Título: AUTOAGREGACIÓN DE TENSIOACTIVOS CATIÓNICOS GEMINI E A SÚA INCORPORACIÓN A FORMULACIÓNS DE NANOVESÍCULAS

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Resumen: The supramolecular field raises a reality that, from the point of view of physics, could be categorized as that of interacting systems in weak coupling. This way of approaching the scenarios at the nano and sub-micro scales is a gateway, in the author's opinion, very suitable for getting into soft matter physics. The nature of this physics lies in its complexity of parameterization, its significant questions, and its versatility and multidisciplinarity. One of the fields in which we find its presence in recent times is nanobiotechnology, an immense field of work in which this and other traditional disciplines converge - biology, medicine, chemistry or mathematics - which is experiencing more and more attention and success. We find in these confluences a magnificent symbiosis: biophysics. This discipline is called to be a space in science with the ability to work on problems and experimental data that would currently represent, in solution, significant advances of humanity. Of all the phenomenologies observed in systems of biophysical interest, self-assembly in the configuration of supramolecular systems turns out to be a common element in countless biophysical processes. Starting from this common position, the author will try to present different results and applications of molecular self-

aggregation systems in this doctoral thesis.

As a first step in the study, it was decided to characterize the self-aggregation of dimeric Gemini surfactants derived from tetradecyltrimethylammonium bromide (C14TAB). To this end, a proposal for modelling the dynamic surface tension recorded with a pending drop sphygmomanometer was put forward. Also, using an isothermal titration calorimeter (ITC), it was possible to analyze the demicelization of the chosen surfactants and propose an alternative criterion for the determination of the critical micellar concentration (CMC). These results were, in part and substantially, collected in an article published in the Journal of Molecular Liquids. Briefly, dynamic surface tension isotherms, for concentrations below CMC, were analyzed from the implementation of a model combining diffusion and adsorption. The diffusion of surfactants inside the droplet was parameterized with the analytical solution of a heat equation (Fick's diffusion law). As for the incorporation of surfactants into the surface, an adsorption model was used that considers the possible lateral interaction between the adsorbate molecules - gemini surfactant - such was the Frumkin isotherm. Finally, a variational relationship between the concentration in the vicinity of the interface and the surface tension has been proposed to complete the mathematical tools that allow the analysis of dynamic stress isotherms. After studying the adsorption at the air/water interface, ITC demicelization measures were used to show the CMC and the different equilibrium regimes in an isothermal titration process. Thus, in addition to identifying the micellar rupture zone below the CMC and the thermodynamic stabilization zone of the micelles above the CMC, an exothermic component was located in the micellar rupture phase associated with a water clustering phenomenon in the vicinity of the broad hydrophobic regions of gemini surfactants. Experiments on demicelization by ITC were performed at different temperatures, verifying classical behaviors in the variation of CMC with temperature and finding the existence of compensation phenomena between enthalpy and entropy in demicelization processes. In this way, a detailed discussion was made on the nature of the different types of energy in demicelization according to the temperature used in the degree process. Thus it was possible to explain phenomena such as endothermic to exothermic energy flow in the mycelial rupture regime.

Two were the techniques used, in detail, in this first block. On the one hand, the pending drop sphygmomanometer, a device optimized to hold a drop of liquid through two coaxial ducts. The droplet formation process is controlled by a titration pump accurately in the sub-microliter. The surface tension information is obtained by applying a mathematical model that analyzes the contour of the droplet, recorded with a camera that is part of the device. A software applies the method of analysis of the perimeter of the drop in real-time, recording the values of surface tension over time, as required by the user depending on the system's behaviour. Also, the drop is generated inside a transparent cell that allows to maintain the liquid-vapour balance with the drop and, at the same time, thermostatize the environment of the drop, thus enabling temperature control in the measurement experiment. On the other hand, the isothermal titration calorimeter is a device that measures the flow of heat between a reference cell and a sample cell in which a titration process is taking place. Due to the titration of a reaction catalyst, the interaction phenomena that occur in the sample cell are recorded by measuring the heat flow between the sample cell and the reference cell, which are also thermostated. A thermocouple-based feedback and compensation device is responsible for recording these heat flows. In this situation, what is titled is a highly concentrated sample of surfactant in a sample cell where there is water. The phenomenon of micellization is subject to the thermodynamic variable of the concentration, and, through consecutive titrations, the concentration of surfactant in the sample cell is increasing. Thus, at first, the recorded heat flux reports demicelization, only to record the micelle dispersion once the CMC is exceeded in the sample

cell. All the titration experience is subjected to the forced and constant thermalization of the reference cell and the sample cell. However, the device will also allow to vary this temperature to set different values of the same, so it was possible to analyze the demicelization process at different temperatures for the same system.

After the study and characterization of the interfacial adsorption properties of gemini surfactants and their micellization, the incorporation of gemini surfactants into lipid bilayers is characterized. On the one hand, the motivation is to analyze the adsorption by changing the air/water interface for a lipid interface; and, on the other hand, to guide the applications derived from the properties of gemini surfactants to the field of nanobiotechnology. The biomimetic and bioapplicability characteristics of lipid bilayers are widely known and reported. Here, different analysis of results showing efficient incorporation of gemini surfactants into the lipid bilayer is presented, and the different characteristics that are imprinted or modified by such insertion or doping are examined in detail.

The lipid bilayer is a phase of self-aggregation that occurs between amphiphilic molecules of a lipidic nature and which differs from the micelle in that there is a break in symmetry concerning it. If in the micelle there is certain isotropy in the distribution of amphiphilic molecules with respect to a centre, in the lipid bilayer there is an anisotropic conformation in which the molecules are distributed along a plane -i.e. XY- and conform in the normal direction to it -i.e. Z- in two layers in opposite directions. This particular form of self-aggregation, with a thermodynamically similar origin to mycelium, represents something as crucial as the first steps in the catalysis of biological systems. This mode of arrangement is what can be found, in general, in the structure of cell membranes of all the primitive biological systems of the realms of nature. More specifically, at present, the application of the generated lipid bilayers in vitro has clear applicability in liposomes.

Liposomes are vesicular structures formed by closed lipid bilayers, and their possibilities are enormous and will be explained in some detail throughout the text. Briefly, liposomes can be used as primitive cell models, as pharmacological transport and release systems, as biosurface functionalizing agents, or as very useful tools in lipoplexing in gene therapies and vaccine technology. In these directions, it is decided to take advantage of the surface activity of gemini surfactants and, including them in the lipid bilayer, to confer properties to liposomes that may be useful in the mentioned fields of applicability.

Using differential scanning calorimetry (DSC), the incorporation of gemini surfactants into the lipid bilayer was tested. Examining the variation of the transition temperature of the different conformational phases of the membrane, it was possible to conclude the role of surfactants in the lipid bilayer due to the variation of their concentration and morphology -varying the length of the gemini spacer-. Transition temperature reflects a property of the membrane that has to do with the distribution of the symmetry of the hydrocarbon chains of lipids. In this sense, a phase transition model based on the Ising paradigm is proposed, and, in this way, the incorporation of the surfactant into the membrane and its role is explained.

Molecular dynamics experiments were performed for the same purpose. Thus, from a molecular point of view, it was possible to determine the different adaptation pathways in the membrane and the different positionings and implications of gemini surfactants in the lipid bilayer by varying their spacer. One of the fundamental variations found by molecular dynamics was the changes in the polarity and electrostatics of the membrane, these being observed and quantified by the technique of elastic and dynamic light scattering (DLS) and application of electric

potentials. This determined the relationship between the adsorption of gemini surfactants on the lipid bilayer and changes in the electrostatic behaviour of liposomes, which resulted in greater colloidal stability even at tiny concentrations of lipid surfactant added to the lipid.

In this second block, we used three experimental techniques to complete the conclusions and modeling of the adsorption of gemini surfactants on the lipid bilayer. The DSC technique examines the heat flow between a sample cell and a reference cell connected to the same thermostatic bath that is varied by following a controlled temperature ramp. Thus, any difference in the heat response of the sample cell to the reference cell will be recorded by a particular implementation of thermocouples between the two cells. In the case of the lipid bilayers studied, there is a divergence in the heat response observed by a representative peak at a specific temperature. This signal stores valuable information on the transition between different conformational phases in the membrane, which will be subjected to a detailed study and detail.

Also, the in silico technique of molecular dynamics was used to obtain information and draw conclusions from the doping of lipid bilayers with gemini surfactants. Thus, computational routines, based on molecular dynamics in the GROMACS programming environment, were used to obtain simulation trajectories during dynamic times on the nanosecond scale. Briefly, computational molecular dynamics solves the equations of motion of parameterized molecules in specific force fields from electrostatic information ¿ i.e. Coulomb- and multipolar -i.e. Lennard-Jones- of the various components of the systems. Similarly, the dynamics of elastic light scattering was used to measure the self-diffusion coefficient and thus the hydrodynamic emberging of liposomes. The coupling of electric fields to DLS also made it possible to measure the electrostatic characteristics of liposomes by analyzing the variation of electrophoretic mobility.

In order to go deeper into the ability to act on the lipid bilayer, results of its interaction with a cationic polymer based on primary, secondary and tertiary amines: polyethyleneimine (PEI) will also be shown. The remarkable ability of this polymer in the lipid bilayer is to generate stable pores that allow water to diffuse between the two layers of the membrane. This phenomenon has been used to hypothesize the PEI's role as an adjunct in transporting complexes based on DNA plexates. Accumulation of PEI was observed in the perinuclear region, of HeLa cells, by confocal fluorescence microscopy. DMPC monolayers were initially used as a nuclear membrane model. Langmuir's balance experiments demonstrated the ability of the PEI to interact with the DMPC. Subsequently, a more evolved membrane model, the lipid bilayer, was used. In this case, the interaction of PEI with liposomes was studied by differential scanning calorimetry and induced release assays measured by carboxyfluorescein fluorescence. Finally, results of molecular dynamics simulation experiments were shown to present details of the dynamics of pore formation in the lipid bilayer induced by PEI.

A third block will be articulated by presenting different nanobiotechnological applications using liposomes doped with gemini surfactants. The gemini surfactants' presence confers positive electrostatic properties to the liposomes used. Once the insertion's dynamics and the conferred properties have been tested, a step further in the system's control is considered. In this sense, different results published in journals such as the Materials Science and Engineering: C will be shown and completed throughout the text. Such an evolution is to propose a supraliposomal system that allows the electrostatic characteristics of liposomes to study their interaction with surfaces. Thus, a system formed by the configuration of a polyelectrolytic substrate, based on the layer-by-layer technique was developed, and the intact incorporation of liposomes into this substrate was shown.

The efficiency in loading and releasing low-solubility molecules has also been proven. As the study of the system progressed, the characteristics of the evolution and cellular interaction of a particular lineage of cells in contact with substrates formed by adsorbed liposomes and buried liposomes were observed and shown. The nature of these polymeric substrates could also be made compatible with systems that mimicked extracellular environments. Thus, different conclusions in the process of cellular communication via extracellular vesicles - represented by liposomes - could be obtained.

Continuing in the progression of the systems' complexity in which these liposomes were used, the adsorption of liposomes to pseudo-three-dimensional systems based on hydrogels was studied and monitored after studying their incorporation into two-dimensional systems. The intention is to use the properties and characteristics of a certain type of hydrogel formed from hyaluronic acid - a negatively charged glycosaminoglycan - to build a surface scaffold that allows the adsorption of cationic liposomes and thus develop a platform to create a determined environment of cell proliferation and differentiation. Thus, the efficient adsorption of liposomes in these hydrogel structures was verified. The drug encapsulation and release that may act as a differentiating factor in mesenchymal cells has also been tested and monitored. Similarly, cytotoxicity measurements were performed using the cationic liposomes studied, all in different cell lines. These processes of increasing the complexity of the systems used were performed using different techniques.

Efficient incorporation of liposomes doped with the surfactant gemini was monitored using the dissipation-measured quartz crystal microbalance (QCM-D) technique. This technique is based on the piezoelectric effect of quartz. This material responds in a certain way by reflecting specific frequencies before applying longitudinal waves. Quartz is functionalized with a gold substrate, which will be modified first with consecutive layers of polyelectrolyte -i.e. poly-L-lysine and hyaluronic acid- and subsequently adsorbing intact cationic liposomes. The ability to store a hydrophobic molecule, as a pharmacological model, was tested using DSC, observing the effects on the transition temperature that are conferred to the bilayer of the liposome by the presence of the model molecule. Encapsulation efficiency and controlled release capacity were determined with dialysis experiments. Detailed specifications and procedures will be explained in detail throughout the text. Exposure to osmotic pressure gradients and different temperatures made it possible to observe the different release regimes in various systems configurations -i.e. liposomes adsorbed to polyelectrolytic substrates or embedded liposomes-. The observed differences between adsorption or incrustation of cationic liposomes in polymeric substrates were also raised in the context of contact with a cell line. MDA-MB-231 breast cancer cells were incubated on different substrates formed as previously described and analyzed with confocal microscopy techniques with fluorescence recording coupling. This technique made it possible to observe the adhesion and proliferation of the cells by observing changes in their morphology and fixation in the substrate with the incubation time.

Concerning the incorporation of liposomes into hydrogel matrices, this fact was observed using DSC, checking for changes in the conformational transition of the lipid bilayer between colloidal dispersion of liposomes or their adsorption to an interface. The efficiency of encapsulation and release of a hydrophobic molecule - dexamethasone - was tested by dialysis experiments and quantified by spectrophotometry coupled to a high-performance liquid chromatography (HPLC-UV) column. The cytotoxicity of gemini doped and dexamethasone-loaded liposomes was evaluated using mesenchymal cells. Taking advantage of the usefulness of

dexamethasone as a differentiating factor in osteoblasts of mesenchymal cells, \textit{in vitro} differentiation experiments were performed.

In a necessarily descriptive way, a block will also be added in which the main experimental techniques and procedures used in the experiments with reported results will be shown. In summary, the methods used were differential scanning calorimetry and isothermal titration, slope drop tensiometry, quartz crystal microbalance with dissipation monitoring, absorption and fluorescence spectrophotometry, dynamic light scattering and simulated molecular dynamics. Also, different experimental procedures, such as dialysis, were made to analyse osmotic pressure drug release profiles. Similarly, extrusion is a high-pressure process in which a dispersion of multilamellar liposomes is forced through a membrane of a given porosity, thus reducing the size and polydispersity of the liposomes. The methodology of liposome synthesis and formulation was assisted by the technique of organic phase roto-evaporation at low pressures and controlled temperature.

In general, the most important conclusions drawn from the results obtained and reported during the text are those related to the properties of physicochemical characteristics that the surfactant gemini implies in its incorporation in the lipid bilayer. Such properties are rapid and efficient electrostatic stabilization, which translates into excellent colloidal stabilization of liposomes doped with gemini surfactant. The main advantage is the tiny amount needed to achieve such a benefit in this sense. More interesting results are those related to samples of the intact incorporation of cationic liposomes into polymeric substrates and their ability in the encapsulation and drug release efficiency. The low cytotoxicity and inferred biocompatibility in cell viability experiments is a result that supports the applicability of these liposomes at the biotechnological level. These overly summarized conclusions, along with the other results shown in the text, present a colloidal system based on liposomes with potential applications in nanobiotechnology; from their use as a model of membranes or extracellular vesicle systems to their use in gene therapies, in controlled release and transport systems, or vaccine technology.

Finally, part of the work was carried out in the Biophysics and Interfaces Group of the Department of Applied Physics of the University of Santiago de Compostela. Another important part was developed in the Colloid and Polymer Physics Group of the Particle Physics department of the same university. Similarly, procedures related to the use of quartz crystal microbalance, polymeric polyelectrolyte layer-to-layer techniques, and cellular experiments with substrate interactions were experiments performed in a research stay at the Biomaterials, Biodegradables and Biomimetics Research Group (i3Bs) from the University of Minho (Portugal). In a parallel, complementary and alternative way, during the work and experimentation of the results compiled in this text, a significant amount of collaborations and scientific work were carried out and supported by this author. In the case of topics related to Soft Matter Physics and nanobiotechnology, the results or procedures used in such work will be cited when necessary because they are considered not only transversal but complementary in practice to everything covered in this thesis.