Adhesion of Arg-Gly-Asp (RGD) Peptide Vesicles onto an Integrin Surface: Visualization of the Segregation of RGD Ligands into the Adhesion Plaques by Fluorescence

Valérie Marchi-Artzner,*[,†,‡] Barbara Lorz,[†] Charlie Gosse,[§,||] Ludovic Jullien,[||] Rudolf Merkel,[†,#] Horst Kessler,[⊥] and Erich Sackmann[†]

Physik Department, Biophysik E22, Technische Universität München, James-Franck Strasse 1, D-85747 Garching, Germany, Laboratoire de Chimie des Interactions Moléculaires, UPR 285, Collège de France, 11 Place Marcelin Berthelot, 75231 Paris Cedex 05, France, Ecole Normale Supérieure, Laboratoire de Physique Statistique, UMR 8550, 24 rue Lhomond, 75231 Paris Cedex 05, France, Ecole Normale Supérieure, Département de Chimie, CNRS UMR 8640, 24 rue Lhomond, 75231 Paris Cedex 05, France, Forschungszentrum Jülich, Institute for Thin Films and Interfaces 4, D-52425 Jülich, Germany, and Institut für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstrasse 4, D-85747 Garching, Germany

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Integrins are adhesion receptors that mediate cell adhesion and play an important function in many biological processes such as morphogenesis and tissue remodeling. These membrane proteins specifically interact with a short tripeptide sequence, RGD (Arg-Gly-Asp), present in numerous extracellular macromolecules. Model systems have been developed in order to understand how membrane adhesion is induced by this specific RGD peptide ligand/integrin recognition system. We have previously shown that RGD giant vesicles selectively adhere to endothelial cells by formation of pinning centers. Nevertheless, the nature of the lipids located in the adhesion contact zone is unknown. One hypothesis is that the lipidic ligands migrate to the contact zone where they are confined after binding to the receptor. To study the possible formation of ligand domains within the vesicle bilayer, we synthesized a fluorescently labeled RGD lipid that can be easily incorporated in giant vesicles. Adhesion of giant RGD vesicles onto an integrin-functionalized surface was followed simultaneously by reflection interference contrast microscopy and fluorescence microscopy. For the first time, it was possible to observe the microsegregation of RGD lipids in the contact zone during adhesion. Additionally, we observed interesting photosensitive properties of the chalcone chromophore that could lead to a new method of analyzing the lipid organization within the membrane during adhesion and to the design of new ligand lipids and vesicle vectors for cell targeting.

Introduction

Direct specific interactions between cells and between cells and extracellular matrixes are critical to the function and the development of multicellular organisms.[1] Cell adhesion to the extracellular matrix is mediated by transmembrane proteins called cell adhesion molecules (CAMs), which implicate specific interactions so that cells adhere only to other cells of certain types. The integrins form one group of such receptors, some of which also regulate cell−cell interactions (e.g., $\alpha V\beta 1$ and VCAM). The integrins bind specifically to the Arg-Gly-Asp tripeptide (RGD), a short amino acid sequence present in several macromolecules of the extracellular matrix including fibronectin, collagen, and laminin. Lipid membranes bearing a RGD peptide headgroup have been built with the aim of better understanding the mechanism of membrane adhesion via specific integrin/RGD molecular recognition systems[2] and of designing new drug delivery systems which may be able to target one specific cell type. In a previous paper, we described the selective adhesion between human endothelial cells and giant unilamellar vesicles (GUVs) containing a synthetic lipopeptide bearing a headgroup composed of the cyclic pentapeptide (c-Arg-Gly-Asp-{Phe}-Ala-Lys), called c(-RGDfK-).[3] The advantage of a cyclic peptide compared to a linear peptide is that the constrained topology involves a selectivity for one subtype of integrin ($\alpha_v\beta_3$ or $\alpha_v\beta_5$).[4] Recently the crystal structure of the extracellular segment of the integrin $\alpha_v\beta_3$ complexed with another cyclic pentapeptide (c-Arg-Gly-Asp-{D-Phe}-{*N*-methyl-Val-}) was determined.[5] We previously observed that the adhesion of vesicles containing RGD lipids onto endothelial cells under a hydrodynamic shear flow induced the formation of pinning centers, which may be explained in terms of a lateral segregation of the ligands within the vesicle membrane and clustering of integrin receptors. The development of selective adhesion, that is, an increase in the number of specific bonds between membranes, depends on the lateral mobility of the bond-

* Corresponding author. Tel: +33 1 44 27 13 50. Fax: +33 1 44 27 13 56. E-mail: v.marchi-artzner@college-de-france.fr.

† Physik Department, Technische Universität München.

‡ UPR 285, Collège de France.

§ UMR 8550, Ecole Normale Supérieure.

|| UMR 8640, Ecole Normale Supérieure.

⊥ Institut für Organische Chemie und Biochemie, Technische Universität München.

Forschungszentrum, Jülich.

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forming species in the plane of the membrane.[6] When the membranes come into contact, complementary molecules have to recognize each other, and this is assumed to depend on their short-range diffusion within the zone of contact. From a theoretical point of view, different mechanisms for lateral phase separation during the adhesion of multicomponent membranes have been proposed.[7−9] The dependence of adhesion on local diffusion was pointed out in various model systems such as (i) rat basophilic leukemia (RBL) cells decorated with anti-dinitrophenyl (a-(DNP)) monoclonal IgE and vesicles exposing DNP-lipid haptens,[10] (ii) membranes containing cationic lipids in contact with a negatively charged surface,[11] (iii) membranes with biotinylated lipids interacting with a streptavidin-coated surface,[12] and (iv) membranes with the homophilic csA (contact site A) receptor from *Dictyostelium discoideum*.[13] However, in all these systems, direct evidence of the ligand microsegregation into adhesion plaques was not provided. Recently however it was shown by the micropipet aspiration technique that a strong lateral attraction of the csA receptors to the adhesion plaque occurs in vesicle−vesicle adhesion.[14]

In this paper, our aim is to detect a possible accumulation of RGD ligands in the zones of strong adhesion in the membrane by simultaneously using fluorescence microscopy and reflection interference contrast microscopy (RICM). First we describe the synthesis of a fluorescently-labeled RGD lipopeptide which was incorporated into GUVs enabling the observation of the reorganization of the RGD-ligands in the adhesion zone. Then we present our results concerning the adhesion of giant vesicles containing fluorescent RGD lipids onto integrin-coated surfaces.

Experimental Section

Materials. All reagents and solvents were at least analytical grade and were used as supplied. The dioleoylphosphatidylcholine (DOPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[poly(ethylene glycol) 2000] (PEG_{2000}−DMPE), and cholesterol (Chol) were purchased from Avanti. HATU[15] was purchased from Biosystems Perseptive GmbH. The cyclic pentapeptides c(-R(Pbf)GD(O^{t}Bu)fK-) and c(-RGDfK-) were prepared as follows:[4b] after the cyclization of the side chain protected linear precursor c(-R(Pbf)GD(O^{t}Bu)fK(Z)-) synthesized by solid-phase synthesis via the Fmoc strategy,[16] the temporary Z-protecting group for lysine was cleaved by hydrogenolysis on a Pd/C catalyst. Pbf and ^{t}Bu can be cleaved by a TFA/$CHCl_3$ (95:5) mixture.[17] All the reactions were monitored by thin-layer chromatography using 0.25 mm silica gel plates (Merck). Column chromatography was performed using silica gel-60 (Merck, 230−400 mesh ASTM). NMR spectra were recorded on a Bruker AM-200 spectrometer (200 MHz). High-performance liquid chromatography (HPLC) was performed using an analytical and a semipreparative column Si-60.

Synthesis of the RGD Fluorescent Lipid. *Chalcone Acid **3***. EO_3 chalcone **1**[18] (1.00 g, 1.22 mmol, 1 equiv) and ethyl diazoacetate (0.153 g, 1.34 mmol, 1.1 equiv) were dissolved in anhydrous dichloromethane (10 mL). Trifluoroboroetherate (3 drops) was then added. The solution turned from a yellow to a red color upon addition of the BF_3OEt_2 catalyst. The mixture was stirred at room temperature for 36 h under argon. After purification on a silica column (eluent AcOEt, Rf = 0.9), the ester **2** (122 mg, 0.134 mmol, yield 11%) was isolated. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.95 (d, J (H,H) = 8 Hz, 2H), 7.73 (d, J_{trans} (H,H) = 15 Hz, 1H), 7.47 (d, J (H,H) = 8 Hz, 2H), 7.27 (d, J_{trans} (H,H) = 15 Hz, 1H), 6.92 (d, J (H,H) = 8 Hz, 2H), 6.57 (d, J (H,H) = 8 Hz, 2H), 4.15 (s, 2H), 3.81 (t, J (H,H) = 7 Hz, 2H), 3.68−3.45 (m, 10 H), 3.26 (t, J (H,H) = 8 Hz, 4H), 1.55 (m, 4H), 1.35−1.1 (m, 55 H), 0.84 (t, 6H). The ester **2** was then saponified in a 10 mM NaOH solution in ethanol for 36 h at room temperature. After neutralization and filtration on a silica column (eluent CH_2Cl_2/MeOH 9:1, Rf = 0.25), the acid derivative **3** (100 mg, 0.114 mmol, yield 9%) was isolated. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.90 (d, J (H,H) = 8 Hz, 2H), 7.73 (d, J_{trans} (H,H) = 15 Hz, 1H), 7.47 (d, J (H,H) = 8 Hz, 2H), 7.27 (d, J_{trans} (H,H) = 15 Hz, 1H), 6.94 (d, J (H,H) = 8 Hz, 2H), 6.57 (d, J (H,H) = 8 Hz, 2H), 4.15 (s, 2H), 3.81−3.51 (m, 10 H), 3.27 (t, J (H,H) = 8 Hz, 4H), 1.55 (m, 4H), 1.35−1.1 (m, 55 H), 0.84 (t, 6H). MS calculated: 877.7; found: 878.5 [M^+ + H].

*c(-R(Pbf)GD(O^{t}Bu)fK-) Chalcone **4***. The acid **3** (30 mg, 34 μmol, 1 equiv), c(-R(Pbf)GD(O^{t}Bu)fK-) (35 mg, 38 μmol, 1.1 equiv), HATU (14.5 mg, 38 μmol, 1.1 equiv), and collidine (9 μL, 68 μmol, 2 equiv) were mixed together in anhydrous DMF (1 mL) and maintained under argon for 6 h. After elimination of DMF and purification on two successive silica plates (eluent CH_2Cl_2/MeOH 9:1, Rf = 0.45) followed by purification by HPLC (eluent: MeOH gradient from 10% to 30% in CH_2Cl_2), pure product **4** was isolated (40 mg, 23 μmol, yield 66%). MS calculated: 1771.13; found: 1171.5 [M^+], 1772.5 [M^+ + H]. Microanalysis calculated for $C_{99}H_{154}N_{10}O_{16}S$, H_2O: 66.41 (% C), 8.78 (% H), 7.82 (% N); found: 66.19 (% C), 8.69 (% H), 7.72 (% N).

*c(-RGDfK-) Chalcone Lipid **5***. The chalcone lipopeptide **4** was deprotected by diluted TFA ($CHCl_3$/TFA 95:5). After crystallization, washing in MeOH, and centrifugation at 0 °C, the product **5** (17 mg, 7 μmol, yield 30%) was isolated. MS calculated: 1462.98 [M^+]; found: 1463.7 [M^+ + H].

Preparation of the Integrin-Functionalized Surfaces. The $\alpha_{IIb}\beta_3$ integrin was isolated from human blood platelets according to the method of Fitzgerald et al. modified by Hu et al.[2] Glass substrates were then coated by $\alpha_{IIb}\beta_3$ integrin according to the procedure previously described.[19] The $\alpha_{IIb}\beta_3$ integrin proteins, solubilized in S300 buffer containing 20 mM Tris pH 7.25, 150 mM NaCl, 1 mM NaN_3, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ completed with 0.01% Triton X-100, were filtered through a 0.2 μm Nucleopore filter. The $\alpha_{IIb}\beta_3$ integrin solution (900 μL) was then spread on a coverslip and incubated for 1 h before repeated washing (6 times) with S1 buffer containing 10 mM HEPES, 100 mM NaCl, 1 mM NaN_3, and 1 mM $CaCl_2$ (osmolarity, 210 mOsm). To prevent nonspecific adhesion, the integrin surface was subsequently incubated with BSA protein: as for integrin, a solution containing 3% of BSA protein (900 μL) in S1 buffer was filtered and incubated on the coverslip before washing 6 times with buffer.

Preparation of the RGD-Doped GUVs. Giant vesicles were prepared by the method of electric swelling.[20] The lipids were dissolved in a (9:1) chloroform−methanol solution, and the solution was spread onto a cover slide covered by indium−tin oxide (ITO) electrodes. A (50:50:3) mixture of cholesterol, DMPC,

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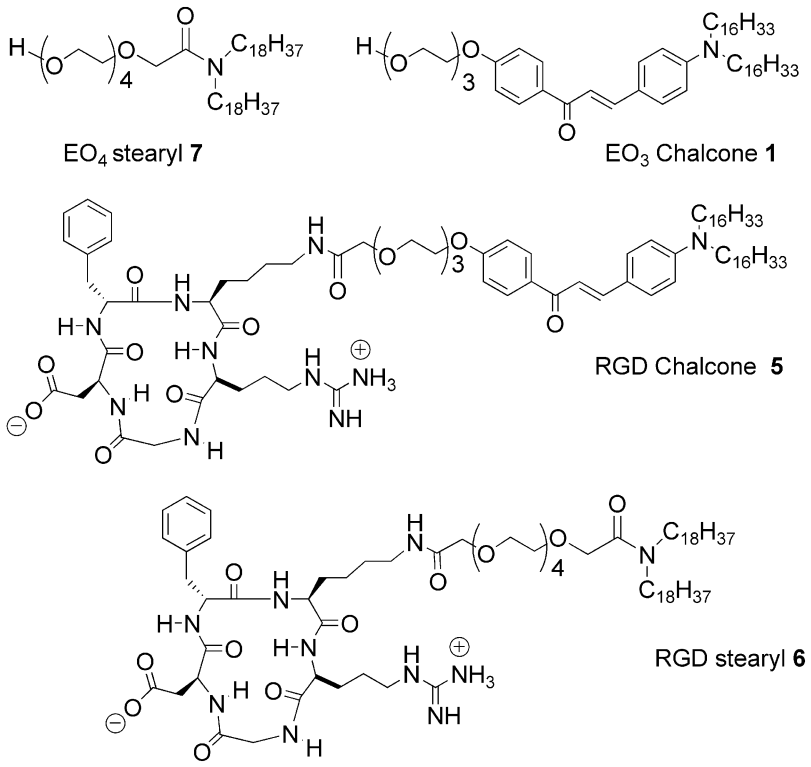

Figure 1. Chemical structures of the various synthetic lipids used in our study. They are either labeled by the chalcone chromophore or not and bear either an RGD peptide headgroup or a control hydroxyl group.

and PEG_{2000}−DMPE was used as a lipid matrix and completed with (i) 2 mol % RGD chalcone **5** or (ii) 2 mol % RGD stearyl **6** and 2 mol % EO_3 chalcone **1**. After drying under vacuum, the coverslips were placed in a closed cell. Vesicles were swollen in a 180 mM sucrose solution containing 0.01 mol % sodium azide; a 10 Hz ac field (20 V/cm) was applied for 2 h.

RICM and Fluorescence Microscopy. Suspensions of the GUVs (100 μL) were diluted with S1 buffer (900 μL) and then deposited onto the receptor-coated coverslip. The vesicle adhesion to the integrin-functionalized surfaces was monitored with a Zeiss Axiovert 100S microscope equipped with an antiflex objective (Plan-Neofluar 63×; numerical aperture 1.25; oil immersion) at room temperature.[21] A high-pressure mercury arc lamp (Osram HBO 100) was used as the light source. Fluorescence was excited and observed by the appropriate filter sets. The 546 nm line of the mercury arc lamp was used for the RICM technique. This allowed us to observe the same sample by both microfluorescence and RICM. A CCD camera (C2400-75i controlled by an Argus 20 image acquisition system, Hamamatsu) that integrates intensities over variable intervals of time permits observation of weak fluorescence signals. The video camera records 25 images per second.

Monolayer Study. A custom-designed film balance combined with a fluorescent microscope was used to observe the lateral organization of the lipid monolayer by microfluorescence. The temperature was maintained at 20 °C.[22]

Results

Synthesis of the Fluorescent c(-RGDfK-) Lipopeptide. To visualize the reorganization of the RGD lipid during the adhesion process through RGD−integrin interaction, we synthesized fluorescently labeled lipids with and without the RGD headgroup. Their chemical structures are presented in Figure 1.

As a chromophore, we chose a chalcone group which was previously used for the labeling of hydrophobic oligonucleotides in order to study their interaction with model membranes.[23] Compared to other conventional chromophores such as NBD used for the labeling of amphiphilic dextran polymers,[24] this inexpensive chalcone can be prepared in large quantities and can be derivatized to couple with various functional groups. In view of its structure, it is rather unambiguously located within the lipid bilayer after membrane insertion. In addition, it possesses suitable photophysical properties that allow the detection of its fluorescence with commercial fluorescence filters for optical microscopy ($\lambda_{exc} \cong 410$ nm; width at half-height of the absorption peak $\cong 70$ nm; $\lambda_{em} \cong 550$ nm).[25] The EO_3 chalcone **1** can be used as a probe for membranes. In fact, solvatochromic properties originating from its donor−acceptor structure facilitate its location in the investigated systems.

A RGD lipid bearing a chalcone chromophore in the alkyl chain was prepared by following the route shown in Figure 2. The EO_3 chalcone **1**, synthesized according to the previously described procedure,[23] was converted to the ester **2** by reaction with ethyl diazoacetate. The low yield of this reaction could be due to the complexation of the catalyst by the chalcone group as revealed by a color change. After saponification, the acid **3** was condensed to the partially protected cyclic peptide c(-R(Pbf)GD(O^tBu)-fK-) using the free amino group of the lysine residue to give the amide **4** with the peptide-coupling reagent HATU.[26] The product **4** could be purified efficiently by HPLC and then deprotected by TFA to give the RGD chalcone **5**. The synthesis of the nonlabeled RGD stearyl **6** and EO_4 stearyl **7** lipids was previously reported.[3]

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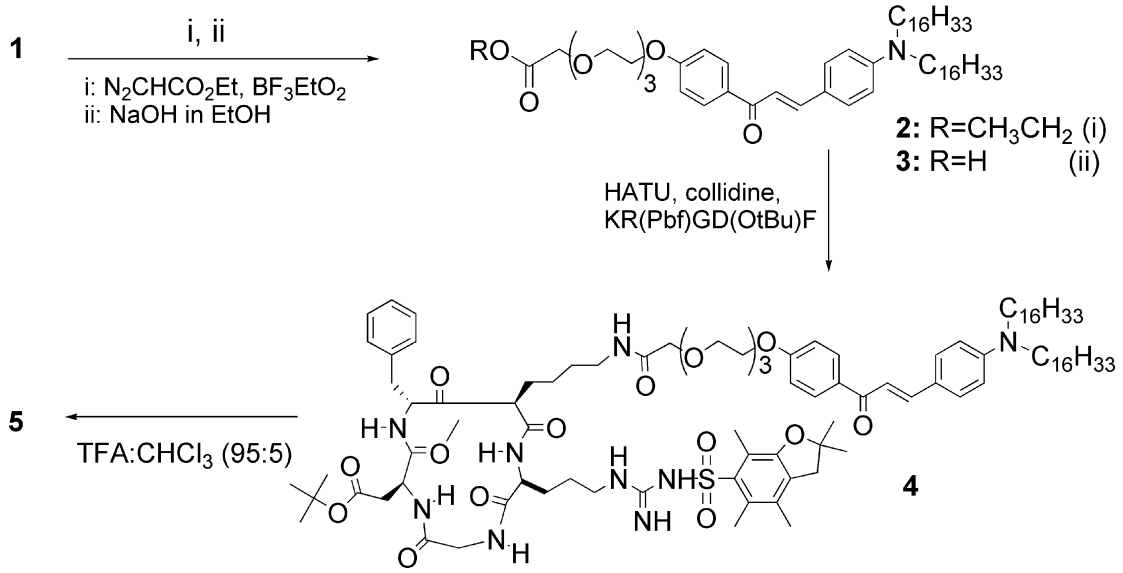

Figure 2. Synthetic route followed for the preparation of the RGD chalcone **5** from the EO_3 chalcone **1**.

Phase Behavior in a Monolayer. To analyze the phenomena occurring during vesicle adhesion, we investigated the phase behavior of pure synthetic chalcone lipids and the lipid mixture used for vesicle preparation. Particular attention was paid to possible phase separation in the vesicle bilayer. In fact, such behavior can be significant if the enthalpy of pinning centers formation can balance the entropic cost associated with a local concentration increase. Pressure−area isotherms of the monolayers of the investigated lipid systems were recorded by using a Langmuir film balance combined with a setup for fluorescent microscopy. The pressure−area isotherms of the monolayer made of pure EO_3 chalcone **1** and RGD chalcone **5** as well as those of RGD stearyl **6** and EO_4 stearyl **7** are presented in Figure 3a.

The EO_3 chalcone **1** monolayer exhibited a plateau corresponding to a first-order transition that could probably be attributed to a transition of the hexadecyl chains from an expanded liquid to a condensed phase, which is consistent with the behavior of the similar EO_4 stearyl **7**. In the case of the RGD chalcone **5**, no such transition was observed in contrast to the case of RGD stearyl **6**. The absence of a transition to a crystalline state in **5** was probably related to the disorder introduced by the chalcone chromophore.

For the giant vesicle preparation, a mixture of DMPC/cholesterol/PEG_{2000}−DMPE (50:50:1) was used. The lipopolymer promotes the formation of GUVs in the presence of the buffer. Moreover, the addition of 1 mol % of PEG_{2000}−DMPE impeded the nonspecific adhesion of the vesicles to the surface. The isotherm of the monolayer containing 2 mol % RGD chalcone **5** did not exhibit any plateau.[27] Epifluorescence microscopy images were recorded during the compression of the monolayer. As shown in Figure 3b, one can observe foamlike microdomains that are either depleted or enriched with chalcone lipid. When the surface pressure was increased, the observed dark domains merged and grew. It was previously reported that similar fluid phases coexist in cholesterol/DMPC mixtures at low surface pressure.[28]

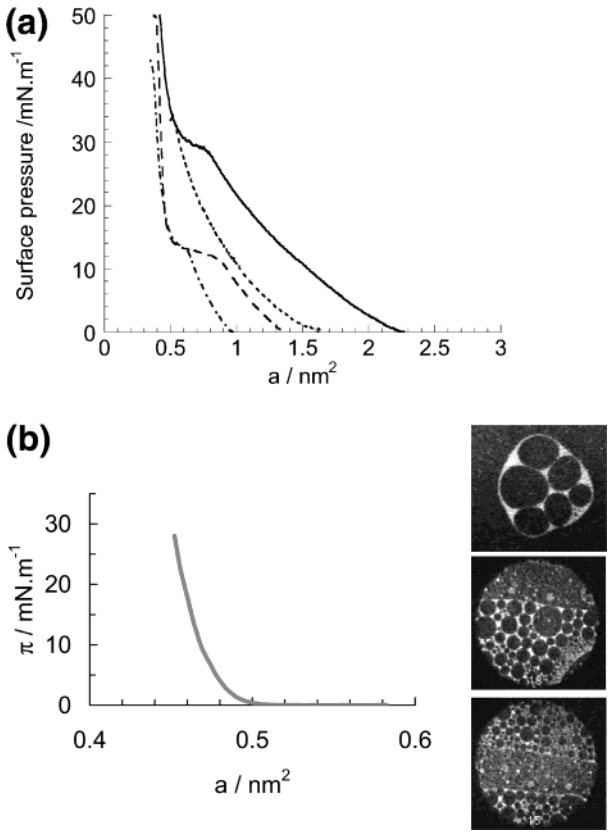

Figure 3. (a) Langmuir isotherms of a monolayer composed of RGD stearyl **6** (solid line), RGD chalcone **5** (dotted line), EO_3 chalcone **1** (dotted−dashed line), and EO_4 stearyl **7** (dashed line). (b) Langmuir isotherms of a monolayer composed of RGD chalcone **5** (2% mol) in a matrix of DMPC/cholesterol/DMPE−PEG_{2000} (50:50:1) and images obtained from optical epifluorescence microscopy at a surface pressure of 35, 14, and 9 mN m^{-1} (from top to bottom).

The present results show that the lipid mixture used for the GUV experiments exhibited the coexistence of domains both rich and poor in fluorescent lipid in the Langmuir monolayer even at a surface pressure of 35 mN

(27) In contrast to the isotherm of the monolayer containing 2% mol EO3 chalcone 1 and 2 mol % RGD stearyl 6 (see Figure A in Supporting Information). In the latter case, the presence of a plateau indicates that a first-order transition occurs as observed in the case of a monolayer of pure EO3 chalcone 1.

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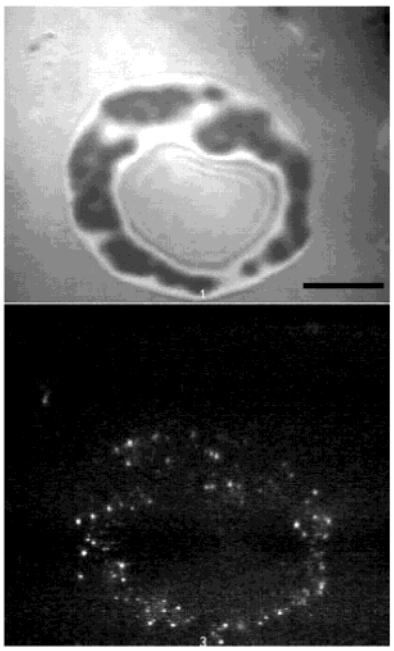

Figure 4. Typical image of a stably adhering vesicle containing RGD chalcone **5** (2 mol %) in matrix DMPC/Chol/DMPE−PEG_{2000} (50:50:3) ($\alpha_{IIb}\beta_3$ integrin surface): (top) by RICM and (bottom) by fluorescence optical microscopy ($\lambda_{exc} = 450$). The fluorescence image was obtained from accumulation of several recorded video images (one image every 40 ms during 500 ms) before the fast bleaching of the probe (around 100 ms). Notice that all the fluorescent spots are localized in the contact zone. The scale bar represents 10 μm.

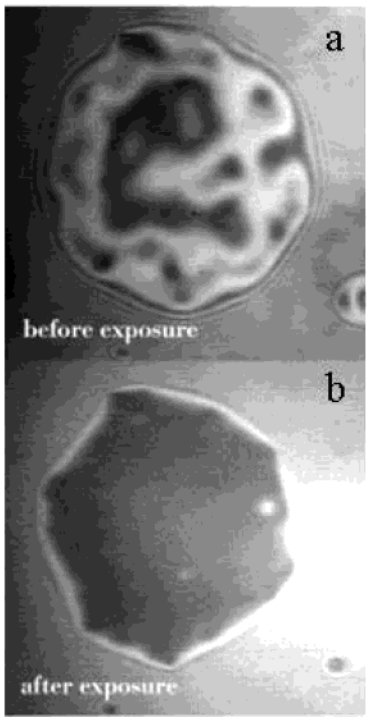

Figure 5. Typical RICM image of an adhering vesicle containing RGD chalcone **5** (2 mol %) in a matrix DMPC/cholesterol/DMPE−PEG_{2000} (50:50:1): images taken (top) before irradiation and (bottom) after irradiation. Notice that the adhesion is strongly reinforced by irradiation (exposure time of about 30 s). The scale is the same as in Figure 4.

m^{-1}. Consequently, this suggests that some heterogeneity in the distribution of the lipid components within the vesicle bilayer can occur. Nevertheless, the RGD chalcone **5** could be preferentially incorporated within the most liquid parts of the membrane.

Adhesion onto an Integrin-Functionalized Surface. To investigate the importance of the lateral redistribution of RGD ligands within a vesicle membrane bound to an immobilized integrin receptor, an adhesion-mimicking system composed of RGD giant vesicles (GUVs) and surfaces presenting adsorbed but functional $\alpha_{IIb}\beta_3$ integrin was designed. We prepared giant vesicles containing 2 mol % of the RGD chalcone **5** and observed their adhesion onto a $\alpha_{IIb}\beta_3$ integrin surface.[29] This smooth biofunctional surface is more appropriate than surfaces coated with endothelial cells for adhesion studies because the surface roughness of the cells complicates the adhesion plaque observation by RICM.

To observe the distribution of the fluorescent ligand in the contact zone, we used a microscope enabling both RICM[21] and fluorescence microscopy observations. After 30 min of incubation, vesicles containing 2 mol % RGD chalcone **5** adhered efficiently onto an integrin-covered surface as manifested by the black zones of contact in the RICM images (Figure 4a). The vesicle shape in the vicinity of the contact zone is delimited by the array of Newtonian fringes. In addition, dark zones appear within the adhesion disk which are attributed to areas of strong adhesion. Once the RICM image of the adhered vesicle remains stabilized, the location of the RGD chalcone lipid **5** was visualized by fluorescence microscopy. Upon a short irradiation of the adhered vesicles (about 500 ms), one can clearly observe fluorescent spots which are concentrated in the adhesion zone appearing as a black zone in RICM as shown in Figure 4b. As a control experiment, GUVs containing 2 mol % of EO_3 chalcone **1** were prepared. These were not found to adhere onto the integrin surface. Furthermore, the fluorescence of these vesicles was homogeneously distributed on the contour of the vesicle. This observation already provides unambiguous evidence for the correlation between the location of the RGD chalcone **5** within the membrane and the sites where the bilayer adheres to the integrin-derivatized surface.

Two other remarkable features emerged during this series of experiments. First, when the RGD chalcone **5** vesicles are adhered onto an integrin-functionalized surface, the fluorescent spots corresponding to the chalcone chromophore disappeared after a very short characteristic time of exposure (about 100 ms). For comparison, the fluorescence of the same GUV in absence of the adhesive integrin surface or the fluorescence of a nonadhesive EO_3 chalcone **1** vesicle can be observed during at least 10 s. Additionally, the same experiment was carried out with giant vesicles containing 2 mol % EO_3 chalcone **1** and 2 mol % nonfluorescently labeled RGD stearyl **6**. Under the same conditions, these vesicles still adhered to the integrin surface. But in contrast to RGD chalcone **5** GUVs, upon irradiation of the sample, their fluorescence was bleached with a characteristic time that was slower by 2 orders of magnitude (about 20 s).

Second, we found that adhesion of vesicles containing 2 mol % RGD chalcone **5** onto the integrin surface became surprisingly much stronger after a 30 s irradiation with the lamp used for fluorescence observation. This was concluded from the large increase of the black area in RICM images after exposure (compare Figure 5a before irradiation with Figure 5b after irradiation). Obviously the system inducing the adhesion process was modified during the irradiation and became more efficient in the adhesion onto the integrin surface.

(29) Boulbitch, A.; Gutenberg, Z.; Sackmann, E. *Biophys. J.* **2001**, *81*, 2743−2751.

To elucidate the mechanism of fluorescence decrease observed during the vesicle adhesion, we carried out two further experiments. We observed the fluorescence change of a solution of a EO_3 chalcone **1** (12.1 μM) in various solutions of OG detergent (see Figure B, Supporting Information). When the concentration of OG in the solution was increased, the fluorescence of EO_3 chalcone **1** was increased. This observation was attributed to the dilution of **1** in the mixed OG/**1** micelles resulting in a decrease of its self-quenching. In a second experiment, the kinetics of the fluorescence bleaching of a solution of EO_3 chalcone **1** was compared in the case of a 1:10 mixture of **1** and OG (where **1** is self-quenched) and in the case of a 1:2000 mixture of **1** and OG (above the critical micelle concentration of OG, around 20 mM,[30] where **1** is not self-quenched) (see Figure C, Supporting Information). The observed characteristic time was the same for the two samples. Therefore, the observed bleaching does not depend on the surface concentration of **1** in OG micelles and is only due to the monomer EO_3 chalcone.

Discussion

In these experiments, the fluorescence spots observed during the vesicle adhesion onto the integrin surface show that the chalcone lipids are concentrated in the adhesion plaques. These experiments also show a specific behavior of the RGD chalcone **5** in pinning centers. The irradiation causes a faster bleaching of the chalcone chromophore affecting the adhesive properties of the vesicle onto the integrin surface. This behavior is related to the very high concentration of RGD chalcone **5** in the contact zone. Indeed, one can envisage that the chalcone fluorescence is self-quenched due to its high concentration in the adhesion zone. However, we observed that the kinetics of the photodestruction of the chalcone monomer in a **1**/OG micellar solution was the same whether there was self-quenching of **1** (1:10 mixture) or not (1:2000). If the very short bleaching time observed for the RGD chalcone **5** adhered vesicles was due to a self-quenching of the chalcone monomer like the one observed in OG micelles, it should be the same as that observed for nonadhered RGD chalcone **5** vesicles or EO_3 chalcone **1** vesicles in solution. Instead of that, we observed a significant difference in the quenching time so that one can exclude a self-quenching of the chalcone monomer for the adhered RGD chalcone **5** vesicles, due to its high concentration in the adhesion plaques. A second mechanism can be envisaged to explain the behavior of the RGD chalcone **5** during vesicle adhesion.

The characteristic short time for fluorescence decrease is in agreement with a photoreaction of the chalcone. It is known from the literature that the chalcone is highly photosensitive. Thus irradiation of chalcone groups can lead to a nonfluorescent cyclobutane dimer through a [2 + 2] cyclodimerization. This process is normally observed in the solid state where the two implicated double bonds have to be parallel and separated by a few angströms.[31,32] To form this chalcone dimer, the local concentration in chalcone lipid has to be very high. The RGD chalcone seems to stay in the fluid phase as suggested by the monolayer study so that its migration within the membrane is not limited by the viscosity or by percolation. In the contact zone, the RGD chalcone groups are expected to form clusters.[33] As a result, the probability of photodimerization under irradiation becomes much stronger than in the case of noninteracting GUVs containing 2 mol % RGD chalcone **5** or interacting ones containing 2 mol % RGD stearyl **6** and 2 mol % EO_3 chalcone **1**. Thus dimerization can occur only in the case of the GUVs interacting with the integrin surface.

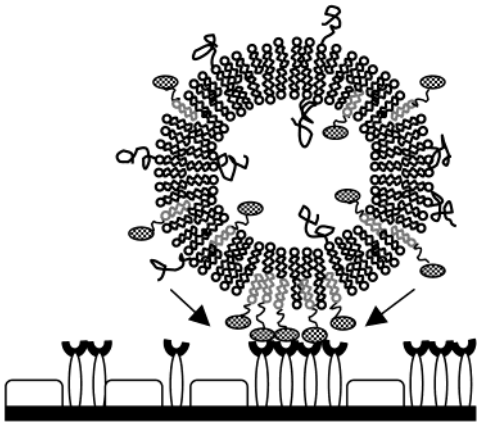

Figure 6. Schematic view of a RGD vesicle interacting with an integrin-covered surface.

In addition, the mechanism of photodimerization also explains why the GUV adhesion was reinforced after irradiation. The affinity for $\alpha_{IIb}\beta_3$ by the cyclic pentapeptide ($\approx$1 μM) is about 10^3 times smaller than for $\alpha_v\beta_3$ (3 nM) so the adhesion is expected to be weak. It is possible that the dimerization enforces binding to such an extent that even $\alpha_{IIb}\beta_3$ also binds strongly. If chalcone dimerization occurs, once the first integrin-RGD complex is formed, the probability for the second RGD of the dimer to bind is greater. The photoinduced dimer bearing two RGD ligands should thus have a stronger affinity for the integrin than the single RGD chalcone lipid.

In conclusion, the experiments show that the RGD chalcone **5** tends to segregate within the adhesion plaques due to ligand−receptor binding as already observed for the case of csA receptors.[14] The RGD chalcone is free to move by lateral diffusion into the contact zone where the integrin surface is very close to the membrane so that the binding reaction with the integrin receptor can take place as schematically shown in Figure 6.

Conclusion

In this paper, we described a new approach to study the local formation of attractive domains during adhesion mediated by integrin/RGD ligand binding. We labeled with fluorescent chalcones the hydrocarbon chains of lipid analogues exhibiting headgroups composed of a triethyleneglycol (EO_3) spacer both with and without exposed cyclic RGD-ligand. We showed that during adhesion the membrane forms zones of tight adhesion separated by unbound areas and that within the tight adhesion zones, arrays of pointlike pinning sites attributed to receptor−ligands pairs are formed.

Furthermore, the concentration of chromophore into small centers leads to a fast bleaching which confirms the ligand segregation. The kinetics and the degree of the local fluorescence decrease are indicators of the local concentration of the chalcone in the adhesion zone of the membrane and enable the visualization of local quasicrystalline lipid clusters associated with the formation of

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adhesion plaques. Interestingly, we also observed that adhesion strength increases drastically upon irradiation. Such a property could be valuable for both studying the physicochemistry of the adhesion and designing new vectors for cell targeting.

The present study was also motivated by attempts to construct carriers for the effective transfer of vectors into cells. The incorporation of RGD-ligands into vesicles isexpected to improve their transfer into cells by binding to specific families of integrins which favor phagocytosis. The preparation of either oligomeric RGD lipid ligands or membranes containing pure RGD lipid domains should improve the efficiency of the adhesion between liposomes and cell membranes.

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Supporting Information Available: Figures showing Langmuir isotherms of a monolayer composed of a lipid matrix, DMPC:chol:DMPE-PEG2000 (50:50:1) completed with 1:6 (2 mol%, 2 mol%) or 1 (2 mol%), evolution of emission intensity with OG concentration, and kinetics of fluorescence bleaching are available free of charge via the Internet at http://pubs.acs.org.

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