Selective Adhesion of Endothelial Cells to Artificial Membranes with a Synthetic RGD-Lipopeptide

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Abstract: A constrained cyclic Arg-Gly-Asp-D-Phe-Lys, abbreviated as cyclo(-RGDfK-), lipopeptide has been synthesized and incorporated into artificial membranes such as giant vesicles with DOPC and solid-supported lipid bilayers. The selective adhesion and spreading of endothelial cells of the human umbilical cord on solids functionalized by membranes with this RGD-lipopeptide have been observed. Furthermore, we have demonstrated strong selective adhesion of giant vesicles to endothelial cells through local adhesion domains by combined application of hydrodynamic flow field and reflection interference contrast microscopy (RICM). The adhesion can be inhibited by competition with a water-soluble RGD peptide. We suggest that this strategy could improve the efficiency of liposomes targeting used as vectors or as drug carriers to cells.

Keywords: adhesion · endothelial cells · liposomes · peptides · vesicles

Introduction

Since the identification of the -Arg-Gly-Asp- (RGD) peptidic sequence as an universal recognition site[1, 2] of various extracellular proteins which interacts with cell-surface receptors of the integrin family,[3] many small RGD-containing peptides have been shown to be able to bind to integrin receptors. In a systematic study of the correlation between the activity and the conformation of small RGD-containing cyclic peptides it has been shown that constrained cyclic peptides mimic the conformation necessary for receptor binding more closely than flexible linear peptides[4, 5] and improve the selectivity for integrin subtypes. Substrates composed of polyacrylamide gels[6] or alkylsiloxanes[7] functionalized by deposition of RGD peptides have been designed as models to study cell adhesion. More recently synthetic oligo(ethyleneglycol)thiolates modified by linear RGD-containing peptides have allowed preparation of self-assembled monolayers (SAMs) with a controlled density of the functional ligands that promoted the bovine endothelial cell attachment and spreading.[8] The cyclic pentapeptide cyclo(-RGDfK-) induces a selective adhesion of osteoblasts[9] onto poly(methylmethacryate) (PMMA) surfaces coated with this acrylamide-modified peptide as a result of its selective interaction with the $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins[10] expressed by these cells.[11, 12]

Despite the extensive research on the RGD peptide and its function as an antagonist to the extracellular matrix (ECM) protein-binding site for integrins, only a few studies have reported on the synthesis of lipidic modified RGD-sequences[13, 37] and their reconstitution to artificial membranes to generate target vesicles for cells.[14–16] Dialkyl lipomodified peptides from extracellular collagen ligand sequences have been synthesized with a very efficient solid-phase approach.[17] Supported membranes and vesicles are suitable model systems to study fundamental features of membrane adhesion such as the measurement of adhesion forces and adhesion-induced clustering of receptors and ligands. Furthermore, the possibility of liposomes targeted selectively to one type of integrin through RGD peptides could be used for cell targeting, and would solve a problem which limits the application of liposomes as vehicles for drug delivery. The strategy to design ligands that promote the selective adhesion of liposomes onto specific sites on the cell surfaces has been proposed previously. It has been applied to target liposomes to cancer cells through folic acid coupled to the lipids.[18] In

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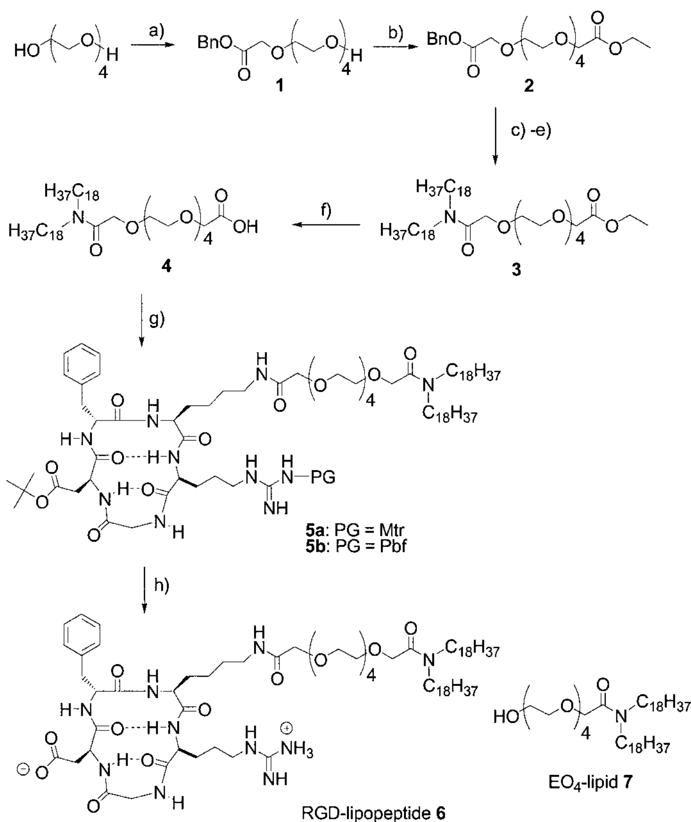
cell targeting through the RGD peptide, a recent communication reports the capacity of surface-modified functional liposomes to bind to NIH3T3 cell membranes.[19]

We prepared an amphiphile with an hydrophilic tetraethylene glycol spacer to improve the accessibility of the RGD-ligand to the protein receptors.[20] To the headgroup of this amphiphile we coupled the cyclic cyclo(-RGDfK-) pentapeptide which is highly selective for $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrin receptors.[10] It was reconstituted into lipid membranes to study the interaction between such functionalized membranes and endothelial cells. The tetraethylene glycol spacer was used to suppress nonspecific binding of the vesicles to cells. It was shown that a triethyleneglycol spacer grafted onto thiolates prevents nonspecific adhesion of cells.[21] Endothelial cells were chosen because they contain the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors[22] and also because of their role in the mechanism of angiogenesis.[23, 24]

In the first part of the present paper, we describe the synthesis of the RGD-lipopeptide and its ability to functionalize either supported membranes or giant vesicles.[25] Experiments on the interaction between giant RGD vesicles and endothelial cells have been carried out with the reflexion interference contrast microscopy (RICM) technique in the presence and absence of a hydrodynamic shearing flow.[26, 27] This technique allows the observation of the adhesion of vesicles onto the substrate and endothelial cells spread on functionalized solids. Moreover by observation of shape changes of the vesicles induced by hydrodynamic flow one can test the binding strength of the vesicles to the cell surface or observe whether the vesicles adhere with large contact areas or are only locally tethered to the cell surface.

Results and Discussion

Synthesis of cyclic RGD lipopeptides: To model the membrane adhesion process mediated by the interaction of the peptidic RGD-sequence and the membrane integrin, a synthetic lipid with a cyclic pentapeptide cyclo(-RGDfK-) headgroup, which is highly active and selective for the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors,[22] has been synthesized following the procedure described in Scheme 1.



Scheme 1. Synthesis of the RGD-lipopeptide **6** and EO_4-lipid **7**. a) N_2CHCO_2Bn, $BF_3\cdot Et_2O$; b) $N_2CHCO_2C_2H_5$, $BF_3\cdot Et_2O$; c) H_2, Pd/C; d) DCC, DMAP, HO-Suc; e) $HN(C_{18}H_{37})_2$; f) KOH/EtOH; g) cyclo(-R(PG)GD(O*t*Bu)fK-); **5a**: TBTU, HOBt, DIPEA; **5b**: HATU, collidine; h) TFA/H_2O 95:5.

Tetraethylene glycol was converted to the diester **2**, which was subsequently debenzylated and treated with distearylamine to yield anchor **3**. Lipid **4** was chosen because of its ability to form stable monolayers and its good solubility in phospholipid membranes. To prepare the lipopeptide **6**, lipid **4** was linked to the partially protected cyclic cyclo(-R(Mtr) GD(O*t*Bu)fK-) or cyclo(-R(Pbf)GD(O*t*Bu)fK-) peptide using the free amino group of the lysine residue to give the amides **5a** and **5b** with the peptide-coupling reagent TBTU and HATU[28] methods, respectively. The removal of the protecting groups of the compounds **5a** and **5b** led to the lipopeptide **6**. The Pbf protecting group of arginine is cleaved off faster than Mtr. The tetraethyleneglycol lipid (EO_4-lipid **7**), which is also very stable during the Langmuir-Blodgett transfer,[29] was prepared following a similar route,[20] and was used to dilute the RGD-lipopeptide **6** in Langmuir monolayers or to serve as a nonactive lipid in control experiments.

Solubility of the RGD-lipopeptide 6 in Langmuir monolayers: To characterize the state of the lipid monolayer deposited on the silanized supports we measured pressure – area isotherms

Abstract in French: *Un lipopeptide possédant un peptide cyclique contraint Arg-Gly-Asp-D-Phe-Lys cyclo(-RGDfK-)a été synthétisé et incorporé dans des membranes lipidiques telles que des vésicules géantes de DOPC ou des films lipidiques supportés. Des cellules endothéliales provenant d'un cordon ombilical humain adhèrent sélectivement sur une surface solide fonctionnalisée par dépôt de membrane contenant ce lipopeptide RGD. De plus, nous avons montré que des vésicules géantes contenant ce lipopeptide adhèrent fortement et sélectivement sur ces cellules endothéliales suivant des domaines d'adhésion locale grâce à l'observation par microscopie à contraste d'interférences par réflexion (RICM) en appliquant simultanément un flux hydrodynamique. L'adhésion peut être inhibée par compétition avec un peptide RGD soluble dans l'eau. Nous suggérons que cette stratégie permettrait d'améliorer l'efficacité du ciblage de cellules par des liposomes utilisés comme vecteurs ou transporteurs de médicaments vers des cellules cibles.*

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of the RGD-lipopeptide **6** and mixtures of this lipid with either the EO_4-lipid **7** or DOPC with a Langmuir film balance. The results are presented in Figure 1. The π–A isotherm of

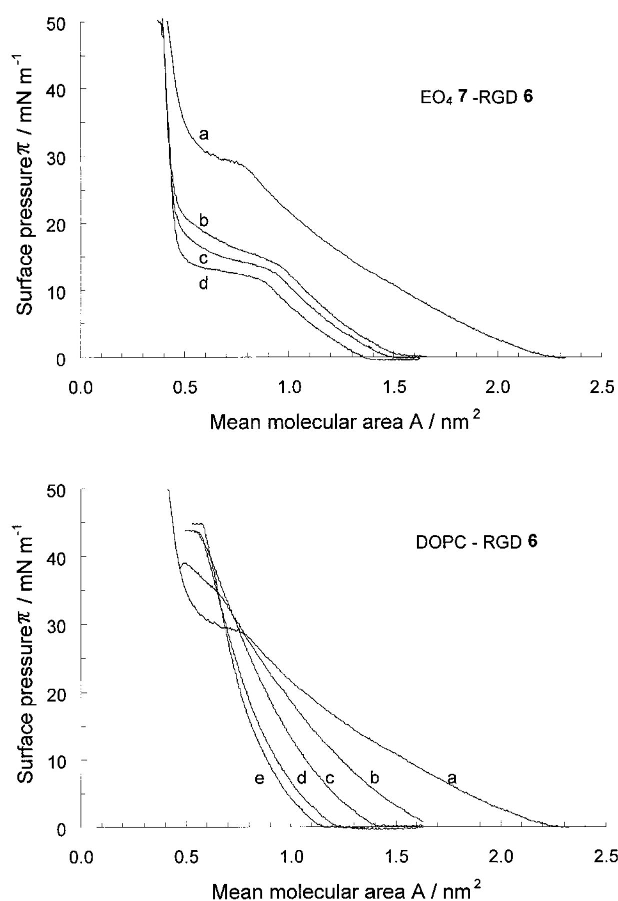

Figure 1. π–A Isotherms of monolayers at 20°C composed of the following mixtures. Top: RGD-lipopeptide **6** and EO_4-lipid **7** with the molar ratios: a) 1:0, b) 1:4, c) 1:9, and d) 0:1; bottom RGD-lipopeptide **6** and DOPC with the molar ratios: a) 1:0, b) 1:1, c) 1:3, d) 1:9, and e) 0:1.

the pure RGD-lipopeptide **6** and EO_4-lipid **7** both exhibit expanded liquid phases at lateral pressures $\pi \leq \pi_m$ (curves a and d in Figure 1) and condensed phases at pressures higher than π_m. A remarkable finding is that the plateau of the RGD-lipopeptide **6** occurs at a much higher surface pressure ($\pi_m = 30\,mN\,m^{-1}$) than for the EO_4-lipid **7**. This high pressure shift is a consequence of the much larger head group of the lipopeptide as well as the surface charge. The expansive lateral pressure associated with the surface charge and the bulkiness of the headgroups leads to lateral expansion of the monolayer. Therefore a higher pressure is required to generate the condensed phase of **6** compared with the EO_4-lipid **7**.[30] This effect was also observed with other lipopeptides.[17] The dipole of the zwitterionic peptide also introduces a repulsive interaction between neighboring molecules within the monolayer and an increase of the mean molecular area. The condensed phase of the RGD-lipopeptide **6** exhibits a considerably larger molecular area than the EO_4-lipid **7**. The mean molecular area of the RGD-lipid in the condensed phase is around 0.7 nm^2 whereas that of the EO_4-lipid **7** is around 0.5 nm^2.[31] Both areas are larger than the value expected for two crystallized alkyl chains (around 0.38 nm^2). For that reason the chains are tilted with respect to the normal of the monolayer and the tilt angle is much larger for the RGD-lipopeptide **6** than for the EO_4-lipid **7**. The transition pressure of the EO_4-lipid **7** increases with increasing concentration of RGD-lipopeptide **6**. However, the transitions remain rather sharp, and the molar area of the condensed phase increases almost linearly with the RGD concentration. This strongly suggests that the RGD-lipopeptide **6** and EO_4-lipid **7** are nearly ideally miscible in the monolayers. This is a consequence of the large repulsive pressure between the RGD-lipopeptide **6** head groups. As in the case of EO_4-lipid **7**, the mixture of DOPC and the RGD-lipopeptide **6** does not show any deviation from the linear variation of the mean molecular area with the molar fraction of the RGD-lipopeptide **6** (see Figure 1, bottom) and the mixture is thus nearly ideal. Again the molar area of the expanded phase decreases drastically with the concentration of DOPC owing to the reduction of the large repulsive pressure between the RGD-lipid headgroups. These results show that it is possible to prepare stable monolayers by mixing the RGD-lipopeptide **6** with EO_4-lipid **7** or DOPC without pronounced phase segregation. The EO_4-lipid **7** or DOPC will be used as matrix lipid to dilute the RGD-lipopeptide **6** both in monolayers and in vesicles. For the 1 to 10% molar fractions of RGD-lipopeptide **6**, phase segregation can be neglected.

Stimulation of cell adhesion on a RGD-functionalized supported membrane: In order to test the functionality of supported membranes functionalized with cyclic RGD peptides, we studied adhesion of endothelial cells on supported monolayers of pure EO_4 **7** or a EO_4 **7**/RGD-lipopeptide **6** (95:5) mixture on a silanized glass cover. For this purpose, the functionalized support was placed in a measuring cell filled with buffer and the system was incubated by a suspension of endothelial cells in a DMEM medium (see Figure 2, top).

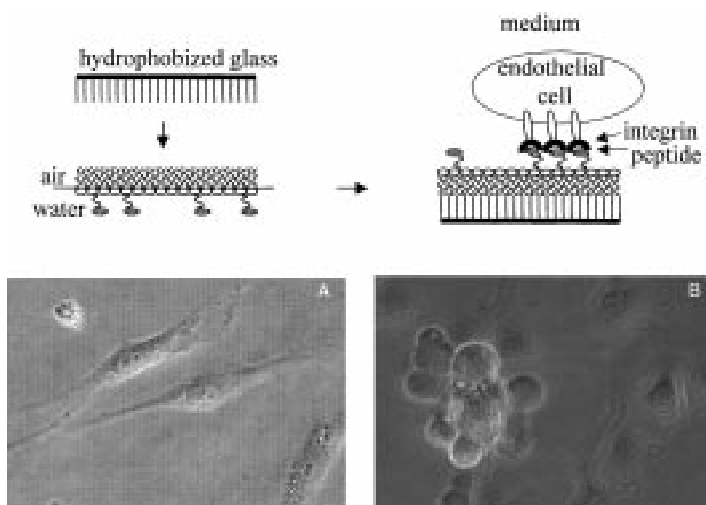

Figure 2. Top) Schematic view of model system studied endothelial cell interaction with supported membrane containing RGD-lipopeptide **6**. Bottom) Contrast phase microscopy images showing adhered cell onto a supported membrane composed of a mixture of RGD-lipopeptide **6** and EO_4-lipid **7** (5:95): A) a spread adhering cell, B) round adhering cell beginning to die as shown by the vesiculation process.

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Table 1. Percentages of cells adhering to the substrates functionalized by deposition of monolayers functionalized by RGD-lipopeptide **6** (composed of 5% mol RGD-lipopeptide **6** and 95% mol EO_4-lipid **7**) and by EO_4 lipid only (100% mol EO_4-lipid **7**), respectively. Measurements were done after incubation of measuring chamber with cells suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C for 90 min (third row) and 180 min (forth row), respectively.

	Pure EO_4-lipid **7**		EO_4-lipid **7**/RGD-lipopeptide **6** (95:5)	
Incubation time	round adhering cell [%]	spread adhering cell [%]	round adhering cell [%]	spread adhering cell [%]
90 min	100	0	80	20
180 min	100	0	43	57

After an incubation of 1.5 h and 3 h, the adhered cells were counted by optical bright-light microscopy. The round and the flat adhering cells are distinguishable by their morphology as can be seen in Figure 2, bottom. This Figure also shows that if the round cells adhere to the substrate they tend to form blisters and seem to die whereas the spread cells maintain their elongated flat form. The percentage of the flat cells and round cells adhered to the substrate was determined. The results of this evaluation for substrates functionalized by RGD-lipopeptide **6** and EO_4-lipid **7**, respectively, are summarized in Table 1. One can clearly see that the presence of the RGD-lipopeptide **6** increases the number of flat adhering cells significantly although there is still nonspecific adhesion of cells onto a pure EO_4 **7** supported membrane. The major effect is that the RGD-lipopeptide **6** induces the spreading of the endothelial cells onto the surface whereas cells do not spread on control surface even after three hours of incubation. A similar observation was made on the attachment of osteoblasts to RGD coated PMMA surfaces.[9] Another interesting observation is that the adherent cells on the control surface remain round and seem to die rapidly by formation of blisters (Figure 2).

In conclusion, our results show that the supported membrane containing the RGD-lipopeptide **6** induces rapid attachment and the spreading of endothelial cells and the cells survive over extended periods of time, whereas adhesion was strongly impeded on surfaces covered by monolayers of EO_4-lipid **7**.

Adhesion of RGD-functionalized vesicles on endothelial cells: As another test of the functionality of RGD lipopeptides as substitutes for vitronectin, we established the reverse system and studied the interaction of giant vesicles with a selective RGD-lipopeptide **6** with endothelial cells by using the RICM technique[26] (see Figure 3).[32]

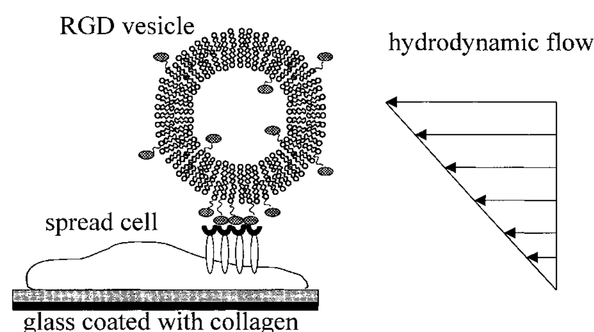

Figure 3. Schematic view of model system studied endothelial cell adhesion to glass substrate coated by collagen and interactation with a giant vesicle containing the RGD-lipopeptide **6**.

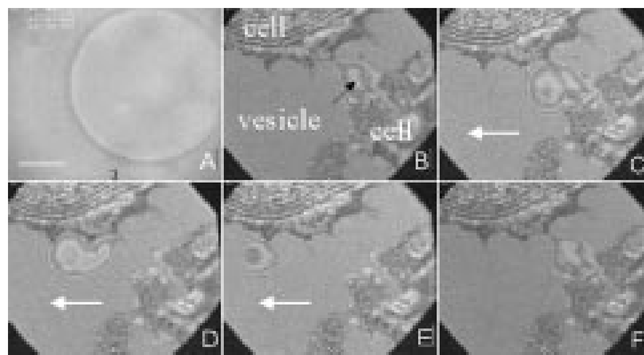

Figure 4. Images of an adhered giant vesicle (diameter 37 μm) onto endothelial cells spread onto a coverslip in the absence and presence of hydrodynamic shear flow: A) Bright field image in absence of flow, B) RICM image of same situation, C–E) RICM images in presence of an increasing hydrodynamic shear flow from the right side, represented by the arrow, F) RICM image in absence of flow. The vesicle is composed of a DOPC/DMPE-PEG2000/cholesterol/RGD-lipopeptide **6** mixture (74:1:10:5). Image B shows the contact zone and the pinning centers of the giant vesicle. Note the microdeformations of the giant vesicle induced by the applied flow in images C to E. The scale bare represents 10 μm. All the panels refer to the same area.

The RICM technique visualizes the contact zone of adhered giant vesicle, and reconstruction of its profile perpendicularly to the surface can be made.[26] First, endothelial cells were deposited onto glass surfaces coated with collagen IV. Remarkably, the cells do not spread as much as on RGD-functionalized surfaces and remain several μm high. However, they spread at the rim of the area of contact and form a flat seam of less than 1 μm height and several μm width. Therefore one can observe the adhering giant vesicles both by phase contrast microscopy (image A in Figure 4a) and by RICM (other images in Figure 4). The latter method suggests that the vesicle adheres locally to the cell surface. In order to verify this local tethering and to measure the relative binding strength of the membrane bound RGD-ligands, we observed the behavior of the vesicles under the action of a hydrodynamic shear flow. A few minutes after the injection of the RGD giant vesicles into the chamber, giant vesicles adhere locally onto the cells as shown in Figure 4 (image B). The vesicle exhibits a well-defined zone of contact with the substrate surrounded by darker fringes, and it appears to be locally attached to the surface of two cells. One can see the bright contact zone surrounded by a dark ring which is stable in time. If an increasing hydrodynamic shear field is applied, the adhered vesicle becomes deformed and elongated in the direction of the flow (images C, D, and E in Figure 4). By following the deformation, one can see that the vesicle stays strongly attached to the cells at three points, and after removal

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of the shear field the vesicle returns to the original position in less than one second (image F, Figure 4). The presence of these pinning centers provides strong evidence that the RGD ligands are strongly bound to the cell membrane by clustering of the integrin receptors. This finding of very strong attachment sites also suggests that this segregation of the receptor–ligand pairs enforces adhesion strength. The reversibility of the vesicle deformation after removal of the hydrodynamic shear field is due to tubelike protrusions tethered to the pinning centers. Indeed these tethers are briefly visible between the images E and F and show that the vesicle stays linked to the cell. As a control experiment the same experiment was performed with pure DOPC giant vesicles. Only very weakly adhered vesicles were observed after the same incubation time, and the vesicles could be removed completely by application of even small hydrodynamic fields. As a conclusion, this experiment shows that the membrane coupled RGD-ligand is functional and generates strong tethering of the vesicle to endothelial cells.

As a further test of the binding of lipid-coupled RGD to the integrins we injected soluble pentapeptide cyclo(-RGDfK-) before addition of the RGD vesicles. No adhered vesicles were observed. The adherent cells retract and tend to detach from the substrate after injection of the soluble cyclo-(-RGDfK-) peptide. If the integrin sites of the cells are saturated by the soluble peptide before addition of the vesicles, adhesion is completely inhibited (see Figure 5). On the other hand, if the soluble cyclo(-RGDfK-) peptide is added after the vesicle injection, all the vesicles can be easily detached from the cells by application of a very small hydrodynamic flow field. This result supports the idea that the observed adhesion is due to the specific interaction between the RGD peptide and the integrin receptors of the cells.

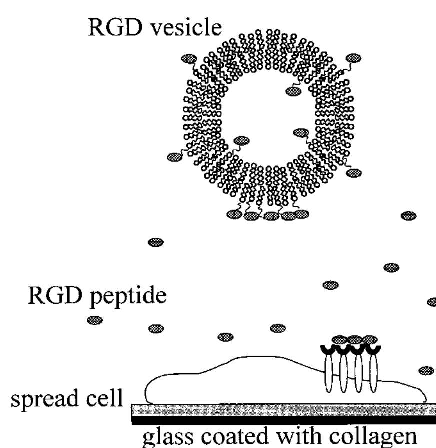

Figure 5. Schematic view of the competitive adhesion between giant RGD-vesicles and soluble cyclic RGD peptide onto an endothelial cell: RGD-vesicle adhesion is inhibited by the soluble cyclic RGD peptide.

Conclusion

A lipid with the cyclic pentapeptide cyclo(-RGDfK-) has been synthesized and built up to artificial membranes presenting accessible RGD sites which are recognized selectively by $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin receptors. The incorporation of this lipid in a supported membrane induces the rapid spreading of endothelial cells. Inversely giant vesicles containing the RGD lipopeptide are selectively tethered to endothelial cells owing to the interaction of the integrin receptors and the RGD peptide. The behavior of the vesicles under a hydrodynamic shear flow shows that the interaction between the RGD vesicles and endothelial cells induces the formation of pinning centers and is most likely a result of the clustering of integrins through the RGD ligands. The strong coupling of RGD-functionalized vesicles to cells could provide a promising way to target vesicles to cells for the purpose of drug delivery.

Experimental Section

Materials: All reagents and solvents were at least analytical grade and were used as supplied. The dioleoylphosphatidylcholine (DOPC) and 1,2-dimyristoyl-*sn*-glycero-3-phophatidylethanolamine-*N*[poly(ethylenegly-col)2000] (PEG-2000-DMPE) and cholesterol (Chol) were purchased from Avanti. HATU was purchased from Biosystems Perseptive GmbH. Cyclic pentapeptides cyclo(-R(PG)GD(O*t*Bu)fK-) (PG = Mtr: 4-methoxy-2,3,6-trimethylbenzenesulfonyl, Pbf: 2,2,4,5,7-pentamethyl-3-hydrobenzofuran-6-sulfonyl) and cyclo(-RGDfK-) were prepared by cyclization of the side chain protected linear precursor which was synthesized by Fmoc-based SPPS.[33, 34] The temporary Z protecting group for lysine was cleaved by hydrogen with the Pd/C catalyst. Pbf/Mtr and *t*Bu can be cleaved with a TFA/H_2O 95:5 solution.[34] All reactions were monitored by thin-layer chromatography using 0.25 mm silica gel covered glass 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).

Synthesis

1-Benzyl 3,6,9,12,15-pentaoxadecanoate ester (1): Benzyl diazoacetate (1.134 g, 6.4 mmol) and tetraethylene glycol (6.8 mL, 39 mmol) were introduced in anhydrous CH_2Cl_2 (8 mL). Two drops of $BF_3 \cdot Et_2O$ were added and the mixture was stirred for 6 h. After extraction with diethyl ether, the organic phase was successively washed with aq sat. NaCl and distilled water and then dried with Na_2SO_4. The crude product was purified on a 60 g silica column (AcOEt) to obtain the title compound (1.168 g, 3.41 mmol, 53 %).^{1}H NMR ($CDCl_3$): $\delta = 7.31$ (s, 5H, C-arom), 5.15 (s, 2H, OCH_2Ph), 4.14 (s, 2H, OCH_2CO), 3.6–3.4 (m, 16H, OCH_2), (t, 3H, $J = 7$ Hz, OH); ^{13}C NMR ($CDCl_3$): $\delta = 169.8$ (CO_2), 135.0, 128.1, 127.9 (C-arom), 72.1, 70.4, 70.1, 70.0, 69.8 (OCH_2), 68.1 (OCH_2CO), 65.9 (OCH_2Ph), 61.0 (CH_2OH); elemental analysis calcd (%) for $C_{17}H_{26}O_7$: C 59.65, H 7.60; found C 59.3, H 7.6.

Benzyl, ethyl tetraethylene glycol ester (2): Compound **1** (3.36 g, 9.8 mmol) and ethyl diazoacetate (1.23 g, 10.8 mmol) were added to anhydrous CH_2Cl_2 (15 mL). Two drops of $BF_3 \cdot Et_2O$ were added and the mixture was stirred for 12 h. Following the same procedure as for **1**, pure product was isolated (2.2 g, 5.14 mmol, 53 %). ^{1}H NMR ($CDCl_3$): $\delta = 7.31$ (s, 5H, C-arom), 5.15 (s, 2H, OCH_2Ph), 4.17 (s, 4H, OCH_2CO), 4.14 (q, 2H, $J = 9.5$ Hz, CO_2CH_2), 3.7–3.6 (m, 16H, OCH_2), 1.26 (t, 3H, $J = 7$ Hz, CH_3); elemental analysis calcd (%) for $C_{21}H_{32}O_9$: C 58.87, H 7.53; found C 58.77; H 7.53.

***N*,*N*-1-Bis(octadecylamide)-15-ethyl 3,6,9,12-tetraoxatetradecanoate ester (3)**: Compound **2** (0.96 g, 2.24 mmol) and 5 % Pd/C (0.45 g) were added to anhydrous dichloroethane (40 mL). After 12 h under a hydrogen atmosphere, the suspension was filtrated. The solution was cooled to 0 °C. Dimethylaminopyridine (DMAP, 395 mg, 3.24 mmol), *N*-hydroxysuccinimide (HO-Suc, 372 mg, 3.25 mmol), and dicyclohexylcarbodiimide (DCC, 668 mg, 3.24 mmol) were slowly added. The mixture was stirred for 1 h. Bisoctadecylamine (1.69 g, 3.24 mmol) was added and the mixture was heated at 30 °C and stirred for 12 h. After filtration of the dicyclohexylurea (DCU) and addition of CH_2Cl_2 (50 mL), the solution was washed successively with aqueous solutions of HCl (0.5 M, 10 mL), aq sat. $KHCO_3$ (10 mL), and aq sat. NaCl (10 mL). After the organic phase was dried with

Na_2SO_4 and purification on a silica column (AcOEt), pure product was isolated (1.213 g, 1.44 mmol, 49 %). ^{1}H NMR ($CDCl_3$): $\delta = 4.2-4.1$ (m, 6H, CO_2CH_2, OCH_2CO_2, OCH_2CON), 3.7 – 3.6 (m, 16H, OCH_2), 3.13 (t, 4H, $J = 7$ Hz, $CONCH_2$), 1.48 (brs, 4H, NCH_2CH_2), 1.4 – 1.1 (m, 63H, CH_2, CH_3), 0.83 (t, 6H, $J = 7$ Hz, 2 CH_3); ^{13}C NMR ($CDCl_3$): 170.15, 168.4, 70.8, 70.4, 70.0, 68.6, 60.5, 46.8, 45.6, 33.8, 31.7, 29.5, 29.2, 28.8, 27.4, 26.9, 26.8, 25.6, 24.8, 22.5, 20.8, 14.0, 13.9; elemental analysis calcd (%) for $C_{50}H_{99}NO_8$: C 71.30, H 11.85, N 1.66; found C 70.71, H 11.13, N 1.45.

Acid 4: Ester **3** (1.213 g, 1.44 mmol) and KOH (720 mg, 12.8 mmol) were dissolved in EtOH (6 mL). The solution was then refluxed for 6 h. The product was then precipitated at 4 °C. Pure product was isolated (892 mg, 1.1 mmol, 76 %). ^{1}H NMR ($CDCl_3$): 4.25 (s, 2H, CH_2CO_2H), 4.0 (s, 2H, OCH_2CON), 3.7 – 3.6 (m, 16H, OCH_2), 3.25, 3.10 (t, 4H, $J = 7$ Hz, $CONCH_2$), 1.5 (brs, 4H, NCH_2CH_2), 1.4 – 1.1 (m, 60H, CH_2), 0.9 (t, 6H, $J = 7$ Hz, 2 CH_3); ^{13}C NMR ($CDCl_3$): 170.15, 168.4, 70.8, 70.4, 70.0, 68.6, 60.5, 46.8, 45.6, 33.8, 31.7, 29.5, 29.2, 28.8, 27.4, 26.9, 26.8, 25.6, 24.8, 22.5, 20.8, 14.0, 13.9; MS calcd for $C_{48}H_{95}NO_8$: 813 $[M]^+$; found 831 $[M+NH_4]^+$.

Protected RGD-lipopeptide 5a: Compound **4** (200 mg, 0.246 mmol), the protected cyclic peptide cyclo(-R(Mtr)GD(O*t*Bu)fK-) (187 mg, 0.205 mmol), *N*-hydroxybenzotriazole ($HOBt \cdot H_2O$) (38 mg, 0.246 mmol), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) (79 mg, 0.246 mmol) were dissolved in anhydrous DMF (2 mL). The pH was adjusted to 8.5 – 9 by addition of DIPEA (2.4 equiv). The mixture was maintained for 24 h at room temperature under argon. After DMF was removed under vacuum, the crude product was purified on silica gel coated glass plates (CH_2Cl_2/MeOH 9:1) to yield the pure product (140 mg, 0.084 mmol, 34 %). MS calcd for $C_{89}H_{154}N_{10}O_{17}S$: 1667.1 $[M]^+$; found 1667.6 $[M+H]^+$.

Protected RGD-lipopeptide 5b: Compound **4** (100 mg, 0.122 mmol), the protected cyclic peptide cyclo(-R(Pbf)GD(O*t*Bu)fK-) (111 mg, 0.122 mmol), *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, 46 mg, 0.122 mmol) and 2,4,6-collidine (30 mg, 0.244 mmol) were dissolved in anhydrous DMF (1 mL) at 0 °C. The mixture was maintained under argon for 10 h at room temperature. After DMF evaporation under vacuum, the crude product was purified on silica gel coated glass plates (CH_2Cl_2/MeOH, 9:1) to yield the title compound (136 mg, 0.08 mmol, 65 %). MS calcd for $C_{92}H_{158}N_{10}O_{17}S$: 1707.1 $[M]^+$; found 1708.5 $[M+H]^+$.

RGD-lipopeptide 6: Compound **5a** (110 mg, 0.064 mmol) was dissolved in a TFA/H_2O mixture 95:5 and stirred for 24 h. The product was precipitated and washed in diethyl ether at 4 °C. After lyophilization from *t*BuOH, the pure product **6** (43 mg, 0.031 mmol, 49 %) was isolated.

Following the procedure for **5a**, the deprotection of **5b** (181 mg, 0.106 mmol) in a TFA/H_2O mixture 95:5 was achieved after 8 h. Pure product **6** (110 mg, 0.078 mmol, 73 %) was isolated after three precipitations in diethyl ether at 4 °C and lyophilization in *t*BuOH. MS calcd for $C_{75}H_{134}N_{10}O_{14}$: 1399.01 $[M]^+$; found 1399.8 $[M+H]^+$.

Cell culture: Cells were obtained from a primary culture of endothelial cells extracted from human navel cord in the Institut für Prophylaxe und Epidemiologie der Kreislauferkrankungen der Universität München. The cells were grown in Dulbecco's modified eagle's medium (DMEM) supplemented by 10 % fetal bovine serum (FBS) (Promo Cell, Heidelberg) at 37 °C and 7.5 % CO_2 in cell culture plates. The cells are used confluent. They are removed from the surface by washing with a PBS buffer and incubating for 2 min with trypsin/EDTA (Life Technologies LDT, Paisley, Scotland). The cell suspension was mixed with cell medium (3:20) and added to the two types of cover slides: i) RGD-functionalized surface, ii) coated with collagen IV.

Preparation of RGD-functionalized surfaces by Langmuir – Schäffer deposition: Monolayer isotherms and deposition of supported membranes by the Langmuir – Schäffer (LS) technique were determined with a computer controlled film balance. The total area of the monolayer was 45 cm^2 and the area was changed with a speed of 0.1 $cm^2 s^{-1}$. The monolayer lateral pressure was monitored with a Wilhemy plate which was calibrated by comparison with the transition pressure of an arachidonic acid monolayer. A mixture of CH_2Cl_2/MeOH 9:1 was used as spreading solvent. Compression was started about 10 min after spreading. The subphase temperature was kept at 20 ± 0.2 °C. The monolayers were deposited onto silanized cover glasses by the LS technique. The monolayer was transferred horizontally through the monolayer into the subphase at 20 $mN m^{-1}$ and the coverslip was maintained within the solution in a petri dish. The dense cell suspension in medium was then added to the petri dish which was incubated at 37 °C. The substrates were observed by using a Zeiss Axiomat microscope equipped with a phase contrast objective. In order to test the effect of the RGD-functionalized surface on the adhesion of endothelial cells the relative number of slightly adhered but round cells and of spread cells was counted.

Giant unilamellar vesicles (GUV): Giant vesicles were prepared by the method of electric swelling.[35] Two solutions chloroform/methanol 9:1 containing: i)cholesterol (20 % mol), PEG 2000-DMPE (1 % mol), DOPC (74 %), and RGD-lipopeptide **6** (5 %) or ii) cholesterol (20 % mol), PEG 2000-DMPE (1 % mol), DOPC (79 %) were used. The lipid solution was spread onto a cover slide covered with indium/tin oxide (ITO) electrodes. After drying the lipid films, the coverslips were placed in a closed cell of a 0.5 mm thickness and allowed to swell in a solution of sucrose (180 mM) containing sodium azide (0.01 % mol) during application of a 10 Hz AC field (20 $V cm^{-1}$) for 5 h. A 100 µL aliquot of the vesicle suspension was then directly injected into the flow chamber or into the culture medium in contact with the endothelial cells adhered onto a glass surface.

RICM measurements of giant vesicle adhesion: The vesicle adhesion of the giant vesicles to endothelial cells was monitored with a Zeiss Axiomat microscope equipped with a RICM objective.[36] The samples were observed by bright field microscopy and by RICM under an hydrodynamic flow. As a control experiment, giant vesicles *without* the RGD-lipopeptide **6** were used. For the competition experiment, cyclo(-RGDfK-) peptide, the soluble analogue of the RGD-lipopeptide **6** was injected at the concentration of 1.3 $mg mL^{-1}$ of the aqueous solution. The experimental setup used for the application of hydrodynamic flow fields was described previously.[27] The flow chamber consisted of a plexiglas and a copper block serving as cover base plate. The coverslip (serving as substrate) formed part of the bottom block. The chamber was sealed with an o-ring. For the generation of the flow we used two syringes filled with a PBS buffer, which are mounted on a slide and then connected to the ends of the measuring chamber to form a closed flow loop. The flow is generated by moving the slide by a stepping motor. The flow can be varied from 0 to 3.46 $mL min^{-1}$ which corresponds to a shear force up to 35 mPa for the chamber of 1 mm height. Both afflux and drain are directed vertical to the channel which guarantees a laminar and almost parallel flow in the chamber at the shear rates we used. In the vicinity of the substrate the profile of the flow can be considered as linear.[24, 38] The cells were spread onto coverslips first coated by collagen IV in petri dishes. The coverslips (24×24 mm) were then placed into the chamber and incubated at about 40 °C in presence of a 100 µL aliquot of giant vesicles containing the RGD-lipopeptide **6**.

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