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Pore spanning lipid bilayers on silanised nanoporous alumina membranes

Abdul Mutalib Md Jani^a, Jinwen Zhou^a, Matthew R. Nussio^a, Dusan Losic^b,
Joe G. Shapter^a, and Nicolas H. Voelcker^a

^aSchool of Chemistry, Physics and Earth Sciences, Faculty of Science and Engineering,
Flinders University of South Australia, Bedford Park 5042 SA

^bIan Wark Research Institute, University of South Australia, Mawson Lakes Campus,
Mawson Lakes 5095 SA

ABSTRACT

The preparation of bilayer lipid membranes (BLMs) on solid surfaces is important for many studies probing various important biological phenomena including the cell barrier properties, ion-channels, biosensing, drug discovery and protein/ligand interactions. In this work we present new membrane platforms based on suspended BLMs on nanoporous anodic aluminium oxide (AAO) membranes. AAO membranes were prepared by electrochemical anodisation of aluminium foil in 0.3 M oxalic acid using a custom-built etching cell and applying voltage of 40 V, at 1°C. AAO membranes with controlled diameter of pores from 30 - 40 nm (top of membrane) and 60 -70 nm (bottom of membrane) were fabricated. Pore dimensions have been confirmed by scanning electron microscopy (SEM) and atomic force microscopy (AFM). AAO membranes were chemically functionalised with 3-aminopropyltriethoxysilane (APTES). Confirmation of the APTES attachment to the AAO membrane was achieved by means of infrared spectroscopy, X-ray photoelectron spectroscopy and contact angle measurements. The Fourier transform infrared (FTIR) spectra of functionalised membranes show several peaks from 2800 to 3000 cm⁻¹ which were assigned to symmetric and antisymmetric CH₂ bands. XPS data of the membrane showed a distinct increase in C1s (285 eV), N1s (402 eV) and Si2p (102 eV) peaks after silanisation. The water contact angle of the functionalised membrane was 80° as compared to 20° for the untreated membrane. The formation of BLMs comprising dioleoyl-phosphatidylserine (DOPS) on APTES-modified AAO membranes was carried using the vesicle spreading technique. AFM imaging and force spectroscopy was used to characterise the structural and nanomechanical properties of the suspended membrane. This technique also confirmed the stability of bilayers on the nanoporous alumina support for several days. Fabricated suspended BLMs on nanoporous AAO hold promise for the construction of biomimetic membrane architectures with embedded transmembrane proteins.

Keywords: Nanoporous alumina membranes, lipid bilayers, artificial membranes

1. INTRODUCTION

Artificial bilayer lipid membranes (BLMs) are important models for experimental studies of biological membranes. In particular, the interaction and insertion of membrane proteins on BLMs has been explored for application in biosensors, advanced drug discovery and protein screening.^[1, 2] Another important aspect of BLMs is their ability to simplify the complexity of the biological membrane whilst providing an experimental platform to study biorecognition processes in a natural environment. One of the most important functions of BLMs is blockage of water and ions as well as polar or charged molecules and hence the ability to control electrolyte levels.^[3] However, the weakness of current BLMs is their fragility and instability over longer period of the time. In order to improve membrane stability, lipid bilayers on solid support surfaces were introduced.^[4]

Supported or s-BLMs have been prepared by depositing a bilayer on a number of substrates such as mica, silica, quartz, oxidised silicon or noble metals.^[5-9] Membrane formation occurs by self assembly, either facilitated by Langmuir-

Blodgett or Langmuir-Schaefer deposition or by spreading of spherical bilayer vesicles (liposomes). The advantages of solid supported membranes are their capability to be analysed by a wide range of surface sensitive techniques such as scanning probe microscopy, quartz crystal microbalance, surface plasmon resonance as well as electrochemical methods.^[10] However, the membranes can be easily detached from the surface. Membrane stability on the surface depends on the pH and ionic strength.^[11] Despite their limitations, s-BLMs have been widely used as model membranes and for analytical applications. As solid supported membranes studies expand rapidly, the requirement for more robust and stable membranes has also increased dramatically. Therefore the search for alternatives to solid supported membranes has received considerable attention by researchers for the past two decades.^[12] Porous materials such as porous silicon and nanoporous anodic aluminium oxide (AAO) platforms are a particularly promising approach.^[13, 14] The pores provide a free volume underneath the bilayer, allowing access to both leaflets of the bilayer and providing space for extramembrane domains of transmembrane proteins. Highly ordered nanoporous AAO membranes have tunable thickness and pore diameters ranging from 10 – 200 nm. AAO membranes have a high surface area and are chemically inert in aqueous medium, biocompatible and allow chemical surface modification.^[15-17] AAO are widely used for many applications including molecular separation, biosensing and templating.^[16, 18] Some of these applications involve surface functionalisation of nanoporous AAO membranes by chemical modifiers with the desired properties. The first report describing the formation of artificial BLMs on nanoporous AAO came from Steinem et al.^[19] BLMs were suspended on AAO covered with a thin film of gold and modified with hydrophilic linker terminated with a self-assembled monolayer (SAM) of alkanethiol terminated with a charged functional group. The charged surface allows the fusion of large unilamellar vesicles and formation of ordered bilayer structure.^[19, 20] The main disadvantage of this approach is related to limitation of gold-thiol chemistry which involved tedious preparations that need to incubate the gold substrate with alkanethiol for a long time.^[21] Another disadvantage of an alkanethiol SAM is its typically more crystalline structure than a normal leaflet of phospholipid bilayer that resulted less fluid environment.^[22]

To avoid these limitations and fabricate more stable artificial membranes based on AAO, we introduce new and simpler approach based on suspending BLMs on silane modified AAO membranes. A schematic of our approach is presented in Fig. 1. We first modify the AAO surface with 3-aminopropyltriethoxysilane (APTES), which provides an amino-functional coating which is positively charged at neutral pH. We then prepare suspended BLMs from negatively charged lipids by vesicle spreading. In this study, we have evaluated fabrication steps and structural properties of suspended BLMs on AAO using surface characterisation techniques including SEM, AFM, FTIR and contact angle measurements.

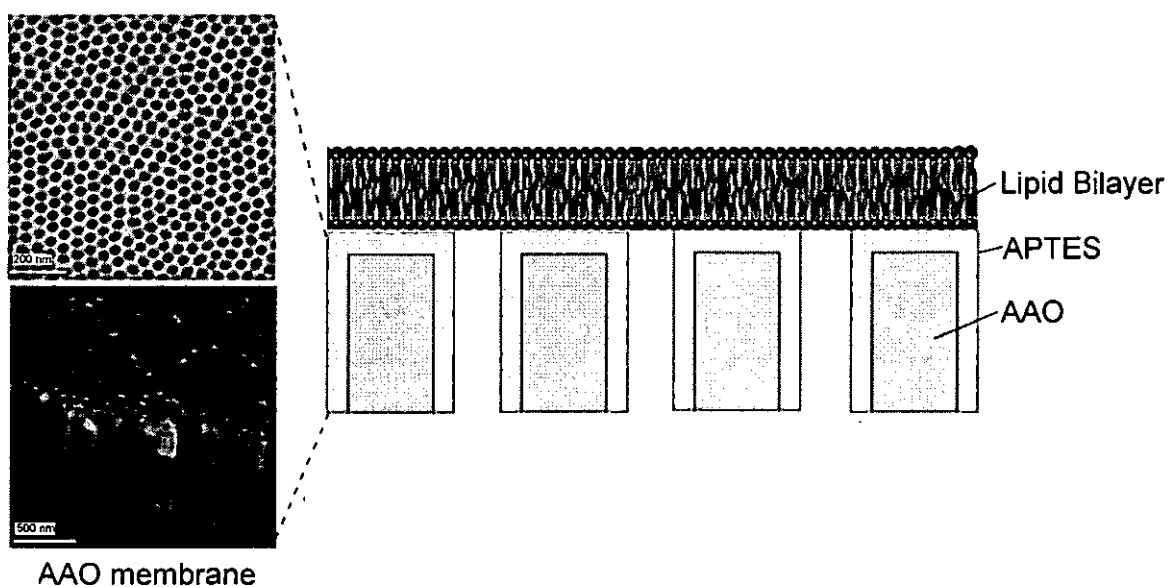


Fig. 1. SEM images of AAO and schematic representation of pore suspended BLMs on silanised AAO.

2. METHODOLOGY

2.1 Materials

Aluminium foil (99.99wt%), with a thickness 0.1 mm was supplied by Alfa Aesar (USA). 1,2-dioleoyl-sn-glycero-3-(phospho-L-serine) (DOPS) was purchased from Avanti Polar (Avanti Polar Lipid Inc.) and used without further purification. Oxalic acid, phosphoric acid, chromic acid, perchloric acid, hydrochloric acid, ethanol, acetone, hydrogen peroxide, sodium hydroxide, copper chloride etc. were supplied from Chem Supply (Australia). 3-aminopropyltriethoxysilane (APTES) and HEPES were purchased from Aldrich (Australia). All solvents and chemicals were analytical grade and purchased from Sigma-Aldrich. Water purified through MilliQ RO 10 Plus and MilliQ Plus 185 (18.2 M Ω m) was used.

2.2 Fabrication of nanoporous AAO membranes

Aluminium foil was degreased in acetone and sonicated for about 30 minutes followed by dipping in sodium hydroxide solution (5%) for few seconds. The cleaned aluminium foil was thoroughly rinsed with water and dried with nitrogen. The foils were then electrochemically polished with perchloric acid/ethanol (1:4 v/v HClO₄:EtOH) for 1 minute at constant voltage 25 V in a custom-built etching cell to reduce the surface roughness. The electropolished aluminium sheets were thoroughly washed with water and dried under a stream of nitrogen. Nanoporous AAO membranes were fabricated using conventional mild anodisation and two-step anodisation process.^[23] Lead was used as cathode electrode and the distance between the electrodes was adjusted to be about 5 cm. A circular area 1.0 cm in diameter was exposed to the electrolyte solution in a thermally insulated bath, the temperature (1°C) of which was controlled by means of refrigeration unit (Julabo PC, John Morris Scientific). Vigorous stirring was maintained during anodising in order to maintain uniform temperature on the electrode surface. The first anodisation lasted for 4 hours under constant voltage of 40 V and electrolyte temperature 1°C, in 0.3 M oxalic acid (C₂H₂O₄) in order to form porous layers. A source meter Keithley 2600 series was used to apply voltage between electrodes during anodisation process. At the end of first anodisation, the formed AAO membranes were immersed for 2 hours on phosphochromic acid (6% H₃PO₄, 1.8% H₂CrO₄) aqueous solution at 80°C, to remove formed porous layer and form a pretextured aluminum surface necessary for the formation of an organised array of pores during the second anodisation step. The second anodisation is performed for more than 8 hours depending on the desired thickness of membranes using the same anodisation conditions as previously described. Finally, free-standing AAO membranes were formed by removing the remaining aluminium layer by CuCl₂/HCl solution followed by a bottom pore opening step carried out with 0.1 M phosphoric acid (H₃PO₄) at room temperature for 2 hours.

2.3 Surface functionalisation of AAO

Alumina membranes were hydroxylated by boiling them in 30% hydrogen peroxide and followed by MilliQ water for 30 minutes. The membranes were then dried under stream of nitrogen and were functionalised with silane by incubating in a 0.5% (v/v) solution of APTES in toluene under room temperature. The silanisation reaction was terminated after 4 hours by extensive rinsing the membrane with toluene, ethanol and several times with MilliQ water and dried under a stream of nitrogen. The dried membrane was left overnight in an oven at 80°C.

2.4 Formation of suspended BLMs

The formation of bilayers of dioleoyl-phosphatidylserine (DOPS) immobilised on the functionalised nanoporous AAO membranes was achieved using a vesicle spreading technique. Lipid stock solutions were prepared by dissolving lipid in chloroform at concentration of 1 mgmL⁻¹. Solvent was removed by evaporation under stream of nitrogen at room temperature. The resulting lipid film was further dried at reduced pressure for 2 hours and then rehydrated in buffer solution containing 150 mM NaCl, 10 mM HEPES/NaOH (pH 7.4). The large multilamellar vesicles formed were extruded 21 times through a 100 nm pore size polycarbonate filters (Avanti Polar Lipid Inc) with Liposfast extruder from Avanti Polar Lipid Inc. The extruded vesicles were then added with CaCl₂ that resulted in 20 mM final concentration of CaCl₂ in the mixture. The required amount (100 μ L – 150 μ L) of this mixture was deposited onto functionalised nanoporous alumina membranes. The DOPS suspension was incubated at room temperature for about 1 hour and washed by changing the buffer solution several times. To facilitate the formation of a BLM, the solution was kept above the phase transition temperature of DOPS during incubation period.

2.5 Surface characterisation

Contact angle measurements were performed by depositing 1.0 μL filtered MilliQ water onto alumina membranes surface at room temperature. Three measurements at different spots were taken on each of the sample. Images were captured with a Panasonic SuperDynamic WV-BP550/G camera with a macrolens. Image processing was analysed using ImageJ software V1.3 and the results reported were based on an average of three measurements.

Analysis of surface chemistry was determined by Fourier-transform infrared (FTIR) spectroscopy conducted on a Thermo-Nicolet Nexus 870 taking 64 scans in transmission mode in air at a resolution of 2 cm^{-1} were done for each sample. Bare porous alumina was used as a background.

XPS analysis of the samples was carried out using an Ultra spectrometer (Kratos Analytical Ltd, GB) with a monochromatic Al $K\alpha$ source. The pressure during analysis was approximately 5×10^{-9} mbar. The elemental composition of samples was obtained from survey spectra, collected at a pass energy of 160 eV. Binding energies were referenced to the aliphatic carbon peak at 285.0 eV. XPS characterisation is performed on both bare AAO and APTES modified AAO membranes

Atomic force microscopy (AFM) topography examinations were performed using a Multimode Nanoscope IV (Veeco Instruments Ltd., Santa Barbara, USA) in tapping and contact modes in either air or buffer. Commercially available silicon cantilevers (Digital Instruments) were used for samples imaged in air at a resonance frequency of 200 - 400 kHz and scan rate of 0.5 - 1.0 Hz. Triangular silicon nitride cantilevers with nominal spring constants 0.15 Nm^{-1} (Veeco Instruments Ltd., Santa Barbara, USA) were used for imaging in fluid at a resonance frequency of 5.0 – 9.0 kHz. The incubation, washing and observation by AFM were performed in the same quartz liquid cell. Image processing was performed using Nanoscope v5.12r3 software (Veeco Instruments Ltd., Santa Barbara, USA).

Scanning electron microscope (SEM) images was measured on a Philips XL 30. To obtain cross-sectional view, the SEM stage was tilted to 40° angle.

3. RESULTS AND DISCUSSION

3.1 Structural characterisation of nanoporous AAO membranes

The SEM images in Fig. 2 shows typical morphologies and pore structures from the top and bottom surface of fabricated AAO membranes etched in 0.3 M oxalic acid under constant voltage of 40 V. Fig. 2(a) shows the top view of the surface. The mean pore diameter of these membranes was about 35 nm. The SEM image reveals the nanopore arrays with an interpore distance is about 100 nm. It should be noted that other pore diameter and hexagonally ordered structure can be obtained by changing the anodisation voltage, choice of electrolyte and temperature.^[24] We found, in agreement with the literature, that two successive anodisations produced a more parallel and ordered pores structure than a single anodisation. The corresponding SEM image of Fig. 2(b) shows a bottom view of the membrane surface. The diameter of the hexagonally arranged pores and interpore distance measured to be 65 nm and 120 nm, respectively. The interpore distance at the top and bottom of the membrane is similar, but the pore diameter at the bottom surface is much larger. The larger pore diameter at the bottom is as a result of longer pore opening process with phosphoric acids which exposing the underlying nanoporous alumina membranes. Fig. 2(c) shows a typical cross-section image of the AAO membranes showing the nonintercrossing and parallel cylindrical holes of the free-standing AAO membranes.

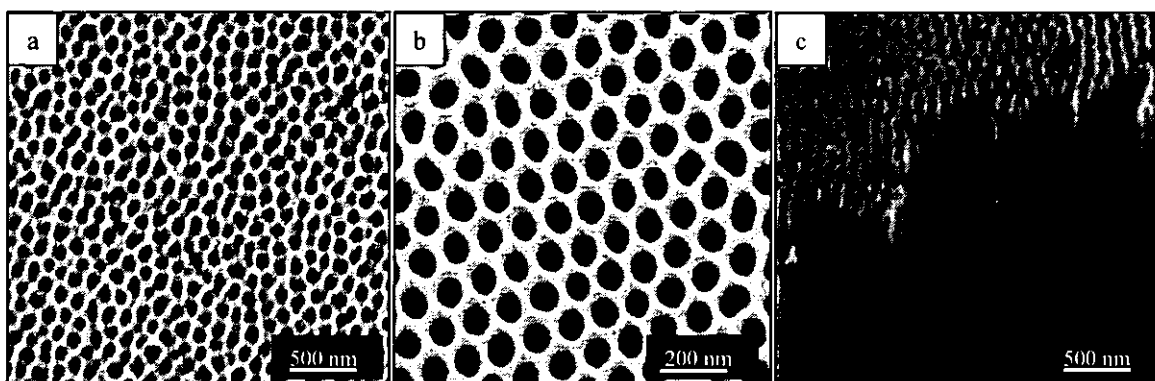


Fig. 2. SEM images of microstructures of nanoporous alumina membranes: (a) top view of nanopore arrays with pore diameters about 30 - 40 nm, (b) bottom view with pore diameter 60 - 70 nm and (c) a cross-sectional view of the membranes show parallel cylindrical holes.

3.2 Surface characterisation of functionalised AAO membranes

3.2.1 Contact angle measurements

One of the simplest methods of confirming the attachment of silanes to the surface of nanoporous AAO membrane is inspection of the static water contact angle on the membrane surface, to determine changes in wettability upon functionalisation. The bare membrane has a water contact angle of $20^\circ \pm 4^\circ$ (Fig. 3a). As expected, freshly hydroxylated membranes were hydrophilic and hydroxylation resulted in a drop of the water contact angle to below 10° , water rapidly spread across membrane surface and filled the pores as depicted in Fig. 3(b). The high hydrophilicity of hydroxylated membranes is due to the surface $-OH$ s, which act as adsorption sites of water molecules mediated by hydrogen bonds. In contrast, the aminosilaned nanoporous AAO membrane was more hydrophobic showing a static water contact angle of $80^\circ \pm 3^\circ$, which remains stable over several weeks (Fig. 3c). The ability of aminosilane to change surface hydrophobicity of the membrane were also observed by previous studies.^[25, 26]

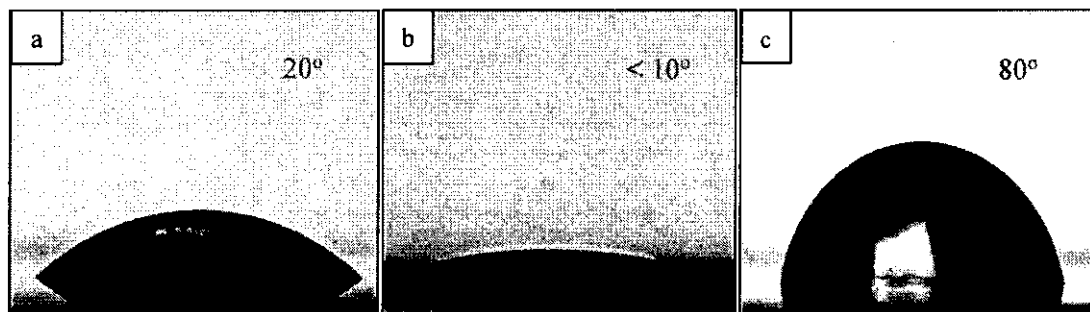


Fig. 3. Images of water droplets (1.0 μ L) on nanoporous AAO membranes, (a) after anodisation, (b) after hydroxylation and (c) after silanisation.

3.2.2 FTIR and XPS Analysis

Infrared spectroscopy was used to detect the presence of the APTES layer on the AAO surface. Indeed, the nanoporous AAO membranes in this study are conducive to simple transmission experiments. A comparison of the infrared spectra for the membranes before and after modification with APTES is shown in Fig. 4, confirming the presence of the APTES layer. The stretching modes at 2850 to 3000 cm^{-1} which were assigned to symmetric and antisymmetric of CH_2 bands were apparent.^[26] These sharp methyl stretches indicate the desirable binding of alkyl silanes on the surface. It also observed that the intense peaks in the regions of double bond vibrations near 1700 cm^{-1} which can be attributed to the adsorption of CO_2 on the membrane.^[27] The spectrum further shows broad binding vibrations centered around 3400 cm^{-1} which were attributed to (Al-OH) stretching.

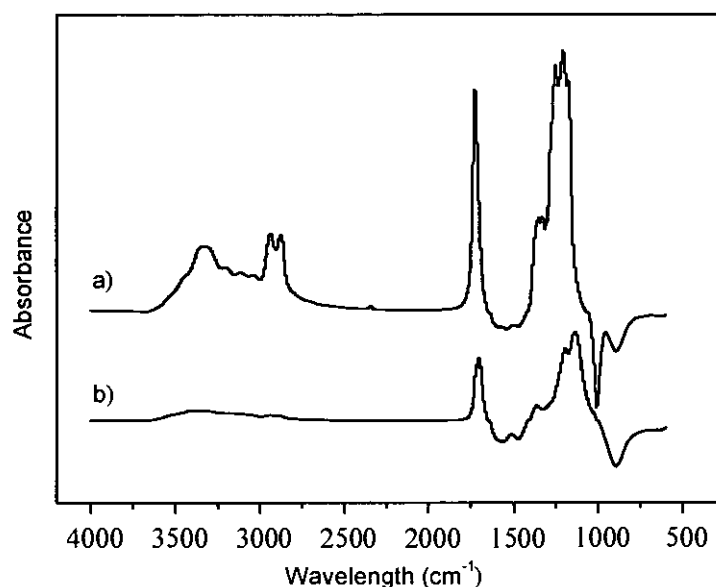


Fig. 4. FT-IR spectra of (a) the functionalised nanoporous AAO membrane and (b) bare nanoporous AAO membrane.

XPS was used to confirm the silane modification of the AAO surface. A typical survey XPS spectrum is shown in Fig. 5. A clear Si2p emission with binding energies of 102 eV is found, indicating the presence of Si-O bonds (Fig. 5a). Survey scans showed there were a distinct increase in C1s (285 eV) and N1s (401 eV) peaks with modification of silane and this was also followed by subsequent decrease in Al2p (75eV) (Table 1). Fig. 5b shows high-resolution C1s scans of major hydrocarbon peaks at 284.1 eV. The binding energy at 284.8 and 285.7 eV are assigned to amines and alkoxy groups respectively. APTES functionalisation is indicated by the asymmetric signal of the positively charged amino tail groups at binding energies of 399 eV (NH_3^+) displayed in Fig. 5(c). The results are consistent with IR spectra results and this behaviour was previously reported and observed on APTES modified porous alumina surfaces.^[28]

Table 1. XPS elemental analysis of bare and modified nanoporous AAO membranes.

| Elements | O (%) | C (%) | Al (%) | N (%) | Si (%) |
|--------------------|-------|-------|--------|-------|--------|
| Bare membranes | 58.1 | 18.7 | 12.5 | - | - |
| Modified membranes | 44.8 | 31.8 | 10.3 | 5.3 | 3.8 |

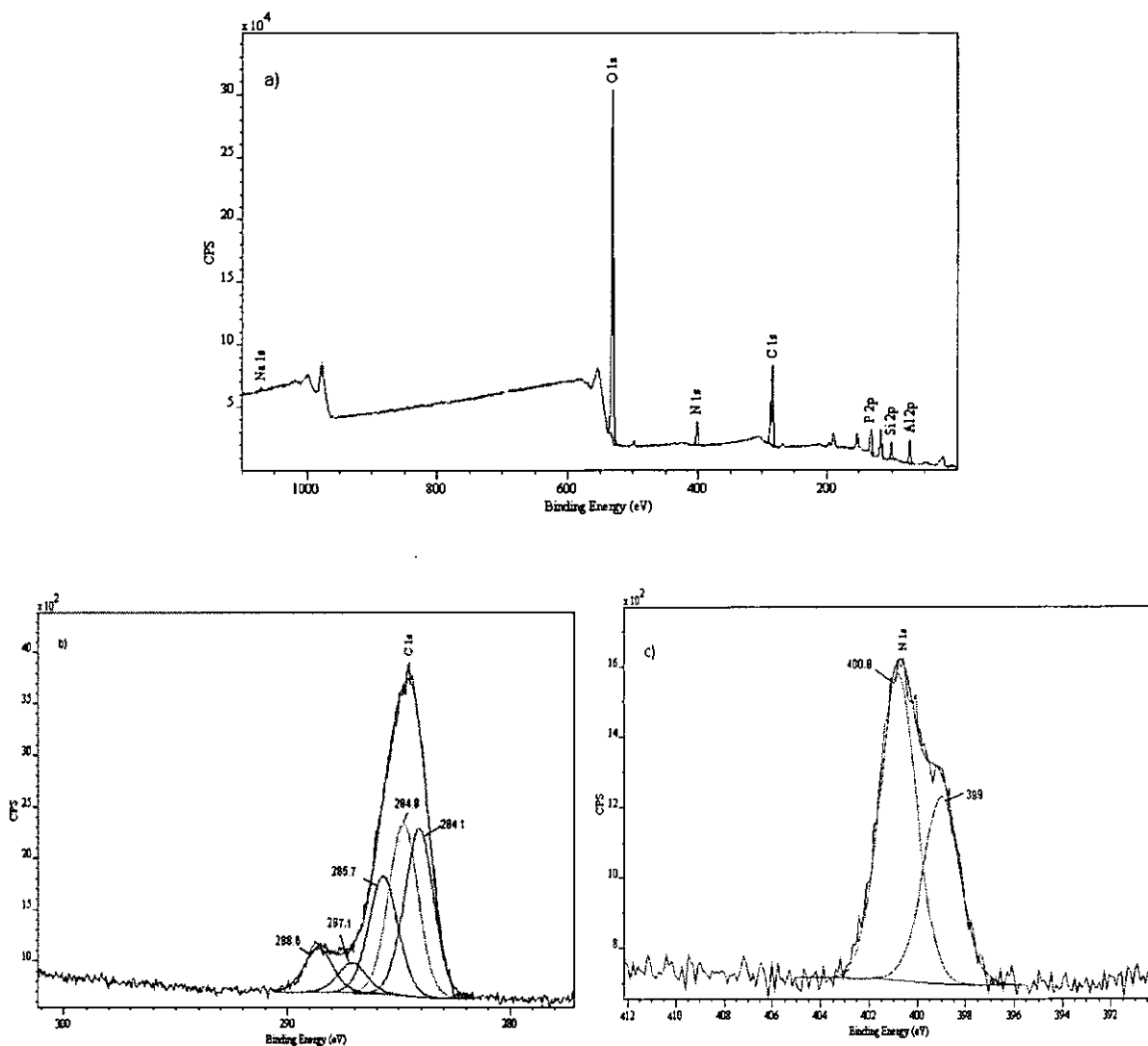


Fig. 5. (a) XPS survey spectrum for the APTES modified nanoporous alumina membrane. High resolution (b) C1s spectrum, and (c) N1s spectrum of the APTES modified nanoporous alumina membrane.

3.2.3 AFM studies of suspended lipid bilayers on functionalised nanoporous AAO membranes

The detailed surface topography of AFM images of the nanoporous AAO membranes before and after BLM deposition is shown in Fig. 6. A tendency of the pores to self-organise in a hexagonal array on top of the membranes is observed in Fig. 6 (a) which is more evident from the inset showing a typical pore structure at the bottom of the surface. Nanoporous AAO membranes were surface modified by hydroxylation, silanised with APTES to improve surface wettability and stability and to impart charged functionalities which promote lipid bilayers deposition on the top surface of the membrane. On APTES functionalised alumina surface (positively charged), the strong electrostatic attractive force between the vesicles and surfaces enhances stable adsorption and rupture of negatively charged large unilamellar vesicles, which promotes the formation of supported lipid bilayers.^[29, 30] AFM studies show a slight change to the

topographical characteristics of freshly prepared AAO membranes and silanised membranes (Fig. 6b). Silanised samples were rougher with root mean squared (rms) values of 70 ± 1.2 nm compared to bare membranes of 20 ± 0.9 nm. Furthermore, the pores appeared to be smaller on the silane functionalised membrane, suggesting formation of silane multilayers on the AAO.

The suspended BLMs on porous alumina surfaces were visualised by AFM. Fig. 6(c) and (d) show AFM images of nanoporous alumina surface covered with APTES before and after spreading of vesicles in situ. A conspicuous change in morphology was observed in Fig. 6(d) consistent with the successful formation of a suspended lipid bilayer. Since the bilayer covers the nanoporous alumina pores, the pores are no longer visible. These results are in accordance with previous studies on alkythiol modified and gold coated AAO confirming the interpretation of AFM data on pores spanning BLM.^[19, 31] The presence of the DOPS bilayer is further confirmed using force analysis. With reference to Fig. 6(e), a discontinuity in the force-distance curve (~ 4 nm) shown in the approaching parts is observed, which is indicative of the tip penetrating the membrane leading to the onset of plastic deformation.^[32, 33] The force at which the membrane breaks is known as the breakthrough force, and this is representative of the membrane's stability.^[34, 35] The same behaviour of DOPS bilayers deposited on support such as mica was observed by previous studies after applying a certain force usually 2 -10 nN indicating the penetration of the tip through a bilayer.^[36, 37] This result confirms that the aminosilanisation of AAO can serve as a starting point for the deposition of suspended BLMs.

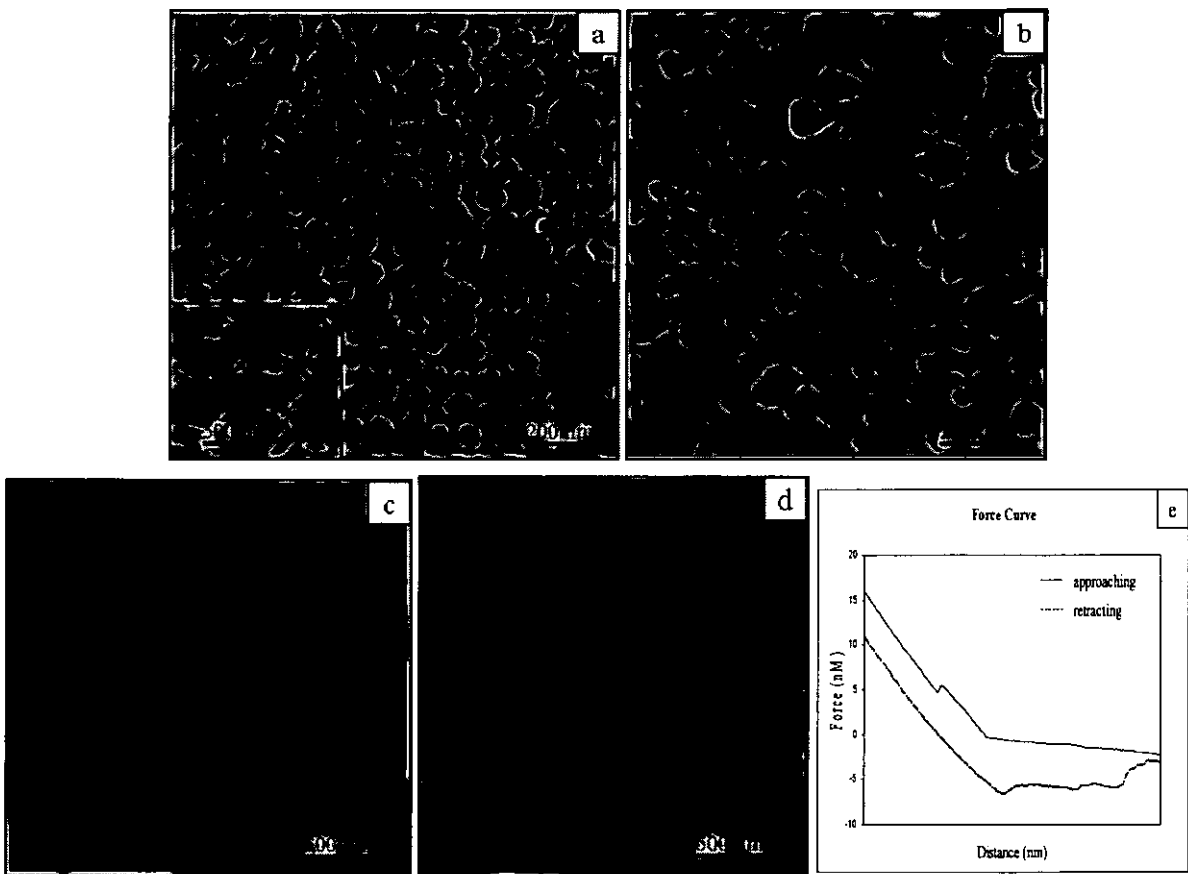


Fig. 6. AFM images of nanoporous AAO membranes and functionalised membranes. (a) top surface of nanoporous alumina membranes, (inset bottom part of the membranes showing hexagonal pore structure), (b) functionalised membranes with APTES under air observations mode, (c) functionalised membranes with APTES under fluid, (d) the same substrates as in (c) after deposition of DOPS vesicles showing pores covered by suspended bilayer and (e) typical force-versus distance curve of suspending lipid bilayer on nanoporous alumina membranes.

4. CONCLUSIONS

In summary, the fabrication and evaluation of structural properties of BLMs suspended on silane functionalised AAO membranes is presented. It was confirmed for the first time that silane functionalised AAO membranes can act as a support for suspending a BLM by the vesicle spreading technique. AFM imaging and force spectroscopy proved that the lipid film was spanning over the pores array and under certain applied forces retains elastic behaviour. Spaces separated by a semipermeable BLM may serve as nanocompartments permitting the incorporation of membrane proteins for biosensor applications. Our approach holds promise for the development of an artificial membrane platform for a diverse range of applications including biosensing, drug delivery and the study of molecular interactions with lipid bilayer membranes.

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