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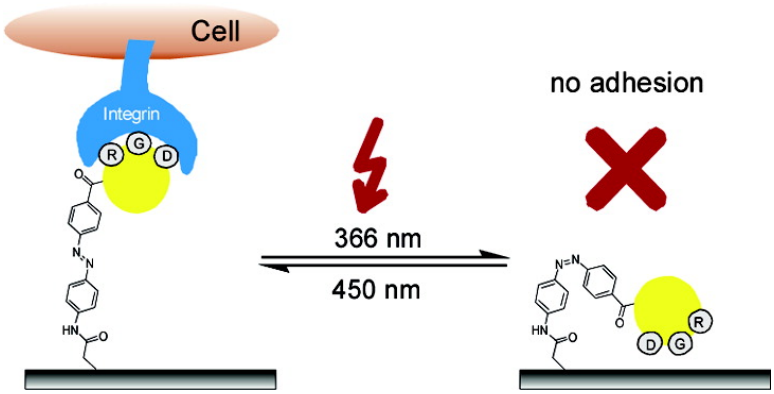

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Photoswitched Cell Adhesion on Surfaces with RGD Peptides

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Abstract: Coating of surfaces by RGD peptides is well-known. Herein we describe the possibility to switch cell adhesion properties by changing the distance and orientation of the RGD peptides to the surface. A set of RGD peptides of the type cyclo(−RGDfK−) was synthesized containing the photoswitchable 4-[(4-aminophenyl)azo]benzocarbonyl central unit as spacer between the acrylamide anchor and the RGD peptide. PMMA (poly methyl methacrylate) surfaces were coated with these peptides. Control of adhesion stimulation by irradiation with 366 or 450 nm light could be achieved.

Introduction

Coating of surfaces with cell-adhesive molecules provides a strong mechanical contact between cells and the surface. Cell adhesion is mediated by integrins,[1] a class of heterodimeric transmembrane cell receptors that bind selectively to different proteins of the extracellular matrix (EMC).[2] Cellular binding sites, like RGD peptides, have been reported to play a major role in mediating cell adhesion through integrins,[3] which transduce information to the nucleus through cytoplasmic signaling pathways. In this study, we have used tailor-made cyclic RGD peptides,[4] developed in our group, which bind specifically to $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, but to $\alpha IIb\beta 3$ with low affinity. The αv integrins are known to adhere to vitronectin. Only the $\alpha v\beta 3$ integrin is found in focal contacts and leads to spreading and migration of (endothelial) cells on vitronectin.[5]

One promising approach to generate cell adhesive and cell repulsive areas on material surfaces is coating with photochemically regulated molecules. This method provides surfaces irreversibly coated with specific photolithographically accessible patterns. However, a transient photochemical alteration of the binding properties of biomolecules can only be achieved by light-induced structural changes.[6]

One of the most commonly used classes of substances containing reversible photoinduced isomerization units are azobenzenes because of the pronounced changes in geometry upon its light-induced $Z-E$ isomerization, the high (photo)-stability, the high quantum yields, as well as of the extremely fast and fully reversible isomerization processes.[7] These are completed within 10 ps for the $E-Z$ and within 1 ps for the $Z-E$ direction.[8] Conversely, thermal $Z-E$ relaxation is a slow process, but leads to 100% E isomer.[7]

Azobenzene derivatives, such as 4-(phenylazo)phenylalanine,[9] 4-[(4-aminophenyl)azo]benzoic acid,[10] 4-(4-aminomethyl)-phenyl)azobenzoic acid,[11] and 3-[(3-aminomethylphenyl)azo]-phenylacetic acid,[12] have been incorporated into peptides and biopolymers to change their structure by photoisomerization.[9,10,12,13] In the field of RGD peptides, 4-[(4-aminomethyl)-phenylazo]benzoic acid has been incorporated into the backbone

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of cyclic peptides, and the effects of light-switched conformational preferences on the binding affinities to integrin have been analyzed.[14]

Surface-immobilized photoswitchable 4-arylazopyridine[15] and azobenzene[16] can be used to form self-assembled monolayers (SAMs) on gold surfaces and Langmuir−Blodgett films (LB films) on glass and silicon. Thereby, it was shown that surface-positioned photoisomerizable substances retain their light-responsiveness. In the dark, at equilibrium, azobenzene is predominantly in the more stable *E* form, and its switching kinetics was found to be altered by immobilization, but both diastereomers could be generated by irradiation with appropriate wavelengths of light. On the other hand, previous studies from our laboratory and others[17] clearly revealed that cell adhesion on different surfaces depends on the spacer length applied between the ligand peptide and the surface. From these experiments, it was compelling to analyze the effect of photoresponsive units in the spacer for anchoring cyclic RGD peptides to surfaces with the working assumption that a shorter spacer would not mediate cell adhesion whereas a longer one would do it. If such concept is realized, photolithographic structuring of surfaces in cell adhesive and cell repulsive areas would become possible.

Materials and Methods

Peptide Synthesis. The cyclic pentapeptide cyclo(−RGDfK−) was synthesized as described previously[4b,17d] and coupled in solution to the spacer/anchor construct with HATU/HOAt/collidine. In one set of peptides (**1**−**4**), the azobenzene moiety was placed near the anchor group and thus in proximity of the solid surface, while in a second set (**5**−**7**), it was grafted directly to the cyclic RGD peptide. Correspondingly, for compounds **1**−**4**, 4-(4-aminophenyl)azobenzoic acid[18,19] was acylated with acryloyl chloride and then C-terminally elongated by coupling to glycine, γ-amino butyric acid, and ϵ-amino hexanoic acid attached to TCP-resin with TBTU/HOBt/DIEA.

For the second set of peptides (**5**−**7**), *N*-acryloylglycine,[20] *N*-acryloyl-δ-amino valeric acid,[21] and *N*-acryloyl-ϵ-amino hexanoic acid[21] were preactivated with HATU/HOAt/collidine for acylation of 4-(4-aminophenyl)azobenzoic acid.

Coating. Twenty microliter solutions of peptides **1**−**7** in 2-propanol/DMSO were placed on PMMA disks (Palacos R, 1 cm)−six for each peptide−followed by irradiation at 254 nm for 2 h. After standing overnight in the dark, every disk was rinsed with 2 mL of PBS buffer (pH 6.0) to remove uncoated peptide. Three disks from every peptide were irradiated at 450 nm for 3 h and stored for 5 days in the dark to ensure that thermal equilibrium is reached even on bound material. The other three disks were irradiated at 366 nm for 12 h directly before seeding with cells. All disks were transferred to cell culture plates (48 wells).

Cell Adhesion Assay. The cell adhesion assays were performed as described by Landegren.[22] MC3T3 E1 mouse osteoblasts were seeded on the BSA blocked substrate at a density of 50 000 cells per well. The cells were allowed to adhere for 1 h under standard tissue culture conditions (37 °C, 5% CO_2) in serum-free culture medium (DMEM) containing 1% BSA (w/v). The wells were washed three times with PBS (pH 7.4) to remove nonadherent cells. Attached cells were quantified by an ELISA detection of the activity of the lysosomal enzyme hexosaminidase. *p*-Nitrophenol-*N*-acetyl-β-D-glucosaminide was cleaved by the enzyme, and the amount of colored *p*-nitrophenol was measured with an ELISA reader (Dynatech Laboratories, MRX) at 405 nm. Results are given as the percentage of the total number of cells seeded (which is considered as 100% of cell adhesion). In all experiments, the mean value of each point given in the figures is the result of triplicates; the error bars represent standard deviations.

Results and Discussion

For this study, 4-[(4-aminophenyl)azo]benzoic acid was chosen as the light switch as it was shown to retain all typical properties of azobenzene derivatives in terms of photoisomerization and photostability.[13c] As known for this and other azobenzene derivatives, at thermodynamic equilibrium in the dark, the *E* isomer, which is about 3 Å longer than the *Z* isomer, is obtained in almost quantitative yield. It can be switched to the *Z* form by irradiation with light at 360 nm, but at the photostationary equilibrium, conversion to the *Z* isomer proceeds only with yields of about 70−90%.[7] Therefore, in cell adhesion assays, the lack of 100% *E* isomer has to be taken into account. However, if the density of RGD is relatively low, the depletion of RGD peptides which are exposed to be recognized by the integrins can switch the recognition from "binding" to essentially "no stimulated binding". It is known from our previous studies that cells need oligomeric binding with a maximum distance of about 65 nm between RGD peptides.[23]

An acrylamide anchor on PMMA was chosen for immobilization of the cyclo(−RGDfK−), as the influence of spacer length on the integrin-mediated cell adhesion is already known for this system.[17d] For an effective spacer, two ϵ-amino hexanoic acids (Ahx) and an acrylamide as anchor was required, which corresponds to a length of 1.7 nm (distance between both external carbonyl carbon atoms), while the acryloyl−Ahx spacer with 0.9 nm length was found to be too short to mediate cell adhesion. Taking this information into account, a set of peptide constructs was designed (Figure 1) containing the photoswit-

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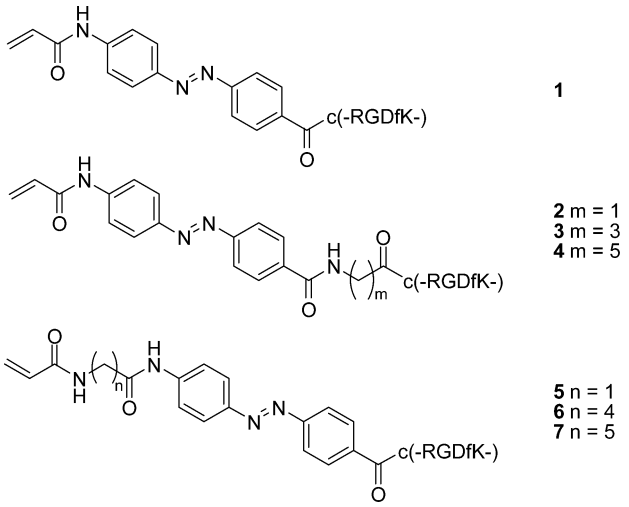

Figure 1. Set of cyclic RGD peptides containing a photoswitchable 4-[(4-aminophenyl)azo]benzocarbonyl unit.

Table 1. Estimated Maximal Spacer Lengths of the Reversibly Photoswitchable Peptides **1**−**7** in *Z* and *E* Configuration Compared to Acrylamide Linkers with and without ϵ-Amino Hexanoic Acid Spacer (the distance between both external carbonyl carbon atoms of the spacer is given)

spacer	length [nm]
Acryl-Ahx-*c*(−RGDfK−)	0.856
Acryl-Ahx-Ahx-*c*(−RGDfK−)	1.723
E isomer	
1	1.25
2	1.73
3	1.82
4	2.06
5	1.65
6	2.01
7	2.13
Z isomer	
1	0.69
2	0.95
3	1.16
4	1.38
5	1.10
6	1.35
7	1.46

chable azobenzene, and the maximum length of the corresponding *Z* and *E* isomers was estimated by using the X-ray structure of *cis*-azobenzene[24] and the standard averaged bond lengths of CH_2-CH_2 and amides (Table 1).

The reversible light-switching of all azo compounds in DMSO solution was confirmed by UV/vis and ^{1}H NMR spectroscopy. The *E:Z* ratio at photostationary equilibrium was calculated from the different chemical shifts of the olefinic anchor protons measured by ^{1}H NMR. The *Z* isomer dominates but in addition between 20 and 30% *E* configuration was present. After light-protected storing of the samples for 5 days, the pure *E* isomers were characterized.

For UV/vis measurements, the samples were stored in the dark for 5 days at room temperature to obtain pure *E* isomer and were then irradiated with UV light at 366 nm for 60 min. After 1, 5, 20, and 60 min, the UV/vis spectra were taken to monitor kinetics of the *E*→*Z* isomerization; in a similar manner, thermal relaxation at room temperature in the dark was

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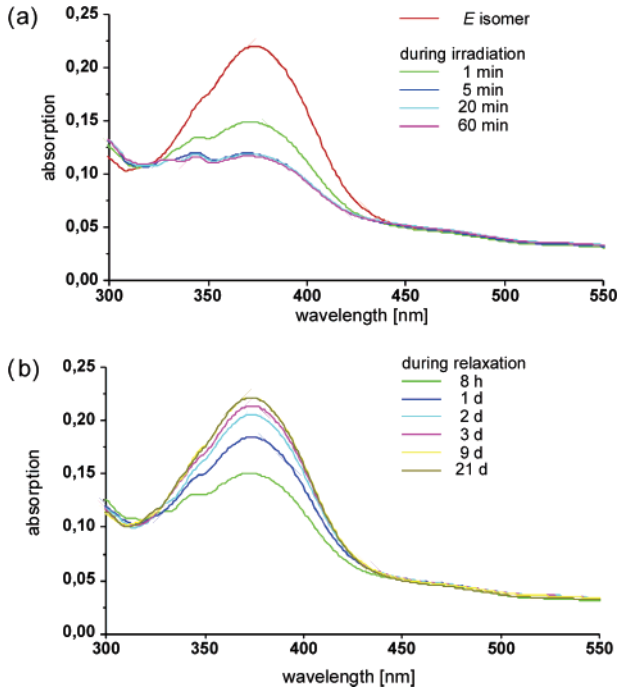

Figure 2. UV/vis absorption spectra from **2** (0.1 mM in DMSO) in *E* configuration, (a) during irradiation with UV light at 366 nm and (b) following thermal relaxation at room temperature in the dark. (Light source was changed at 350 nm while spectra were recorded. Spectra were smoothed according to the Savitzky−Golay method (2 × 13 data points) to reduce noise.)

monitored by UV spectra. Figure 2 shows the isomerization behavior of peptide **2**. The maximum population of *Z* isomers is reached 5 min after irradiation of the *E* isomer. As known for azobenzene, thermal relaxation is much slower than the photoinduced isomerization. When a sample is kept in the dark for more than 3 days, the initial state is regained (pure *E* isomer). The absorption maximum of the *E* isomers of all peptides is at about 370 nm, which allows a relatively selective irradiation of the *E* configuration at 366 nm. Reversibility of the photochemical-induced isomerization was demonstrated by repeated switching (at least two times), followed by spectroscopic characterization. The photostability of the peptides in solution was verified by ^{1}H NMR, ESI-MS, and HPLC.

By confirming the slow kinetics of thermal relaxation for all peptides compared to the time scale of cell adhesion assays, at least from this point of view, all peptides are potentially suitable for the reversible photocontrol of cell adhesion on artificial surfaces.

Peptides **1**−**7** were immobilized on PMMA disks by irradiation with UV light at 254 nm. Then one part of the disks was irradiated at 450 nm for 3 h and then stored in the dark for 5 days to switch the peptides completely into the *E* configuration, while the other part was irradiated overnight at 366 nm directly before the cell adhesion test. All peptide-coated surfaces showed an increase of cell adhesion after irradiation at 450 nm and storage in the dark (Figure 3). Disks irradiated at 366 nm showed lower enhancements of cell adhesion, but only for peptide **5** was cell adhesion by light-induced *E,Z* isomerization reduced to the same level as that for uncoated material. On the other hand, peptide **1**, which has the shortest spacer in *Z* configuration, still exhibits increased cell adhesion compared to uncoated PMMA, even after irradiation at 366 nm. A rational explanation

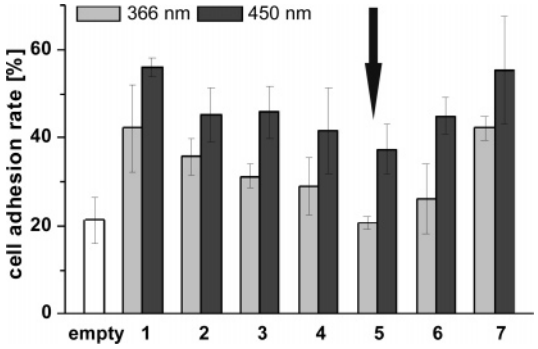

Figure 3. Cell adhesion of MC3T3 E1 mouse osteoblasts on PMMA. The peptide concentration in the coating solution was 1 mM. Samples were irradiated for 3 h at 450 nm and overnight at 366 nm, respectively.

of this phenomenon is difficult, even taking into account the changes observed in photoisomerization of azobenzene derivatives on solid surfaces.[15,16]

Summary

The distance of the RGD ligand from the PMMA surface could be switched in the peptides **1**−**7** by light-induced *E*,*Z* isomerization. All peptides lead to enhanced cell adhesion on PMMA disks in their *E* form, whereas the plating efficiency was decreased after irradiation at 366 nm, which shortens the distance of the RGD-containing peptides to the surface by *E*,*Z* isomerization. For acryloyl-Gly-[4-(4-aminophenyl)azo]benzocarbonyl-*c*(−RGDfK−) **5**, the cell adhesion in the *Z* configuration could be reduced to almost the same level as that on uncoated PMMA disks, while in the *E* conformation, the plating efficiency was still enhanced by 17%. Therefore, photochemical control of cell adhesion was achieved.

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Supporting Information Available: Synthetic procedures and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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