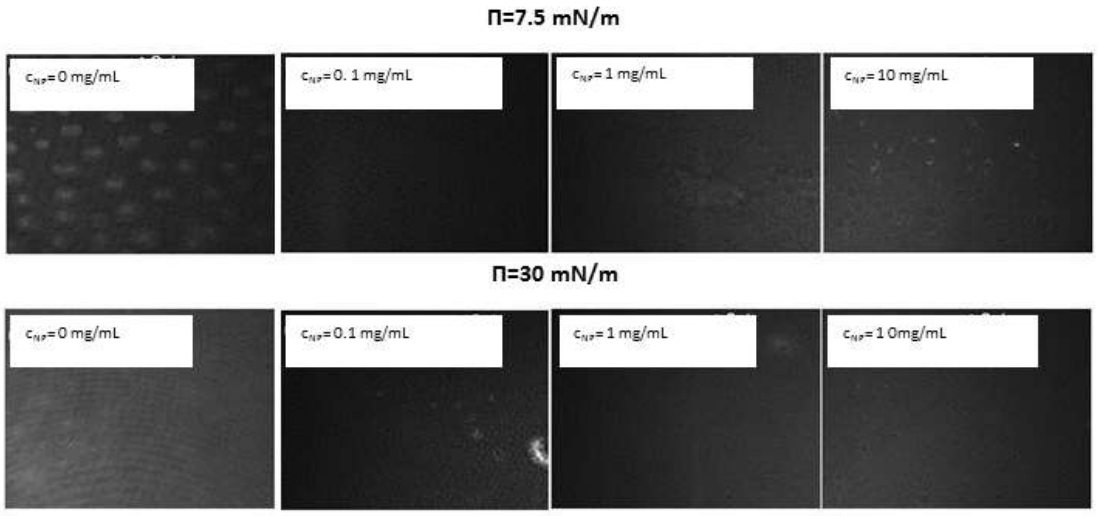

Figure 2. BAM image sequences (images size = 155 μm x 210 μm) of two different *Π* states for pristine DPPC monolayers and DPPC monolayers spread onto subphases with different TiO_2 concentrations.

Paying attention to the concentration effects, no dependence on the NPs concentration was found for the incorporation of TiO_2 into DPPC monolayers. This can be rationalized considering that the effect of the NPs is purely interfacial and the bulk composition does not modify significantly the influence of NPs. Thus, it is possible to consider the existence of a trapping phenomenon of the NPs passing close to the lipid layer, being the maximum capacity of the lipid layer to capture NPs reached for NPs concentration relatively small. Furthermore, the absence of a concentration dependence for the NPs penetration allows one to conclude that the NPs attachment to the lipid layers is not diffusion controlled or, alternatively that the characteristic diffusion time is shorter than the time assumed, in all the conditions studied, for the equilibration of the mixed layers (~ 1 hour). The analysis of the concentration dependence of the collapse pressure, Π_c, of the monolayer (Figure 1b) shows a decrease of Π_c with the incorporation of NPs into the monolayer, with again a rather limited dependence on the nanoparticle concentration. This decrease of Π_c, meaning

that the cohesion of the monolayer is reduced by the presence of the nanoparticles, can be explained assuming that NPs are strongly embedded in the monolayer, causing the disruption of the monolayer structure. NPs, in fact, may act as nucleation sites for the collapse, which is related to the steric hindrance, induced by their incorporation, and to the reduction of the lateral cohesion of the lipid tails. Similar disordering effects have been previously reported for mixed monolayers of DPPC and carbon nanoparticles or fumed silica.[67, 73] However, the strength of TiO_2 as disordering agent seems to be apparently stronger than those found for other particles, independently of their hydrophobicity degree. Despite NPs are strongly embedded at the interfacial layer some refinement of the monolayer composition is found. This means that part of the material (lipid molecules or nanoparticles) is squeezed out from the mixed monolayers to the subphase upon compression. This squeezing-out phenomena is evidenced from the possibility of compression of the mixed monolayer till higher packing density than the pure DPPC.[74-75] This implies that above a compression degree threshold material starts to be expelled from the monolayer (particles, lipid molecules or particle – lipid complexes). However, the monolayer composition refinement is not complete as was found for the case of DPPC monolayers with embedded silica NPs,[71] which is related to differences in the strength of the attachment of the nanoparticles to the fluid interface. The strong embedding of the TiO_2 into the lipid layer can be explained due to the complex interplay of multiple interactions including electrostatic attraction, hydrogen bonding, and direct coordination of the phosphate or carbonyl oxygen of the lipids with available titanium dioxide sites. This confers an extraordinary strength to the TiO_2 NP – lipid bonds,[48, 54, 76-77] being reported in the literature a binding between DPPC and TiO_2 nanoparticles four times higher to that found for DPPC and silica nanoparticles.[54]

The non-efficient re-spreading of the materials expelled from the interface to the subphase

during the compression is associated with the appearance of hysteresis on the monolayer isotherm upon expansion.[78] Figure 3 shows for sake of example the isotherm of a complete compression - expansion cycle for one of the monolayers studied in this work. The information content in these cycles can have an important physiological relevance since the dynamic character of the biological processes. Note that for all the monolayers the compression was carried out until the point in which Π remains constant or starts to decrease. Thus, it is avoided the over-compression of the monolayer.

During the expansion of the surface area, it is expected that the aggregates expelled during the compression tend to re-spread at the water/vapor interface with a slow dynamics, leading to the appearance of hysteresis in the traces of the $\Pi - A/A_0$ curves. The presence of nanoparticles alters this hysteresis due to the fact that the interaction lipids – nanoparticles must be considered when the refinement process of the monolayer is analyzed. Note that the hysteresis observed on the compression – expansion cycles provides information about the expelling of material from the interface during the compression, but such hysteresis does not allows quantifying the existence or absence of complete re-spreading during the expansion step of the expelled materials. The effect of the penetration of nanoparticles in the monolayer during the compression – expansion cycles can be effectively investigated through the normalized hysteresis area, HA_n[79]

$$HA_n = \frac{HLA}{A_{max} - A_{min}} \tag{2}$$

where A_{max} and A_{min} are the maximum and minimum areas of the compression - expansion cycle, and HLA is the area of the hysteresis loop defined as

$$HLA = \left[\int_{A_{min}}^{A_{max}} \Pi(A)dA\right]_C - \left[\int_{A_{min}}^{A_{max}} \Pi(A)dA\right]_E \tag{3}$$

where $\Pi(A)$ denotes temporal surface tension corresponding to the surface area A. The indices E and C refer to surface expansion and compression, respectively. This parameter provides a quantification of the effect of the nanoparticles in the refinement process of the monolayer. Figure 3b shows the values of HA_n referred to that corresponding to pure DPPC monolayers ($(HA_n)_{DPPC}$) for the different monolayers studied in this work. The penetration of nanoparticles into DPPC monolayers increases the hysteresis of the compression – expansion cycle, as evidenced the values of normalized hysteresis area that are almost twice than this corresponding to pristine DPPC monolayers. This is explained considering that the incorporation of nanoparticles modify the refinement of the pristine DPPC monolayer, probably due to the formation of lipid – nanoparticle supramolecular aggregates, slowing down the re-entrance kinetics of the material expelled from the interface during the compression and consequence the re-equilibration process of the monolayer.

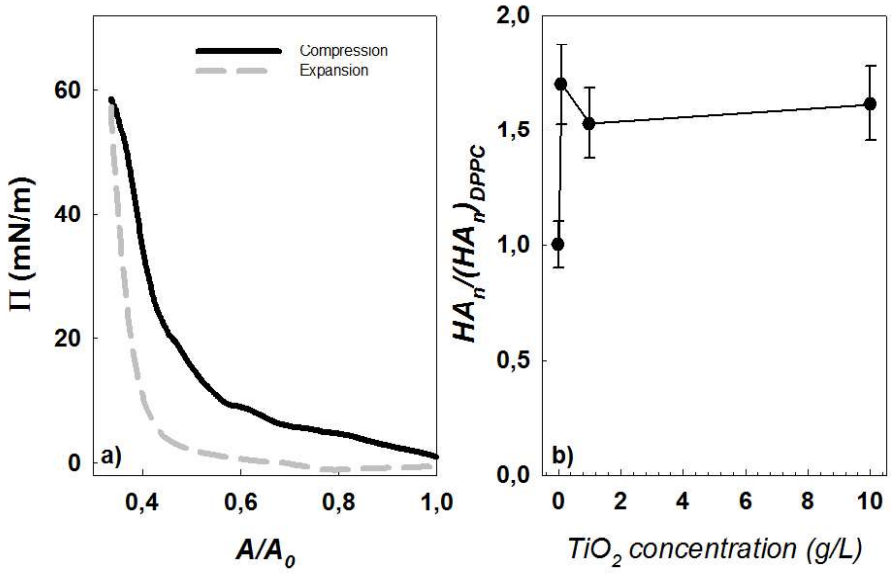

Figure 3. (a) Compression – expansion hysteresis cycle for DPPC monolayer spread onto a subphase containing TiO_2 in concentration 10 mg/mL. Note that the compression – expansion cycles are the average of three measurements with reproducibility better than 2 mN/m. (b) Dependence of the normalized hysteresis area referred to that corresponding to

pristine DPPC monolayers, $(HA_n)_{DPPC}$, on the titania concentration.

Rheological response of DPPC + TiO_2 mixed layers

The analysis of the dependence of the quasi-equilibrium dilatational elasticity, ε_0, on the particle concentration can provide important insights on the interaction between particles and DPPC. For isothermal compression in a Langmuir trough, the quasi-equilibrium dilatational elasticity can be written as[68]

$$\varepsilon = -A\left(\frac{\partial \Pi}{\partial A}\right)_T \tag{4}$$

and can be evaluated by the numerical derivate of the $\Pi - A$ isotherms. The quasi-equilibrium dilatational elasticity for DPPC monolayers spread onto sub-phases containing different NPs concentration is shown in Figure 4a, for sake of comparison the results for pristine DPPC monolayers are also included.

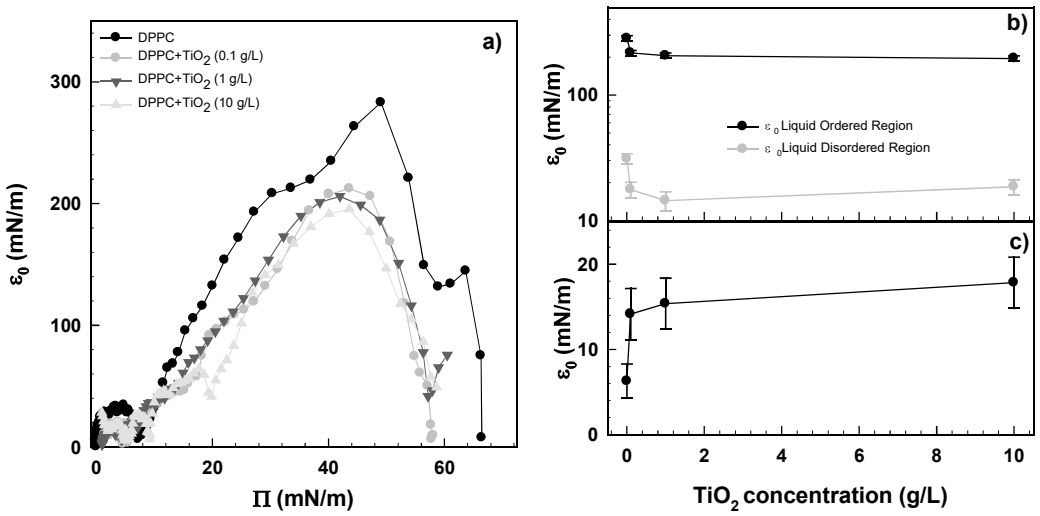

Figure 4. (a) Quasi-equilibrium dilatational elasticity, ε_0, calculated from Π -A isotherms for pristine DPPC monolayers and monolayers spread onto subphases with different TiO_2 concentrations. (b) Dependence of the maximum values of the quasi-equilibrium dilatational elasticity, ε_0, calculated from Π -A isotherms for the Liquid Disordered and the Liquid Ordered Phases phases on the TiO2 concentration. (c) Dependence of ε_0 for Π values similar to those of the coexistence region of pristine DPPC monolayers on the TiO_2

concentration.

Pristine DPPC monolayers present two well-differentiated maxima of the quasi-equilibrium dilatational elasticity against surface pressure. The maximum observed at the lowest surface pressure corresponds to the intrinsically disordered Liquid Expanded (LE) phase whereas the second larger one, at the highest surface pressures, is due to the formation of highly ordered Liquid Condensed (LC) phase. The LC-LE coexistence region of DPPC monolayers presents a typical quasi-null elasticity value.[33, 80]

The incorporation of TiO_2 into the DPPC monolayer does not modify the qualitative feature of the elasticity against the surface pressure, even if the values of both the Liquid Expanded and Liquid Condensed phases are appreciably reduced (see Figure 4b). This is ascribable to the alteration of the packing of the molecules, which reduces the cohesion between the molecules. The modification of the elasticity in the two phases is characteristic of TiO_2 since other types of particles do not affect to the elasticity of the expanded liquid phase, e.g. hydrophilic and fumed silica or carbon black, altering only the elasticity corresponding to the condensed liquid ones,[30] which can be explained again considering the high affinity of lipids for TiO_2 surface.[54] Thus, the effect of TiO_2 on the LC phase is easily explainable considering that the nanoparticles affect strongly the reorientation of the lipid molecules at the interface. This reduces the packing degree of the lipids and consequently the cohesion of the monolayer. The decrease of the elasticity of the LE phase can be also ascribable to a delayed interfacial packing. Moreover, the general hindering of the packing associated with nanoparticles penetration leads to the disappearance of the LE-LC coexistence region found on pristine DPPC monolayers (see Figure 4c), with values of elasticity significantly different to zero.

The points discussed so far concern the effect of the penetration of TiO_2 nanoparticles on the equilibrium properties of the system. However, most of the physiological processes involving biologically relevant systems are dynamics process. In particular, biological systems, e.g. lung surfactant in the alveoli, are subjected to oscillatory compressions and tensions associated with a liquid outflow from the interfacial layer, which plays a central role on the interfacial clearance and ordering of the molecules at the interface.[81-82] Thus, the study of the dependence of the complex modulus of the dilatational viscoelasticity, $|E|$, on the deformation frequency obtained by the oscillatory barrier method can contribute to understand the effect of nanoparticles incorporation on the behavior of lipid layers. Figure 5 shows the dependence of $|E|$ on the deformation frequency, v, at different values of Π for pristine DPPC monolayers and DPPC monolayers spread onto TiO_2 nanoparticles dispersion with different concentration. It is worth mentioning that the low frequency limit is provided by the quasi-equilibrium dilatational elasticity obtained from the numerical derivate of the surface pressure – area isotherm. The frequency associated with this quasi-equilibrium dilatational elasticity can be estimated from the compression velocity $d(\Delta A)/dt$ (2 cm^2/min), with ΔA being the change of the area and t the time, and the total area of the trough A (243 cm^2) as $d(\Delta A/A)/dt \sim 10^{-5}$ Hz. The dynamics, within the frequency range evaluated, of the different monolayers studied is characterized by relaxation processes evidenced by inflection points in the best fit curve. The results point out that the experimental results are well-described by a theoretical model involving an interfacial reorganization process within an insoluble interfacial layer as defined the following expression[68]

$$|E| = \sqrt{\frac{E_I^{\,2} + E_0^{\,2}\lambda_I^{\,2}}{1 + \lambda_I^{\,2}}} \qquad (5)$$

where $\lambda_1 = \nu_1/\nu$ and ν_1 is the characteristic frequency of the process, i.e. the time scale in which the reorganization of material at the interface occurs.[83] E_1 and E_0 are the high frequency elasticity, within the explored range, and the low frequency limit of the viscoelasticity modulus, respectively. The best-fit curves from Equation (4) for different monolayers are reported in Figures 6.

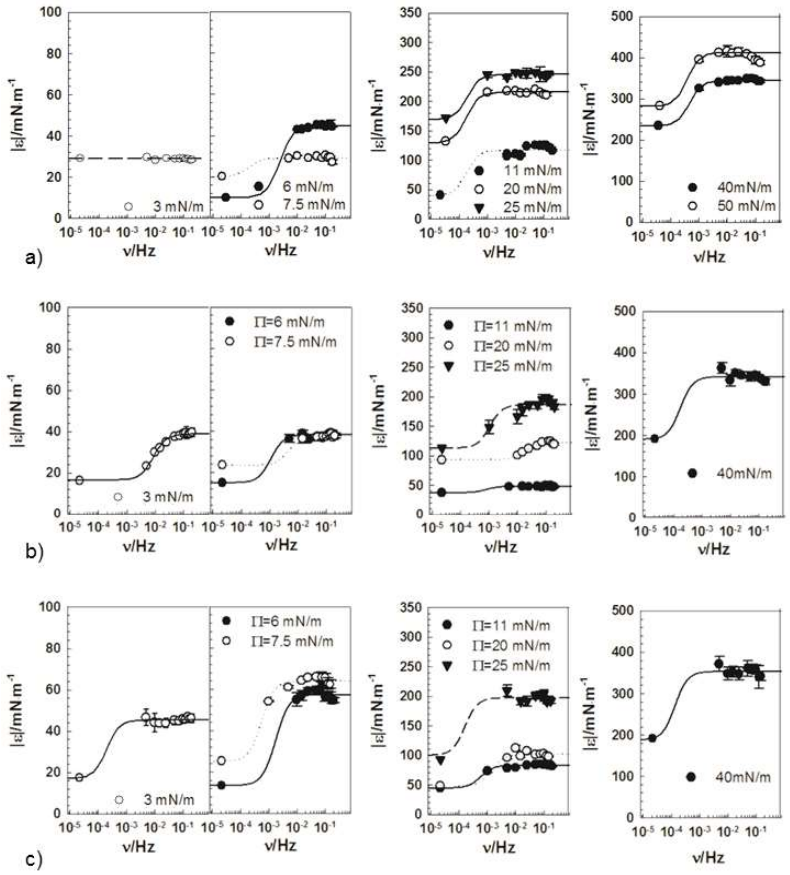

Figure 5. Dilatational viscoelasticity modulus against deformation frequency at different values of surface pressure obtained by oscillatory barrier experiments for pristine DPPC monolayers and DPPC monolayers spread onto TiO_2 dispersion with different nanoparticles concentration. Lines represent the best fit theoretical curves according to Eq. (4). (a) c_{NPs} = 0 mg/mL. (b) c_{NPs} = 0.1 mg/mL. (c) c_{NPs} = 10 mg/mL.

It is important to notice that the penetration of nanoparticles into DPPC monolayers leads to the emergence of a relaxation process for surface pressures around 3 mN/m, which was

not found in pristine DPPC monolayers. This process can be associated with a possible exchange of DPPC molecules between the water/vapour interface and the surface of the nanoparticles that penetrate into the lipid monolayer. The absence of such process in pristine DPPC monolayers is ascribable to the expanded character of the DPPC which can favour a fast molecular reorganization at the interface, being the time-scale of such reorganization beyond the experimental window accessible to our experiments. It is worth mentioning that NPs incorporation into DPPC monolayers modifies the dilatational viscoelasticity limits but, except for, the incorporation of NPs into DPPC layers does not induce the appearance of additional relaxation processes, at least within the frequency range explored. Figure 6 shows the surface pressure dependences of the characteristic relaxation frequency, ν_1 for different NPs concentrations. The evolution of this parameter is rather important since allows evaluating the influence of NPs on the dynamics of biological relevant systems.

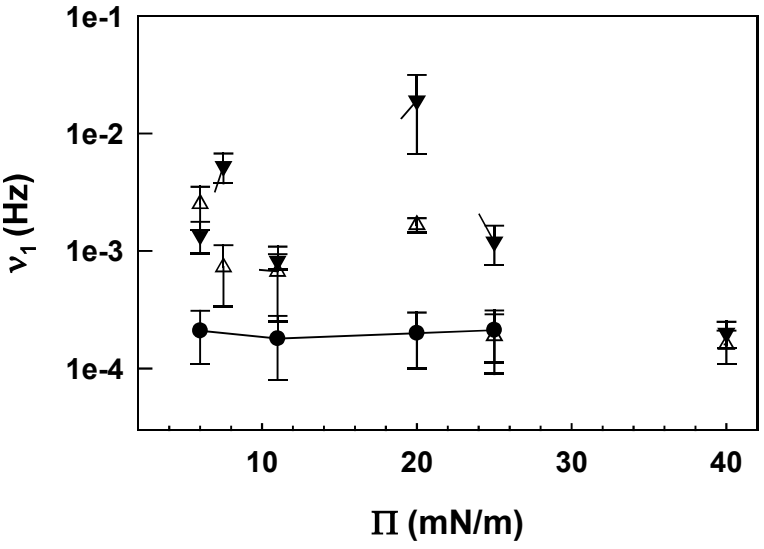

Figure 6. Surface pressuredependences of the relaxation frequency against surface pressure. (●) c_{NPs} = 0 mg/mL. (▼) c_{NPs} = 0.1 mg/mL. (Δ) c_{NPs} = 10 mg/mL.

The penetration of the TiO_2 nanoparticles into DPPC monolayers modifies slightly the characteristic frequency of the relaxation processes found within the monolayer. In most cases, NPs penetration increases the value of ν_1 in almost one order of magnitude, passing from values about 10^{-4} Hz for pristine DPPC monolayers to values around 10^{-3} Hz after nanoparticles incorporation. This increase of the frequency could be associated with the different interfacial processes occurring in pristine DPPC monolayers and monolayers spread onto TiO_2 dispersions. For pristine monolayers, the occurring relaxation processes within the entire phase diagram are associated with the reorganization of lipid molecules within the monolayers, i.e. interfacial diffusion and molecular reorganization. On the other side, after incorporation of NPs, the exchange of DPPC molecules between the particles surface and the interface must be also considered for the explanation of the relaxation of the interfacial layers and consequently the time-scale in which the relaxation occur is slightly modified. It is worth mentioning that for condensed monolayers, the reorganization processes in absence and presence of nanoparticles seem to be similar, this could be associated probably with the modification of the characteristic frequency of the exchange of lipid molecules from the interface and the particle surfaces, appearing decoupled to the other interfacial processes. Thus, for the highest surface pressure both pristine DPPC monolayers and DPPC monolayers spread onto particles dispersions present a relaxation governed by processes with an interfacial origin. Figure 7 shows the values obtained for the elasticity limits.

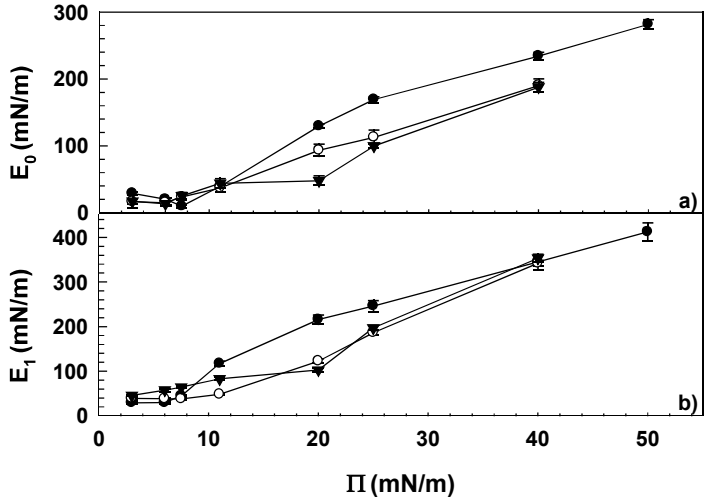

Figure 7. Concentration dependences of the low frequency (a), E_0, and high frequency (b), E_1, viscoelasticity modulus limits obtained from the fitting of the results in Figure 6 to equation (4). (●) c_{NPs} = 0 mg/mL. (▼) c_{NPs} = 0.1 mg/mL. (Δ) c_{NPs} = 10 mg/mL.

The penetration of TiO_2 nanoparticles into the DPPC monolayers reduces the value of E_0 with respect to that of pristine DPPC monolayers. This can be easily understood, considering that, E_0 corresponds to the quasi-equilibrium elasticity, strictly related to the resistance of the monolayer to a slow compression, and such resistance is reduced by the presence of NPs. Also E_1 is found to decrease with the incorporation of nanoparticles. However, the increase of the compression degree of the monolayer leads to values of E_1 similar to that found for pristine DPPC monolayers, without NPs. This can be explained considering the existence of a refinement process of the monolayer composition with the increase of the compression degree.

The rheological investigation presented so far concerns the response of the monolayers to small amplitude of dilatational deformation, ensuring that the response of the system remains in the linear regime. However, most of the processes involving biological relevant systems are non-linear. Thus, the study of the response of the system under non-linear

conditions presents a key role on the understanding the effect of the NPs incorporation on the system behaviour. In order to model this aspect, the monolayers were studied against dilation at a fixed frequency (0.050 Hz), for deformation amplitudes, $\Delta u = A/\Delta A_{0,}$ in the range 0.01 – 0.40. These studies were carried out at a fixed value of surface pressure (~ 40 mN/m) that is considered a reference state for the study of bio-systems such as lung surfactant or biomembranes.[61-63] Figure 8 shows the dependence of the THD on the deformation amplitude for DPPC monolayers spread on pure water or dispersions with different nanoparticle concentration.

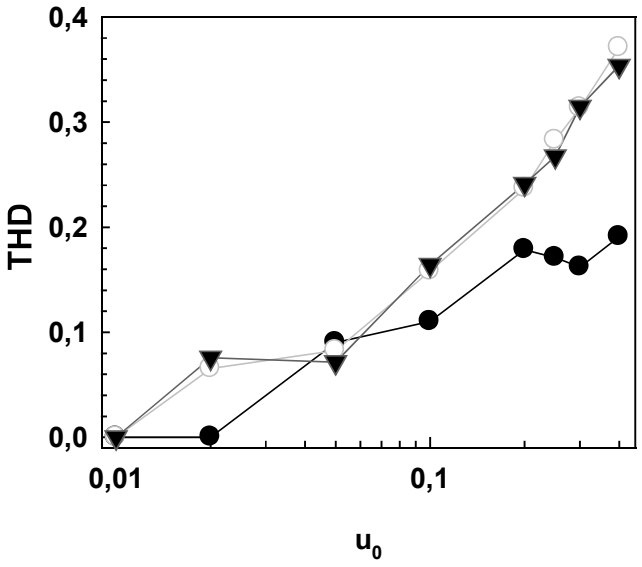

Figure 8. Amplitude deformation dependence of the THD for DPPC monolayers spread at water/vapor interface with different nanoparticles concentrations.(●) c_{NPs} = 0 mg/mL. (▼) c_{NPs} = 0.1 mg/mL. (Δ) c_{NPs} = 10 mg/mL.

As expected, the increase of the amplitude of the dilatational deformation leads to the increase of the THD.[30, 58, 64] Furthermore, the penetration of nanoparticles also leads to the increase of the THD. The increase of the non-linearity in presence of nanoparticles is due to the larger inhomogeneity of the monolayers that leads to the hindering of the lateral

redistribution of the material at the interface.[84] Again no dependence on the NPs concentration was found for the THD. Thus, it is possible to assume THD as a useful parameters for the quantification the potential adverse effects of the nanoparticles on the physiological function.

Conclusions

The effect of the TiO_2 nanoparticles incorporation into DPPC monolayers have been studied using a combination of interfacial sensitive techniques (Langmuir trough and Brewster Angle Microscopy). The study of the equilibrium isotherms of the lipid monolayers in presence of nanoparticles has shown the penetration of the nanoparticles into the lipid layer, without appreciable dependence on the NPs concentration in the range investigated. This induces important excluded area effects that modify the packing of the molecules at the interface and reduces consequently the cohesion between the lipid molecules. In addition, even if some of the trapped particles are squeezed-out from the interface during the compression of the monolayer, the absorbed TiO_2 nanoparticles remains strongly embedded in the lipid layers, affecting both the interfacial structure and the mechanical properties of the lipid monolayers. Furthermore, the incorporation of the nanoparticles alters the viscoelastic response of the lipid layers, modifying the dynamic of redistribution of the interfacial components and the linearity range of the response.

The results presented here have to be considered in a wide framework of investigation focused on the interaction of nanoparticles with model interfaces, relevant for biological systems. In previous works[50-51, 60, 67, 71, 73] particles of different chemical nature and surface state, presenting hydrophilic or hydrophobic character, have been investigated by means of the same surface sensitive techniques used here. The results obtained there evidenced

similar effects to those found in the present work for TiO_2 nanoparticles, e.g. the modification of the monolayer phase behavior with the hindering of the LC domain growth, the increase of the resistance to compression and the lowering of the collapse pressure. In addition, also the effects on the dynamic properties of the monolayer, such as that on the dilatational rheology response, due to the occurring of surface relaxation processes involving particles, and that on the linearity of the monolayer response to area deformation, were already observed. It is worth to notice that these effects presented different degree of importance depending on the nature of the nanoparticles. For examples, the effect on the linearity of the hydrophilic particles is greater than that observed for the hydrophobic ones. Again, the LE-LC coexistence phase tends to disappear for hydrophilic particles while the hydrophobic ones tend to shift the isotherms towards larger surface area (excluded area effects). TiO_2 nanoparticles present feature similar to the previously investigated silica nanoparticles as concerns the hydrophilicity, the size and the surface charge. Nevertheless, even if from a qualitative point of view the same effects were observed for these two kinds of nanoparticles, TiO_2 nanoparticles seem to be much more perturbative of the behavior and properties of the DPPC monolayers. For example, the same concentration of NPs in the sub-phase affects much more the phase behavior of the monolayer with respect to Silica and decrease more the collapse pressure.

From the results of the present work, one can conclude that, the peculiarities of the TiO_2 with respect to other nanoparticles are their complete hindering of the LE-LC coexistence, and the induction of a more disordered structure of the monolayer with lower cohesion between the lipid molecules.

Concluding, even though the lipid system investigated in this work was simply DPPC monolayer, which is a model system rather far from any real system, these results may contribute to the understanding of some general aspects related to the physico-chemical

bases of this type of interactions and may be applied to quantify the potential impact to more realistic systems.

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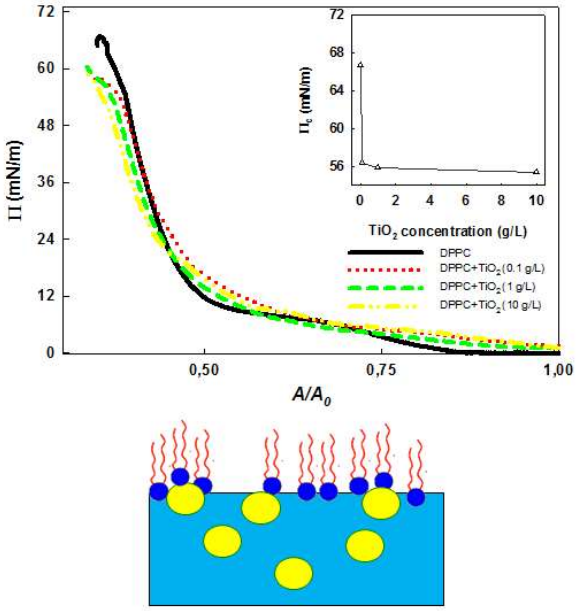
1 **TOC Graphics (For Table of Content Use Only)**



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