To tailor cell–surface interactions, precise and controlled attachment of cell-adhesive motifs is required, while any background non-specific cell and protein adhesion has to be blocked effectively. Herein, a versatile and highly reproducible antifouling surface modification based on “clickable” groups and hierarchically structured diblock copolymer brushes for the controlled attachment of cells is reported. The polymer brush architecture combines an antifouling bottom block of poly(2-hydroxyethyl methacrylate) poly(HEMA) and an ultrathin azide-bearing top block, which can participate in well-established “click” reactions including the highly selective copper-catalyzed alkyne-azole cycloaddition (CuAAC) reaction under mild conditions. This straightforward approach allows the rapid conjugation of a cell-adhesive, alkyne-bearing cyclic RGD peptide motif, enabling subsequent specific attachment of NIH 3T3 fibroblasts, their extensive proliferation and confluent cell sheet formation after 48 h of incubation. The generally applicable strategy presented in this report can be employed for surface functionalization with diverse alkyne-bearing biological moieties via CuAAC or copper-free alkyne-azole cycloaddition protocols, making it a versatile functionalization approach and a promising tool for tissue engineering, biomaterial implant design, and other applications that require surfaces supporting highly specific cell attachment.

1. Introduction

During the last decades significant effort has been made toward understanding how cellular processes are influenced by chemical and physical properties of the extracellular environment, as these interactions are vital to the development of novel biomaterials for tissue engineering.[1] Cell adhesion, spreading, proliferation, differentiation and apoptosis depend on many factors, such as i) mechanical properties of the surface or the matrix, ii) the concentration, type, and distribution of adhesive ligands, and iii) presence of other signaling molecules in the culture medium, among other factors.[2–5] Therefore, the precise tailoring of materials’ surface properties is of paramount importance to facilitate effective interactions between cell receptors (e.g., integrins) and artificial scaffolds or biomaterial implants. This generally requires the immobilization of small peptide motifs onto the surface, which mimic those found in extracellular matrix proteins. In particular Gly-Arg-Asp (RGD) was identified as a minimal essential cell-adhesion peptide sequence, which is analogous to a cell binding sequence found in fibronectin, vitronectin, laminin or osteopontin, and acts as a signal to regulate cell adhesion, spreading, differentiation, and survival.[6–7]

Nevertheless, in order to control the fate and the behavior of cells on the surface, the surface must not only support these desired specific interactions, but also suppress unwanted non-specific cell adhesion and protein adsorption (fouling), since protein fouling will inevitably lead to uncontrolled cell attachment.[8] The design of surface modifications that can effectively prevent protein fouling and non-specific cell adhesion (i.e., “antifouling”) has therefore received considerable attention. In this context, polymer brushes consisting of polymer chains densely tethered to the surface by one end show superior performance as antifouling coatings.[9,10] They are prepared by “grafting from” appropriate surface-immobilized initiators through one of several currently available controlled polymerization techniques (CRP).[11] Among them, atom transfer radical polymerization (ATRP) has been used extensively due to its advantages, such as compatibility with relevant monomers and numerous functional groups, mild polymerization conditions, and tolerance to impurities and residual traces of oxygen.[12] In particular, surface-initiated ATRP (SI-ATRP), in which polymer chains grow from an ATRP initiator immobilized on a substrate surface, is frequently used for the preparation of functional interfaces of various architectures.[13,14]

More recently, surface modifications based on hierarchically structured copolymer brushes comprising an underlying
passivating layer and functionalizable top-block have gained increasing importance, since they allow combining different functions concurrently.[15,16] Lately, we exploited this strategy to couple passivating characteristics of underlying poly(AAm) block with a second cell-adhesive RGD peptide functionalized poly(AA) top-block, which effectively decoupled the control determinants of cell–surface interactions.[17]

While many techniques have been applied to immobilize cell-adhesive peptides onto artificial surfaces, they are generally incompatible with fouling resistance and therefore do not provide specificity to cell adhesion. In general, the choice of the immobilization strategy for biochemical cues depends primarily on the type of reactive groups present on a polymer brush surface. Whereas for the carboxylic acid side chain functional polymers the surface activation is accomplished via EDC/NHS chemistry, activation of the hydroxyl side groups can be realized by p-nitrophenyl chloroformate or N,N′-disuccinimidyl carbonate protocols.[11,18] Although both methodologies are among the most frequently used strategies, they require additional blocking or passivation steps, which are usually of limited effectiveness and may lead to substantial reduction or even complete loss of their initial antifouling properties.[19]

In this regard, reactions meeting the criteria of “click” chemistry appear to be advantageous owing to the mild reaction conditions, high efficiency and selectivity, as well as tolerance to the presence of other chemical groups.[20] Moreover such reactions enable the modification of surfaces without impairing their antifouling properties without the requirement for additional deactivation or passivation steps after the surface biofunctionalization.[6,21] In this context, the functionalization of antifouling polymer brushes using alkyne-azide cycloadditions, with a hierarchical architecture containing an azide-functional polymer top block, was demonstrated recently to benefit affinity biosensing applications as it minimizes the changes to the properties of the brush. More importantly, this approach could even be applied to micropattern functionalities over the surface, which could be beneficial for cell migration and dynamics studies.[22,23]

Herein, we report on the preparation of a combined antifouling and “clickable” surface modification, based on hierarchically structured diblock copolymer brushes and their biofunctionalization with cell-adhesive peptides. This approach affords a versatile and generally applicable platform to promote peptide-mediated cell adhesion.[24–26,27] The ability of such functional surfaces to still prevent fouling in relevant cell culture media is also critical for the precise control over cell attachment, as non-specifically deposited proteins would act as ligands for cells and lead to undesired cell binding.[28]

The preparation of the antifouling and “clickable” block copolymer brushes was achieved by a sequential polymerization technique from ATRP initiator coated microscopy glass slides or silicon wafers. These consisted of a diblock polymer architecture, with bottom passivating block composed of poly(HEMA) layer[29,30] while the top block was a few-nanometer-thin poly(glycidyl methacrylate) poly(GMA) layer endowed with azide moieties in every side group, which affords surface biofunctionalization with alkyne-conjugated cyclo(Arg-Gly-Asp-D-Phe-Lys), hereafter referred to as c(RGD) peptide sequences. The strategy reported herein provides an avenue for studying specific cell–surface interactions, by decoupling the interference of protein adsorption from a culture medium, while offering a tunable surface density of immobilized peptides to promote cell binding.

2. Results and Discussion

To achieve a coating that allows controlled and selective attachment of desired cells, while preventing undesired protein and cell attachment, hierarchically structured diblock copolymer brushes that can be functionalized via “click” chemistry were investigated. Their synthesis was tackled by growing two polymer blocks in sequential SI-ATRP polymerization steps and selectively endowing the ultrathin top-block with “clickable” azide moieties, as schematically illustrated in Figure 1.

2.1. Preparation of Azide-Bearing “Clickable” Block Copolymer Brushes

The bottom poly(HEMA) layer was prepared by SI-ATRP from silicon and glass substrates functionalized with self-assembled monolayers of the silane-containing ATRP initiator, namely 11-(trichlorosilyl)undecyl-2-bromo-2-methylpropanoate.[31] The polymerization mixture did not include free “sacrificial” initiator, but contained approximately 10 mol% of Cu(II) with respect to Cu(I) salt. Addition of the Cu(II) as deactivator at the beginning of the reaction helps to quickly establish the steady-state ATRP equilibrium and hence to suppress the termination reactions.[12] Such termination reactions must be minimized, as they would create a fraction of polymer which forms loops, thus reducing the number of –Br end group and their ability to act as macroinitiators for the formation of a top polymer block.

Following the polymerization of the poly(HEMA) block, the preserved –Br end groups of the polymer chains, acted as macroinitiators in the subsequent ATRP step (Figure 1). Thereby, the growth of the poly(GMA) block proceeded from the bottom poly(HEMA) layer, in a manner analogous as previously reported.[16,33] The increase in dry layer thickness after each polymerization step was confirmed by ellipsometry, thus assigning a thickness to each polymer block even though they are likely not physically segregated. The dry thickness of poly(HEMA) block was tuned by controlling the polymerization time to 15.0 ± 1.0 nm and the ultimate dry thickness of the upper poly(GMA) block was 2.0 ± 0.3 nm in all cases.

To follow changes in the surface composition taking place during each polymerization or modification step, we employed X-ray photoelectron spectroscopy (XPS) and GASR-FTIR spectroscopy. The immobilization of the ATRP initiator onto the substrates was evidenced by the appearance of the expected Br 3d signal assigned to C–Br peak in the high resolution spectrum (Figure 2A) as well as characteristic signals in the C 1s spectrum (Figure 2B; Figure S1, Supporting Information). The chemical structure of poly(HEMA) layer was confirmed by the presence of C–O and O–C=O contributions in the high-resolution XPS C 1s spectrum, which is typically found in poly(HEMA) brushes (Figure 2B.2).[34] Chain extension
of the bottom poly(HEMA) layer with the 2.0 ± 0.3 nm thick poly(GMA) block did not change the C=O and O=C=O signal ratio significantly, as shown in Figure 2B,3. The chemical composition of the final diblock poly(HEMA-b-GMA) copolymer brushes was also evidenced by the FTIR spectroscopy. The FTIR spectrum obtained for poly(HEMA-b-GMA) copolymer brushes features several distinctive and sharp absorption bands characteristic for both poly(HEMA) and poly(GMA) polymers, as shown in Figure 2D. While the absorption band present at 1728 cm⁻¹ corresponds to the stretching vibrations of the ester carbonyl C=O groups, the stretching C=O=C mode of the ester groups is found at 1150 cm⁻¹.[35,36] Asymmetric stretching vibrations of the epoxy rings in the top poly(GMA) block are seen as a peak of moderate intensity at around 910 cm⁻¹.[37]

Decoration of the antifouling diblock copolymer brushes with azide (−N₃) moieties capable of participating in subsequent CuAAC reactions was achieved by ring-opening of the epoxy groups present in each side-group of the ultrathin top poly(GMA) block with sodium azide NaN₃. This was achieved in a similar fashion as described previously.[26] Controlling the thickness of the poly(GMA) top block allows tuning of surface density of the reactive azide moieties and thus the amount of immobilized biomolecules, as was previously shown.[21]

The FTIR spectra along with high-resolution XPS spectra of the poly(HEMA-b-GMA) brushes taken before and after azidation confirm the success of the reaction (Figure 2C,D). As shown in Figure 2D, the azide stretching vibration is visible as a single band of moderate intensity in the FTIR spectrum at approximately 2110 cm⁻¹. Additionally, two strong peaks appear, in the N 1s range of the XPS spectrum of the azide-bearing poly(HEMA-b-GMA)-N₃ brushes, having their origin in N−N=≡N at 400.3 eV as well as positively charged nitrogen N−N=≡N at 404.2 eV (Figure 2C,4). The recorded intensity ratio (2:1) corresponded to the theoretical expectations. In
2.2. Surface Functionalization with Cell-Adhesive Peptides

The presence of highly reactive azide moieties on the ultrathin top block provides a convenient means for straightforward surface immobilization of selected cell signaling peptides via one of the well-established “click” chemistry protocols. To demonstrate this, we immobilized an alkyn-bearing c(RGD) peptide on to the surface via CuAAC chemistry from an aqueous peptide solution at a concentration of 100 µg mL\(^{-1}\).

In comparison with conventional ligation protocols currently being in use such as active-ester chemistry through EDC/NHS, endowing brushes with azide moieties that participate in “click” reactions is advantageous. The reactivity of azides is highly specific and will not interfere with other biomolecules or non-specifically bind components of cell culture media and thereby cells. Therefore no deactivation step of unreacted azide moieties is necessary after the functionalization step to maintain the antifouling properties of the surface.[19] Also no reaction side products that could contaminate the surface are formed.

Successful functionalization of the azide-bearing block copolymers with the cell-adhesive c(RGD) peptide was confirmed by XPS and GASR-FTIR measurements. As can be seen in the XPS spectra in Figure 3A, the attachment of biorecognition element to the brush layer was accompanied by new contributions arising from the amide \(-\text{NH}-(\text{C}=\text{O})\) groups and charged arginine guanidinium ion at 399.6 and 401.0 eV, respectively, along with a significant increase of the C=O contributions to the C 1s signal (originating from the PEG spacer, Figure 1 and Figure S1, Supporting Information).

The peptide immobilization was also accompanied by the disappearance of the azide peaks (Figure 3B) in the N 1s region, which were replaced by a contribution assigned to the amide \((\text{C} (=\text{O})-\text{NH})\) groups at 399.7 eV. This particular signal originates from the peptide bonds present in the c(RGD) structure, as shown in Figure 1. Presence of the c(RGD) at the polymer brush surface was also confirmed by the appearance of two weak bands in the FTIR spectrum at 1660 and 1550 cm\(^{-1}\), corresponding to the amide I and amide II vibrations of the peptide sequence, respectively (Figure 3).[38]

The azide band still visible in the FTIR spectrum of the peptide-functionalized surface arises from groups in the initiator monolayer not leading to polymer chains, which are subsequently converted to azide. Since they are buried deep within the brush, they are not accessible for peptide binding and they are beyond the penetration depth of XPS analysis. Based on the increase of thickness measured by ellipsometry after functionalization with peptide, we estimated the peptide surface density to be \(\sigma_{\text{RGD}} = 1.5 \pm 0.7\) peptides nm\(^{-2}\) (Equation (S1), Supporting Information).

2.3. Attachment and Proliferation of Cells

The diblock copolymer brush surfaces functionalized with cell-adhesive c(RGD) were subsequently used in cell culture experiments to assess the potential of the strategy for biological research and tissue engineering applications. For this purpose...
and also to obtain a benchmark we used NIH 3T3 mouse fibroblasts suspended in a serum-containing culture medium. The ability of the non-functionalized and the peptide-functionalized polymer brush surfaces to prevent or promote cell adhesion was compared by quantifying the number of adhered cells 24 h after cells seeding. The adhering cells were counted in optical microscopy images taken for 3 individual samples at 5 different spots each. As a reference, standard tissue culture polystyrene (TCPS) was used and analyzed.

As is evident from Figures 4 and 5C, cells adhered readily onto the TCPS, reaching a surface concentration of 190 ± 17 cells mm\(^{-2}\) after 24 h of incubation at standard conditions (37 °C, 5% CO\(_2\)). Cell attachment to TCPS is promoted by non-specifically adsorbed proteins from the serum-containing medium, which provide abundant sites for the cells to adhere.\(^{[39,40]}\)

A high number of cells (270 ± 43 cells mm\(^{-2}\)) attached to the block copolymer brushes functionalized with cell-adhesive c(RGD) (poly(HEMA-b-GMA)-c(RGD)), when cells were seeded at the same concentration as for TCPS under standard conditions. This observation reflects successful attachment and enhanced bioactivity of the c(RGD) employed. Notably, the number of attached cells exceeded the overall number of cells present on the TCPS by about 40% on average, which is significantly higher than in other biofunctionalization strategies employed previously.\(^{[15,41]}\) The high number of adhered cells also confirms that peptide molecules are displayed at sufficient surface density and are accessible to be recognized by fibroblasts and support their adhesion.

At the same time, we found that the number of cells attached to non-modified, azide-bearing “clickable” surfaces was on average 2 ± 1 cells mm\(^{-2}\), which constitutes only 1% of cells attached to the peptide-functionalized surfaces. Beside the absence of immobilized peptide, the lack of cell adhesion also indicates that there are no cell binding sites on the surface that could be provided by non-specifically adsorbed proteins.

This confirms that pristine, non-biofunctionalized azide-bearing poly(HEMA-b-GMA)-N\(_3\) brushes effectively resist fouling from proteins present in the culture medium. The fouling resistance of the non-functionalized brushes can be compared to the abundant, non-specifically adhered cells observed on TCPS, caused by protein fouling.\(^{[21]}\) Thus, cell attachment on the peptide-functionalized polymer brushes is specifically elicited by the immobilized c(RGD) peptide.

To analyze differences in the cellular response between various surface modifications, optical microscopy images were captured after 24 h of incubation at 37 °C. As can be seen in Figure 5A, the immobilization of the cell-adhesive c(RGD) peptide to the azide-bearing substrates led not only to attachment, but also distinctive spreading of the attached cells. Moreover, cells were found to be evenly distributed over the entire poly(HEMA-b-GMA)-c(RGD) surface. This observation further highlights sufficient concentration and accessibility of the

![Figure 3. A) High-resolution XPS spectra (N 1s region) and B) FTIR spectra of 4 azide-bearing diblock poly(HEMA-b-GMA)-N\(_3\) polymer brushes before and after immobilization of cell-adhesive c(RGD) 5 poly(HEMA-b-GMA)-c(RGD); the corresponding chemical structures are shown in Figure 1.](image-url)

![Figure 4. Quantitative analysis of NIH 3T3 fibroblasts attached to peptide-functionalized poly(HEMA-b-GMA)-c(RGD) brushes, azide-bearing diblock copolymer poly(HEMA-b-GMA)-N\(_3\) brushes, and TCPS dishes as positive control, 24 h of incubation at 37 °C. The calculated cell number density on the peptide-functionalized surfaces were significantly higher than those found on TCPS (*p < 0.005) and azide-bearing diblock copolymer brushes (**p < 0.001) used as control.](image-url)
c(RGD) peptide sequences for the integrin-mediated cell attachment, not being buried inside of the brush layer. On the other hand, there are very few cells present on the non-functionalized azide-bearing surfaces, as shown in Figure 5B. Moreover, these cells do not appear to be spreading on the surface, highlighting their poor ability to adhere. As previously mentioned, standard TCPS substrates served as a positive control supporting adhesion of fibroblasts. The cell-binding sites on proteins adsorbed from the cell culture medium enable extensive cell attachment and spreading. This is in sharp contrast with the antifouling properties and cell-repellency of the surfaces protected by the azide-bearing polymer brushes.

Further confirmation that the cell binding is promoted specifically only by covalently bound peptide was obtained in a control experiment in which the peptide functionalization was carried out in the absence of Cu catalyst (Figure S2, Supporting Information). This led to only a small number of cells adhering ($26 \pm 15$ cells mm$^{-2}$), probably as a result of non-specific peptide adsorption. These cells appear rounded in shape, suggesting very poor surface attachment.

For obtaining better insight into the cell morphology, cells were fixed at 37 °C after 24 h of incubation at standard conditions and were subsequently stained at 22 °C with phalloidin-rhodamine for actin cytoskeleton (red) and Hoechst 33258 for the cell nucleus (blue). The specimens were then imaged by fluorescence microscopy at ambient conditions (Figure 6).

Cells that adhered to the peptide-functionalized substrates (Figure 6A) were well spread with their cytoskeleton stretched.

Figure 5. Optical microscopy images of NIH 3T3 cells seeded onto A) peptide-functionalized poly(HEMA-b-GMA)-c(RGD), B) azide-bearing poly(HEMA-b-GMA)-N$_3$ brushes, C) TCPS dishes as control.

Figure 6. Merged brightfield fluorescence microscopy images (left) and magnified sections (right) of attached fibroblasts, fluorescently stained for actin cytoskeleton (red) and cell nucleus (blue) on A) poly(HEMA-b-GMA)-c(RGD) and B) azide-bearing diblock poly(HEMA-b-GMA)-N$_3$ brushes after 24 h of incubation at standard conditions.
along the surface. Particularly, the magnified sections show the spreading of each cell to be anchored on distinctive spots, which coincide with focal adhesions formed by the cell integrins attached to the surface-immobilized peptide motifs. By contrast, cells observed on the non-functionalized azide-bearing diblock copolymer poly(HEMA-b-GMA)-N₃ brushes showed a rounded shape, lacking in all cases any hint of spreading. Indeed the magnified sections show that the adhering entities on the surface formed cell aggregates of various sizes, seen by the presence of various nuclei in blue.

Encouraged by the excellent cell adhesion onto the c(RGD) peptide-functionalized polymer brushes, we decided to prolong the incubation time on those substrates. Extension of cells incubation period to 48 h resulted in further fibroblast proliferation (cell doubling time ≈ 20 h), reaching the formation of a confluent cell sheet covering the entire surface, as seen in the optical microscope image shown in Figure 7B. This further underlines the cell-adhesive character achieved by the peptide immobilization on the otherwise cell-repellent polymer brushes.

The effectiveness and mild conditions employed together with its simplicity make our approach an interesting candidate for applications in biological research and tissue engineering. While peptide-functionalized polymer brushes supported cell proliferation into a confluent cell sheet already within 48 h, the same type of polymer brush without the conjugated c(RGD) peptide prevented cell adhesion (Figures 5B and 6B).

Importantly, the diblock copolymer strategy presented here did not require any further chemical activation or deactivation of the azide groups involved in the peptide immobilization, nor any passivation steps, which are necessary in other methods, e.g., reacting remaining active esters or adsorption of thiols or bovine serum albumin.²⁸⁻⁴⁴

We further envision that 2D surface patterning of biomimicking motifs on such polymer brushes will facilitate the spatially resolved triggering of specific regions from cell-repellant to cell-adhesive in a simple one-step process. In this regard, polymer brushes based on similar principles were previously successfully applied for the patterning of fluorophores or biotin.²¹ Moreover, the CuAAC protocol employed may be replaced by a catalyst-free ligation involving azide groups such as the strain-promoted alkyne-azole cycloaddition (SPAAC), if interaction of the Cu-catalyst with the cell signaling molecules to be immobilized is a concern.

3. Conclusions

In this contribution, we reported on the preparation and application of a cell-seeding platform based on antifouling yet “clickable” diblock copolymer brushes, allowing integrin-specific cell attachment promoted by surface biofunctionalization with a cell-adhesive peptide motif. For this purpose we employed an alkyne-modified c(RGD) peptide sequence, which was covalently immobilized onto the brush surface via CuAAC under mild conditions. The main advantage of the developed “clickable” platform compared with other frequently employed systems based on, for example, EDC/NHS chemistry lies in its selectivity and simplicity, as there is no need for deactivation of unreacted azide moieties or background passivation following surface biofunctionalization. Immobilization of the cell-signaling c(RGD) peptide enabled extensive attachment and proliferation of NIH 3T3 fibroblasts leading to confluent sheet formation after only 48 h of incubation at standard conditions (37 °C, 5% CO₂). We also envision the utilization of copper-free strain promoted alkyne-azole cycloaddition (SPAAC) reaction, if compatibility with Cu-catalyst used during the CuAAC “click” reaction is a concern. The strategy presented in this report, is a versatile, straightforward and generally applicable approach for the preparation of biologically inert surfaces that can be easily functionalized using different cell-adhesive entities depending on the targeted cell types required by specific application.

4. Experimental Section

Materials: All the chemical reagents were used without further purification. 2-Hydroxyethyl methacrylate (97%), glycidyl methacrylate (≥ 97%), CuBr (99.999%), CuBr₂ (99.999%), 2,2’-dipyridyl (BiPy), 2-bromoisobutyl bromide, 11-mercapto-1-undecanol, were purchased from Sigma-Aldrich. HPLC grade absolute ethanol (≥ 99.8%), anhydrous toluene (≥ 99.8%) were purchased from VWR Chemicals. Dulbecco’s phosphate saline buffer (PBS) (10x, without Mg²⁺ and Ca²⁺) was acquired from Lonza. 11-(trichlorosilyl) undecyl-2-bromo-2-methylpropanoate ATRP initiator was prepared according to the literature.²¹ In this study, Milli-Q water with a resistivity of 18.2 MΩ cm was drawn from a Millipore Direct-Q8 system (Millipore advantage A10 system, Schwabach, with Milli-Mark Express 40 filter, Merck, Germany).

Synthesis of 4-Pentynoic-PEG₁₃-c(RGDfK): The peptide sequences were assembled on solid phase (Tenta gel R RAM) by automatic solid phase peptide synthesis using a Liberty Blue microwave peptide synthesizer.
at 25 bromo-2-methylpropanoate in dry toluene. Silanization of silicon wafers
′-bipyridyl (143 mg, 917 mmol) and CuBr₂ (16.3 mg, 73 mmol) in
brushes, with the ultrathin top poly(GMA) block, were prepared according
slides were stored in vacuum before each subsequent modification step.
30 ATRP-activated glass/silicon substrates. Polymerization proceeded at
1:1) was degassed by Ar bubbling for 1 h in an ice bath. Subsequently
and 2,2-dipyridyl (219 mg, 1.4 mmol), in water/ethanol mixture (15 mL,

CuBr (81.7 mg, 0.57 mmol) was added under inert atmosphere and
107 glass substrates were removed from the polymerization solution, rinsed

Functionalization of the 4-Pentynoic-PEG₂₋(c(RGDfK)) Peptide Stock Solution:
Preparation of the adhesive peptide stock solution in Milli-Q water
surfaces functionalization with cell-adhesive peptide via CuAAC was performed according to the protocol found elsewhere.[45] Briefly, to a 50 µL of the initial 4-pentynoic-PEG₂₋(c(RGDfK))
solutions (aliquots of 1 mg mL⁻¹), 950 µL of Milli-Q water was added to obtain 1 mL of the final solution at the peptide concentration of 0.1 mg mL⁻¹. Such prepared stock solution was degassed by Ar bubbling for 15 min followed by addition of 10 µL of 0.2% sodium ascorbate. At the end, 4 µL of an oxygen-free CuSO₄ (0.05 mol) in water was added immediately before the peptide immobilization reaction. To assess the degree of non-specific cell adhesion, a negative control experiments in the absence of Cu catalyst were also conducted (Figure S2, Supporting Information).

Functionalization of Polymer Brush Surfaces with 4-Pentynoic-PEG₂₋
c(RGDfK) via CuAAC Reaction: 90 µL of the reaction mixture at peptide concentration 0.1 mg mL⁻¹, was placed over individual glass substrates (10 × 10 mm²) endowed with azide moieties and they were rapidly covered with small parafilm sheets to prevent water evaporation during the functionalization step. Peptide immobilization via CuAAC proceeded at room temperature for 60 min, and was kept constant in all cases. After the peptide immobilization substrates were copiously rinsed with fresh portion of MilliQ water, ethanol and blow-dried with nitrogen. Surface concentration of immobilized c(RGD) peptide was estimated based on high-resolution XPS results and spectroscopic ellipsometry experiments.

Cultivation and Seeding of NIH 3T3 Mouse Fibroblasts: NIH 3T3 fibroblasts provided by Dr. Jürgen Schnekenburger (Biomedical Technology Center of the Medical Faculty Münster, Germany) were cultured at standard conditions (37 °C, 5% CO₂ in Dubibeco’s modified Eagles media (DMEM; Gibco, Life Technologies) supplemented with 10% fetal bovine serum (Gibco, Life Technologies), 2 × 10⁻³ M l-Glutamine, penicillin (100 U mL⁻¹; Gibco, Life Technologies) and streptomycin (100 µg mL⁻¹; Gibco, Life Technologies). For passaging the fibroblasts were rinsed with PBS, detached by using 0.05% trypsin/EDTA (Gibco, Life Technologies), resuspended, and diluted in fresh cell medium. Then the cell pellets were collected by centrifugation (Heraeus Megafuge 16 Centrifuge, Thermo Scientific) (1200 rpm for 4 min) in a conical tube. The final cell concentration was calculated with an improved Neubauer counting chamber (Brand, Wertheim, Germany). Before cells seeding, polymer brush modified glass substrates were immersed into 70% ethanol for 15 min and rinsed twice with PBS. Subsequently, they were transferred into a 6-well plate and NIH 3T3 fibroblasts cells were seeded at a density of 1.5 × 10⁴ cells cm⁻² in 3 mL medium. Cell culture was performed at 37 °C using air enriched with 5% CO₂ in a humidified incubator. The cell morphologies were observed by optical light microscopy (Primovert, Carl Zeiss, Oberkochen, Germany).

Fluorescence Staining of Cell Actin Cytoskeleton and Nucleus: For fluorescence staining, cells attached to the substrates were washed with prewarmed (37 °C) PBS, fixed with paraformaldehyde (4% in PBS, 30 min; VWR) at 37 °C. The fixed cells were then permeabilized with Triton X-100 (0.2% in PBS, 10 min; VWR), and unspecific binding sides were blocked for 45 min (2% BSA in PBS; Sigma-Aldrich) at room temperature (22 °C). Afterward, samples were incubated in a phalloidin–rhodamine solution for 30 min (5 µL mL⁻¹ in 1% BSA; Invitrogen, Life Technologies) as well as Hoechst 33258 (1 µg mL⁻¹, Bisbenzimide H33258, Sigma-Aldrich) for 20 min. Subsequently, samples were coated silicon and/or glass slides. Surface azidation was achieved by nucleophilic ring-opening reaction of the top-poly(GMA) block with sodium azide NaN₃ at 60 °C for 12 h. After the top-block poly(GMA) azidation process glass substrates were immersed in ethanol and rinsed several times with fresh portion of ethanol, Milli-Q water and dried in a nitrogen stream. Caution, sodium azide reacts violently with several common laboratory organics and inorganics such as CS₂, bromine, bromoacetic acids, and heavy metals. Chlorinated solvents should never be used as reaction media as it might result in the formation of explosive compounds.

Preparation of Br-End Capped Poly(HEMA) Brushes by SI-ATRP: Surface-initiated atom transfer polymerization (SI-ATRP) of poly(HEMA) brushes from silicon wafers and Menzel glass slides were rinsed with UV/HPLC spectrograde ethanol, Milli-Q water and dried in a stream of nitrogen. Subsequently, their surface was activated in UV-ozone cleaner (Jetlight) for 20 min, and then immediately immersed in a freshly prepared (1 mL) solution of 1-(2-chloroethyl)undecyl-2-bromo-2-methylpropanoate in dry toluene. Silanization of silicon wafers and hydroxyl groups on glass surfaces was allowed to proceed for 3 h at 25 °C in a dry atmosphere. The substrates were subsequently rinsed with dry toluene, acetone, absolute EtOH, and Milli-Q water and blow dried in a stream of N₂.

Preparation of Br-End Capped Poly(HEMA) Brushes by SI-ATRP: Surface-initiated atom transfer polymerization (SI-ATRP) of poly(HEMA) brushes from silicon/glass functionalized substrates was carried out according to the modified procedure reported previously.[24] A solution of 2-hydroxyethyl methacrylate (HEMA) (6.66 g, 66.6 mmol), CuBr₂ (12.3 mg, 0.05 mmol), and 2,2-dipyridyl (219 mg, 1.4 mmol), in water/ethanol mixture (15 mL, 1:1) was degassed by Ar bubbling for 1 h in an ice bath. Subsequently CuBr (81.7 mg, 0.57 mmol) was added under inert atmosphere and stirred until complete dissolution. The polymerization solution was transferred under Ar protection to the degassed reactors containing ATRP-activated glass/silicon substrates. Polymerization proceeded at 30 °C and was ceased by ethanol addition and subsequent rinsing with copious amount of fresh ethanol and Milli-Q water. Such prepared glass slides were stored in vacuum before each subsequent modification step. SI-ATRP of the Top-Poly(glycidyl methacrylate) Block: Diblock copolymer brushes, with the ultrathin top poly(GMA) block, were prepared according to a slightly modified procedure reported previously.[27] A 50 mL round-bottom flask loaded with glycidyl methacrylate (GMA) (6 g, 42 mmol), 2,2'-bipiridyl (143 mg, 917 mmol) and CuBr₂ (16.3 mg, 73 mmol) in anhydrous N,N-dimethylformamide (DMF, 7.5 mL), was deoxygenated by Ar bubbling for 45 min. Subsequently CuBr (52.6 mg, 367 mmol) was added to the flask under Ar protection and the mixture was vigorously stirred until complete dissolution. Afterward, polymerization solution was transferred under Ar protection to oxygen-free reactors containing silicon and/or glass substrates coated with the bottom poly(HEMA) block layer. Polymerization reaction proceeded at 35 °C for 5 h resulting in diblock copolymer poly(HEMA-b-GMA) brushes with the oxirane-bearing top-poly(GMA) layer. After the reaction course silicon and/or glass substrates were removed from the polymerization solution, rinsed twice with ethanol and Milli-Q water and blow dried with nitrogen.

Functionalization of Diblock Copolymer Brushes with NaN₃; Na₃ solution (5.3 mg mL⁻¹) in anhydrous DMF was added to Ar purged reactors containing diblock poly(HEMA-b-GMA) polymer brush

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mounted with Mowiol mounting medium (Carl Roth GmbH) on cover glass slides and attached to microscope slides. Brightfield fluorescence microscopy images were acquired with an Axiovert 135, Carl Zeiss, Oberkochen, Germany. Cells were counted from microscopic contrast images with ImageJ software (version 1.52j). To obtain statistically valid results, all cell-related experiments and analyses were conducted at least in triplicate on independently prepared samples and pictures taken at randomly selected areas.

Spectroscopic Ellipsometry: The dry thicknesses of the polymer brush layers after each polymerization and/or modification step was measured using a J.A. Woollam M-2000X Spectroscopic Ellipsometer. Data were acquired in the wavelength range \( \lambda = 245–1000 \text{ nm} \) at angles of incidence of 60°, 65°, and 70°. The raw data were fitted with multilayer models in CompleteEASE software, using a Cauchy dispersion relation for the polymer layers.

Grazing-Angle Specular Reflectance Fourier Transform Infrared Spectroscopy: The chemical structures of the polymer brushes were assessed by Grazing-Angle Specular Reflectance Fourier Transform Infrared (GASR-FTIR) spectroscopy using a Nicolet Nexus 870 FTIR spectrometer equipped with a SAGA GASR attachment (ThermoFisher Scientific). The spectra were collected at 256 scans and 4 cm\(^{-1}\) resolution with an aperture of 16 mm, under continuous purging with dry air.

X-Ray Photoelectron Spectroscopy: XPS measurements were performed using a K-Alpha+ XPS spectrometer (ThermoFisher Scientific, UK) operating at a base pressure of 1.0 × 10\(^{-9}\) Pa. The data acquisition and processing were performed using the Thermo Avantage software. To limit the X-ray induced destruction of the thin polymer films and maximize the signal-to-noise ratio, individual points were measured within areas covering 8 × 8 mm\(^2\). At each point high-energy resolution core level spectrum was measured using a microfocused, monochromated Al K\(\alpha\) X-ray radiation (spot size of 400 μm, pass energy of 100 eV). All reported XPS spectra were averages of 64 individual measurements. The spectra were referenced to the C 1s peak of hydrocarbons at binding energy of 285.0 eV controlled by means of photoelectron peaks of PET and metallic Cu, Ag, and Au standards. The atomic concentrations of the different chemical moieties were determined from the respective photoelectron peak areas of levels Br 3d, Si 2p, C 1s, N 1s, O 1s and Cu 2p high resolution spectra after modified Shirley’s inelastic background subtraction. Assuming a simple model of a semi-infinite solid of homogeneous composition, the peak areas were corrected for the photoelectric cross-sections, the inelastic mean free paths of the electrons in question, and the transmission function of the spectrometer used.\[^{44}\] All high resolution spectra were fitted using Voigt profiles. The experimental uncertainties in the quantitative analysis of XPS were assessed to be below 10%. The value covers the overall uncertainties due to variations connected to sample preparation and the XPS background subtraction.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Keywords
antifouling, copolymer brushes, controlled-cell attachment, surface biofunctionalization

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