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Towards bio-silicon interfaces: Formation of an ultra-thin self-hydrated artificial membrane composed of dipalmitoylphosphatidylcholine (DPPC) and chitosan deposited in high vacuum from the gas-phase

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The recent combination of nanoscale developments with biological molecules for biotechnological research has opened a wide field related to the area of biosensors. In the last years, device manufacturing for medical applications adapted the so-called bottom-up approach, from nanostructures to larger devices. Preparation and characterization of artificial biological membranes is a necessary step for the formation of nano-devices or sensors. In this paper, we describe the formation and characterization of a phospholipid bilayer (dipalmitoylphosphatidylcholine, DPPC) on a matrix of a polysaccharide (Chitosan) that keeps the membrane hydrated. The deposition of Chitosan (~25 Å) and DPPC (~60 Å) was performed from the gas phase in high vacuum onto a substrate of Si(100) covered with its native oxide layer. The layer thickness was controlled in situ using Very High Resolution Ellipsometry (VHRE). Raman spectroscopy studies show that neither Chitosan nor DPPC molecules decompose during evaporation. With VHRE and Atomic Force Microscopy we have been able to detect phase transitions in the membrane. The presence of the Chitosan interlayer as a water reservoir is essential for both DPPC bilayer formation and stability, favoring the appearance of phase transitions. Our experiments show that the proposed sample preparation from the gas phase is reproducible and provides a natural environment for the DPPC bilayer. In future work, different Chitosan thicknesses should be studied to achieve a complete and homogeneous interlayer.

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I. INTRODUCTION

The recent combination of nanoscale developments with biological molecules for biotechnological applications, giving birth to the field of bionanotechnology, has produced applications as diverse as transport devices for drug delivery, carriers of small molecules and radioactive material, templates for pharmaceutical design, antioxidant, chemotactic, and neuroprotectants, among others.1–6 Thus, the final aim of bionanotechnology could be defined as the creation of highly functional nano-scale systems with broad applications in biotechnology.3,7 In spite of its promising applications, extensive basic research needs to be done in order to obtain a detailed understanding of the physicochemical properties that govern the interactions between inorganic and organic/biological molecules.8 Also, the biological effects of these nanoscale developments need to be addressed before full integration with living tissues can be achieved. The design of interfaces between solid surfaces and biological molecules such as membranes and/or proteins using Si(100)/SiO2, also known as bio-silicon interfaces, is an important and rapid developing area of both scientific and applied research. The main goal of these research efforts is related to the design and implementation of solid-supported systems that could serve as non-denaturing matrices for the immobilization of soluble and membrane-spanning proteins under native conditions such as enzymes, receptors, and ion channels. Current research has shown that when proteins are directly supported over solid surfaces, partial unfolding of the protein may occur, due to non-bonding interactions with the inorganic support, impairing protein function.9–11 Thus, a “bio-mimetic” environment for the membrane-spanning protein must be provided. A soft hydrophilic polymer cushion could help to provide such an environment by keeping the lipid-protein membrane hydrated and separating it from the solid support12 (and references therein). Several candidates to be used as soft-cushion polymers are currently under research, such as dextran, hyaluronic

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acid, and other polysaccharides.\textsuperscript{10,12} Also, several methods have been proposed for the preparation of stable polymer-membrane composite films and for the anchoring or coupling of membrane proteins to these bio-mimetic interfaces on solid surfaces.\textsuperscript{12–14}

Chitosan (CH) is a linear polysaccharide obtained by the deacetylation of chitin, which can be found in the shells of crustaceans, exoskeletons of insects, fungi, and plants,\textsuperscript{15} thus being very easy to obtain from nature at low cost. This compound has interesting features, such as biocompatibility,\textsuperscript{16–18} biodegradability,\textsuperscript{17,18} non-toxicity,\textsuperscript{18} high molecular weight,\textsuperscript{19} and antimicrobial activity,\textsuperscript{20} among others.\textsuperscript{21–24} These characteristics have been widely used in various fields of applications: manufacture of artificial skin,\textsuperscript{24} wound healing,\textsuperscript{25} biosensors,\textsuperscript{26,27} controlled drug-release,\textsuperscript{26,28} agriculture,\textsuperscript{29,30} and water treatment.\textsuperscript{20} The porous nature of CH\textsuperscript{31,32} together with its insolubility in water\textsuperscript{33,34} permits its application as a hydrating support. In accordance, a hydrated CH matrix could be used to stabilize a phospholipid bilayer. One of the most studied phospholipids is DPPC, because it is the major constituent of the pulmonary surfactant,\textsuperscript{35} being also one of the favorite constituents of liposomes used as biological membrane models\textsuperscript{36,37} (and references therein). The structure and temperature-dependent phase transitions of DPPC are well known. These phases are: crystal phase subgel (L\textsubscript{c}), gel phase (L\textsubscript{g}), ripple phase (P\textsubscript{r}), fluid crystalline (L\textsubscript{c})\textsuperscript{38,39} and fluid disordered.\textsuperscript{40} Different phases of DPPC can be easily detected by observing enthalpy variations under temperature changes while embedded in a hydrating environment, which could be provided by CH. The most common techniques for the formation of CH films are spin coating and dip coating\textsuperscript{31,41,42} both lacking fine thickness control ($\geq$500 Å). Electro-spinning is another technique used for the formation of highly porous fibers, characteristics that could be used in different applications.\textsuperscript{43–45} However, until now it has been very difficult to find a technique that easily permits the formation of thin films under precise thickness control.

The focus of this research is to use CH to produce a hydrating support that could maintain the structure of a DPPC bilayer while using an inorganic surface as basal support. In order to achieve this goal, both thin films of CH and DPPC were evaporated over a silicon wafer via Physical Vapor Deposition (PVD) to produce the Si/SiO\textsubscript{2}/CH/DPPC membrane shown in Fig. 1.\textsuperscript{46} The film thickness was monitored by Very High Resolution Ellipsometry (VHRE). The use of CH as a hydrating matrix, due to its porous structure, provides a suitable environment for the maintenance of the structure of the DPPC bilayer. These conditions also allowed us to conduct a thermal characterization of phase transition temperatures for the DPPC bilayer. Structural characterization was carried out using VHRE and Atomic Force Microscopy (AFM), applying heating and cooling cycles.

II. EXPERIMENTAL METHODS

A. Materials

Low viscosity CH from shrimp shells (deacetylation degree $\geq$75\%) and DPPC, 1,2-dipalmitoyl-sn-3-phosphoglyceroychololene, (\textsuperscript{≥}99\%) was acquired from Sigma Aldrich (St. Louis, MO and Milwaukee, WI, USA). Pure water for chromatography was imported by Merck (Darmstadt, Germany).

Silicon wafers (100) covered with their native oxide layer (15 to 25 Å) were obtained from Virginia Semiconductor Inc. For silicon wafer cleaning, H\textsubscript{2}SO\textsubscript{4} (sulfuric acid 95\%–97\%) and H\textsubscript{2}O\textsubscript{2} (hydrogen peroxide 30\%), both acquired from Merck, were used in a 7:3 ratio (piranha chemical bath).\textsuperscript{48} A jet of ultra-pure nitrogen gas, N\textsubscript{2} (99.995\% purity) (AGA/Linde, Santiago, Chile) was used for substrate drying.

B. Film deposition

CH was allocated in a Knudsen cell inside the evaporation chamber, without any further treatment (out of the box). The evaporation process was performed in high vacuum ($\sim$10\textsuperscript{−6} Torr) and at 90 °C approx. DPPC evaporation was performed under the same conditions but the cell temperature was changed to 80 °C.\textsuperscript{49} Substrate temperature ($\sim$21 °C) and deposition rate ($\sim$1 Å/min) have been experimentally studied previously in order to obtain homogeneous films (data not shown). The sample topography was determined by AFM and the non-decomposition of CH and DPPC was verified by Raman spectroscopy. The optical thickness of each thin film was monitored in situ during depositions with VHRE by tracing the absolute ellipsomeric polarizer angle P of the sample and by comparing it to the corresponding angle P\textsubscript{0} of the clean substrate, using the Drude model for single layers and the ellipsometric parameters $\Delta$ and $\psi$, which are related to the measured polarizer P ($\Delta = 2P + 90^\circ$) and analyzer A ($\Delta = \psi$) angles.\textsuperscript{50} After deposition, the vacuum chamber was vented to atmospheric pressure at a low rate, in order to assure that no compound detachment occurs due to strong air flow.

C. Characterization

The VHRE with a single wavelength laser (He–Ne, 632.8 nm) in polarizer, compensator, sample, analyzer
The objective lens of the microscope was an Olympus Mplan a backscattering geometry, where the incident beam is linear. Data acquisition time was 10 s. This Raman microscope uses a 5.5 mW, 632.8 nm He–Ne laser in combination with a visible light spectrometer and an Olympus optical microscope. The homemade scanning probe microscope at Pontificia Universidad Católica de Chile (Professor Dr. Guido Tarrach), based on hardware and software developed at the Güntherodt Group at Basel University, Switzerland, was operated in intermittent contact mode. Pyramid-shaped tips were used, with dimensions: height 7 μm, length 225 μm, and width 38 μm, resonance frequency 190 KHz, force constant 48 N/m, and tip radius <10 nm (model Tap190-G), obtained from Budget Sensors, Sofia, Bulgaria. To obtain the highest lateral resolution, the image was taken with 256 × 256 pixels and a scan rate of one line/2 s. In addition, we used the new commercial AFM NanoWizard 3 from JPK Instruments, available at the Physics Institute of the P. Universidad Católica de Chile.

III. RESULTS AND DISCUSSION

A. Raman spectroscopy

Raman spectra were taken to assure that the evaporated CH and DPPC layers did not suffer decomposition during deposition from the gas phase. Figures 2(a)–2(c) compare fresh “out of the box” bulk CH with evaporated CH on a Si substrate and intentionally burned CH from an evaporation process in air. The spectra (Fig. 2(b)) indicate that the evaporated thin CH film shows some characteristic peaks of fresh bulk CH. The peak at 1115 cm⁻¹ can be attributed to vibrations of saturated ester groups or C–C stretching vibrations,⁵¹, ⁵² the 1449 cm⁻¹ peak corresponds to methyl group CH₃ bending⁵² the 1607 cm⁻¹ peak corresponds to C–C ring stretching vibrations,⁵¹ while the 2921 cm⁻¹ peak is caused by C–H symmetric stretching modes of vibration.⁵¹, ⁵² Following the same logic, in Figures 2(d)–2(f) we compared the Raman spectra of fresh “out of the box” bulk DPPC with an evaporated DPPC film and with the Si substrate. Again, for the evaporated DPPC film (Fig. 2(e)) we obtained the same characteristic Raman peaks as for fresh bulk DPPC plus the characteristic peak of the Si-substrate at 950 cm⁻¹. The Raman peaks detected with our instrument for both bulk DPPC and evaporated thin-film DPPC, coincide very well with the Raman spectrum and peak assignment reported by Fox et al.⁵⁹ For comparison, Figure 2(f) shows the Raman peaks of the clean Si-substrate coated with its native oxide layer. These tests are the first confirmation that it is possible to deposit CH and DPPC from their gas phase onto a solid substrate without decomposition of the corresponding molecules.

B. Very high resolution ellipsometry

In Fig. 3(a), we show the variation of the polarization angle (dP) as a function of temperature in the artificial
The transition temperatures at \(\sim 28^\circ C\), \(\sim 42^\circ C\), and \(\sim 55^\circ C\) correspond to the gel to ripple (33 \(^\circ C\)), \(40\) ripple to fluid crystalline (41 \(^\circ C\)), \(40\) and fluid-disordered phase (53 \(^\circ C\) to 60 \(^\circ C\)), \(40\) respectively (Table I).

Phase transitions in the Si/SiO\(_2\)/CH/DPPC artificial membrane can only take place in a moist or wet environment. That is valid especially for the ripple phase (metastable phase), which is characterized by a change from an almost flat surface to a washboard-like structure.\(^{49,55-60}\)

In a previous publication, we studied the hydrated DPPC bilayer without CH (Si/SiO\(_2\)/DPPC).\(^{49}\) In this case, dP decreased during a temperature ramp from room temperature to \(\sim 49^\circ C\) and started to grow as temperature was further increased. We relate this observation to the evaporation of water, thus indicating that hydrated DPPC deposited on Si/SiO\(_2\) has a low capability to retain water. However, in the current system (Si/SiO\(_2\)/CH), we observe that a 25 Å CH film does not increment the dP value during a heating ramp (Fig. 3(b)). This means it is able to retain water even at high temperatures (150 \(^\circ C\)). From these measurements we conclude that the steps observed in the decreasing dP curve in Figure 3(a) are related to phase transitions in the DPPC bilayer, without any important background—increasing of the dP curve—due to loss of water. From previous studies with VHRE,\(^{61-63}\) we know that the Si/SiO\(_2\)/DPPC system without hydration does not show any phase transitions neither at temperatures above \(\sim 49^\circ C\) nor during cooling ramps, due to dehydration. However, measurements on the Si/SiO\(_2\)/CH/DPPC artificial

### Table I. Phase transitions temperatures obtained with VHRE.

<table>
<thead>
<tr>
<th>Artificial membrane</th>
<th>Thickness (Å)</th>
<th>VHRE (^{\circ C})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Si/SiO(_2)/CH/DPPC</td>
<td>Gel/ripple</td>
<td>Ripple/fluid</td>
</tr>
<tr>
<td>Heating ramp</td>
<td>26–56</td>
<td>28</td>
</tr>
<tr>
<td>Cooling ramp</td>
<td>29</td>
<td>41</td>
</tr>
</tbody>
</table>
FIG. 4. Topographies measured with a homemade AFM in intermediate contact mode. Above: topographical images. Below: AFM cross-sections. The topographical images show the behavior of the Si/SiO₂/CH/DPPC membrane’s surface during the heating cycle. The images clearly show a decrease in surface roughness, which we attribute to phase transitions.

membrane show that CH is capable to retain water and also to hydrate the DPPC bilayer, which otherwise would not show any phase transitions or steps in the ellipsometric dP.

C. Atomic force microscopy

The observed topographical changes in the AFM images confirm the phase transition temperatures obtained from ellipsometric measurements. The images in Figs. 4(a) and 4(b) (27 °C and 33 °C, respectively) show the DPPC membrane in its ripple phase. Increasing temperature causes the cluster’s height to decrease, while the “foot-print” or diameter increases due to the raise in molecular movement, indicating the beginning of cluster melting. In Fig. 4(c) (43 °C), most of the clusters have melted, indicating the transition to the fluid phase while it is also possible to observe an important decrease in roughness, where the agglomerations or clusters begin to disappear. In Fig. 4(d) (47 °C), the film appears very
flat and only few clusters can be observed. We ascribe this to a type of intermediate ordering of the molecules between the fluid phase and the fluid-disordered phase.

Another approach to detect phase transitions is the observation of interference patterns in AFM images. The infrared AFM-laser light reflected from the tip of the cantilever and from the sample underneath the cantilever causes the interference in the electronic feed-back loop, observed in Figs. 4(c) and 4(d). This interference phenomenon appears in our home-made setup only for very smooth surfaces. In this way, we see that Fig. 4(c) (43 °C) shows areas of the surface—which are somewhat smoother than others—where no interference is observed. In Fig. 4(d) (47 °C), the surface appears totally flat as the sample is in the fluid crystalline phase, with the exception of a few small clusters. However, in Fig. 4(e) (54 °C), it is not possible to observe any particle, cluster, or any type of ordering of the molecules. We ascribe this behavior to the complete melting of the membrane, attributed to the transition from the fluid crystalline phase to the fluid disordered phase, where the tip of the cantilever—even in AC mode—is capable to scratch the surface and cannot detect any difference in the height of this fluid surface.

In Fig. 5, we present two images taken before and after a temperature change at room temperature. AFM images taken with the JPK device show that the CH/DPPC samples do not change their overall morphology. We can observe that the surface of the clusters is not modified and only the roughness decreases along the entire surface. The latter could be caused by an annealing process, which can also be observed in Figs. 4(c) and 4(d) (43 °C and 47 °C, respectively).

IV. CONCLUSION

This article is the first report of CH being deposited from the gas phase onto a solid substrate and under precise in situ thickness control by VHRE. This is a methodological change from the usual “top-down” approach (i.e., from thick to thinner films) to a “bottom-up” one (i.e., starting from the clean substrate). The traditional “top-down” methods are not appropriate for our purposes, because the thinnest films produced with other methods are still too thick (∼500 Å). Our method allows us to deposit CH films of any thickness, starting from a clean substrate. With this technique we were able to build a self-hydrated artificial membrane (Si/SiO2/CH/DPPC), formed by a DPPC phospholipid bilayer (∼60 Å thickness) and a CH layer (25 Å average thickness).

The characterization of these systems was obtained by VHRE and AFM, applying heating and cooling cycles to determine both stability and phase transition temperatures of the DPPC bilayer. The existence of the observed phase transitions in DPPC bilayers indicates that this method is appropriate to build an artificial support that simulates natural conditions for such bilayers. These examples probably hold as well for other types of phospholipids (e.g., 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-palmitoyl,2-oleoyl-sn-glycero-3-phosphocholine (POPC), and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC)).

The DPPC film shows phase transitions only while hydrated. Therefore, the observation of the gel/ripple (∼28 °C), ripple/fluid crystalline (∼42 °C), and the fluid crystalline/fluid disordered (∼55 °C) phase transitions during controlled temperature cycles indicates that the system Si/SiO2/CH/DPPC (humidified) forms a stable artificial membrane. VHRE measurements confirm that the hydrated artificial membrane—containing CH—does not lose water during the heating cycle, because the otherwise expected increase of dP is not observed, even at temperatures above 100 °C. In our VHRE setup, hydrating the fresh Si/SiO2/CH/DPPC membrane leads to a decrease of the absolute P angle. In a previous publication for hydrated Si/SiO2/DPPC—without CH—we show that during the heating cycle water evaporates, because the measured dP angle increases.

AFM images taken during heating cycles confirm the transition temperatures—previously obtained with VHRE—of the DPPC bilayer. Furthermore, our AFM images show that although phase transitions are observable during heating cycles, the topography of the artificial membrane at room temperature does not change. In other words, the artificial membrane resists temperature changes, returning to its initial structure after each cycle. For this reason, we consider the Si/SiO2/CH/DPPC artificial membrane as a good prototype for the insertion of a wide range of proteins.

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