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Patterned domains of supported phospholipid bilayer using microcontact printing of Pll-g-PEG molecules

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ABSTRACT

In this work, we propose a reliable microcontact printing (μCP) process for generating Patterned Supported Phospholipids Bilayer (P-SPB) confined by Poly-L-(lysine)-grafted-polyethylene(glycol) (Pll-g-PEG) molecular barriers. The efficiency of Pll-g-PEG for inhibiting the fusion process of incubated liposome was first analyzed by Quartz Micro Balance (QCM) measurements. The quality and stability of Pll-g-PEG patterns were then both verified by fluorescence microscopy and Atomic Force Microscopy (AFM) in liquid media. The micro domains of P-SPB produced were stable in liquid environment during several weeks and also during AFM imaging. This exceptional stability is a clear improvement compared to previous studies involving proteins as confinement barriers.

1. Introduction

Studying biological structures and organization of cell membranes is a major challenge for understanding cell's behavior in response to their external environment. Supported Phospholipids Bilayer (SPB) is a good experimental model for in vitro studies, due to the reproduction of a native environment in a controlled manner [1]. For limiting the surface covered by the SPB and confining lipid diffusion, several methods have been elaborated but the main principle remains the same. It consists of a local and controlled deposition of molecules which will play the role of diffusion barriers: Patterned SPB (P-SPB) can been obtained using photolithography [2], coupled with a lift off process [3], microfluidics [4] and microcontact printing (μCP) [5]. Different molecules have been used as barriers, from proteins [6], metal thin layer [7], polymers [3] and recently polyethyleneglycol (PEG) [8]. Other works have also demonstrated the formation of SPB directly on a polymer stamp followed by transfer to a surface [9]. Among the patterning methods developed for P-SPB generation, microcontact printing (μCP) has appeared as one of the most promising. μCP is a well known and useful way to pattern a wide range of molecules into microscale and nanoscale features on different types of substrates [10]. It exhibits major advantages such as low cost and versatility [11] and easy implementation[12]. After molecular pattern generation, phospholipids pre-formed into liposomes spontaneously adhere to the surface and form the SPB through a "fusion" process [13] into the patterns. The fusion is inhibited onto the printed molecules acting as a barrier for the SPB. A very simple design thus allows us to create isolated domains of SPB. By coupling surface patterning to self-assembly, complex biochemical structures can be generated, opening the possibility of studying in vitro membrane behavior and membrane proteins using imagery tools like fluorescence or Atomic Force Microscopy [14]. A series of fluorescence results have been obtained using bovine serum albumin (BSA) as a barrier material but to our knowledge, no AFM studies gave significant results using this method due to the instability of BSA molecules under AFM imaging. In order to employ both fluorescence and AFM imaging, we have thus identified two majors constraints for the choice of the barriers molecules: Antifouling behavior and stability under liquid imaging. In this perspective, our attention was attracted by the poly-L-lysine-polyethylene(glycol) (Pll-g-PEG) molecules. Polyethylene(glycol) (PEG) has already been identified as an efficient antifouling molecule but its grafting onto surfaces remained problematic. PEG chains may be immobilized

Abbreviations: μCP, microcontact printing; PDMS, polydimethylsiloxane; PMMA, polymethylmethacrylate; RIE, reactive ion etching; OTS, octadecyltrichlorosilane; EggPC/FITC-DHPE, egg-phosphatidylcholine/*N*-(5-fluoresceinthiocarbamoyl)-1,2-dihexa-decanoyl-*sn*-glycero-3-phosphoethanolamine; MOPS, 3-morpholino-propanesulfonic acid; DLS, dynamic light scattering; AFM, atomic force microscopy; Pll-g-PEG-TRICT, poly-lysine(20)-grafted[3,5]-polyethyleneglycol(2)-TRITC; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethansulphonic acid; QCM, quartz micro balance.

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on surfaces using covalent coupling [15–17], via functionalization with ethylene glycol-terminated alkanethiols [18,19,20] or silane [21,22] or via the adsorption of a block copolymer. PEG molecules are currently used over large spectrum of applications, mainly chemical surface coating [23], due to their well known anti-adhesive properties against proteins [15,24] and cells. Large numbers of PEG formulations are currently commercially available and their utilisation mainly depends on the final application. Different PEG solutions, including silane-PEG or poly(TMSMA-r-PEGMA)-PEG have already been used for the formation of patterned membrane using μCP or microfluidics, and the presence of proteins or membranes was totally avoided on PEG patterns [25,26]. A family of PEG molecules grafted on poly-lysine chains (Pll-g-PEG), commercially available, emerged recently and presented advantages compared to other formulations [27]. μCP of these molecules and anti-adhesive properties were demonstrated on metal oxide TiO_2, SiO_2 where no external chemical preparation was needed [28]. Adhesion of the coating to the substrate is due to the presence of poly-lysine chains grafted to PEG molecules, inducing an electrostatic interaction with negatively charged surfaces. Interactions of the Pll-g-PEG and lipid bilayer were studied using Quartz Micro Balance (QCM) coupled to fluorescence [28]. SPB was preformed on a SiO_2 cleaned surface and Pll-g-PEG was added resulting in a disruption of SPB structure.

In this work, we first monitored the possible interaction between Pll-g-PEG and lipids using QCM to confirm that liposomes do not adsorb on Pll-g-PEG films and also that Pll-g-PEG patterns were not altered by the presence of a lipid membrane. μCP of Pll-g-PEG on a clean SiO_2 surface was then achieved at the microscale. We evaluated the stability of these patterns by Atomic Force Microscopy (AFM) in liquid medium. Stability is essential for maintaining the confinement of lipids within each pattern and for the minimization of image artifacts due to tip contamination. We then added a lipid mixture based on EggPC (egg yolk phosphatidylcholine) coupled to DHPE-FITC (*N*-(biotinoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine-fluorescein isothiocyanate) in order to create P-SPB through a selective fusion process on the SiO_2 areas inhibited on the Pll-g-PEG zones. The presence of the FITC dye on the membrane mixture and TRICT (tetramethylrhodamine isothiocyanate) dye on the PEG chains allows fluorescence characterization to be performed. Atomic Force Microscopy in liquid was then pursued on the patterned surface. By coupling patterning and self-assembly, we obtained domains of SPB, from 8 μm^2 to 4 μm^2 with a high reproducibility. These P-SPBs remained stable for several days in liquid. The choice of Pll-g-PEG provided many advantages over previously tested molecules used to construct of repulsion barriers, such as BSA proteins. Indeed, the resulting patterns were both very stable and also did not contaminate AFM tips when conducting imaging in liquid. The technology process we propose is therefore very useful for the investigation of membrane proteins by AFM in supported bilayer membrane configuration.

2. Experimental

2.1. Materials and experimental systems

Resist AZ1529 was purchased at CIPEC (Paris, France). Sussmicrotech 150 system exposure was used for photolithography. Octotrichlorosilane (OTS) was bought from Sigma–Aldrich (France). Poly-dimethyl-solixane (PDMS) material (sylgard 184) was purchased from Dow Corning [29]. Poly-L-grafted-polyethylene(glycol) (Pll-g-PEG) and Poly-L-grafted-polyethylene(glycol)-tetramethylrhodamine isothiocyanate (Pll-g-PEG-TRITC) was bought in powder form from Surface Solution (Switzerland). Phosphatidylcholine from egg yolk (EggPC), were purchased from Sigma–Aldrich (Alabaster, AL), *N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phospho-ethanolamine (DHPE-FITC) was purchased from Avanti [29]. Sonication was employed using a VCX500 model (Sonics and Material, USA) mounted with a micro tip probe 421 (Misonix Sonicator, USA). MOPS (3-morpholino-propanesulfonic acid). Cleaned surface glass slides were bought from VWR seller (France). QCM interactions were monitored using the QSense-E4 system with SiO_2 quartz (QSX303) purchased from Qsense (Sweden). Fluorescent pictures were captured using an inverted microscope IX70 (Olympus, Japan) mounted with a series of objectives from ×5 to ×100. Fluorescence dyes were excited using a Mercury lamp. Pictures were captured with an Andor Lucas camera (Ireland). All AFM images were obtained using AFM-JPK NanoWizard II System (Berlin, Germany). Silicon Nitride cantilevers (MLCT 0.05–0.01 N m^{-1}) were purchased from Veeco (Santa Barbara, USA).

2.2. Vesicle preparation

We prepared EggPC/FITC-DHPE (0.97/0.03 molar ratio) (Egg yolk phosphatidylcholine/*N*-(biotinoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine-fluorescein isothiocyanate) lipids solution following an established procedure [30]. Lipid powder was dissolved and stored at −20 °C in chloroform. A desired volume of each lipid type was mixed and dried under nitrogen for several minutes and solutions were placed overnight in a vacuum chamber to remove any trace of solvent. Buffer MOPS (10 mM pH 7.4) was added and liposomes were formed by sonication for five minutes. Liposome radius was measured using Dynamic Light Scattering (DLS) Zetasizer Nano ZS (Malvern instruments Ltd., UK, He–Ne laser 633 nM) and gave an average radius of 100 nm. We arbitrarily choose a final concentration of 0.1 mg/ml for the entire experiment.

2.3. Pll-g-PEG solution

Pll-g-PEG molecules were received in powder and stored at −20 °C to avoid chemical degradation. Dilution was made using Hepes at 10 mM, Nacl 150 mM pH 7.4 (Hepes 1) as a buffer diluted to a concentration of 1 and 0.1 mg/ml. Solutions were stored at −20 °C for several weeks and were used only once to avoid aggregation at room temperature.

2.4. QCM characterization

Basic principles of QCM technology consist in a real time measurement of both dissipation and resonance frequency of an oscillating quartz crystal within a controlled microfluidic chamber. QCM monitors the response of the oscillating crystal to the addition of mass as it adsorbed to the sensor surface. Therefore, detection of shift in the resonance frequency indicates adsorption to the sensor surface. QCM technology can be used to characterize the formation of thin films (nm) such as proteins [31], polymers, supported phospholipids bilayer [32,33] and cells [29] onto surface, in liquid and also for studying various kinds of interactions: protein/DNA [34,35], protein interactions [35], PEG [36]. We selected only the results of the 7th order for presentation in this article because similar results were obtained for the other orders. All measurements were performed at a temperature of 25 °C. Two quartz sensors "SiO_2" were cleaned and activated using an O_2 plasma chamber for 5 min to enhance hydrophilic behavior of the surface. The interaction between Pll-g-PEG and EggPC liposomes was monitored in chamber 1 while the formation of EggPC SPB was used as a negative control in chamber 2. Buffer Hepes 1 was injected into the fluidic chamber 1 followed by Pll-g-PEG injection at 1 mg/ml. After rinsing