Surface-Attached PDMAA−GRGDSP Hybrid Polymer Monolayers that Promote the Adhesion of Living Cells

S. Loschonsky,[†] K. Shroff,[†,‡] A. Wörz,[†] O. Prucker,[†] J. Rühe,[†] and M. Biesalski*[,†]

Department of Microsystems Engineering (IMTEK), University of Freiburg, Georges-Köhler-Allee 103, 79110 Freiburg, Germany

Received September 26, 2007; Revised Manuscript Received October 30, 2007

Peptide−polymer hybrid molecules are being introduced, where one part of the molecule (i.e., the peptide) promotes the adhesion of living cells, whereas the other part of the molecule (i.e., the synthetic polymer) is known to prevent cell adhesion. The hybrid copolymer, poly(dimethylacrylamide) (PDMAA)−glycine-arginine-glycine-aspartic acid-serine-proline (GRGDSP) was synthesized by first preparing an initiator-modified peptide and in a second step growing the PDMAA block directly off the peptide through atom transfer radical polymerization (ATRP). The PDMAA block length can be varied by adjusting appropriate polymerization conditions, thereby changing progressively the amount of the cell-repelling part of the molecule. The hybrid copolymer was further used to prepare surface-attached peptide−polymer monolayers at planar solid glass substrates through a photochemical immobilization process. By blending of the hybrid copolymer with PDMAA homopolymer (i.e., without peptide), the apparent peptide film concentration can be varied in a very simple manner. The adhesion of human skin fibroblast cells in serum-free medium was investigated as a function of the amount of peptide−polymer in the solution used for film preparation. Cells do not adhere to a pure PDMAA monolayer; however, already 0.02 wt % of peptide in the film is enough to induce cell adhesion, and 0.1 wt % promotes stress-fiber formation within adherent cells. Using lithographical means, chemically micropatterned peptide−polymer films were prepared that allow for a spatial control of the adhesion of living cells and thus they constitute a simple platform for the design of live-cell biochips.

Introduction

There is a growing interest in the development of bioinspired hybrid polymers for the use as novel functional biomaterials.[1–6] Interesting materials comprise the design of peptide−polymer conjugates in which a bioactive peptide sequence is linked to a synthetic (bioinert and nontoxic) polymer. Such hybrid polymers are promising materials for the design of targeted carrier systems in the area of drug delivery,[7] as matrices in tissue engineering,[8–10] or for the design of so-called polypeptide drugs.[7,11] If used as surface-coating materials,[12] polymers, and peptide−polymer conjugates may improve the biocompatibility of implants or they may be useful in the microengineering of cell-based diagnostic tools such as, for example, live-cell chips.[12–16]

In principle, defined peptide−polymer hybrid materials can be designed by linking functional, preformed peptides to preformed polymers. Although conceptionally simple, a limitation of this "grafting to" approach concerns the variety of polymers that can be used as well as their molecular size. Functional groups in the polymer may interfere with the reactivity of the chosen end groups, or they can interact with functional sites on the peptide itself during the coupling step. The latter may limit the regioselectivity of the attachment of the polymer to the peptide as well as it may affect the coupling kinetics. Additionally, if the molecular size of the end-functional, synthetic polymer is too large, the probability that the reactive end-group is buried inside the polymer coil and therefore does not find its counterpart in the peptide is increasing. Thus, up to now, low molar mass polymers are typically employed in such convergent approaches for preparation of peptide−polymer hybrids.

Some of the above-mentioned limitations may be overcome by preparing the synthetic macromolecule directly on a predesigned functional peptide (i.e., divergent "grafting from" approach). To this, a few interesting novel examples of hybrid block copolymers have been recently reported where sequence-defined peptides were first modified with a functional group that can initiate a polymerization reaction (i.e., formation of an initiator-modified peptide), and subsequently synthetic polymers were grown in situ "from" the peptide. The latter results in defined peptide−polymers of adjustable molecular size and functionality of the polymer block.[17–22] Polymerization methods that have been employed with initiator-modified peptides include nitroxide-mediated polymerization (NMP),[21] reversible addition fragmentation transfer polymerization (RAFT),[23] and atom transfer radical polymerization (ATRP).[17–20,22] All three methods are known as "controlled radical polymerization" techniques, and each of them has its own unique advantages, and disadvantages, which are discussed in a wealth of review articles and books. To this, the reader is referred to a handbook of radical polymerization and articles therein.[24]

As far as ATRP with initiator-modified peptides is concerned, Washburn and co-workers reported the synthesis of a peptide-poly(hydroxyethylmethacrylate) (PHEMA) hybrid copolymer.[22] A peptide that carries the well-known cell-recognition sequence "RGD" (arginine-glycine-aspartic acid)[25] was prepared by solid-phase peptide synthesis, and the *N*-terminus of the peptide was modified with a functional group that can be used to initiate an ATRP reaction. In a subsequent step, the peptide−PHEMA was prepared directly on the solid support swollen in the monomer,

* Corresponding author. E-mail:biesalski@imtek.de.
[†] Department of Microsystems Engineering (IMTEK), University of Freiburg.
[‡] Present address: Department of Chemical Engineering, University of Minnesota, 151 Amundson Hall, Minneapolis, MN 55455.

10.1021/bm7010714 CCC: $40.75
Published on Web 01/08/2008

solvent, and polymerization additives. After cleavage from the solid support, this peptide−polymer hybrid material was then coated (i.e., physically adsorbed) onto the bottom of six-well plates and the adhesion of mouse fibroblast cells was studied. It was shown that the cells adhered to the peptide−polymer but did not attach to pure PHEMA surfaces.

When designing peptide−polymers for the use as surface-coating material, one should take a few very important issues into account. First, ATRP reactions performed on solid phase, i.e., using a resin-bound initiator-modified peptide, may face several severe limitations: with increasing amount of grafted polymer, it may become likely that the diffusion of polymerization additives to the active centers of the growing chains will be kinetically hindered, which may directly interfere with the ATRP equilibrium, thus limiting the control over the reaction kinetics and the molecular size as well as uniformity of the resulting polymers. Second, and more importantly, any mismatch of the peptide sequence generated throughout the design of the initiator-modified peptide will be directly built in into the final peptide−polymers, and purification of the target molecule is not trivial, as individual molecules only differ in the chemistry of the end group with the largest part of the hybrid material (i.e., the synthetic polymer block) being essentially identical. Finally, if RGD−polymer hybrids are designed to be used as bioactive surface coatings, not only the precise synthesis of the bioactive polymers itself is important, but also the thermal/chemical stability of the attachment of the molecules to a solid surface is of utmost importance for any application. If the bioactive polymers are only physically adsorbed, such as the PHEMA−RGD polymers outlined above, they may be displaced by other substances (solvents, salts, proteins, etc.) during a cell adhesion assay. The formation of covalent bonds between the peptide−polymer and the surface may overcome this limitation and enhances long-term stability of such peptide−polymer films. Several techniques have emerged over the past decades in order to chemisorb macromolecular films to solid substrates, and the reader is referred to books that describe a large number of different approaches.[26,27] A very simple, but versatile photochemical method to link polymer molecules permanently to solid substrates was introduced by Prucker et al.[28] Here, a benzophenone-function was modified with a monochloro-silane anchor group, and so-prepared benzophenone−silanes were linked to oxidic glass surfaces through a condensation reaction under mild conditions. Subsequently, polymers of varying chemistry and molecular dimensions can be coated on top of the benzophenone−silane monolayer, and molecules in close contact with the surface-immobilized benzophenone-functions are covalently attached to the latter by a simple photochemical process. Details of this chemisorption process will be given further below in the text.

In the following, we report the synthesis of poly(dimethylacrylamide) (PDMAA)−peptide hybrid copolymers, which carry an RGD cell-recognition motif within the predefined peptide sequence. The molecules are designed by first preparing an initiator-modified peptide and subsequently using this initiator-modified peptide to polymerize DMAA monomer by ATRP. We then use the prepared hybrid copolymers as a coating material to design surface-attached peptide−polymer monolayers that specifically promote the attachment of living cells. In addition, we vary the apparent peptide concentration in the films by a simple blending of PDMAA homopolymer with PDMAA−glycine-arginine-glycine-aspartic acid-serine-proline (GRGDSP) hybrid polymer. Finally, we investigate the influence of the peptide concentration in the film on the respective cellular response (i.e., adhesion and spreading) as well as show the first results of using the peptide−polymer to create chemical micropatterns that promote cell adhesion in a spatially controlled manner.

Experimental Section

Materials and Instrumentation. All solvents and reagents were purchased from Aldrich and were used as received (p.a. grade or higher), if not otherwise stated. *N*,*N*-Dimethylacrylamide (DMAA) monomer was passed through a basic alumina column and distilled under reduced pressure prior to its use. Toluene was dried and distilled over sodium. Triethylamine was stirred over calcium hydride and distilled prior to its use. Both reagents were stored in an inert gas atmosphere at room temperature.

Atomic force microscopy (AFM) analysis was performed on a Nanoscope IIIa microscope (Digital Instruments, Santa Barbara, CA). All images were obtained in a tapping mode.

X-ray photoelectron spectroscopy (XPS) measurements were carried out on a Perkin-Elmer PHI 5600 spectrometer using Mg Kα radiation. The step width during accumulation of the spectra was set to 0.8 eV (survey) and 0.1–0.13 eV (detail scans), respectively. The analyzer angle was set to 90° relative to the plane of the substrate.

The dry thickness of surface-attached polymer monolayers was measured by ellipsometry (Riss EL-X1, Germany, incidence angle: 70°). Microstructures were visualized using an imaging ellipsometer I-Elli 2000 (Nanofilm, Germany, incidence angle: 65°), and analysis was performed using LabView software.

Synthesis of the Initiator-Modified Peptide. The resin-attached peptide Resin-Wang-O-PSDGRG-NH-Fmoc (3 g, max load: 0.8 mmol/g) was prepared by BioLux GmbH, Stuttgart, Germany using standard Fmoc solid phase protocols. The purity, as proven by test cleavage and LC-MS analysis, was above 95% for the targeted sequence H_2N-GRGDSP-OH. After swelling the resin in DMF for 20 min, the Fmoc protecting group was removed by shaking the swollen resin with piperidine (20 vol % in DMF), three times for 20 min, with intermediate DMF and methanol washing steps. The attachment of the initiator-function (2-bromo-2-methylpropionic acid) to the free amine group was performed using a standard *N*-hydroxybenzotriazole (HOBt)/*O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (HBTU) coupling protocol. To this, 5 equiv of 2-bromo-2-methylpropionic acid (2 g, 12 mmol) were activated with 5 equiv of HOBT (1.6 g, 12 mmol), 5 equiv of HBTU (4.55 g, 12 mmol), and 10 equiv (4.1 mL, 24 mmol) of di-isopropyl-ethylamine (DIPEA) in 45 mL of DMF at room temperature by stirring this solution for 5 min. The activated reagent was subsequently added to the resin-bound peptide. After shaking at room temperature for 45 min in 45 mL of DMF, the solution was discarded and the resin was washed three times with DMF and methanol. The coupling procedure was repeated three times to ensure quantitative linkage of the initiator function to the amine terminus of the resin-bound peptide. The so-prepared initiator-modified peptide was cleaved off of the resin by treatment with 95% TFA (in water) for 2 h at room temperature. Excess TFA was removed under reduced pressure, and the peptide was precipitated as a white solid in cold *tert*-butyl-methyl ether. The initiator-modified peptide was characterized by standard analysis techniques, as outlined in the Supporting Information.

Polymerization of *N*,*N*-Dimethylacrylamide in DMSO. The polymerization of DMAA using the initiator-modified peptide was carried out according to the following protocol: monomer (15.45 mL, 150 mmol) and CuCl (20 mg, 0.2 mmol) were degassed by three freeze–pump–thaw cycles. Separately degassed DMSO (8 mL) was added to this and stirred for 5 min at room temperature. Then, Me_6TREN (55 μL, 0.2 mmol) was added and the mixture was stirred for another 5 min at room temperature. To start the polymerization, a stock solution of initiator-modified peptide (147 mg, 0.2 mmol) in DMSO (7.45 mL) was added to the Schlenk flask. Aliquots (4 mL) were taken at regular time intervals for further analysis. The polymerization was stopped by opening the flask and exposing the catalyst to air, and the mixture was directly added to a solution of 50 mL of diethyl ether for precipitation

of the polymer. The ether phase was decanted, and the polymer was redissolved in methylene chloride and passed through a neutral alumina column to remove the catalyst. The polymer was recovered by removing the solvent in vacuum.

Polymer Characterization. The analysis of the monomer-to-polymer reaction conversion by HPLC, and NMR is not trivial, as solvent and peptide signals were overlapping with the monomer signals. Thus we determined the latter gravimetrically. The number-average molar mass and the polydispersity (PDI = M_w/M_n) were determined by gel permeation chromatography (GPC) on a Hewlett-Packard Agilent 1100 series using a refractive index detector at $T = 25$ °C. Three columns (PSS GRAM gel, 10^4, 10^3, 30 Å; Polymer Standard Service, PSS) in series were used. DMF (containing 70 mmol/L LiCl) was used as mobile phase with a flow rate of 1 mL/min. The system was calibrated with narrow-disperse poly(methyl methacrylate) standards. Data acquisition and analysis were performed using PSS software.

Synthesis of 4-(3′-Chlorodimethylsilyl)propyloxybenzophenone. The benzophenone-silane (4-(3′-chlorodimethylsilyl)propyloxybenzophenone), which was used for the surface attachment of polymer monolayers, was prepared according to a protocol by Prucker et al.[28] The purified silane was dissolved in dry toluene at a concentration of 0.05 mol/L and stored in an inert gas atmosphere until further use.

Preparation of Surface-Attached Polymer Films. Silicon wafers and microscopy glass slides were cut to a size of 1.5 cm × 1.5 cm and treated with piranha solution (3:1 (v/v) H_2SO_4/H_2O_2) for 15 min to remove organic impurities. The substrates were thoroughly rinsed with distilled water, dried at 120 °C, and placed in Schlenk tubes. Dry toluene was added until complete coverage of the substrates (typically 50 mL), and 10 mL of the prepared benzophenone−silane solution (50 mmol) as well as 1 mL (7.2 mmol) of dry triethylamine were subsequently added under inert gas atmosphere. The silanization was carried out at room temperature overnight in the dark. The substrates were intensively rinsed with toluene, methanol, and acetone to remove excess silane and dried in vacuum.

The deposition of the polymer film on the benzophenone−silane modified substrates was performed by spin-casting (3000 rpm for 1 min.) of a polymer solution, prepared in 2-propanol (concentration: 5 mg/mL). The solid substrates were exposed to 400 mJ/cm^2 of UV light at 254 nm to form covalent bonds between the benzophenone−silane monolayer and the polymer. Only polymer chains that are in close vicinity to the surface can be linked to the benzophenone. All other polymer chains, which are not bound onto the solid support, were then removed by extracting the resulting monolayers in 2-propanol. The films were subsequently analyzed with respect to chemical identity (FTIR and XPS), thickness (ellipsometry), and homogeneity/roughness (imaging ellipsometry, AFM).

Preparation of Micropatterned Polymer Films. A PDMAA layer was deposited on a benzophenone-modified glass substrate as described before and was subsequently illuminated through a photomask, leading to covalent attachment of a monolayer of the polymer in the illuminated areas (quartz glass covered with 100 nm of chromium; the layout and a micrograph of the mask are shown in the Supporting Information). After the development (i.e., washing off of all nonbound PDMAA molecules), a peptide−polymer solution (100 mol % PSDGRG−PDMAA) was spin-cast onto the substrate. The resulting polymer film was irradiated with UV light (now without mask, i.e., flood exposure). After extraction of the substrate with methanol and water, only polymer remains on the surface that was in direct contact with the benzophenone monolayer. PDMAA−GRGDSP, which is deposited in areas that were blocked during the first illumination step, is thus bound firmly to the substrate.

Cell Adhesion Assays. The substrates coated with surface-attached polymer and peptide−polymer monolayers were first sterilized by dipping two times in nondenatured ethanol and dried in a biosafety cabinet. The substrates were then dipped into phosphate-buffered saline (PBS, pH 7.4) and subsequently in 1% bovine serum albumin (BSA) solution in PBS. The substrates were then rinsed two times with pure PBS and placed in 6-well plates containing prewarmed (37 °C) basal medium (without serum). The treatment of the substrates with BSA was performed in order to avoid cells sticking to the back side of the substrate during cell-adhesion assay.

Fibroblast cells were released from culture plates and washed with PBS. The cells were suspended in known amount of basal medium, counted, and diluted to a final concentration of 10^6 cells/mL. One mL of this cell suspension was added to each well that contained the prepared substrates. The cells were mixed by gentle swirling, and cells were allowed to adhere to the substrates for 1 h (incubation at 37 °C and 5% CO_2). After this time, the substrates were removed from the 6-well plates and washed several times with PBS in order to remove any nonadherent cells. Optical micrographs were taken on an inverted microscope (Nikon TS-F 100), and cells were counted as a function of surface area at four different spots on the substrate in triplicate samples. The substrates were placed into new 6-well plates, basal medium containing 10% fetal calf serum (FCS) was added, and the substrates were incubated at 37 °C for 24 h, after which further micrographs were taken and the adherent cells were prepared for stress fiber staining.

Fixation of Adherent Cells and Staining of Actin Stress Fibers. The adherent cells were washed twice with prewarmed phosphate-buffered saline, pH 7.4 (PBS), and treated with 3.7% formaldehyde solution in PBS for 10 min at room temperature. After washing with PBS, the cover slips were placed in a glass Petri dish and extracted with a solution of cold acetone ($T = -20$ °C) for 3−5 min. Five μL of dilute methanolic stock solution (6.6 μM) of NBD phallacidin was added into 200 μL of PBS for each sample for staining purposes. Bovine serum albumin (BSA, 1%) was added to the staining solution to reduce nonspecific background staining with these conjugates. The staining solution was placed onto the cover slips and left for about 20 min at room temperature. After this time, samples were washed again with PBS and analyzed by fluorescence microscopy using a Nikon TS-F100 instrument ($\lambda_{ex} = 465$ nm; $\lambda_{em} = 536$ nm).

Results and Discussion

Synthesis of Peptide−PDMAA Hybrids by In Situ ATRP. The combination of cell-adhesive and cell-repellent properties in a single hybrid molecule is of great interest for the design of materials that specifically interact with living cells and at the same time largely suppress nonspecific interactions, e.g., with proteins. A number of hydrophilic polymers have proven as interesting candidates for this purpose. For example, poly(dimethylacrylamide) (PDMAA) films, anchored to planar substrates, possess excellent protein- and cell-repellent properties.[29–32] As acrylamides can be polymerized by ATRP,[33–35] we decided to prepare PDMAA−peptide hybrid molecules. In a first step, we modified the cell-recognition motif glycine-arginine-glycine-aspartic acid-serine-proline (GRGDSP), derived from the extracellular matrix protein fibronectin, at the *N*-terminus with an ATRP initiator function by using a solid phase chemistry protocol, as shown in Figure 1. The prepared initiator-modified peptide was cleaved off of the resin by treatment with 95% TFA in water, followed by precipitation in cold ether and final lyophilization. The characterization with respect to its chemical identity is outlined in detail in the Supporting Information.

The ATRP of DMAA using the initiator-modified peptide was performed in DMSO solution. DMSO was chosen as solvent because it is a good solvent for the initiator-modified peptide, the monomer, and the catalyst, as well as the polymer. In addition, DMSO is known to prevent undesired peptide–peptide interactions, which ensures homogeneous dissolution of the peptide during in situ polymerization of the PDMAA block. The polymerizations were performed at 25 °C, and samples were taken at regular time intervals to analyze the polymerization kinetics and the evolution of the apparent molar mass, as well

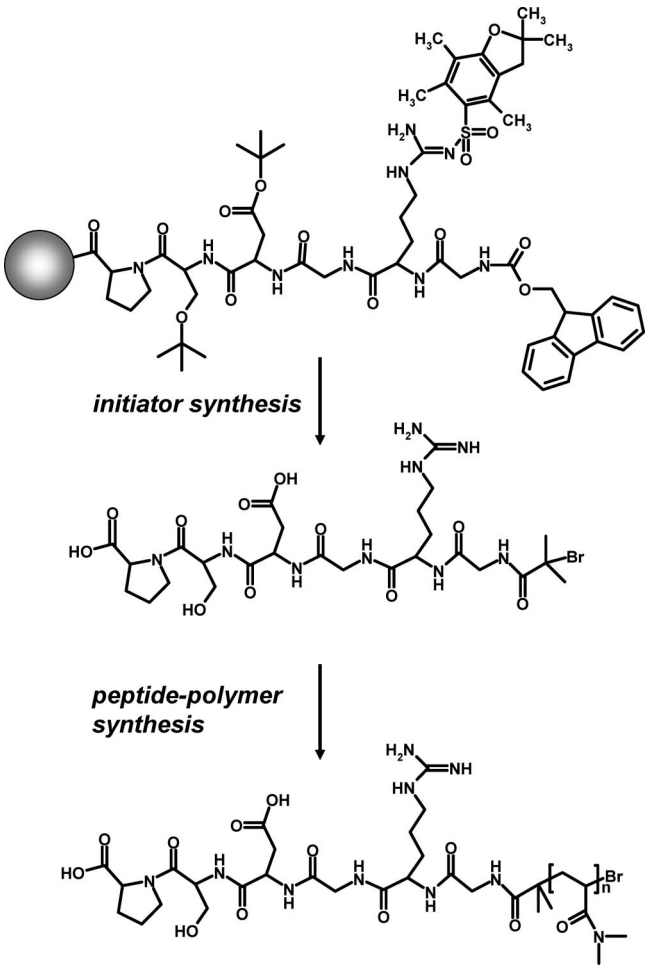

Figure 1. Schematic illustration of the synthesis of an initiator-modified bioactive peptide ligand and the subsequent use of this peptide for the in situ preparation of a PDMAA−GRGDSP hybrid copolymer.

as polydispersities of the PDMAA−GRGDSP hybrid copolymers. The kinetics of the polymerization reaction is shown in Figure 2. The conversion achieved after 60 min of polymerization time was about 35%. Already after reaction times of about 20 min, the reaction rate shows a nonlinear behavior. This finding is similar to observations by other groups, when polymerizing amide-functional monomers by ATRP.[33–35] The decrease in the apparent rate of the polymerization may be caused by interactions of the amide groups with the copper catalyst system, which can interfere with the ATRP equilibrium and which slows down the reaction kinetics.[33,34] In addition, the displacement of the terminal bromine group during an intramolecular cyclization reaction may terminate the growth of a PDMAA chain.[33] Finally, the nonlinear first-order kinetics may be caused by radical recombination and disproportionation reactions, which both yield a final termination of a growing polymer chain.[36]

Next we investigated the molar mass and the molar mass distribution of the PDMAA−GRGDSP. Initial NMR investigations were not useful, as the apparent molar mass of all samples was too high. Thus, we used GPC with DMF as the mobile phase and narrow dispersed PMMA as a standard. LiCl was added to the mobile phase in order to avoid unspecific aggregation of the polymer. Figure 3 shows examples of elution curves of PDMAA−GRGDSP, obtained at different polymerization times denoted in the figure. With increasing polymerization time (i.e., increasing conversion of the reaction), the

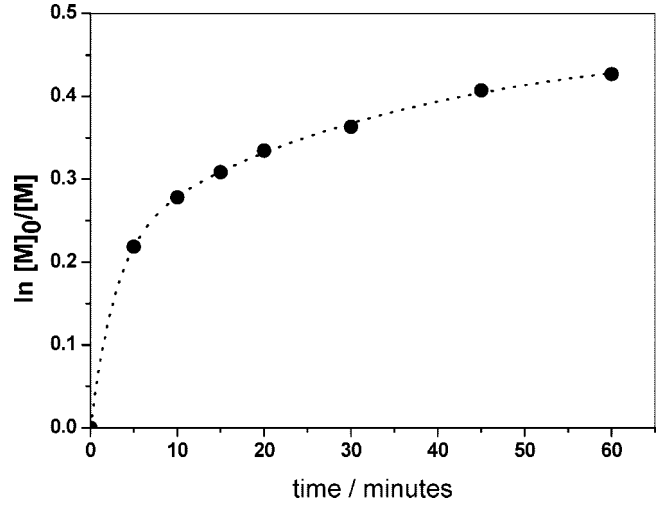

Figure 2. First-order kinetic plot ($\ln[M_0]/[M_t]$ as a function of polymerization time, with $[M_t]$ being the respective monomer concentrations at time $t = 0$ and t) of the polymerization of DMAA in DMSO at 25 °C. Polymerization conditions: [DMAA]:[initiator-modified peptide]:[CuCl]:[Me_6TREN] 750:1:1:1 in 50 vol % DMSO. The dotted line is a guide to the eye.

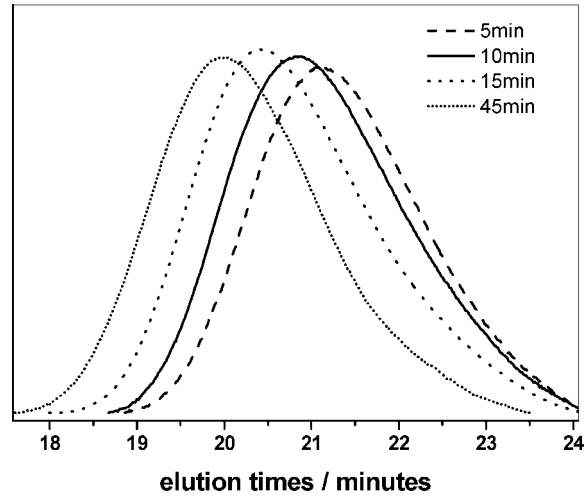

Figure 3. GPC elution traces of PDMAA−GRGDSP obtained through polymerization of DMAA with the initiator-modified peptide in DMSO solution at varying polymerization times denoted in the figure (GPC mobile phase: DMF(70 mM LiCl); standard: PMMA). Polymerization conditions are given in Figure 2.

elution traces shift to lower retention times, indicating an increase in the apparent molar mass. In addition, a tailing of the peaks in the elution traces toward higher elution times indicates the presence of termination reactions.

Figure 4 shows the number average molar mass as a function of the reaction conversion, as derived from the GPC measurements. The apparent molar mass increases with increasing conversion from about 15 000 to more than 30 000 g/mol. The polydispersity was about $M_w/M_n = 1.4-1.6$. The expected molar mass (calculated from ATRP kinetics) is shown as a solid line. It can be seen that the experimentally derived molar mass and the expected molar mass are very similar, which indicates a high level of reproducibility of the polymerizations. Note, narrow dispersed PDMAA standards were not available, thus we used PMMA standards for GPC calibration, which may result in incorrect absolute values of M_n. Thus, presently, we refer to the values shown in Figure 4 as to be apparent molar masses. The linear increase of the molar mass as a function of the reaction conversion may suggest a "poisoning" (i.e., removal

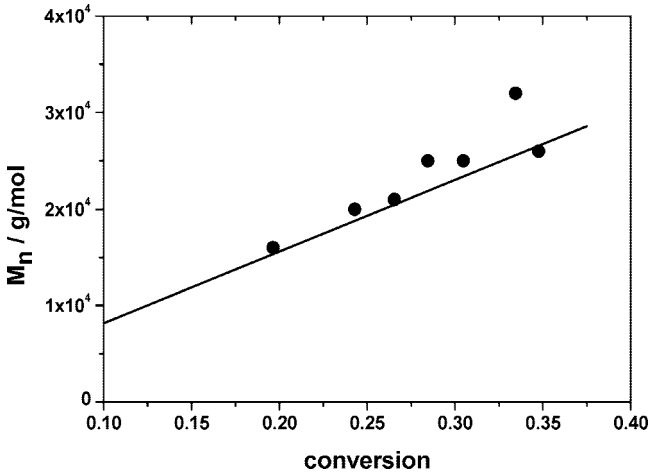

Figure 4. Number-average molar mass as a function of the reaction conversion. Polymerization conditions are given in Figure 2. The solid line represents the expected molar mass, according to $M_n = M_{ini} + \{([M_0]/[I_0])pM_{mon}\}$, where M_{ini} is the molar mass of initiator-modified peptide; M_{mon} is the molar mass per PDMAA repeat unit, $[M_0]$ and $[I_0]$ are the initial concentrations of the monomer and the initiator-modified peptide, respectively, and p is the reaction conversion.

of the catalyst from the ATRP equilibrium by interaction with the amide functions) of the catalyst as to be the major source that causes a nonlinear first-order kinetics shown in Figure 3 and thus limits the final conversion to about 35%. The hybrid copolymers were further used for the preparation of bioactive surface coatings that promote the attachment of living cells.

Substrate-Attached Bioactive Peptide−Polymer Monolayers. To investigate the bioresponsive properties of the prepared PDMAA−GRGDSP hybrid copolymers, we followed a very simple, yet very effective technique that was developed by Prucker et al. in order to bind these molecules in a thin film configuration onto solid glass substrates (Figure 5). In brief, a monochlorosilane, carrying a benzophenone group, is linked to oxidic surfaces by a base-catalyzed condensation reaction. By illumination with UV light ($\lambda = 254$ or 365 nm), the carbonyl group of the surface-attached benzophenone group is converted through an n−π^* or $\pi-\pi^*$ transition into a reactive triplet state, which is essentially a "biradical" in nature. The latter may abstract a proton from an aliphatic group in close vicinity (e.g., from a nearby polymer chain). The resulting radicals (located on the polymer chain and the carbonyl carbon of the surface-attached benzophenone) may than recombine to form a covalent bond between the surface-attached benzophenone and the polymer chain. For more details of this photoimmobilization procedure, the mechanism, and the binding-efficiency, the reader is referred to the literature.[28,32]

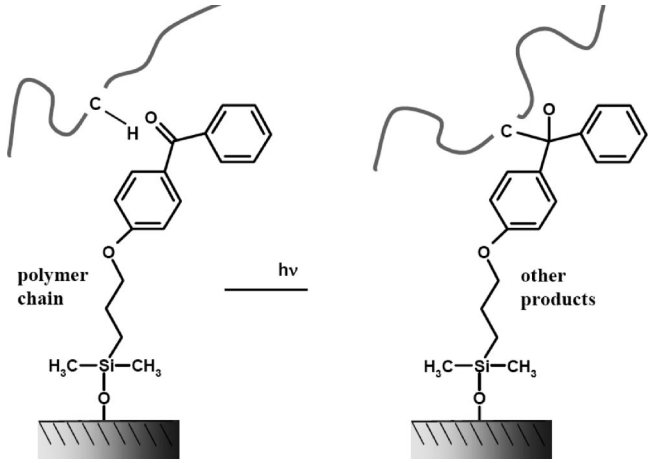

Figure 5. Schematic illustration of a photochemical route to covalently attach polymer molecules to solid surfaces using a surface-immobilized benzophenone−silane. The mechanism is briefly explained in the text and can be found in detail in ref 28.

The deposition of the polymer film on the benzophenone−silane modified substrates was performed by spin-casting (3000 rpm for 1 min) of PDMAA−GRGDSP (5 mg/mL in 2-propanol) onto planar glass slides (BK7 glass) and Si wafers. The solid substrates were exposed immediately after deposition to 400 mJ/cm^2 of UV light ($\lambda = 254$ nm), which covalently links the polymer to the immobilized benzophenone. Only polymer chains that are in close vicinity to the surface can be linked; thus after extraction of the layer with a good solvent, a polymer monolayer covers the surface.[28] In addition, Prucker et al. have shown that the resulting dry thickness scales with the molecular size of the polymer (i.e., with R_g).[28]

Besides surface-attached PDMAA−GRGDSP monolayers, we also prepared reference surfaces by using PDMAA homopolymers in a similar fashion. The PDMAA homopolymer was prepared under identical conditions, as compared to the PDMAA−GRGDSP. Synthesis, and characterization of this homopolymer are outlined in the Supporting Information. To obtain films with varying peptide contents, we finally prepared surface-attached monolayers by codeposition of PDMAA homopolymer and PDMAA−GRGDSP. To this, solutions having different concentrations of the respective polymers (M_n(PDMAA) = 26 000 g/mol, M_n(PDMAA−GRGDSP) = 25 000 g/mol) were prepared. Solutions (5 mg/mL in 2-propanol) containing 1, 5, 10, 25, 50, and 100 mol % of PDMAA−GRGDSP, respectively, were then spin-cast and linked to the substrate as outlined above. Provided that neither component preferentially adsorbs to the benzophenone surface, homogeneously distributed mixtures of PDMAA−GRGDSP/PDMAA may be found in the prepared monolayers. Note, we do not expect that all peptide ligands will be presented at the outer surface but rather assume that the peptide will be randomly distributed in the film.

After preparation of the surface-attached monolayers, the films were characterized with respect to dry thickness (ellipsometry), chemical identity (XPS, FTIR), and surface roughness, as well as homogeneity on the micrometer scale (imaging ellipsometry, AFM). Because the two base polymers had roughly the same molar mass, the monolayer thicknesses of PDMAA−GRGDSP (M_n = 25 000 g/mol), PDMAA (M_n = 26 000 g/mol), and PDMAA−GRGDSP/PDMAA blends was in all cases after extraction with 2-propanol about 4.0 ± 0.5 nm.

The chemical identity of the surface-attached monolayers was proven by X-ray photoelectron spectroscopy (XPS) and FTIR. Details are shown in the Supporting Information. The XPS and FTIR spectra of the PDMAA and the PDMAA−GRGDSP monolayers show all expected signals, indicating the presence of the polymers at the interface. As both the PDMAA and the GRGDSP−peptide carry a large number of amide groups, a quantitative evaluation of the peptide surface concentration from these spectra is not trivial and is not attempted here.

Bioactivity Studies on Peptide−Polymer Monolayers. The bioactivity of the polymer monolayers was investigated by cell-adhesion assays in serum-free culture media. Human skin fibroblast cells were used as model cell lines. In brief, PDMAA, PDMAA−GRGDSP, and mixed PDMAA/PDMAA−GRGDSP monolayers were prepared as described above. As substrates, transparent BK7 glass slides were used. For the cell-adhesion assays, substrates were placed in 6-well plates and covered with

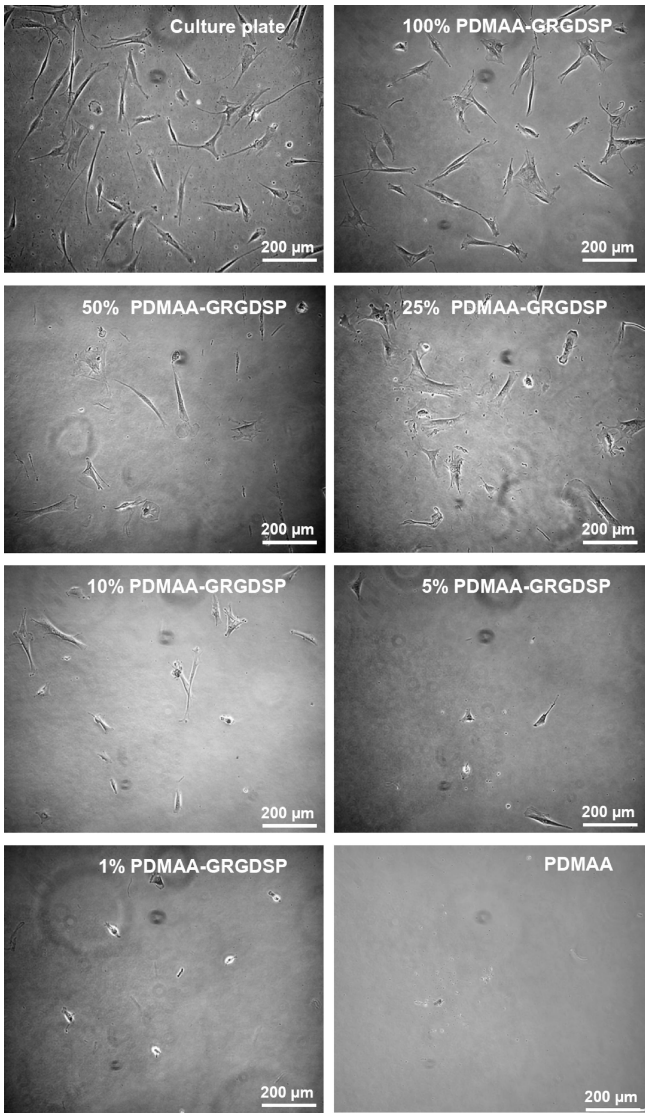

Figure 6. Optical micrographs of adherent human skin fibroblast cells on surface-attached polymer monolayers. The monolayers were prepared by spin-casting mixed solutions of PDMAA and PDMAA−GRGDSP in 2-propanol at molar concentrations of the hybrid copolymer denoted in the figure. In addition, an optical micrograph of cells adhering to a poly(lysine) coated tissue culture plate is shown.

prewarmed medium. A known amount of cells were suspended and incubated with substrates for 1 h at 37 °C (5% CO_2). After this time, nonadherent cells were washed off of the surface by repeated extensive washings with phosphate-buffered saline (PBS). Optical micrographs were taken, and adherent cells were statistically analyzed with respect to the amount of attached cells relative to a positive control (cells seeded on a poly(lysine) coated culture plate in medium containing 10% FCS). After this, the medium was replaced by fresh medium having 10% FCS, and the substrates were further incubated at 37 °C for several days.

After 24 h, further micrographs were taken, which are shown in Figure 6, and the cells were visually inspected with respect to morphology as well as statistically analyzed with respect to the relative number of adherent cells per surface area. Significant differences in these representative micrographs of the coatings with respect to number and morphology of the surface-attached cells were observed. No cells adhered to the neat PDMAA monolayer, most likely due to the protein-repellent properties of PDMAA films.[29,30,32]

Already with 1 mol % of PDMAA−GRGDSP in the spin-cast solution, a few cells adhered to the polymer film. This finding is very remarkable, as in this case the peptide constitutes only about 0.02 wt % of a 4 nm thin monolayer. However, the morphology of the cells adhering to the polymer monolayer, which consisted of 1 mol % peptide−polymer, looked significantly different if compared to cells adhering to a respective positive control (i.e., cells on culture plate, upper left micrograph). The cells stay rather round, and they do not spread. When increasing the peptide concentration in the spin-cast solution, the number of adherent cells on the prepared monolayers increases. Above 5 mol % peptide−polymer (corresponding to 0.1 wt % peptide in the film), the morphology of the attached cells was similar to cells that adhered to the culture plate. The cell spreading, which is accompanied by intracellular actin stress-fiber formation, was further investigated using a standard fixation/staining protocol with NBD phallacidin. In Figure 7, fluorescent micrographs of cells adhering and spreading on a tissue culture plate (left) and on a PDMAA−GRGDSP monolayer (right) are shown. In both cases, actin stress-fiber formation is visualized, and no significant differences in the appearance of the stress fibers of the cells adhering to the respective surfaces can be observed.

The amount of adherent cells, which is obtained through statistical analysis of a large number of optical micrographs, was normalized to the number of cells that adhered to the positive control (i.e., the poly(lysine) coated culture plate). Figure 8 shows this relative number of adherent cells as a function of the PDMAA−GRGDSP concentration in the spin-cast solution. The number of adherent cells increases with increasing peptide concentration. Above 50 mol % peptide−polymer, the increase in relative cell adhesion levels off and the number of adherent cells stays almost constant at a value

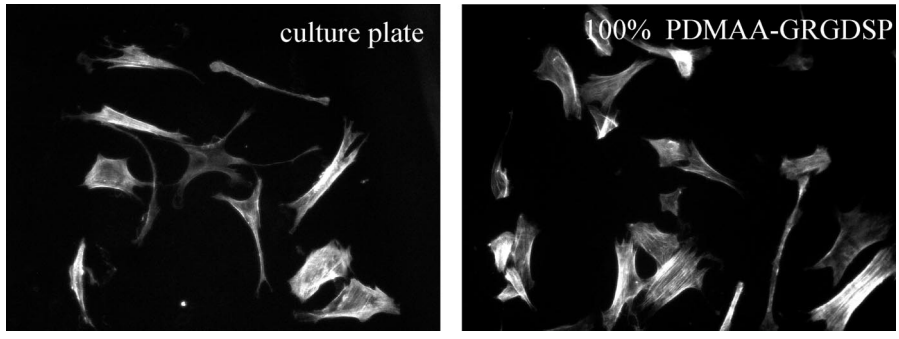

Figure 7. Fluorescent micrographs of adherent human skin fibroblast cells fixed and stained for actin filaments (white) using fluorescent phallotoxins. Details of the fixation and staining of the cells are given in the Experimental Section. The micrographs are taken from cells adhering to a tissue culture plate (left) and to a surface-immobilized PDMAA−GRGDSP polymer monolayer (right).

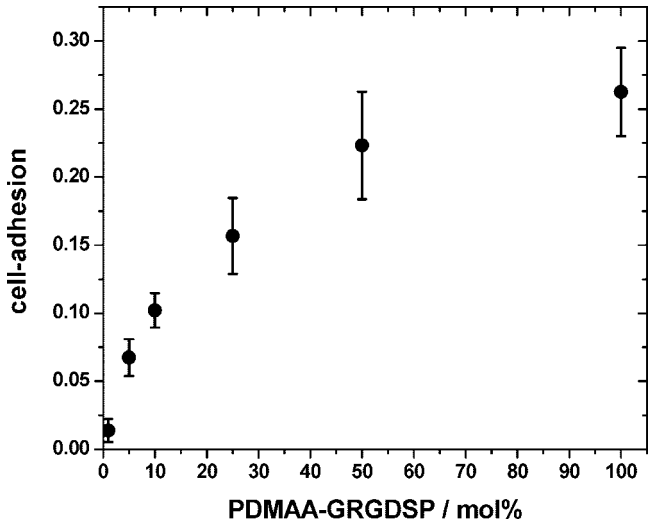

Figure 8. Relative amount of adherent cells as a function of the PDMAA−GRGDSP hybrid copolymer concentration in the spin-cast solution, which was used in the preparation of the surface-attached monolayers.

of about 30% with respect to the number of seeded cells. This result is at a first view qualitatively very similar to results reported by other groups, who showed that the amount of ligands[8,37–39] and the availability of ligands[40–42] strongly affect cell adhesion at artificial interfaces and that a minimum peptide surface concentration is required to promote both cell adhesion and cell spreading (i.e., cytoskeleton formation inside the cells). To this, Massia, and Hubbel reported adhesion and spreading (i.e., stress-fiber formation) with human foreskin fibroblast cells above a surface peptide concentration of about 0.01 pmol/cm^2 (ligand: GRGDY, grafted onto glass).[37] Spatz and co-workers reported focal contact formation and spreading of various cell types if cyclic-RGD ligands are placed at grafting distances below about 80 nm, from which an apparent peptide surface concentration of about 0.03 pmol/cm^2 can be calculated.[38] Finally, Griffith and co-workers as well as our own group reported minimum peptide surface concentrations for cell spreading of about 0.03[40] and 5 pmol/cm^2 [42] when using RGD-modified hydrogel surface coatings, and substrate-supported polymerized peptide−amphiphile monolayers, respectively.

In the present experiments, we adjusted the peptide content within the hybrid copolymer through controlled polymerizations and within the surface-attached films by blending the PDMAA−GRGDSP molecules with the corresponding PDMAA homopolymers of similar molar mass, as outlined above. By measuring the number-average molar mass of the respective polymers (M_n) as well as measuring the dry thickness of the monolayers h_{dry}, one can calculate the peptide surface (i.e., film) concentration c_{surf} according to the following equation:

$$c_{surf} = \frac{\rho \cdot h_{dry}}{[(M_{n,PDMAA-GRGDSP} + M_{n,PDMAA})/2]} \cdot \frac{n_{PDMAA-GRGDSP}}{n_{PDMAA}} \quad (1)$$

Here ρ is the density of the polymer (for calculations, we used a density of $\rho = 1$ g/cm^3 for all blends), and $n_{PDMAA-GRGDSP}/n_{PDMAA}$ is the molar composition of the respective polymers in the spin-cast solution. Table 1 shows the peptide surface concentration for varying molar compositions of the respective polymers in the spin-cast solution, as calculated by using equation 1. The ligand concentration varies for the respective

Table 1. Apparent Peptide Film Concentrations Calculated from Eq 1.

$n_{PDMAA-GRGDSP}/n_{PDMAA}$	c_{surf} [pmol/cm^2]
0.00	0.0
0.01	0.2
0.05	0.8
0.10	1.6
0.25	3.9
0.50	7.8
1.00	15.7

films from about 0.2 pmol/cm^2 up to about 20 pmol/cm^2. Thus by simple blending the two polymers, we can generate polymer monolayers with a wide range of apparent ligand concentrations. Surfaces that consist of more than 0.8 pmol/cm^2 peptide ligand (corresponding to about 5 mol% PDMAA−GRGDSP in the spin-cast solution) promote cell adhesion and spreading from serum-free solution. The latter ligand surface concentration is quite remarkable, as not all peptides presumably may be accessed by the integrin receptors.

Micropatterned Bioresponsive Monolayers. As the peptide−polymer molecules are linked to the surface by a photochemical step, we were further interested in generating chemically micropatterned surfaces that spatially control the attachment of

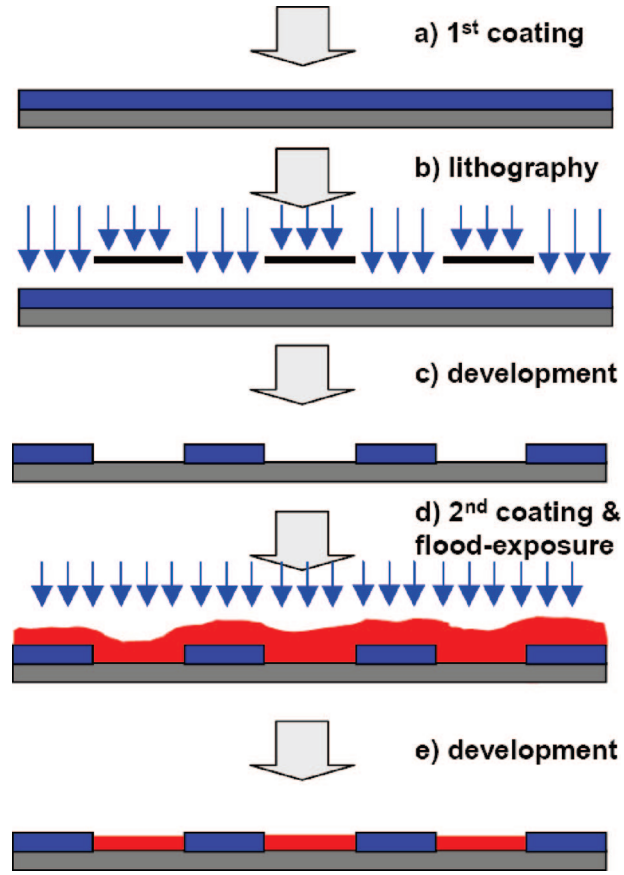

Figure 9. Schematic illustration of the design of chemically microstructured surface-attached polymer monolayers by lithographical means: (a) deposition of a PDMAA homopolymer film (by spin-casting); (b) irradiation of the PDMAA film through a photomask; (c) extraction of the nonbound PDMAA homopolymer (i.e., development of a positive resist); (d) deposition of a PDMAA−GRGDSP hybrid copolymer film on top of the developed PDMAA monolayer (by spin casting); (e) illumination of the substrate (without mask, i.e. flood exposure), and extraction of nonbound polymer (development of the chemical micropattern).

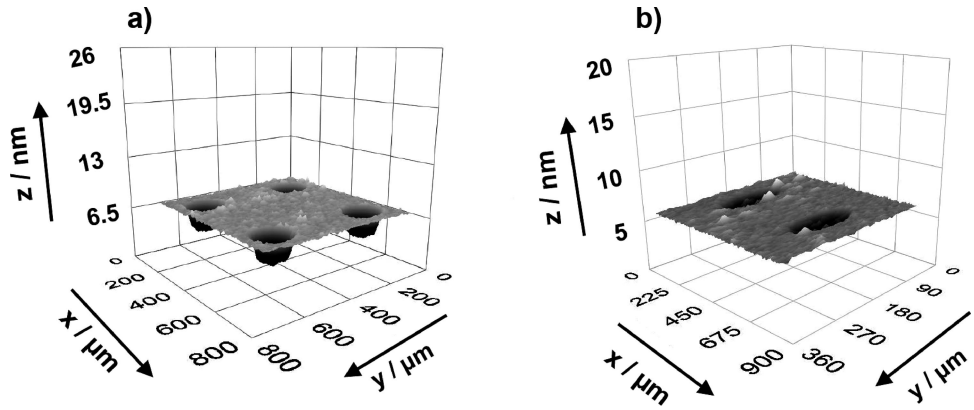

Figure 10. Imaging ellipsometry detail micrographs (surface plots) of the microstructured surface-attached PDMAA/PDMAA−GRGDSP monolayers: (a) PDMAA homopolymer monolayer, after development (step (c) in Figure 10). (b) PDMAA/PDMAA−GRGDSP micropattern after attachment and development of the second, PDMAA−GRGDSP monolayer (step (e) in Figure 9).

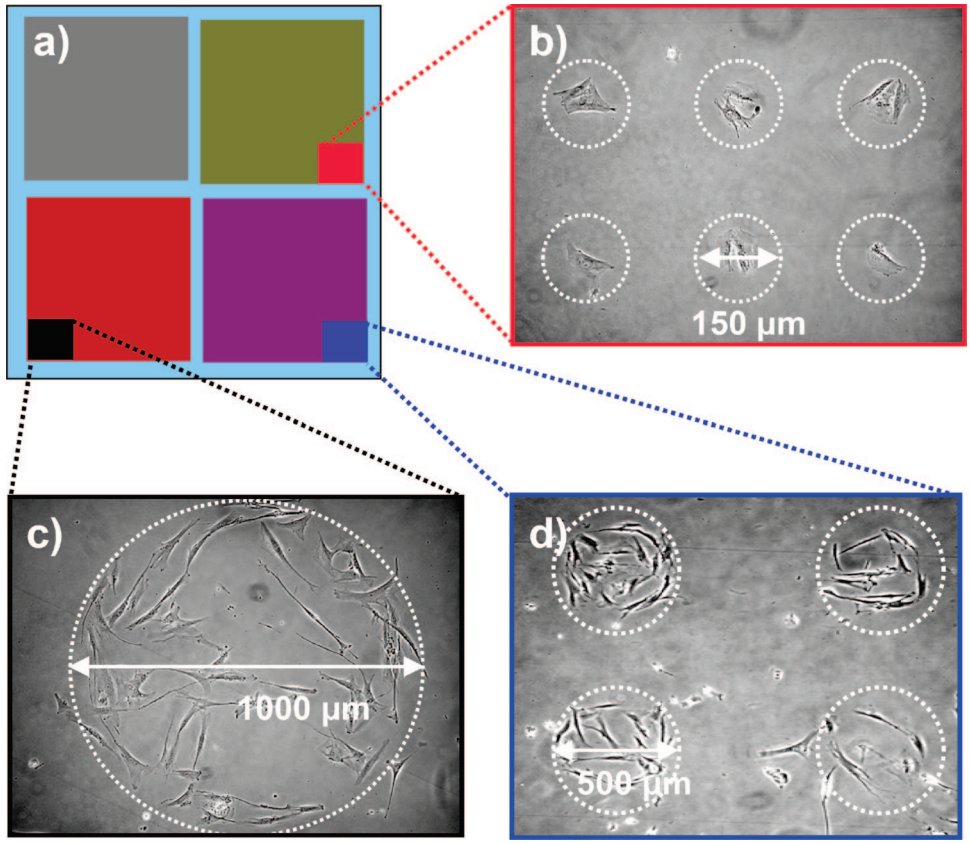

Figure 11. (a) Schematic illustration of the lithographical mask layout comprising four different areas, where spots of different sizes have been blacked out during UV illumination for the microstructuring of the PDMAA homopolymer monolayer. Details: see text. (b−d) Optical micrographs of adherent fibroblast cells on chemically micropatterned PDMAA/PDMAA−GRGDSP monolayers. The white dotted circles are guides to the eye and represent spot areas to which cells preferably adhere.

living cells. As schematically outlined in Figure 9, we followed a lithographical approach to design surfaces where the peptide−polymer is linked in distinct areas, whereas all other areas are covered with a pure PDMAA monolayer. A challenging question is whether also peptide−polymer molecules may be able to penetrate the existing PDMAA monolayer during the second coating process and may also be linked to the surface. To overcome possible drawbacks from such a situation, the illumination time during the first surface attachment of the PDMAA monolayer was set to multiple half-lifetimes of the excited benzophenone, thus making it statistically unfavorable that in the second step a PDMAA−peptide molecule, which eventually has penetrated the PDMAA layer, will come into contact with an excited benzophenone group and thus will be attached to the surface.

During the preparation of the chemically micropatterned surfaces, the surface topography was studied at individual steps using imaging ellipsometry (i-elli). As an example, Figure 10 shows i-elli detail micrographs of a positive resist after development (corresponding to part (c) in Figure 9) and the final micropatterned surface after the last development step (corresponding to part (e) in Figure 9). The film thickness of the pure PDMAA layer (light-gray areas in the micrograph) is about 3–4 nm, as determined from the depth of the holes, and it is in good agreement with the thickness determined by conventional external reflection ellipsometry. In the right micro-

graph, the apparent optical height difference of the "holes" and the surrounding continuous polymer monolayer is reduced to less than 1 nm, which indicates the successful attachment of the peptide−polymer in this area. The two polymers are essentially of the same chemical nature, and only one carries a small peptide unit at one end. If now, for whatever reason, the benzophenone units in the area outside of the holes should not be completely used up, this imperfection will only cause a small deviation in the chemical structure of the final film.

Using the chemically micropatterned surfaces, we investigated the response (i.e., adhesion) of living cells to the so-prepared microarrays. In first experiments, we used a mask that generates microarrays, where circular spots of varying size were established on the same substrate. This is in particular interesting, as one may be able to tailor the available surface area for individual cells (or cell sheets) on the same surface, thus eventually generating cell chips with tunable amounts of adherent cells per spot.

Figure 11 schematically illustrates the mask layout, which was used to generate the chemical micropatterns, as well as optical micrographs of fibroblast cells that adhered to the prepared surfaces following the above outlined standard cell-adhesion assay. The concentration of the PDMAA−GRGDSP in the spin-cast solution that was used to fill the open spots in the positive resist, was 100 mol %, which corresponds to a ligand surface concentration of about 16 pmol/cm^2. Spot diameters (i.e., areas where cells possibly find peptide ligands) of the individual squares are defined by the mask to 1000 μm (red, lower left), 500 μm (violet, lower right), 300 μm (gray, upper left), and 150 μm (green, upper right), as shown in Figure 11a. Cells adhered to the peptide−polymer presented in the spots (Figure 11b−d), but no cells adhered to the surrounding PDMAA background monolayer. Most remarkably, with spot sizes of 150 μm in diameter (Figure 11b), on average, a "one-cell-per-spot" array could be established. These results suggest that a spatial control of the cell growth on artificial surfaces can be achieved by a selective deposition and photoattachment of designed peptide−polymer hybrid copolymers in a very simple, yet very controlled fashion.

Conclusion

In situ ATRP of DMAA using an initiator-modified GRGDSP-peptide has been employed to generate a water-soluble, neutral peptide-modified polymer. To this novel hybrid, copolymers were designed that combine cell-adhesive and protein-repellent properties in a single molecule. PDMAA−GRGDSP hybrid copolymers were synthesized having molar masses of up to 30 000 g/mol, with polydispersities of about $M_w/M_n = 1.4–1.6$. The hybrid copolymer can be attached to solid (glass) surfaces through a simple photochemical process. The obtained monolayers are excellent candidates to establish stable bioactive polymer coatings, as they showed a great potential to direct the adhesion and growth of living cells. Human skin fibroblast cells adhered and spread from serum-free medium to surfaces that carried the bioactive ligand but did not adhere to surfaces that consisted of a PDMAA monolayer alone. Pure PDMAA monolayers swell in the aqueous environment and suppress nonspecific protein adsorption, including that of proteins, which are required for the mediation of specific cell-matrix adhesion. While pure PDMAA does not support the adhesion of living cells, the addition of very small amounts (0.02–0.1 wt %) of a bioactive peptide into the surface-attached polymer film already induced cell adhesion and spreading. A wide spectrum of composition of the monolayers can be easily obtained by simply blending the hybrid copolymer with pure PDMAA homopolymer of similar molecular weight. Finally, taking advantage of the surface attachment using benzophenone photochemistry, chemically micropatterned surfaces can be designed that contain areas of the PDMAA−GRGDSP hybrid copolymer on a PDMAA background. Such surfaces direct the adhesion of living cells in a spatially controlled way, which renders these hybrid copolymers interesting candidates for the generation of cell chips.

Acknowledgment. We acknowledge financial support through the Emmy Noether Program of the DFG (grant BI738/1-3/1-4), as well as through the Landesstiftung Baden Württemberg GmbH, and the Fonds der Chemischen Industrie. We thank N. Schatz for technical assistance, as well as J. Couet for synthesizing and providing the ligand Me_6TREN. The BMBF is thanked for financial support within the project "Mikrostrukturen und Methoden für die Zelluläre Bioanalytik−MIBA" (16SV2337).

Supporting Information Available. Outline of NMR and ESI-MS characterization of the initiator-modified peptide, preparation of PDMAA homopolymer using a low molar mass amide-functional initiator, details of the characterization of the surface-attached polymer films by XPS and FTIR, and a photomicrograph of the used lithographical mask for the design of the chemically patterned surfaces. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Vandermeulen, G. W. M.; Klok, H. A. *Macromol. Biosci.* **2004**, *4*, 383.
(2) Löwik, D. W. P. M.; Ayres, L.; Smeenk, J. M.; van Hest, J. C. M. *Adv. Polym. Sci.* **2006**, *202*, 19.
(3) Klok, H. A. *J. Polym. Sci. Part A: Polym. Chem.* **2005**, *43*, 1.
(4) Couet, J.; Biesalski, M. *Soft Matter* **2006**, *2*, 1005.
(5) Tirrel, M.; Kokkoli, E.; Biesalski, M. *Surf. Sci.* **2002**, *500*, 61.
(6) Hersel, U.; Dahmen, C.; Kessler, H. *Biomaterials* **2003**, *24*, 4385.
(7) Wang, C.; Stewart, R. J.; Kopeček, J. *Nature* **1999**, *397*, 417.
(8) Hubbell, J. A. *Biotechnology* **1995**, *13*, 565.
(9) Shin, H.; Seongbong, J.; Mikos, A. G. *Biomaterials* **2003**, *24*, 4353.
(10) Langer, R.; Tirrell, D. *Nature* **2004**, *428*, 486.
(11) Duncan, R. *Nat. Rev. Drug Discovery* **2003**, *2* (5), 347.
(12) Falconnet, D.; Csucs, G.; Grandin, H. M.; Textor, M. *Biomaterials* **2006**, *27*, 3044.
(13) Folch, A.; Toner, M. *Annu. Rev. Biomed. Eng.* **2000**, *2*, 227.
(14) Whitesides, G. M.; Ostuni, E.; Takayama, S.; Jiang, X.; Ingber, D. E. *Annu. Rev. Biomed. Eng.* **2001**, *3*, 335.
(15) Park, T. H.; Shuler, M. L. *Biotechnol. Prog.* **2003**, *19*, 243.
(16) Revzin, A.; Tompkins, R. G.; Toner, M. *Langmuir* **2003**, *19*, 9855.
(17) Couet, J.; Samuel, J. D. J. S.; Kopyshev, A.; Santer, S.; Biesalski, M. *Angew. Chem., Int. Ed.* **2005**, *44*, 3297.
(18) Couet, J.; Biesalski, M. *Macromolecules* **2006**, *39*, 7258.
(19) Rettig, H.; Krause, E.; Börner, H. G. *Macromol. Rapid Commun.* **2004**, *25*, 1251.
(20) Becker, M. L.; Liu, J.; Wooley, K. L. *Biomacromolecules* **2005**, *6*, 220.
(21) Becker, M. L.; Liu, J.; Wooley, K. L. *Chem. Commun.* **2003**, *2*, 180.
(22) Mei, Y.; Beers, K. L.; Byrd, H. C. M.; VanderHart, D. L.; Washburn, N. R. *J. Am. Chem. Soc.* **2004**, *126*, 3472.
(23) Cate, M. G. J.; Rettig, H.; Bernhardt, K. N.; Börner, G. *Macromolecules* **2005**, *38*, 10643.
(24) Matyjaszewski, K., Davis, P. D., Eds. *Handbook of Radical Polymerization*; Wiley: Hoboken, NJ, 2002.
(25) Pierschbacher, M. D.; Ruoslahti, E. *Nature* **1984**, *309*, 30.
(26) Rühe, J.; Knoll, N. *J. Macromol. Sci., Part C: Polym. Rev.* **2002**, *42*, 91.
(27) Rühe, J.; Biesalski, M. Biocompatibility of Microsystems. In *Bioengineered Microsystems*; Zappe, H., Ed.; Elsevier: New York, 2007.
(28) Prucker, O.; Naumann, C. A.; Rühe, J.; Knoll, W.; Frank, C. W. *J. Am. Chem. Soc.* **1999**, *121*, 8766.
(29) Matsuda, T.; Sugawara, T. *J. Biomed. Mater. Res.* **1995**, *29*, 749.

(30) Hirose, M.; Kwon, O. H.; Yamato, M.; Kikuchi, A.; Okano, T. *Biomacromolecules* **2000**, *1*, 377.
(31) McClain, M. A.; Culbertson, C. T.; Jacobson, S. C.; Ramsey, J. M. *Anal. Chem.* **2001**, *73*, 5334.
(32) Berchtold, B. Ph. D. Thesis, Department of Microsystems Engineering, University of Freiburg, 2005.
(33) Teodorescu, M.; Matyjaszewski, K. *Macromolecules* **1999**, *32*, 4826.
(34) Teodorescu, M.; Matyjaszewski, K. *Macromol. Rapid Commun.* **2000**, *21*, 190.
(35) Neugebauer, D.; Matyjaszewski, K. *Macromolecules* **2003**, *36*, 2598.
(36) Matyjaszewski, K.; Xia, J. *Chem. Rev.* **2001**, *101*, 2921.
(37) Massia, S. P.; Hubbell, J. A. *J. Cell. Biol.* **1991**, *114*, 1089.
(38) Arnold, M.; Cavalcanti-Adam, E. A.; Glass, R.; Blümmel, J.; Eck, W.; Kantlehner, M.; Kessler, H.; Spatz, J. P. *Chem.Phys.Chem.* **2004**, *5*, 383.
(39) Irvine, D. J.; Ruzette, A. V. G.; Mayes, A. M.; Griffith, L. G. *Biomacromolecules* **2001**, *2*, 545.
(40) Maheshwari, G.; Brown, G.; Lauffenburger, D. A.; Wells, A.; Griffith, L. G. *J. Cell Sci.* **2000**, *113*, 1677.
(41) Dori, Y.; Bianco-Peled, H.; Satija, S. K.; Fields, G. B.; McCarthy, J. B.; Tirrell, M. *J. Biomed. Mater. Res.* **2000**, *50*, 75.
(42) Biesalski, M.; Knaebel, A.; Tu, R.; Tirrell, M. *Biomaterials* **2006**, *27*, 1259.

BM7010714