Peptide-presenting two-dimensional protein matrix on supported lipid bilayers: An efficient platform for cell adhesion

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Understanding and controlling cell adhesion to biomaterials and synthetic materials are important issues in basic research and applied sciences. Supported lipid bilayers (SLBs) functionalized with cell adhesion peptides linked to lipid molecules are popular platforms of cell adhesion. In this paper, an alternative approach of peptide presentation is presented in which peptides are stereo-selectively linked to proteins self-assembling in a rigid two-dimensional (2D) matrix on SLBs. Annexin-A5 (Anx5) was used as prototype protein for its known properties of forming stable and rigid 2D matrices on lipid surfaces. Two types of Anx5-peptide complexes, containing either a RGD or an IKVAV sequence, were synthesized. The authors show that both Anx5-peptide complexes present the same properties of binding and 2D organization on lipid surfaces as Anx5, when investigated by quartz crystal microbalance with dissipation monitoring, atomic force microscopy, and transmission electron microscopy techniques. Anx5-RGD and Anx5-IKVAV 2D matrices were found to promote specific adhesion of human saphenous vein endothelial cells and mouse embryonic stem cells, respectively. The influence of the surface density of exposed peptides on cell adhesion was investigated, showing that cells attach to Anx5-peptide matrices when the average distance between peptides is smaller than about 60 nm. This cell adhesion platform provides control of the orientation and density of cell ligands, opening interesting possibilities for future applications. [DOI: 10.1116/1.2821954]

I. INTRODUCTION

Understanding and controlling cell adhesion to biomaterials and synthetic materials are important issues in basic research and applied sciences, e.g., for the development of artificial tissues, the biointegration of medical implants, or the development of cell chips for drug screening assays.[1–4] A wide range of methods has been developed for inducing cell adhesion on a variety of substrates (see the review in Ref. 5). These methods rely principally on biomimetic functionalization of substrates with extracellular matrix proteins or peptides. The most commonly used cell adhesion ligand is the Arg-Gly-Asp (RGD) peptide,[6,7] which is present in several extracellular matrix proteins and is recognized by integrins.[8,9] Typical procedures for coupling cell recognition ligands to solid substrates involve physical adsorption, either directly or via polymer linking,[10] covalent coupling via self-assembled monolayers,[11] affinity interaction using the streptavidin-biotin system, or supported membranes formed either by vesicle deposition[5,12] or above a polymer cushion.[4,13]

Functionalized supported lipid bilayers (SLBs) have become increasingly popular as a cell adhesion platform, because they constitute a natural cell environment in which membrane proteins and peptides can be incorporated. In addition, the formation of SLBs is simple[14] and they are highly resistant to nonspecific adsorption by proteins or cells.[12,15–18] SLBs consist of a continuous lipid bilayer, 5 nm thick, separated from the substrate by a ~1 nm thin water cushion. Previous studies of cell adhesion with SLBs have relied on the use of lipids functionalized with peptides.[18–26] The influence of the orientation,[20,22,23] accessibility,[18,26]

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conformation,[20,22] or mobility[27] of lipo-peptides on cell attachment and spreading have been characterized, thanks to the availability of a wealth of surface-sensitive techniques adapted to the SLB format.

In this paper, we present a novel type of cell adhesion platform, in which peptides are linked stereo-selectively to proteins forming a rigid 2D matrix on a SLB surface. The basic concept was to exploit the property presented by several proteins of self-assembling in 2D matrices on a lipid surface.[28–30] The main potential advantages of this mode of peptide presentation are the control of the orientation and of the density of peptides. In addition, this system allows addressing questions of interest in cell adhesion, such as the influence of the mobility of the ligands, by comparing the behavior of immobile protein-peptides with mobile lipo-peptides on SLBs.

This approach is presented here with Annexin-A5 (Anx5) as a prototype of 2D matrix-forming protein. Anx5 is known to self-assemble and form 2D matrices covering lipid surfaces containing anionic phospholipids, in the presence of Ca^{2+} ions.[30–36] The structure of Anx5 2D crystalline assemblies has been extensively characterized, both by transmission electron microscopy (TEM) (Refs. 31–33 and 36) and atomic force microscopy (AFM).[30,34,35] We have shown that Anx5 forms 2D crystals of trimers on lipid monolayers and on SLBs prepared on mica, and 2D close-packed assemblies of trimers on SLBs prepared on glass substrates.[34,37] The surface density of Anx5 molecules in crystalline or close-packed assemblies is basically the same, about 33 000 molecules/μm^2, which corresponds to a distance of about 6 nm between adjacent Anx5 molecules.[35]

This paper describes first the synthesis and the characterization of the binding and 2D organization properties of two types of Anx5-peptide complexes, containing either a RGD or an IKVAV motif.[38,39] The IKVAV sequence is found in laminin,[40] and it has been shown that IKVAV peptides were able to promote cell adhesion.[24,38] Then, we show that the 2D matrices of Anx5-peptides are able to promote cell adhesion with two cell types, namely human saphenous vein endothelial cells (HSVE cells) and mouse embryonic stem cells (MES cells), which are pluripotent cells that can give rise to any cell type.[41] Finally, we show how the surface density of Anx5-peptides influences cell attachment.

II. MATERIALS AND METHODS

A. Materials

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine] (DOPS) were purchased from Avanti Polar Lipids (AL, USA). All other chemicals were of ultrapure grade. Water was purified with a RiOs system (Millipore, France).

Muscovite mica plates of 12 mm diameter were purchased from Metafix (Montdidier, France). Glass cover slips of 12 mm diameter were from Fisher Scientific (Illkirch, France). QCM-D sensor crystals, covered with 100-nm evaporated gold and reactively sputter-coated with 50-nm silicon oxide, were purchased from Q-Sense (Gothenburg, Sweden).

B. Expression and purification of recombinant rat Anx5-SH and Anx5-SS-Anx5 dimers

The rat Anx5 coding sequence was excised by NcoI digestion from the pKK233–2-Anx5 expression vector[42] and cloned into the pGELAF+ expression vector[43] between two NcoI restriction sites, resulting in the pGEF-A5 expression vector. A double mutant (C314S, T163C) was constructed, in which the single cysteine-SH residue present in native rat Anx5 was deleted (C314S), while a cysteine-SH was introduced in position 163 (T163C), resulting in the pGEF-A5B expression vector. The protein coding sequence of the double-mutant Anx5 (C314S, T163C), referred to hereafter as Anx5-SH, was amplified by PCR, excised by NcoI/BamHI digestion, and cloned into the expression vector pET11b (GE-Healthcare), resulting in the pET11b-Anx5-SH expression vector. *Escherichia coli* BL21(DE3) cells were transformed by heat shock with pET11b-Anx5-SH plasmid.

Cells were plated on LB medium containing 100 μg/mL ampicillin and incubated overnight at 37 °C. A single colony was collected and grown in 25 ml LB-ampicillin medium overnight at 37 °C. An aliquot was diluted with 400 mL LB-ampicillin medium to give an OD_{600} of 0.1. The culture was incubated at 30 °C until OD_{600} reached 0.7. Protein expression was then induced by addition of 0.4 mM IPTG and growth was continued for 16 h at 30 °C.

Cells were harvested by centrifugation (10 min, 4.5 krpm, 4 °C). The pellet was resuspended in an appropriate volume of 10 mM Tris, 1 mM EGTA, 0.01% NaN_3, *p*H 7.5, to give a calculated OD_{600} of 60. The cell suspension was sonicated at 4 °C with a model 250 Branson sonicator operated in a pulse mode consisting of five steps of sonication at 13 W for 1 min with 15 s intervals. Membrane fragments and large debris were removed by centrifugation at 12 000g for 2 h at 4 °C. The supernatant, referred to as a soluble extract, was collected and stored at 4 °C until use.

For purification, the soluble extract was filtered over 0.22 μm filters and applied in 4 mL fractions on a Superdex-200 column (GE-Healthcare) pre-equilibrated and eluted with a buffer containing 20 mM Tris, *p*H 8, and 0.02% NaN_3 (buffer A). The fractions containing Anx5-SH were pooled and purified by anion-exchange chromatography with a MonoQ HR5/5 column (GE-Healthcare) pre-equilibrated with buffer A. Elution was performed with a linear NaCl gradient in buffer A. Anx5-SH eluted at approximately 230 mM NaCl. The yield was about 25 mg pure Anx5-SH protein per 400 mL of culture.

Although Anx5-SH proteins have a natural tendency to oxidize and form Anx5-SS-Anx5 dimers, called hereafter $(Anx5\text{-}S)_2$ dimers, the yield in $(Anx5\text{-}S)_2$ dimers by spontaneous oxidation was highly variable. An optimized method of production of $(Anx5\text{-}S)_2$ dimers was developed, consisting of mixing Anx5-SH with dithiodipyridine (DTDP) (Sigma Aldrich) at an Anx5-SH/DTDP molar ratio of 2/1, followed by overnight incubation at room temperature.

$(Anx5\text{-}S)_2$ dimers were purified on a MonoQ column eluted with a NaCl gradient in buffer A, and stored at 4 °C until use.

C. Synthesis of Anx5-RGD and Anx5-IKVAV complexes

The RGD peptide (sequence: GCRGYGRGDSPG) and IKVAV peptide (sequence: CSRARKQAASIKVAVSADR) were synthesized by solid phase Fmoc chemistry[44] with an Applied Biosystems 431A synthesizer (Courtaboeuf, France). They were purified by reverse phase chromatography on a C-18 column eluted with a water/acetonitrile gradient containing 0.1% trifluoroacetic acid (TFA) and lyophilized.

The covalent coupling of each peptide to Anx5-SH involved the formation of a disulfide bond between the sulfhydryl group of Anx5-SH and the sulfhydryl group from the cysteine residue contained within either the RGD or IKVAV peptide; it was realized as follows: 75 nmol $(Anx5\text{-}S)_2$ dimers were reduced by addition of 10 mM dithiothreitol (DTT) for 30 min at room temperature. Reduced Anx5-SH was purified on a HiTrap desalting column (GE-Healthcare) eluted with buffer A. 63.5 nmol An5-SH in buffer A were mixed with 254 nmol adhesion peptide in 0.1% TFA and then with 63.5 nmol DTDP. The mixture was incubated overnight at room temperature. The Anx5-peptide complexes were purified on a MonoQ column, eluted with a NaCl gradient in buffer A. Their purity and mass were analyzed by SDS-PAGE and MALDI-TOF mass spectrometry, respectively.

D. Preparation of small unilamellar lipid vesicles

DOPC and DOPS lipids were dissolved in chloroform, mixed in desired amounts to give a DOPC/DOPS mixture of 7:3 (w:w), dried in a rotary evaporator, resuspended at 2.5 mg/mL final lipid concentration in a buffer solution made of 10 mM Hepes, 150 mM NaCl, 2 mM NaN_3, *p*H 7.4 (buffer B), and vortexed. To form small unilamellar vesicles (SUVs), the lipid suspension was first homogenized by five cycles of freeze-thawing and vortexing, and then sonicated with a tip-sonicator (model-250, Branson, USA) operated in a pulse mode at 18% duty cycle for 30 min with refrigeration over ice. The sample was centrifuged for 10 min at 16 000 g in an Eppendorf centrifuge in order to remove titanium particles. SUV suspensions were stored at 4 °C until use.

E. Functionalization of glass supports for cell adhesion

Glass cover slips (12 mm diameter) were cleaned by exposure to 2% SDS for 30 min, then rinsed with ultrapure water, blow-dried with nitrogen, and exposed to UV/ozone for 10 min.[34] Substrates cleaned in this way were stored in air. Prior to use, they were re-exposed to UV/ozone for 10 min, placed over a piece of parafilm, and immediately covered with 150 μL of a buffer containing 10 mM Hepes, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM NaN_3, *p*H 7.4 (buffer C). A SLB was formed by deposition of 150 μL of 0.1 mg/mL SUVs, diluted in buffer C, over the glass substrate.[34,45] After 20 min, the excess of vesicles was removed by gently exchanging the chamber liquid with buffer C by ten cycles of addition/withdrawal of 100 μL aliquots. Anx5 or Anx5-peptide solutions were added at 20 μg/mL final protein concentration. After 30 min incubation, time sufficient for saturation, the excess of protein was removed as described before. The functionalized glass supports were used extemporaneously.

F. Cell adhesion experiments

Primary HSVE cells were harvested, amplified, and characterized as described by Fernandez *et al.*,[46] slightly modified from Golledge *et al.*[47] Cells grew in M199 medium (Invitrogen Corp, Cergy Pontoise, France) supplemented with 20% fetal calf serum (Eurobio, Les Ulis, France), 50 IU/mL heparin (Sanofi Aventis, Paris, France), 10 ng/mL bFGF (Sigma Aldrich, St Quentin Fallavier, France), and 50 μg/mL gentamycin (Biomedia, Boussens, France).

HSVE cells from passages 3 to 4 were detached from culture flasks by trypsin (0.125%)-EDTA (0.0625%), collected by centrifugation for 7 min at 1200 rpm, and suspended in Dulbecco's modified eagle's medium (Invitrogen) containing 1.8 mM Ca^{2+} and 1 mM Mg^{2+}. The protein-SLB-functionalized glass supports were placed in a 12-well plate coated with an agarose layer to prevent cell adhesion to the plastic according to Amédée *et al.*,[48] and 200 000 cells/well were seeded. Cell adhesion experiments were performed at 37 °C and 5% CO_2 for 3, 6, or 24 h.

The extent of cell adhesion was determined by means of a colorimetric assay according to Verrier *et al.*[49] Briefly, the samples were transferred, after rinsing with Hanks' balanced salt solution (Invitrogen) to a 48-well plate without agarose. The cells were imaged by phase contrast microscopy and then supplemented with 0.1 M sodium citrate, 7.5 mM *p*-nitrophenyl-*N*-acetyl-β-*D*-glucosaminide, 0.5% (v/v) Triton X-100, *p*H 5, and placed at 37 °C and 100% humidity.[50] After 2 h, the enzymatic reaction was stopped by addition of a solution composed of 50 mM glycine, 5 mM EDTA, *p*H 10.4, and absorbance was measured at 405 nm using a microplate reader (Dynex MRX).

Cell adhesion was also investigated with an R1 MES cell line.[41] Cells were grown and passaged for few days in the following medium: knockout DMEM, knockout serum replacement 15%, *L*-glutamine 2mM, pen/strept 100 u/100 μg/mL, nonessential amino acids 100 μM (Gibco-BRL/Invitrogen), 2000 u/mL LIF (Leukemia Inhibitory Factor, Chemicon/Millipore), and 100 μM β-mercaptoethanol (Sigma). Cells were trypsinized, counted, and 5×10^4 cells in 0.4 mL were seeded per well in a 24-well plate containing SLB-functionalized glass slides corresponding to the different conditions to analyze (Anx5-RGD, Anx5-IKVAV, Anx5 alone), and three control conditions (plastic, nontreated glass, and 0.1% gelatinized glass). MES cells are routinely cultured on gelatin to increase adhesion to the plastic surface. E14 and R1 ES cells were incubated overnight at

37 °C with 5% CO_2, fixed the next day with 4% paraformaldehyde, washed with PBS, and finally incubated at room temperature with the nuclear dye 4′6′-diimidazolin-2-phenylindole (DAPI) for 10 min to quantify the number of cells attached in each condition.

G. Liposome binding assay

DOPC/DOPS (4:1, w:w) multilamellar lipid vesicles (MLVs) were prepared as follows: the desired amounts of lipids dissolved in chloroform were mixed, the solvent was eliminated in a rotary evaporator, a volume of buffer B was added to give a 10 mg/mL lipid suspension, and the suspension was homogenized by vortexing. Aliquots of 100 μg MLVs were incubated for 20 min with mixtures of known Anx5/Anx5-peptide ratio containing a total amount of 40 μg protein (expressed in the equivalent amount of Anx5) in a total volume of 200 μL buffer C. Unbound proteins were separated from MLV-bound proteins by centrifugation at 100 000 rpm for 45 min in a Beckmann Ultima centrifuge. The pellet was resuspended in 300 μL buffer C and a second centrifugation step was carried out to wash the pellet. The final pellet was resuspended in a total volume of 40 μL buffer C. The relative amount of Anx5 and Anx5-peptide was analyzed by SDS-PAGE.

H. Quartz crystal microbalance with dissipation monitoring

QCM-D measurements were performed with a Q-SENSE D300 system equipped with a QAFC 302 axial flow chamber (Q-SENSE, Gothenburg, Sweden), as described in detail elsewhere.[51] In brief, the adsorption of matter at the surface of a sensor crystal induces changes in the resonance frequency, F, of the crystal and in the energy dissipation, D. The frequency change, ΔF, is related to the adsorbed mass, including coupled water, and the dissipation change, ΔD, is related to energy losses in the adsorbed layer and provides information on its viscoelastic properties. The adsorbed mass, m, can be deduced from the Sauerbrey equation,[52] $m = -C \times \Delta F$, with $C = 17.7$ ng cm^{-2} Hz^{-1}, which has been demonstrated to be valid for SLBs and protein assemblies on SLBs.[45] In the QCM-D curves presented in the figures, the ΔF and ΔD jumps associated with buffer exchanges were corrected for.

I. Atomic force microscopy

AFM experiments were performed in liquid using a Nanoscope IV-Multimode (Veeco, Dourdan, France), equipped with a J-scanner (120 μm). Oxide-sharpened silicon nitride cantilevers with a nominal spring constant of 0.06 N/m (Digital Instruments, CA) were exposed to UV/ozone (BHK, CA, USA) for 10 min prior to use.[34] The liquid-covered substrates were installed in a contact mode fluid cell equipped with an O-ring, and a sample solution or buffer was injected with a syringe. Contact mode images were recorded at a scanning rate of 4–6 Hz and a scanning angle of 0°. Images were flattened.

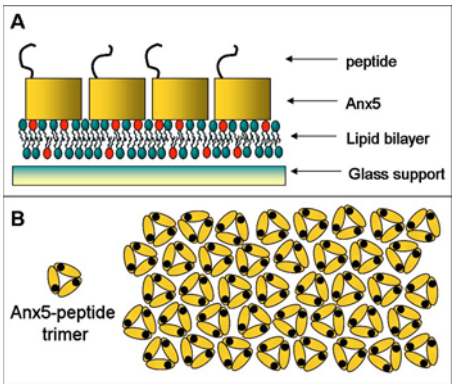

FIG. 1. Scheme of the cell adhesion platform based on a 2D matrix of Anx5-peptides on a SLB. (A) Side view: Anx5-peptide protein complexes self-assembled in a rigid 2D matrix over a SLB formed on a glass support. Cell-adhesion peptides are exposed to the aqueous solution. (B) top view: 2D close-packed assembly of trimers of Anx5-peptides.

J. Transmission electron microscopy

Protein 2D crystallization experiments were performed as described in detail in Ref. 29. Briefly, a lipid monolayer was formed by applying 0.6 μl of a lipid mixture containing 250 μM DOPC and 125 μM DOPS dissolved in chloroform:hexane (1:1, v/v) over a 17 μL droplet of 25 μg/mL Anx5-peptide in buffer C. After 1 h incubation necessary for crystallization, the material present at the air-water interface was transferred onto a perforated carbon-coated 200-mesh copper EM grid by horizontal lifting. The grid was washed with water, negatively stained with 1% uranyl acetate, and coated with a thin layer of carbon to increase stability. TEM observations were performed with a CM-120 FEI microscope operated at 120 kV. Images were recorded on a $2k \times 2k$ USC-1000 slow-scan CCD camera (Gatan, CA, USA) at a nominal magnification of $\times 40\,000$. Images were processed with the image processing GRIP software.[53]

III. RESULTS AND DISCUSSION

Our goal was to develop a platform for cell adhesion consisting of a 2D matrix of Anx5-peptide complexes self-assembled on a SLB, as illustrated in Fig. 1.

A. Synthesis of Anx5-peptide complexes

Two cell-adhesion peptides, containing either a RGD or an IKVAV motif, were synthesized. Both sequences contained in addition a cysteine residue, in order to form a covalent disulfide-bond between the peptides and Anx5-SH, a double mutant of Anx5. The single cysteine residue of Anx5-SH was stereo-selectively inserted in a solvent-exposed loop on the concave face of Anx5, opposite to the membrane-binding face, in order to ensure high accessibility of the coupled elements.[54,55]

The Anx5-RGD and Anx5-IKVAV disulfide-linked complexes were produced at close to 100% purity with an overall yield of 20% (data not shown). Molecular masses of 36 738 and 37 608 Da were determined by MALDI-TOF mass spec-

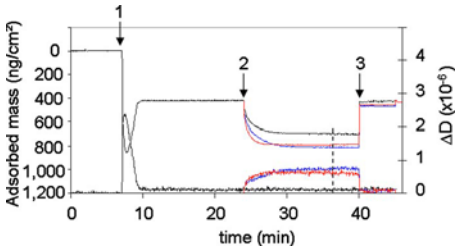

FIG. 2. Adsorption of Anx5-RGD, Anx5-IKVAV, and Anx5 on DOPC/DOPS (7/3) SLBs, by QCM-D. Changes in adsorbed mass (upper curves; increasing values downwards) and dissipation (bottom curves) measured upon addition of DOPC/DOPS (7/3) vesicles (arrow 1), followed by the addition of 20 μg/mL Anx5-RGD (blue), Anx5-IKVAV (red), or Anx5 (black) (arrow 2). Rinsing the QCM-D chamber with protein-free buffer is indicated by a dashed line. At arrow 3, the chamber was rinsed with an EGTA-containing buffer. Biphasic aspect of the mass and dissipation curves observed after the addition of lipid vesicles is characteristic of the formation of a SLB on SiO_2, stabilizing at about 440 ng/cm^2 and exhibiting no dissipation.[45,60]

trometry for Anx5-RGD and Anx5-IKVAV, respectively. These values are in good agreement with the theoretical values of 36 759 and 37 628 Da, respectively.

B. Binding and organization of Anx5-peptide 2D matrices on SLBs

The behavior of Anx5-peptide complexes on lipid surfaces was investigated by QCM-D, AFM and TEM, in order to check whether the covalent coupling of peptides to Anx5-SH affected Anx5 properties of binding and 2D self-assembly on SLBs.[29,30,33–35]

1. Characterization of the binding of Anx5-peptide complexes to SLBs, by QCM-D

Figure 2 presents the QCM-D adsorption responses obtained with both Anx5-RGD and Anx5-IKVAV, as well as with Anx5, on SLBs. Saturation of the SLB surface was obtained with about 20 μg/mL Anx5-RGD, similarly to Anx5.[35] At saturation, Anx5-RGD and Anx5-IKVAV adsorbed monolayers are characterized by mass and ΔD values of $\sim$350 ng/cm^2 and $\sim 0.8 \times 10^{-6}$, respectively. In comparison, a saturating monolayer of Anx5 has a mass of 280 ng/cm^2 and a ΔD value close to 0.[35] As expected, binding of Anx5-peptide complexes is Ca^{2+}-dependent, as shown by the instantaneous and complete release of Anx5-peptide upon rinsing with a buffer containing 2 mM EGTA (arrow 3 in Fig. 2). The 2D Anx5-peptide matrices are stably bound to the SLB, as shown by the absence of release upon rinsing with a protein-free solution (dashed line in Fig. 2). This property, which is characteristic of Anx5,[35] is essential for the present application as cell adhesion experiments must be performed in the absence of Anx5-peptide in solution.

The relative mass difference of $\sim$25% observed between Anx5-peptide ($\sim$350 ng/cm^2) and Anx5 (280 ng/cm^2) matrices is large in comparison with the relative mass increment of the peptides ($\sim$4–6%). This, together with the high dissipation values of Anx5-peptide matrices as compared to Anx5, indicates that the peptides are flexible and highly hydrated. The RGD and IKVAV peptides are thus favorably exposed for interacting with integrins.

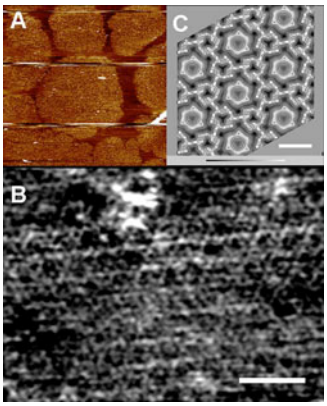

FIG. 3. 2D organization of Anx5-RGD on lipid surfaces, by AFM (A), (B), and TEM (C). A) Domains of Anx5-RGD formed on a DOPC/DOPS (7/3) SLB on mica. Formation of domains is characteristic of the formation of 2D crystalline domains of Anx5 on mica-SLBs.[29] Image size: 20 μm. (B) High-magnification AFM image revealing the hexagonal crystalline organization of Anx5-RGD molecules. Scale bar: 40 nm. (C) 2D projection map of an Anx5-RGD 2D crystalline area from a TEM image, calculated at 2.5 nm resolution, with $p3$ symmetry imposed ($a=b=17.7$ nm, $\gamma=120°$).

2. Characterization of the 2D organization of Anx5-peptide matrices by AFM and TEM

The structure of Anx5-peptide 2D matrices was further studied by AFM on SLBs and by TEM on lipid monolayers, following procedures established with Anx5.[30,33] The results obtained with both peptides were strictly similar; for simplicity, the results obtained with Anx5-RGD are presented here.

Anx5-RGD complexes self-assemble on SLBs in 2D domains, as shown by AFM [Fig. 3(A)]. At saturation, the domains cover entirely the SLB surface (data not shown), as previously observed for Anx5. Observed at higher resolution, these domains present a 2D crystalline organization with hexagonal symmetry [Fig. 3(B)], which is characteristic of Anx5 2D crystals.[30,35] However, the crystalline order is less clear in images of Anx5-RGD crystals than on Anx5 crystals' images. This is most likely due to the interaction of the AFM tip with RGD peptides, which are flexible and protrude above the rigid Anx5 matrix.

High-resolution structural information was obtained by TEM on Anx5-RGD 2D assemblies formed on lipid monolayers. Two-dimensional crystals of Anx5-RGD were obtained in the same conditions as for Anx5.[29,33] Figure 3(C) presents a 2D projection map of an Anx5-RGD 2D crystal, calculated by Fourier analysis at 2.5 nm resolution. The 2D crystals of Anx5-RGD are made of trimers arranged with $p6$ symmetry (unit cell dimensions: $a=b=17.7$ nm, $\gamma=120°$), as Anx5 2D crystals.[33]

In conclusion, these results demonstrate that the presence of a peptide covalently linked on the concave face of Anx5 opposite to the membrane-binding face does not affect its properties of binding and 2D organization on lipid surfaces. We consider therefore most likely that on glass substrates,

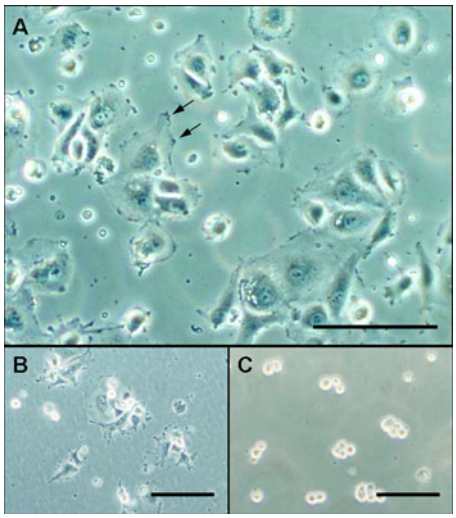

FIG. 4. Adhesion of HSVE cells on various supports. Phase contrast micrographs recorded after 3 h seeding HSVE cells. (A) HSVE cells homogeneously spread on a 2D matrix of Anx5-RGD (at a maximal density of 33 000 Anx5-RGD/μm^2). Dense focal contact points are observed at the cell periphery (arrows). (B) Cells spread on gelatin, exhibiting similar size and morphology as on Anx5-RGD. (C) HSVE cells do not adhere to an Anx5 matrix and exhibit a rounded shape. Scale bars: 50 μm.

which were used in cell adhesion experiments, Anx5-peptides behave also like Anx5 and form close-packed trimers[34] (Fig. 1).

C. Adhesion of HSVE cells and MES cells on 2D matrices of Anx5-peptides

The use of Anx5-peptide 2D matrices on SLBs as a platform for cell adhesion was then investigated with two cell types, HSVE cells and undifferentiated MES cells.

HSVE cells were found to adhere to 2D matrices of Anx5-RGD, as shown in Fig. 4(A) recorded after 3 h incubation. The cells were homogeneously spread, with an average diameter of about 20 μm. The cell nuclei were well-resolved and cytoplasmic extensions with focal contact points observed at the cell periphery [arrows in Fig. 4(A)]. The cell density was about 1400 cells/mm^2. This value is slightly higher than the value obtained on gelatin, which is the reference substrate for these cells [Figs. 4(B)].[56,57] HSVE cells do not adhere to pure Anx5 matrices [Fig. 4(C)] and only weakly to Anx5-IKVAV matrices (Fig. 5). On these substrates, quiescent cells were observed, presenting a characteristic rounded shape and intense light scattering, with a diameter of 2–5 μm [Fig. 4(C)].

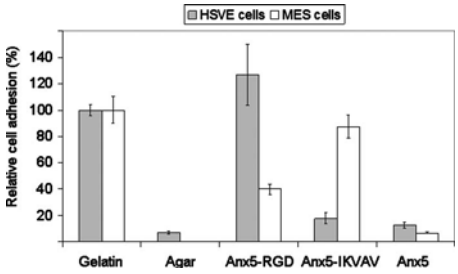

FIG. 5. Adhesion of HSVE cells and MES cells on various supports. The 100% relative adhesion values correspond to values measured on gelatin, which is the reference substrate for both cell types.

With MES cells, we found that Anx5-IKVAV 2D matrices promote cell attachment almost as efficiently as gelatin, which is the reference substrate for these cells (Fig. 5). Previous reports have shown that peptides containing the IKVAV sequence were able to promote the adhesion of neural stem cells,[24,38] stem cells committed to differentiate only into neural cell types, yet this is the first time that this effect is described for MES cells, pluripotent cells that can give rise to any cell type. MES cells were also observed to attach to Anx5-RGD 2D matrices, yet to a lesser extent than to Anx5-IKVAV (Fig. 5). No attachment was detected on pure Anx5 matrices.

These results demonstrate that the Anx5-peptide 2D matrices constitute an efficient platform for cell adhesion.

D. Influence of the density of Anx5-peptide complexes on cell attachment

We investigated then the influence of the peptide density on cell adhesion. As Anx5 and Anx5-peptides present almost identical properties of binding and 2D organization, we considered that the peptide density could be varied in a controlled manner by using mixtures of Anx5 and Anx5-peptides of known Anx5/Anx5-peptide ratios. To verify this hypothesis, we developed the following liposome-binding assay: (1) liposomes were incubated in the presence of protein solutions containing known Anx5/Anx5-peptide ratios, with an excess of Anx5 moieties with respect to the available lipid surface; (2) liposomes were separated from unbound proteins by centrifugation; (3) the respective amount of liposome-bound Anx5 and Anx5-peptide was evaluated by SDS-PAGE. The results presented in Fig. 6 show qualitatively that, for both RGD and IKVAV peptides, the respective amount of Anx5 and Anx5-peptide bound to liposomes was indeed in agreement with the original Anx5/Anx5-peptide ratio.

The surface density of Anx5-RGD peptides was shown to influence the adhesion of HSVE cells (Fig. 7). The maximal density of Anx5-RGD molecules in a 2D matrix made of pure Anx5-RGD is about 33 000 Anx5-RGD/μm^2; the corresponding RGD-to-RGD distance is about 6 nm.[33,35] Decreasing the RGD density down to 6000 RGD/μm^2 had only a minor effect on cell adhesion. Cell attachment was observed down to 1% Anx5-RGD, which corresponds to 300 RGD/μm^2.

Similar results were obtained when the attachment of MES cells was studied at various densities of Anx5-IKVAV peptides. The critical IKVAV density required for initial cell attachment was close to 300 IKVAV/μm^2 (data not shown).

A density of 300 Anx5-peptide/μm^2 corresponds to an average distance of 58 nm between ligands. This number is strikingly close to the critical distance of 58–73 nm determined by Spatz and colleagues[58] with nanopatterned RGD-linked gold particles, below which cells do not adhere. We

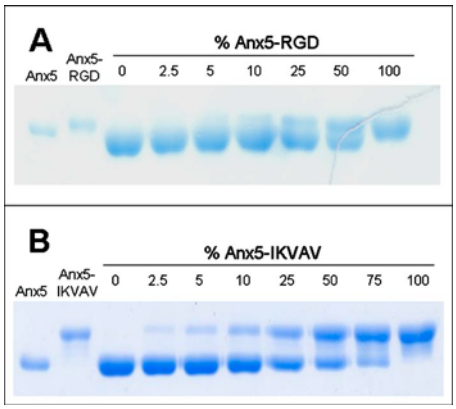

FIG. 6. SDS-PAGE analysis of the relative amount of Anx5-peptides and Anx5 bound to liposomes. Two left lanes correspond to pure Anx5 and Anx5-peptides [RGD in (A), IKVAV in (B)] in the absence of liposomes. The other lanes correspond to liposome pellets obtained after incubation with the following (Anx5-peptide/Anx5) mass ratios: 0/100; 2.5/97.5; 5/95; 10/90; 25/75; 50/50; 75/25 (for IKVAV), 100/0. Relative intensities of the Anx5-peptide and Anx5 bands in samples ranging from 25/75 to 100/0, estimated visually, are in good agreement with the protein ratios. For the three lower ratios (2.5/97.5, 5/95, and 10/90), the large difference in the absolute amounts of deposited proteins prevents direct quantitative estimation. Amount of Anx5-peptide was estimated by comparison with reference gels of known Anx5-peptide amounts (data not shown).

must say however that the 58-nm distance determined in our study corresponds to an average distance between ligands, and not an absolute distance as in the case of RGD-gold particles.[58] Nevertheless, the critical ligand density determined in our study is significantly lower than values previously reported with SLBs functionalized with lipo-peptides.[23] These two modes of peptide presentation differ mainly in terms of lateral mobility of ligands, as ligands diffuse freely in 2D in the case of lipo-peptides incorporated in SLBs,[23] while they are immobile in Anx5-peptide matrices, and also in the case of nanopatterned gold colloids. Other possible differences are the homogeneity, orientation, and/or accessibility of the peptides, which are well controlled in the case of a protein 2D matrix.

IV. CONCLUSIONS

This paper presents a platform for cell adhesion, consisting of a 2D matrix of Anx5-peptide complexes self-assembled on a SLB. The property of Anx5 to self-assemble at lipid surfaces into a rigid platform is exploited for presenting cell adhesion peptides. All known advantageous properties of Anx5 are shown to be conserved in Anx5-peptide complexes. Cell attachment is demonstrated with two different peptides and two different cell types. The efficiency of the platform in promoting cell adhesion is likely to result from the homogeneity, orientation, and accessibility with which peptides are presented. This paper also shows that undifferentiated MES cells attach specifically to IKVAV peptides, opening up the possibility of cell separation.

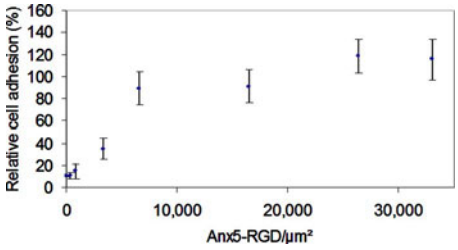

FIG. 7. Influence of the RGD peptide density on the adhesion of HSVE cells. Mean values and SD from six experiments. The 100% relative adhesion values correspond to values measured on gelatin.

The proposed strategy opens promising perspectives, for example for the sorting of cells via Anx5-peptide-SLB-functionalized glass beads, for the transfer of cells between supports, e.g., via Langmuir-Blodgett techniques, or for the development of cell chips via lipid bilayers suspended over holes.[59] Anx5 2D matrices may also be used for presenting other molecular entities, e.g., antibodies or proteins for the development of biosensors or microarray systems.

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