The Formation of Chimeric Nanomorphologies, as a Reflection of Naturally Occurring Thermodynamic Processes

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The Formation of Chimeric Nanomorphologies, as a Reflection of Naturally Occurring Thermodynamic Processes

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Abstract. The self-assembly process of different in nature biomaterials leads to the morphogenesis of various nano-structures, where the individual molecule properties (*e.g.* hydrophilic-to-hydrophobic balance and elasticity), profoundly affect the intermediate surfaces' interfacial thermodynamics. Herein, the mixing of a phospholipid and an amphiphilic block copolymer, through the thin-film hydration method, gave different morphologies, among which there were vesicles (*i.e.* liposomes and polymersomes), micelles and worm-like structures. The formation of such variety of structures is attributed to divergent entropic pathways, which are determined by a number of parameters, such as the lipid:polymer molar ratio and the polymer composition. The developed nanosystems are considered as chimeric/mixed, because of the two different in type biomaterials that compose them. The vesicles also exhibited membrane "irregularities", which are connected with their biophysical behavior. Nature has "chosen" vesicular forms to be the thermodynamically stable "biological apartments", in which life was enclosed and additionally, vesicles provided compartmentalized systems, where the intracellular environment was built. Phospholipid properties result in membranes/bilayers that harmonically assimilate other molecules, like proteins and retain their integrity and functionality, while gaining additional features. A cause that alters this relationship might induce changes in the membrane composition and morphology, with respect to lipid rafts/domains, what has been linked with the activation and development of certain human disorders/diseases. The self-assembly of two different biomaterials into various structures that present distinct membrane phenomena is believed to simulate these natural processes.

1. Introduction

Since the introduction of the fluid mosaic model of the cell membrane structure by Singer and Nicolson in 1972, it is generally accepted that the gross structure of biomembranes is a bilayer of various phospholipids, mainly in fluid/liquid crystalline state, with incorporated (i) mobile globular proteins and (ii) glycoproteins. Recently, the term "fluid-mosaic membrane model" was proposed by Nicolson, to underline the importance of mosaic, aggregate, raft and domain structures, as well as restraints on the lateral mobility of proteins. Physiological membranes are the result of thermodynamic equilibrium between hydrophobic and hydrophilic interactions of molecules. Moreover, due to their composition, they exhibit functionality, responding dynamically to external stimuli, while retaining their structural integrity [1,2]. However, these stimuli, such as temperature, ionic strength or lateral pressure fluctuations, but also compositional alterations in the membrane e.g. cholesterol content,

through integration, might induce phase segregation and final domain/raft formation. In certain cases, this mechanism is associated with various diseases, like cancer [3].

Liposomes, apart from drug delivery nanosystems, have long been considered as models for biomembranes and are widely utilized in this manner. Because of their simple composition and structure, they facilitate the study of the various functions and interactions that occur in nano-scale, for example how ions and molecules impact on the liposomal membrane, also providing a picture of the pharmacological action of many drug molecules [4]. In addition, important is the fact that liposomes form compartmentalized systems, where transmembrane exchange of components is studied, while copolymers, proteins and transporters can be incorporated to modify the membrane permeability [5].

This paper discusses the effect that certain biomaterials, for example block copolymers, have on the liposomal membrane thermodynamic formation, morphology and final biophysical behavior, based on a previous publication and how these mechanisms and effects might reflect various phenomena occurring on natural biomembranes [6].

2. Experimental Details

2.1. Materials

The phospholipid L-α-phosphatidylcholine, hydrogenated (Soy) (HSPC) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA) and used without further purification. Chloroform and other reagents were of analytical grade and purchased from Sigma-Aldrich Chemical Co. The poly(2-(dimethylamino)ethyl methacrylate)-b-poly(lauryl methacrylate) (PDMAEMA-b-PLMA) amphiphilic diblock copolymer was provided in two different molar compositions, 70-30 for PDMAEMA-b-PLMA 1 and 60-40 for PDMAEMA-b-PLMA 2.

2.2. Methods

The methods utilized for the study, including the polymer synthesis, the preparation of vesicular nanosystems, as well as their morphology elucidation through electron microscopy, are briefly presented here. Full information on the methods are available in the previously published work [6].

2.2.1. Polymer Synthesis. The PDMAEMA block was prepared first and then used as a macromolecular chain transfer agent for the synthesis of the second PLMA block, through RAFT polymerization. The molecular weight (M_w) of the diblock copolymer was 8,900 and 10,800 and the M_w/M_n value was 1.16 and 1.19 for PDMAEMA-b-PLMA 1 and PDMAEMA-b-PLMA 2 respectively, determined by size exclusion chromatography (SEC).

2.2.2. Vesicle Preparation. Different chimeric formulations of HSPC:PDMAEMA-b-PLMA 1 and 2 were prepared, using the thin-film hydration method. Briefly, appropriate amounts of HSPC and PDMAEMA-b-PLMA 1/2 (9:0.1 and 9:0.5) were dissolved in chloroform/methanol (9:1 v/v), to form the film, which was then hydrated with PBS (pH = 7.4), above the phase transition temperature of the lipid (52 °C for HSPC), for a total concentration of 5 mg/mL. The resultant chimeric systems were subjected to probe sonication and allowed to anneal.

2.2.3. Cryogenic transmission electron microscopy (cryo-TEM). Cryo-TEM analysis of the chimeric systems was performed with a Tecnai F20 TWIN microscope (FEI Company, USA). Specimen preparation was done by vitrification of the aqueous suspensions on grids with holey carbon film. Prior to use, the grids were activated for 30s in oxygen plasma, using a Femto plasma cleaner (Diener Electronic, Germany). Cryo-samples were prepared by applying a droplet (2.1 μL) of the solution to the grid, blotting with filter paper and immediate freezing in liquid ethane, using a fully automated blotting device Vitrobot Mark IV (FEI Company, USA). After preparation, the vitrified specimens were kept under liquid nitrogen until they were inserted into a cryo-TEM holder and analyzed at -178 °C. Pictures were processed using ImageJ software.

3. Results and Discussion

3.1. Classes of polymer-induced self-assembled nanomorphologies and membrane phenomena, observed by cryo-TEM

The types of nanostructures that we observed were dependent on the nanosystem composition and included mainly vesicular conformations (*i.e.* liposomes and polymersomes), but also disk-like structures and worm-like micelles (Figure 1 and 2). In fact, it was not until very recently that the latter were observed for chimeric/mixed nanosystems of phospholipid and polymer [7,8]. In addition, in terms of membrane electron beam contrast, the vesicles were further categorized to "homogeneous membrane" or "heterogeneous membrane" ones and the membrane morphology was assessed, with respect to biophysics and thermodynamics of membranes [9].

Concerning vesicular membrane morphology, two phenomena were observed, which were interpreted based on either the experimental conditions or the intermixing of the two different biomaterials and the interactions between their instantly formed surfaces during self-assembly. In particular, liposomes of diameter between 70 and 80 nm, presumably possessing anchored polymer chains on their outer surface, presented angle-shaped/faceted membrane, while darker membrane segments were also visualized. By combining these phenomena with previously extracted differential scanning calorimetry (DSC) results on the incorporation of block copolymers inside the phospholipid membrane, we can assume that binary and higher systems might exhibit altered membrane morphologies in different physicochemical conditions [10]. This mimics the responsive natural membrane, which has achieved and maintains an integral structure, in spite of its great complexity, which leads to thermodynamic instability and the great complexity in the surrounding environment, which constantly bombards the membrane with stimuli fluctuations.

Apart from vesicles, worms emerged in most nanosystems, depending on the polymer content and hydrophobicity, highlighting the strong lyotropic effect of biomaterials on membrane morphogenesis, even at low concentrations (Figure 1c, 1d, 2b, 2c, 2d). This effect is defined by the hydrophilic-to-hydrophobic balance, molecular weight and elasticity of the polymer, which in turn affect the energy and tension of resulting nanosurfaces, leading to mismatch between lipid and polymer surfaces [7,8]. It is important to point out that the self-assembled process and the resulting morphologies depend on the concentration of the biomaterials used. In nature and in the development process of artificial cell membranes, like liposomal membranes, there is a hierarchical selection of which particular biostructures are predominant, based on that concentration. According to the information theory by Shannon and to the entropic approaches, information is the statistically predominant direction of the organization process of the system, based on the hierarchical selection of which biosystem is considered as necessary for survival [11]. By changing the concentration of the biomaterials, the information approach changes and consequently, this selection is reconsidered.

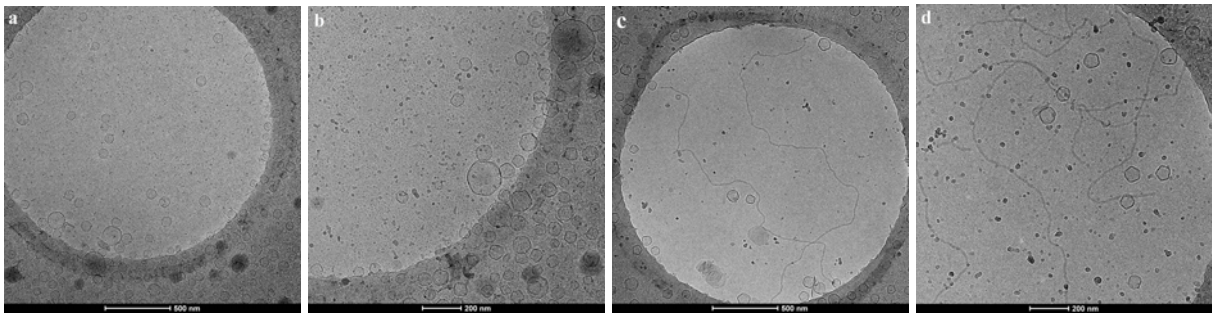

Figure 1. Formation of nanostructures by mixing phospholipid and the more hydrophilic polymer PDMAEMA-b-PLMA 1, in 9:0.1 (a,b) and 9:0.5 (c,d) molar ratios.

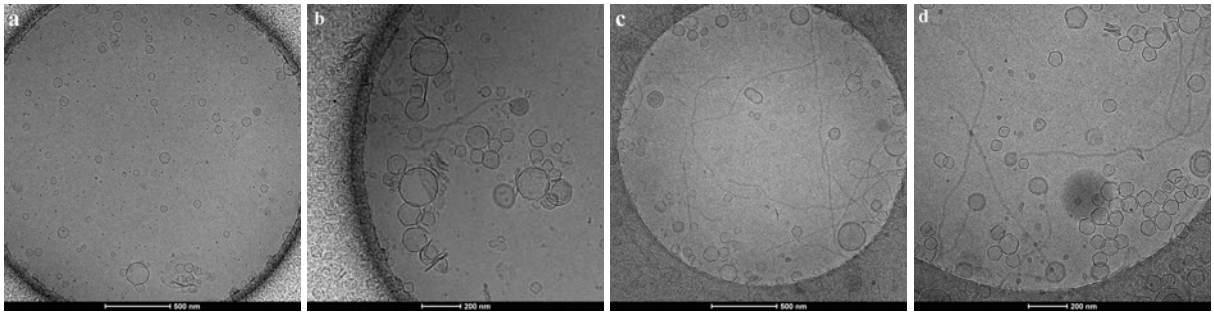

Figure 2. Formation of nanostructures by mixing phospholipid and the more hydrophobic polymer PDMAEMA-b-PLMA 2, in 9:0.1 (a,b) and 9:0.5 (c,d) molar ratios.

3.2. The insertion of foreign molecules in the liposomal bilayer, as a driving force for the thermodynamic formation of divergent membrane morphologies and irregularities, associated with their biophysical behaviour

Our theory is that the hydrophobic parts of the polymers tend to segregate inside the membrane and this leads to the formation of raft-like nanodomains, which might alter the apparent morphology of the membrane and induce for example angles and flat surfaces. Of course, it has been reported that vesicles form these highly curves areas, which were proposed to accommodate fluid lipids, depending on their size and lipid length. However, we propose that the hydrophobic and lyotropic effect induced by the incorporated polymer chains modify this behavior, maybe promoting it [12,13]. A specific study suggested that high curvature (small size) hinders the packing of vesicles in the $P_{\beta'}$ rippled phase and drives them directly to the gel-like $L_{\beta'}$, during freezing from the L_a liquid crystalline phase [14]. At the same time, DSC experiments have already led to a plethora of results, which prove that, depending on the lyotropism of biomaterials, amphiphilic block copolymers' and various other molecules' insertion inside the liposomal membrane diminishes the pretransition, that is the intermediate $P_{\beta'}$ phase, over a certain threshold of molecule concentration [10,15]. This approach, combined with the high main transition temperature T_m of HSPC, could mean that the polymers are inserted inside the membrane during hydration, form polymer rafts and during relaxation/cooling, promote the formation of these fluid lipid-containing angular segments, even in larger vesicles.

On the other hand, polymer insertion has also been reported to produce various metastable phases in the lipid membrane. These metastable phases are responsible for the variability of biosystems' parameters that cause biophysical and thermodynamic "irregularities". These "irregularities" could be defined as *"biophysical disease factors"*, as mentioned in the recently published monograph by Demetzos [16].

The morphological configuration of nanosystems emanates from the thermodynamics of the intermixed biomaterials and defines their biophysical behavior, affecting their biological stability, since they interact with circulating components in the organism and as a result, their final biological effectiveness. An example for that is the impact of nanoparticle shape on the organ accumulation and toxicity over time [17]. The other discussed aspect of their biophysics is how domain/raft formation affects their interactions with other membranes and substances, rendering them appropriate for studying various human diseases [3]. Hence, the same phenomenon that might facilitate or hinder effective drug delivery, might as well shed light on how physiology creates normal membranes and why/by what ways it activates surface abnormalities, which induce pathogenesis.

4. Conclusion

Domains, rafts and metastable phases in phospholipid bilayers play an important role in their biophysical behavior. Proteins have also been detected to be associated with rafts and are found to be related with human diseases. From a biological point of view, it can be assumed that innovative approaches for fighting human diseases (*i.e.* viral infections, lipid storage diseases, cancer etc.) will arise through the understanding of rafts in biological membranes [3]. The complex behavior of lipid

mixtures in membranes and the additional action of molecules (*e.g.* polymers and proteins) that leads to the formation of membrane domains/rafts and metastable phases have been postulated for a long time. Today, it is known that both the hydrophobic lipid chains, as well as their hydrophilic head groups, together with membrane proteins, are important in inducing *clustering effects* on membranes. Such *clustering effects* can also be mimicked by introducing non-lipid synthetic molecules, making liposomes even more attractive models for studying the thermodynamic formation of natural membranes.

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