Supplementary Information

Cell Adhesion through Clustered Ligand on Fluid Supported Lipid Bilayers

Ludivine Sandrin, Liliane Coche-Guérente, Amandine Bernstein, Hajra Basit, Pierre Labbé, Pascal Dumy, and Didier Boturyn **

Department de Chimie Moléculaire, UMR CNRS/UJF 5250, ICMG FR 2607, 301, rue de la chimie, BP53, 38041 Grenoble cedex 9, France. E-mail: didier.boturyn@ujf-grenoble.fr

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Materials and equipments

Protected amino acids were obtained from Activo tec (Cambrigde, UK), Bachem Biochimie SARL (Voisins-les-Bretonneux, France), France Biochem SA (Meudon, France), Merck Eurolab (Fontenay-sous-Bois, France) or Calbiochem-Novabiochem (Merck Biosciences - VWR, Limonest, France). PyBOP® was purchased from Calbiochem-Novabiochem and PyAOP® from PerSeptive Biosystems (Foster City, Canada). NovaSyn® TGR resin were obtained from Calbiochem-Novabiochem, Fmoc-Gly-SASRIN® resin from Bachem Biochimie SARL and 2-chlorotritylchloride® resins from Advanced ChemTech Europe. Other reagents were obtained from Aldrich (Saint-Quentin Fallavier, France) and Acros (Noisy-le-Grand, France). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was obtained from Avanti Polar Lipids. PBS, DMEM, Foetal calf serum, penicillin and streptomycin for cell culture were obtained from Gibco. Ultrapure water was obtained from a Milli-Q Purelab UHQ (Elga) with a resistivity of 18.2 MΩ/cm. All reactants were analytical grade, anhydrous $MnCl_2$, Na_2HPO_4 and KCl came from Acros organics, NaCl from Fluka, $MgCl_2$ from Aldrich, sodium dodecyl sulphate (SDS) from Panreac and Tris[hydroxymethyl]aminomethane hydrochloride (Tris) from Sigma. The Tris buffer consisted of Tris HCl, 50 mM ; NaCl, 150 mM ; $MgCl_2$, 2 mM and $MnCl_2$, 1 mM. The PBS buffer used in QCM experiments consisted of Na_2HPO_4 0.01 M ; NaCl 0.137 M ; KCl 0.0027 M ; pH = 7.4.

RP-HPLC analyses were performed on Waters equipment consisting of a Waters 600 controller, a Waters 2487 Dual Absorbance Detector and a Waters In-Line Degasser. The analytical column used was the Nucleosil 120 Å 3 µm C18 particles, 30×4 mm^2 operated at 1.3 mL.min^{-1} with linear gradient programs in 15 min run time (classical program 5 to 100 % B in 15 min). UV monitoring was performed most of the time at 214 nm and 250 nm. Solvent A consisted of H_2O containing 0.1% TFA and solvent B of CH_3CN containing 9.9% H_2O and 0.1% TFA. Water was of Milli-Q quality and was obtained after filtration of distilled water through a Milli-Q® cartridge system. CH_3CN and TFA were of HPLC use quality. RP-HPLC purifications were performed on Waters equipment consisting of a Waters 600 controller and a Waters 2487 Dual Absorbance Detector. The preparative column, Delta-Pak™ 300 Å 15 µm C18 particles, 200×25 mm^2 was operated at 22 mL.min^{-1} with linear gradient programs in 30 min run time. Solvents A and B were the same than the ones used in RP-HPLC analysis.

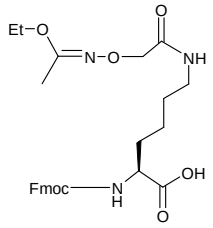
Electron spray ionization (ESI-MS) mass spectra were obtained on an Esquire 3000 (Bruker). NMR spectra were recorded on BRUKER Avance 300 spectrometers. Chemical shifts are expressed in ppm and calculated taking the solvent peak as an internal reference.

General procedures for peptide syntheses

Assembly of all linear protected peptides was performed manually or automatically by solid-phase peptide synthesis (SPPS) using the standard 9-fluorenylmethoxycarbonyl/tertiobutyl (Fmoc/*t*Bu) protection strategy. In manual SPPS, device consisted in a glass reaction vessel fitted with a sintered glass frit. The latter allowed elimination of excess reagents and solvents under compressed air. Before use, the vessel was treated for 12 h (typically overnight) with $(CH_3)_2SiCl_2$ as lubricant to prevent resin beads from sticking to the glass inner wall during the synthesis. It was then carefully washed with CH_2Cl_2 until complete acid trace clearance. At the beginning of the synthesis and after each ether washing, the resin was washed and swollen twice with CH_2Cl_2 (20 mL/g resin) for 15 min and once with DMF (20 mL/g resin) for 15 min. Coupling reactions were performed using, relative to the resin loading, 1.5-2 eq. of N_α-Fmoc-protected amino acid *in situ* activated with 1.5-2 eq. PyBOP and 3-4 eq. DIPEA in DMF (10 mL/g resin) for 30 min. The resin was then washed twice with DMF (20 mL/g resin) for 1 min and twice with CH_2Cl_2 (20 mL/g resin) for 1 min. The completeness of amino acid coupling reaction was checked by two tests: Kaiser and TNBS. *N*α-Fmoc protecting groups were removed by treatment with piperidine/DMF (1:4) (10 mL/g resin) for 10 min. The process was repeated three times and the resin was further washed five times with DMF (10 mL/g resin) for 1 min. The completeness of the deprotection was checked by UV measurement (λ = 299 nm, ε = 7800 $M^{-1}.cm^{-1}$).

Synthesis of lipopeptide 1

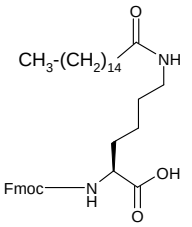
Building block 2.



2

Amino acid **2** was prepared following the procedure previously described.[1]

Building block 3.



3

To a stirred solution of palmitic acid (1 g, 3.9 mmol) and *N*-hydroxysuccinimide (0.6 g, 5.2 mmol) in ethyl acetate/dioxane (12 mL, 1:1) at 4 °C was added DCC (0.8 g, 3.9 mmol) in one portion. The resulting mixture was stirred at room temperature for 3 h. The formed DCU was filtered off and the filtrate concentrated under vacuum. The obtained residue was dissolved in CH_2Cl_2 (80 mL), and the solution was washed with 5% aqueous $NaHCO_3$ (75 mL), and water (3 x 75 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, filtered and concentrated. NHS ester of palmitic acid was obtained as white powder which was used without further purification (1.2 g, 3.4 mmol, 87 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.90 (3H, t, J = 6.7 Hz), 1.28 (22H, m), 1.77 (2H, m), 1.45 (2H, t, J = 7.5 Hz), 2.85 (4H, m).

To a stirred mixture of Fmoc-lysine (0.82 g, 1.7 mmol) and DIPEA (0.3 mL, 1.7 mmol) in CH_2Cl_2 (14 mL) at room temperature was added dropwise over 10 min a solution of NHS ester (0.6 g, 1.7 mmol) in CH_2Cl_2 (6 mL). The pH of the resulting mixture was regularly adjusted to pH 8-9 by further additions of DIPEA. After 1 h of reaction, the reaction mixture was concentrated under vacuum providing an oily residue. After addition of CH_2Cl_2 (15 mL), the organic phase was washed with hydrochloric acid solution (1 M, 2 x 12 mL), water (2 x 12 mL), and brine (12 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, filtered and concentrated. Lyophilization from water/CH_3CN afforded the building block **3** as a white solid which was used without further purification (0.82 g, 1.4 mmol, 82 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.80 (3H, t, J = 6.7 Hz), 1.15-1.18 (22H, m), 1.33-1.56 (10H, m), 2.06 (2H, t, J = 7.7 Hz), 3.16 (2H, m), 3.60 (1H, m), 4.14 (1H, t, J = 7.4 Hz), 4.28 (2H, m), 5.70 (1H, d, J = 6.8 Hz), 7.23 (2H, m), 7.30 (2H, m), 7.53 (2H, m), 7.68 (2H, m).

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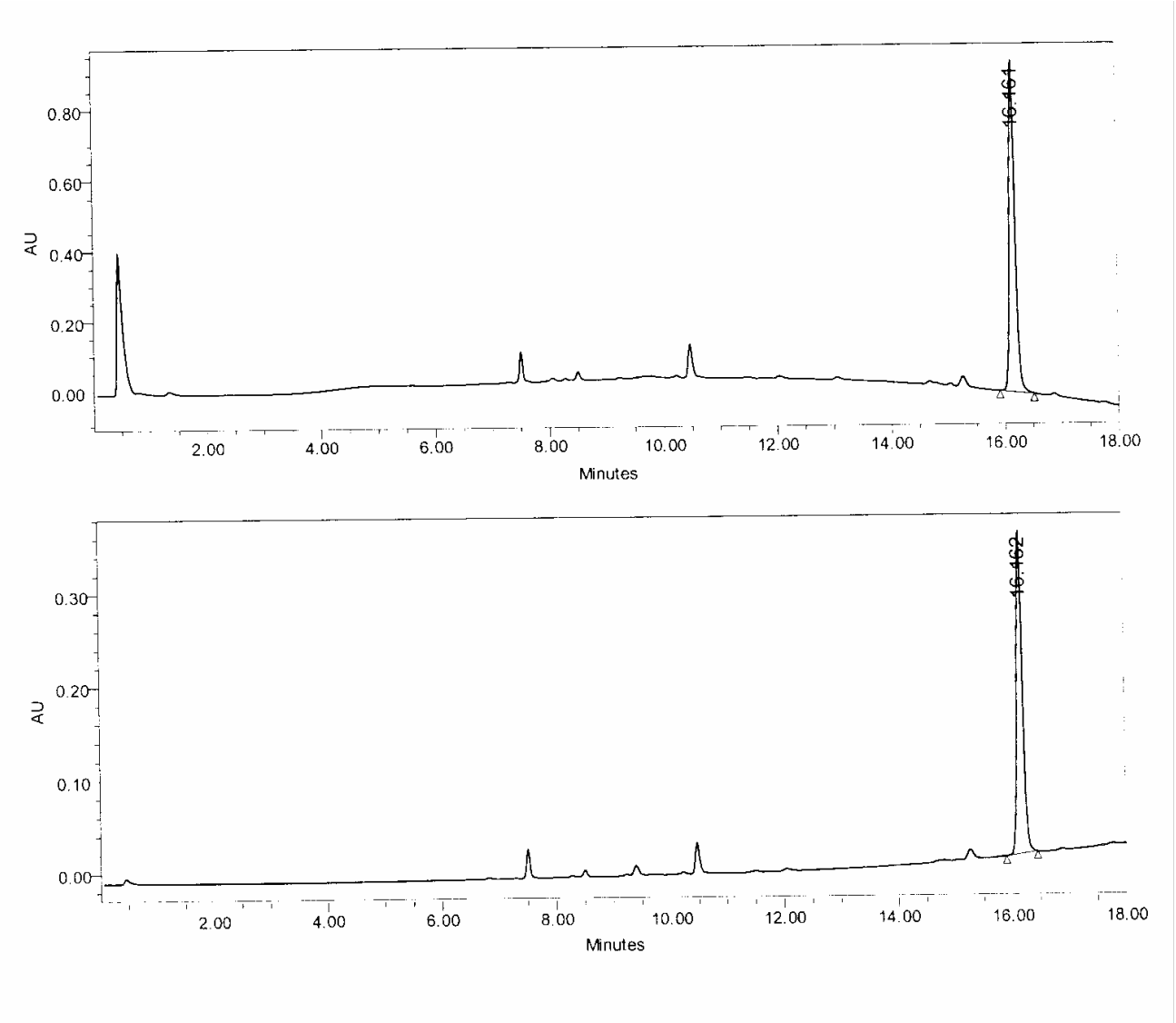
1 S. Foillard, M. Ohsten Rasmussen, J. Razkin, D. Boturyn and P. Dumy, *J. Org. Chem.*, 2008, **73**, 983.

RP-HPLC profile of 3

(Nucleosil 120 Å 3 µm C18 particles, 30×4 mm^2)

Conditions: see Materials and equipments pS2

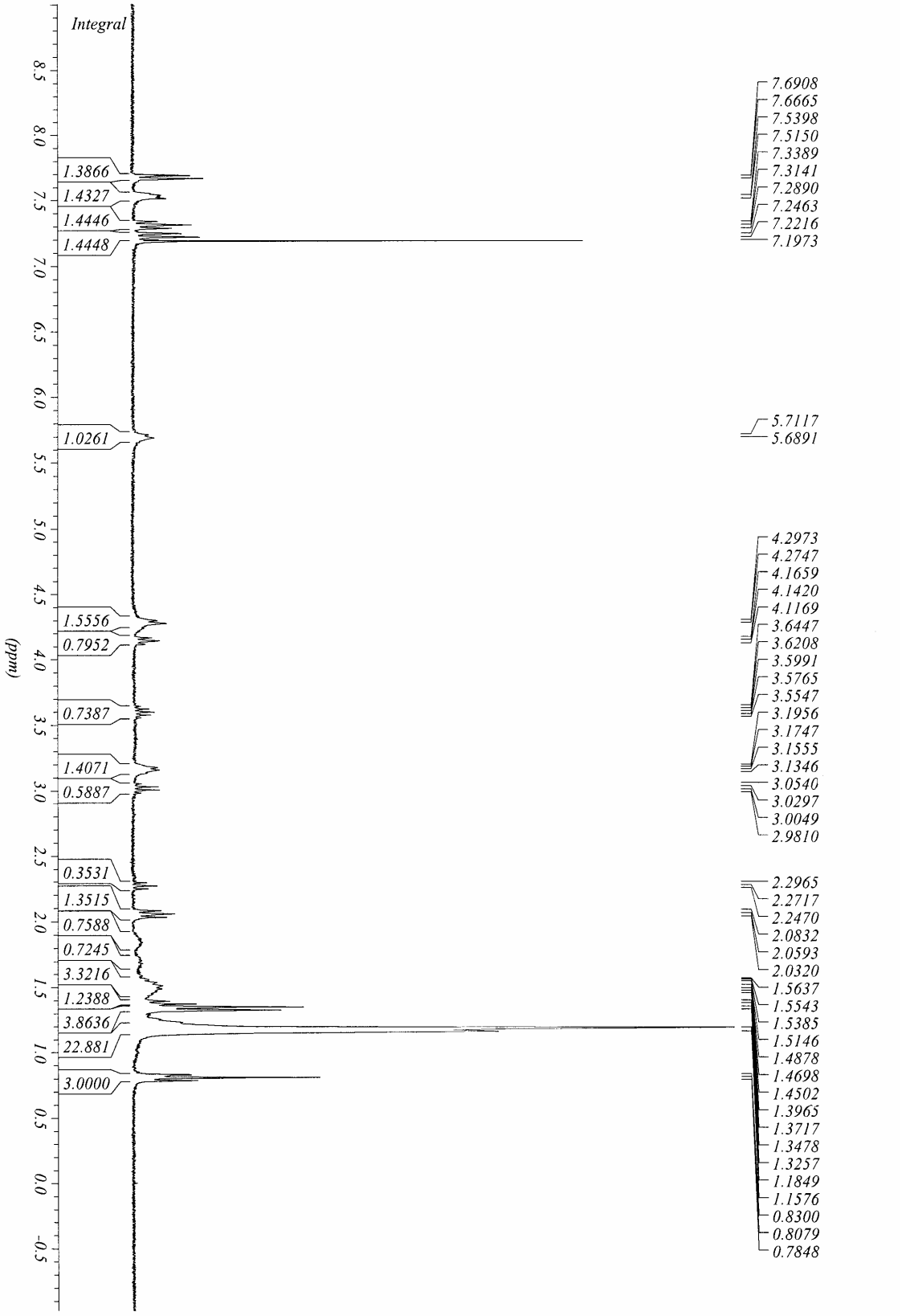
214 nm and 250 nm



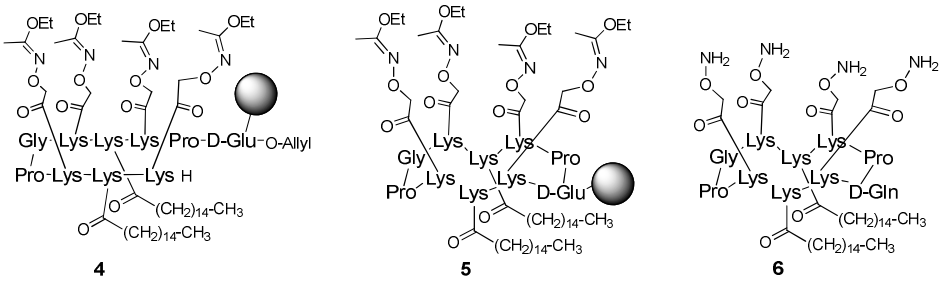
1H-RMN of 3

($CDCl_3$)

Conditions: see Materials and equipments pS2



Lipodecapeptide 5.



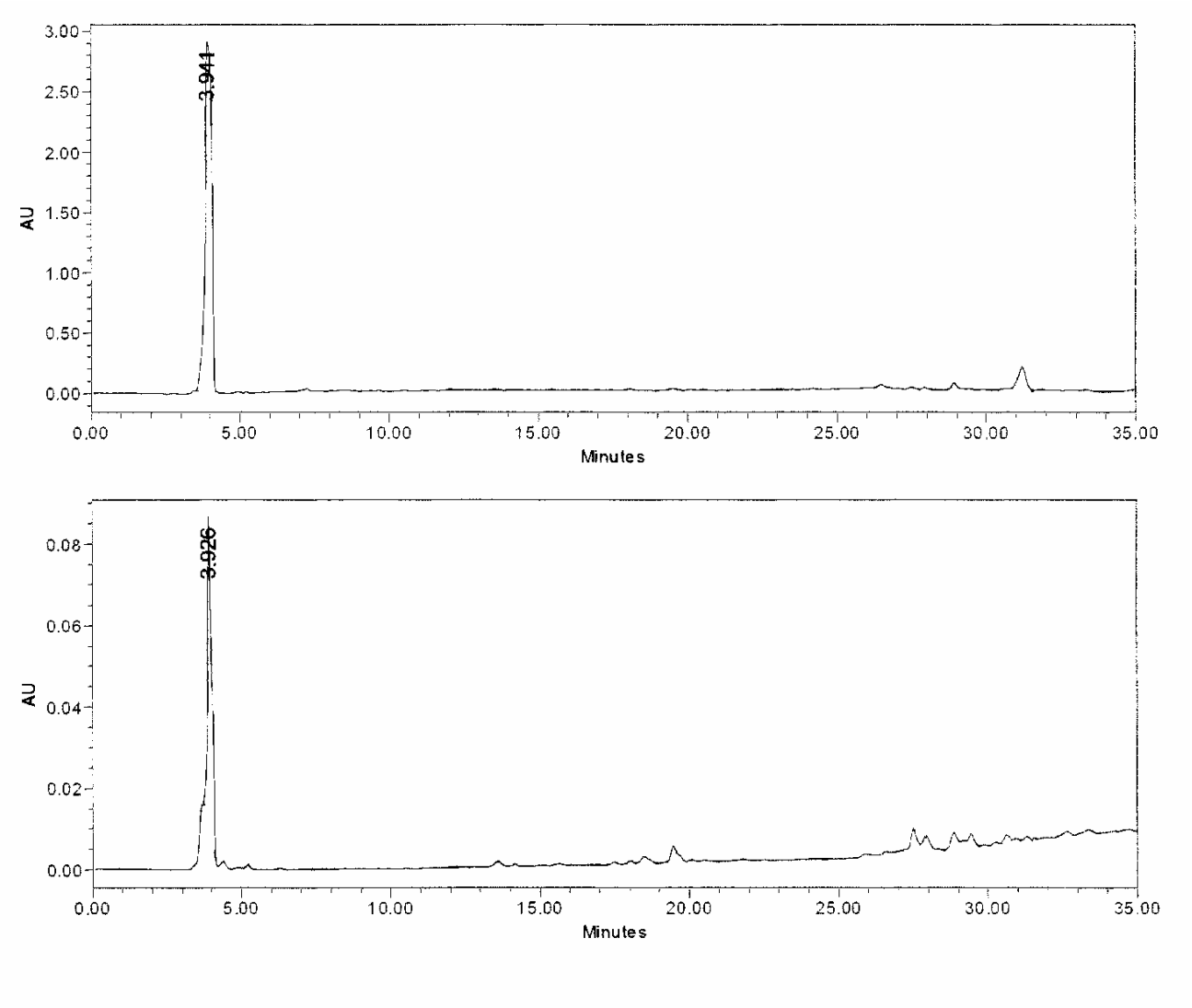
Lipopeptide **4** synthesis was carried out on Rink-amide resin (300 mg, loading 0.2 mmol/g) as described in the general procedure. Then, allyl group was removed: the linear decapeptide **4** on resin (100 mg, 0.02 mmol), previously swelled in dry CH_2Cl_2 under argon (5 mL, 15 min), was treated by adding successively phenylsilane (2.2 mL, 1.3 mmol) followed after 3 min by $Pd(PPh_3)_4$ (15 mg, 0.013 mmol). The reaction mixtures were stirred for 30 min at room temperature. The resin was washed with CH_2Cl_2 (2 x 10 mL), dioxane/H_2O (9/1, 10 mL), DMF (10 mL) and CH_2Cl_2 (10 mL). Peptide cyclization was performed in DMF (10 mL). pH was adjusted to 8-9 by addition of DIPEA, then PyAOP (10 mg, 0.02 mmol) was added and the mixture was stirred at room temperature for 30 min. The resin was washed with DMF (5 x 10 mL) and CH_2Cl_2 (10 mL). Then, resin was treated with a solution of trifluoroacetic acid/H_2O/triisopropylsilane (90/5/5, 10 mL) for 30 min. Solvent was removed under reduced pressure and residue dissolved in the minimum of CH_2Cl_2. Ether was added to precipitate the crude lipopeptide **6**. The latter was triturated and washed three times with ether affording lipopeptide **6** as a white powder (47.8 mg, 0.019 mmol, 95%). ESI-MS calc for $C_{93}H_{170}N_{21}O_{21}$ 1917.3, found 1917.7.

RP-HPLC profile of 6

(Supelco 300 Å 10 µm C5 particles, 250 × 4.6 mm^2)

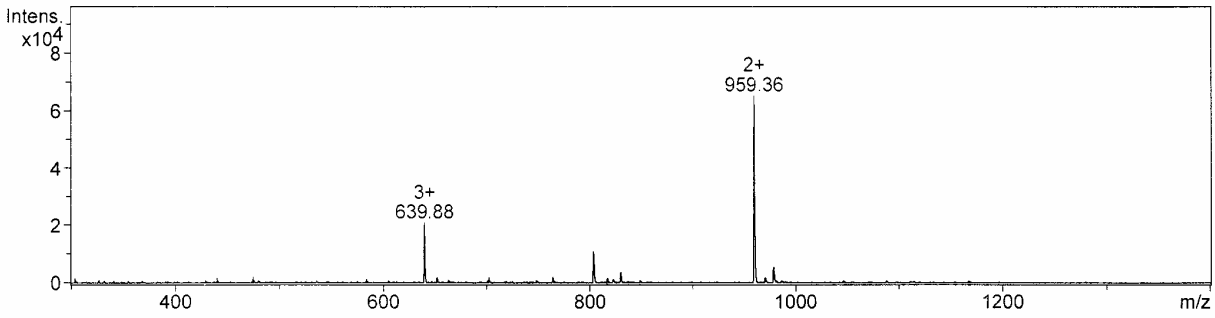
Conditions: linear gradient 5 to 100 % B in 30 min

214 nm and 250 nm



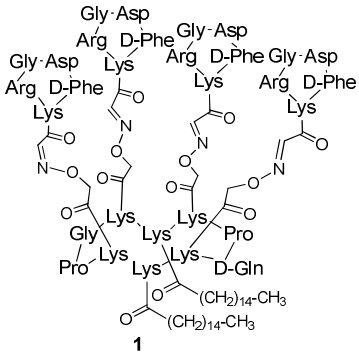
ESI-MS analysis of compound 6

Conditions: see Materials and equipments pS2



Component	Deconvoluted Mass	Molecule	Absolute Abundance	Relative Abundance
A	1917.70	[M + H]+	84909	98.98

RGD-lipopeptide 1.



1

Cyclo[-Arg-Gly-Asp-D Phe-Lys(-CO-CHO)-] was prepared as previously described.[2] To a stirred solution of lipopeptide **6** (10 mg, 4.2 µmol) in acetic acid/H_2O/CH_3CN (50/25/25, 0.5 mL) was added Cyclo[-Arg-Gly-Asp-D Phe-Lys(-CO-CHO)-] (15 mg, 18.5 µmol). After 5 h stirring at room temperature, RP-HPLC purification afforded the compound **1** as a white powder (1 mg, 0.22 µmol, 5%). ESI-MS calc for $C_{209}H_{325}N_{57}O_{53}$ 4484.3, found 4484.6.

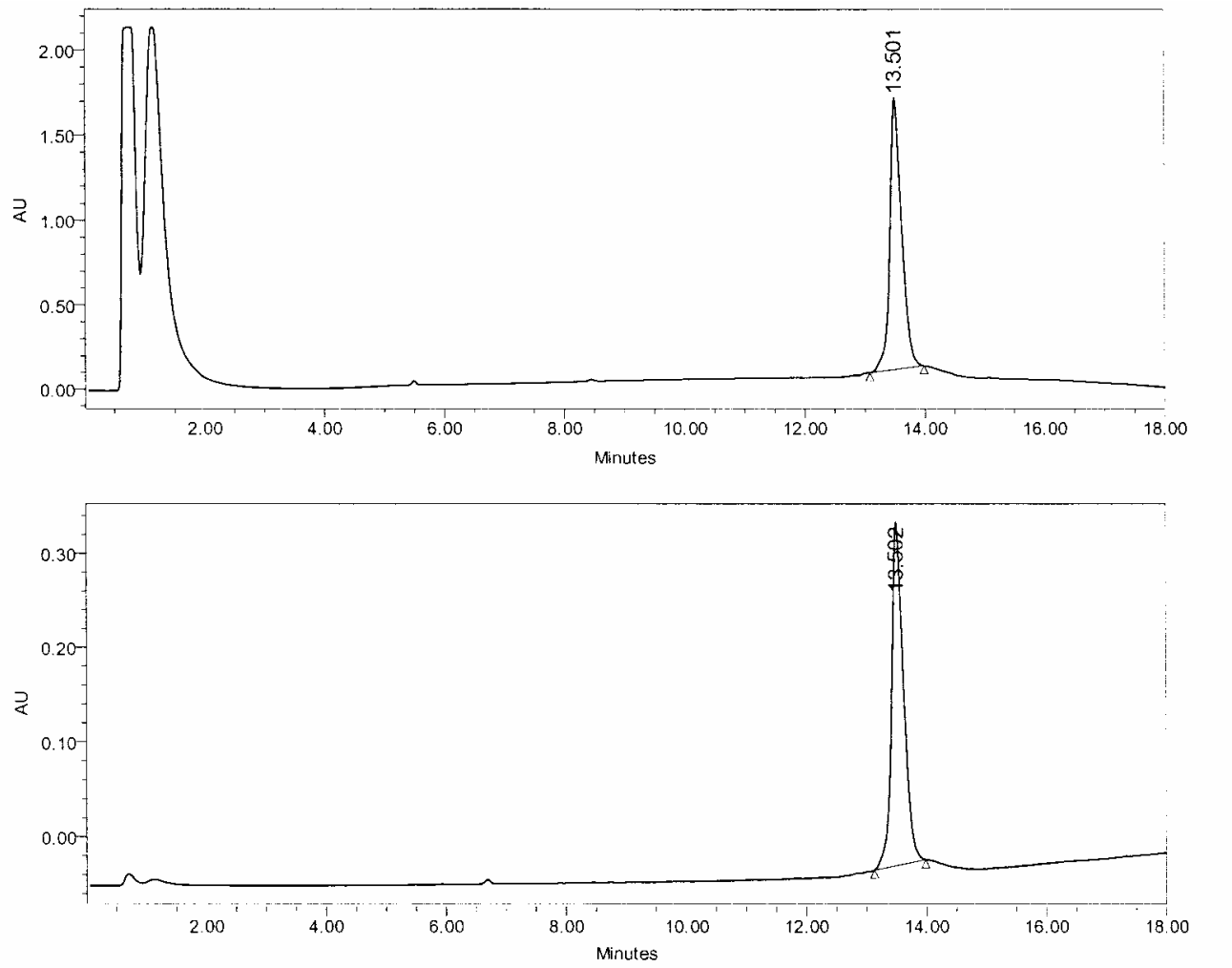
2 D. Boturyn and P. Dumy, *Tetrahedron Lett.*, 2001, **42**, 2787.

RP-HPLC profile of 1

(Nucleosil 120 Å 3 µm C18 particles, 30×4 mm^2)

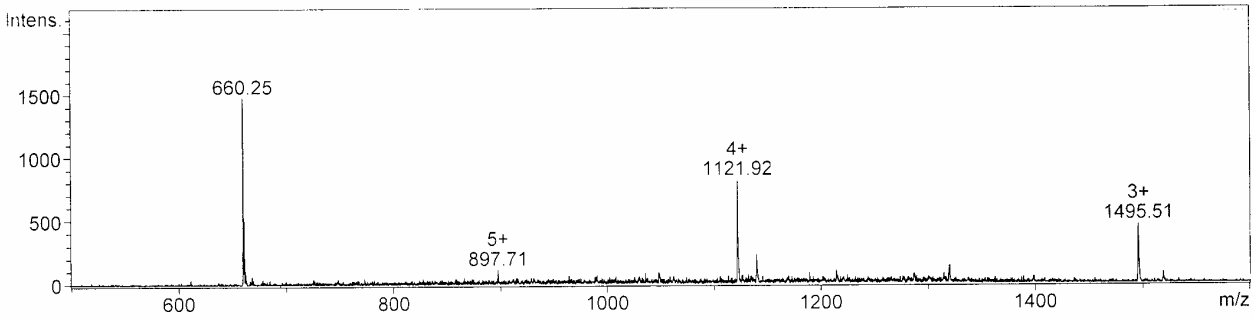
Conditions: see Materials and equipments pS2

214 nm and 250 nm



ESI-MS analysis of compound 1

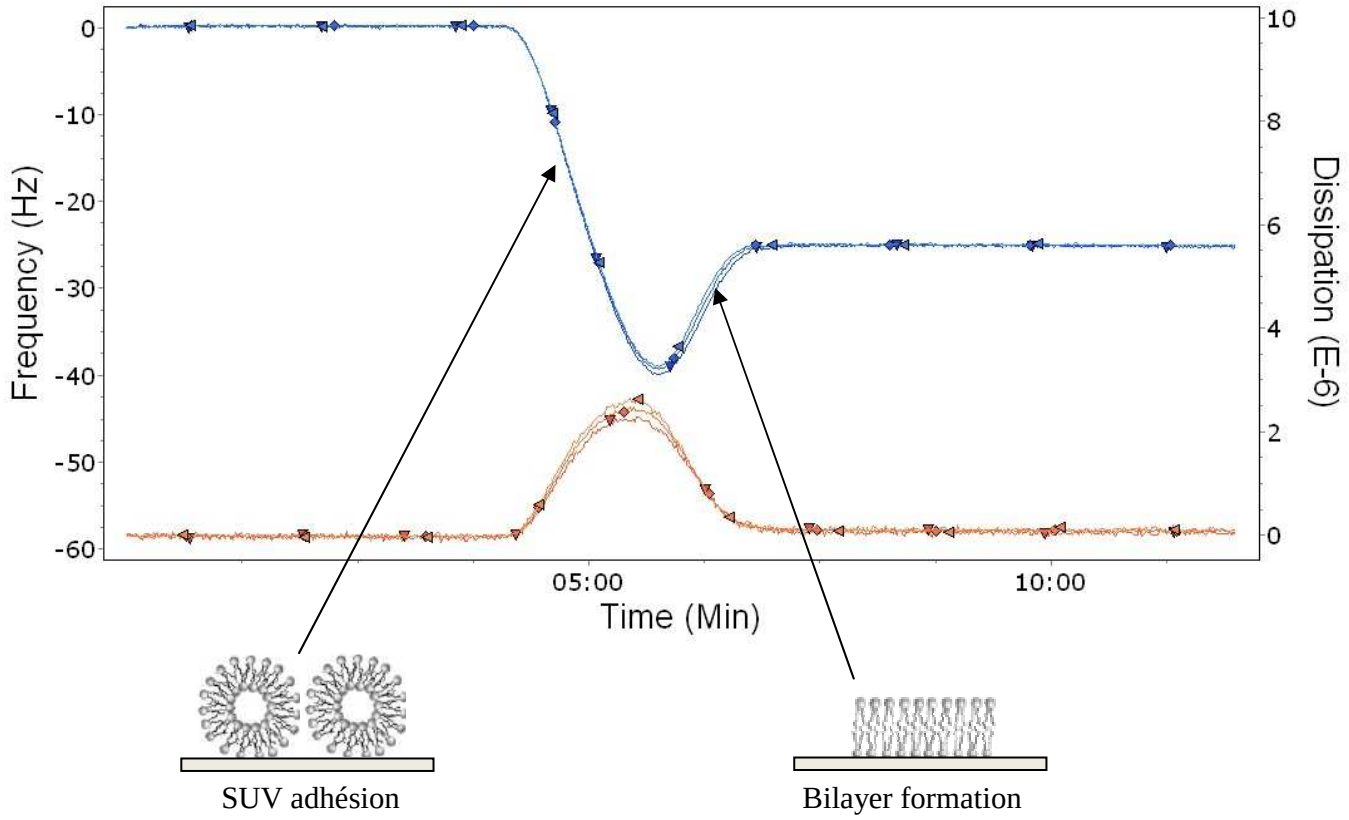
Conditions: see Materials and equipments pS2



Component	Deconvoluted Mass	Molecule	Absolute Abundance	Relative Abundance
A	4484.61	[M + H]+	1382	99.38

Vesicles formation

Lipid stock solutions were prepared in chloroform, 13 10^{-3} M for POPC and 0.15 10^{-3} M for lipopeptide **1**. A mixed solution at the desired percentage was prepared and evaporated under nitrogen and then under reduced pressure for few minutes. Lipids were resuspended in Tris buffer and sonicated for thirty minutes at 10°C to form the small unilamellar vesicles (SUVs). The SUVs were purified by ultracentrifugation for 4 h at 4°C at 47 000 rpm. The size of SUV was measured by a Zetasizer nano (Malvern, USA), typically around 40 nm. SUVs were stored at 4°C at 0.2 mg/ml, stable for several weeks. Then, planar supported lipid bilayer was obtained *in situ* by fusion of SUV on QCM-D silica substrate (See Figure below).



Cell culture

HEK293(β3) and HEK293(β1) are subclones of the human embryonic kidney HEK293 cell line, stably transfected by a plasmid encoding respectively the human integrin αVβ3 or the human integrin αVβ1. Cells were cultured as adherent monolayers in DMEM (Dulbecco's Modified Eagle's Medium) enriched with 4.5 g.L- 1 glucose and supplemented with 1 % glutamine, 10 % FBS (Fetal bovine serum), penicillin (50 U.mL-1), streptomycin (50 µg.mL-1) and G418 (700 µg.mL-1). All cells were maintained at 37°C under an atmosphere of 5 % CO2. After washing with PBS, cells were detached with Trypsin (Gibco) from the plate. After counting and centrifugation, the cells were resuspended in DMEM free serum at the interest concentration 100 000 cells/mL.

QCM-D measurements

Experiments were performed on a quartz crystal microbalance with dissipation monitoring (QCM-D) Q-Sense E4 system equipped with flow chambers from Q-sense AB (Q-Sense AB, Göteborg, Sweden). Monitoring the resonance behavior of piezoelectric oscillators allows measurement of the mass adsorbing at the surface of the oscillator in real time. Changes in the resonance frequencies (Δf) are related to attached mass (including coupled water), and its dissipation (ΔD) are related to frictional (viscous) losses in the adlayer. This QCM-D instrument was operated with the use of AT-cut single crystal quartz sensors with 5 MHz resonant frequency. The overtones n = 3, 5, 7, 9, 11 and 13 are also recorded to have better stability and higher sensitivity than that obtained with the fundamental resonance. In the case of homogeneous, quasi-rigid films with low thickness, the areal adsorbed masses, $\Delta\Gamma$, could be calculated according to the Sauerbrey equation, $\Delta\Gamma = -C\,\Delta f n/n$ with the mass sensitivity C = 17.7 ng cm^{-2} Hz^{-1} for f_1 = 5 MHz. QCM-D measurements were operated in flow mode. The measurement chamber and all solutions via a Thermomixer (Ependorf) were stabilized at 37 °C to ensure stable operation. All buffers were previously degassed in order to avoid bubble formation in the QCM-D measuring chamber. SiO_2-coated QCM sensors (Q-Sense AB, Göteborg, Sweden) was activated by immersion in a 2% SDS solution for 20 minutes, rinsed with water and dried with nitrogen followed by exposure to UV-ozone for 10 minutes. Then, the surfaces were docked in the QCM measurement chamber. All the solutions were flowed through the system using a four channels peristaltic pump (Ismatec SA). A background of Tris buffer was injected at 50 µL/min until a stable baseline was reached. A solution of SUV at 0.2 mg/mL in Tris buffer was injected until stabilization. During this time, the bilayer was formed on the sensors. After the rinsing in Tris buffer during 15 minutes and in PBS buffer during one hour, DMEM was passed through the chamber to obtain a stable baseline in adhesion buffer during approximately 30 min prior to the injection of the cell suspension (100 000 cells/mL) for 1 hour at 100 µL/min. Finally, the measurement chamber was rinsed with DMEM at 50 µL/min.

AFM characterization of SLBs

AFM experiments were performed on a Pico plus (Molecular Imaging) commercial instrument. Topography pictures were obtained in a liquid cell using magnetic taping mode (MAC Mode) with type II MAC Levers tips (0.4 Nm^{-1}). Data treatment (height measurements after baseline correction only) and presentation were realized with the help of Gwyddion Software. Correlation length and rms roughness was evaluated from 2 × 2 μm^2 images.

Figure 1A shows the AFM topography image of a pure POPC SLB obtained by fusion of small unilamellar vesicles on a Si/SiO_2 silicon wafer. Rms roughness of 0.25 nm and correlation length of 188 nm completely agrees with the formation of a supported lipid bilayer. Similar features (rms = 0.17 nm and correlation length 389 nm) were obtained after fusion of small unilamellar vesicles of POPC in combination with 5% of RGD-lipopeptide **1** (Figure 1B). Since the height of the four RGD ligands on the cyclodécapeptide scaffold is estimated at 4.5 nm from molecular modelisation, whereas the height of the polar head of POPC is only 1 nm, it can be concluded that AFM images do not reveal lipopeptide aggregation but an homogenous dispersion of compound 1 within SLB.

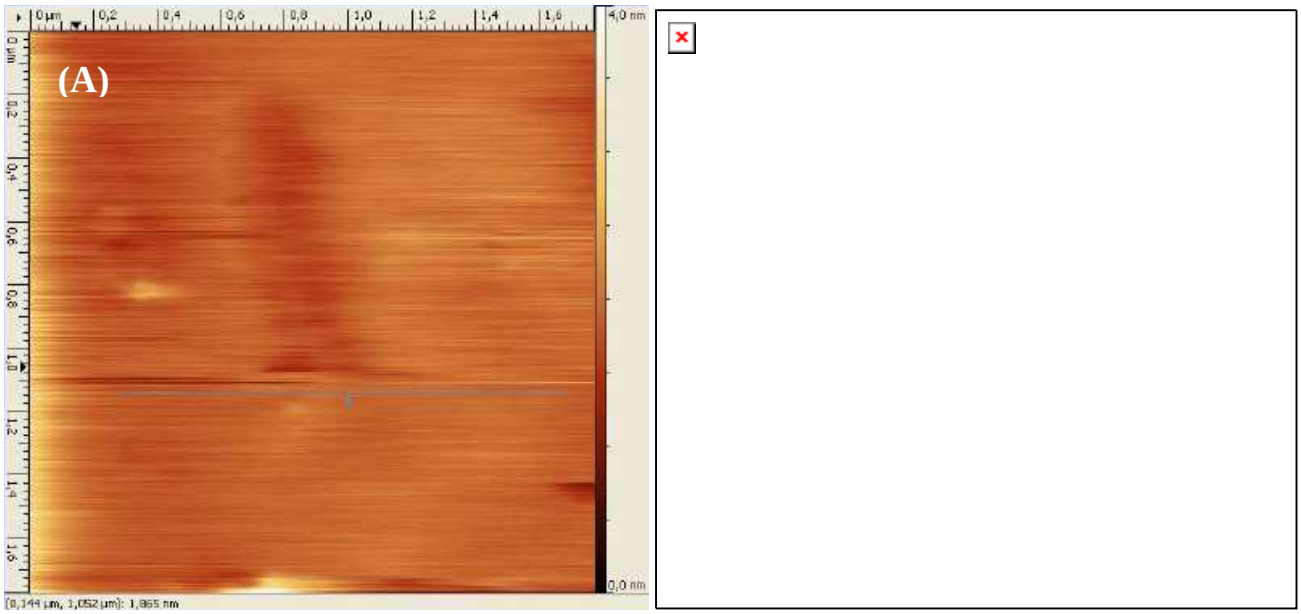


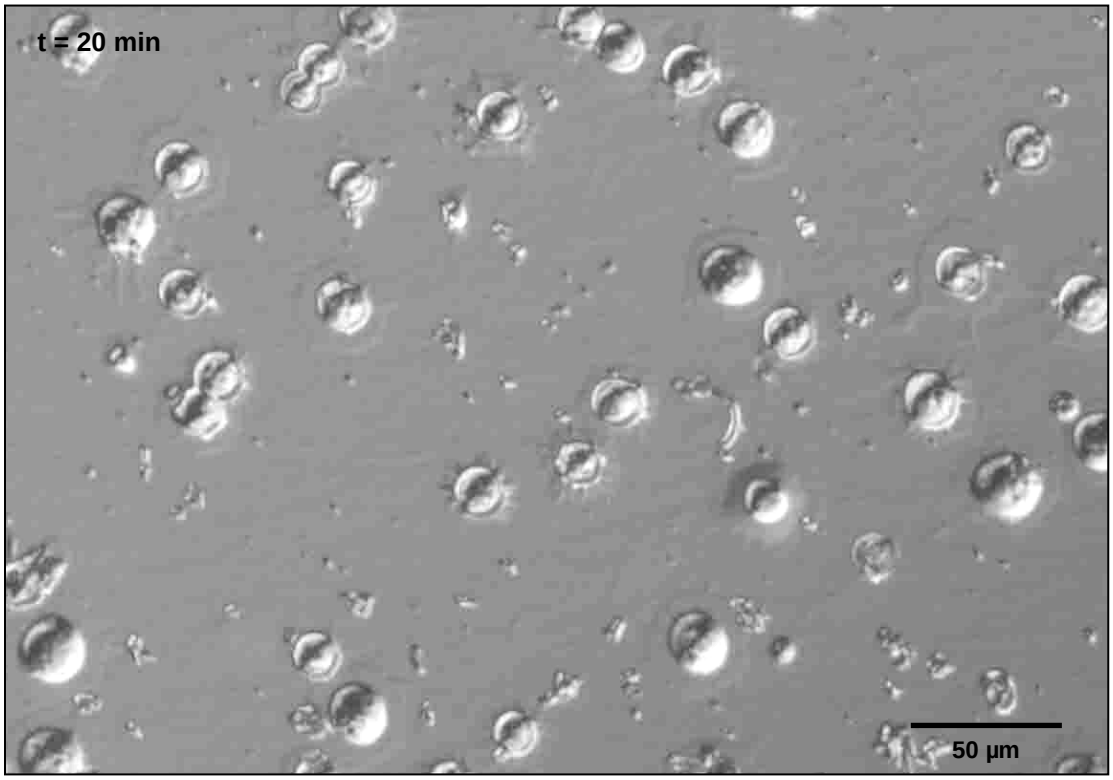
Figure 1: Representative topography AFM images (2 µm × 2 µm) of SLBs on silicon wafers prepared by small unilamellar vesicle fusion of (A) POPC and (B) POPC in combination with 5% of RGD-lipopeptide 1.

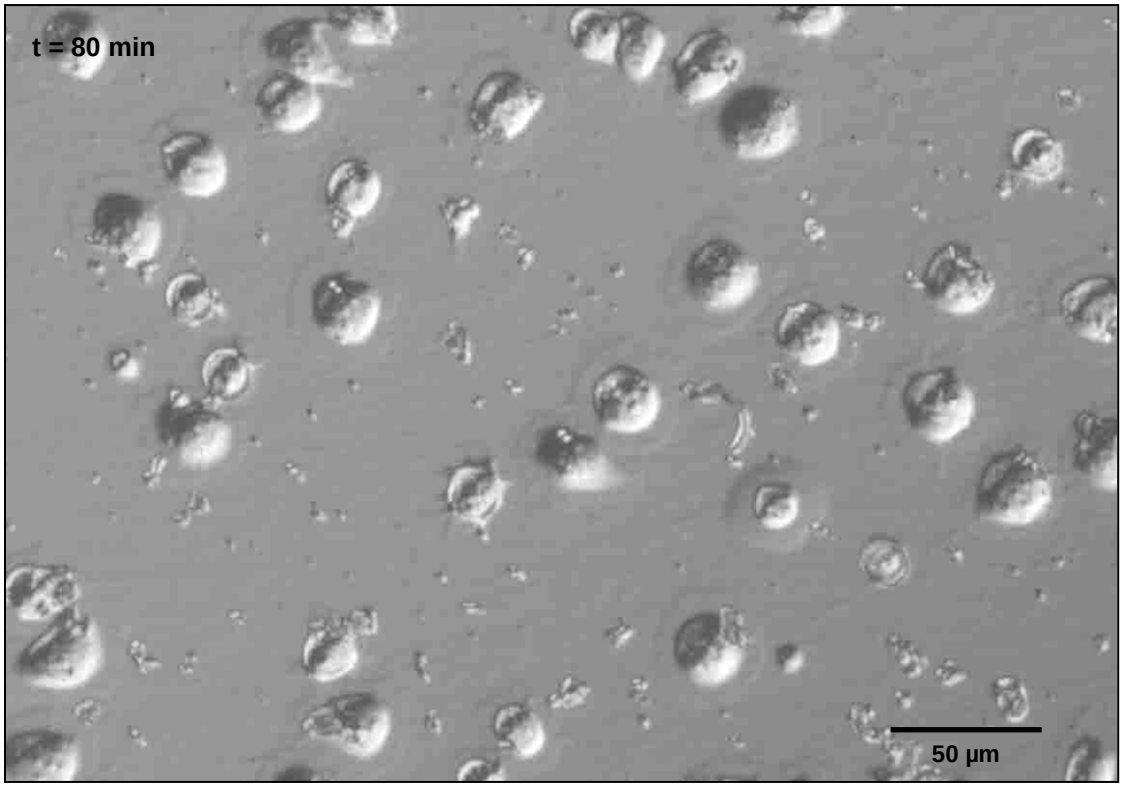
Microscopy experiments in combination with QCM-D

Microscopy experiments coupled with QCM-D were performed using the Q-sense window module 401 (Q-Sense AB, Göteborg, Sweden) and a microscope Axio Imager A1m (Carl Zeiss S.A.S., France). Cell experiments were carried out as described above. Injection of the cell suspension (100 000 cells/mL) was performed during 15 min at 100 µL/min, then the measurement chamber rinsed with DMEM at 50 µL/min. The images were registered and treated using the software Axiovision (Carl Zeiss S.A.S., France).

Optical microscopy images of cell adhesion using 0.1% lipopeptide 1

Images were recorded after injection of cells for 15 min at 100 µL/min then DMEM at 50 µL/min.





Optical microscopy images of cell adhesion using 1% lipopeptide 1

Images were recorded after injection of cells for 15 min at 100 µL/min then DMEM at 50 µL/min.