Cell Adhesion Hot Paper

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Multifunctional Coating Improves Cell Adhesion on Titanium by using Cooperatively Acting Peptides

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In memory of Klaus Braun

Abstract: *Promotion of cell adhesion on biomaterials is crucial for the long-term success of a titanium implant. Herein a novel concept is highlighted combining very stable and affine titanium surface adhesive properties with specific cell binding moieties in one molecule. A peptide containing* L*-3,4-dihydroxyphenylalanine was synthesized and affinity to titanium was investigated. Modification with a cyclic RGD peptide and a heparin binding peptide (HBP) was realized by an efficient on-resin combination of Diels–Alder reaction with inverse electron demand and* Cu^{I} *catalyzed azide–alkyne cycloaddition. The peptide was fluorescently labeled by thiol Michael addition. Conjugating the cyclic RGD and HBP in one peptide gave improved spreading, proliferation, viability, and the formation of well-developed actin cytoskeleton and focal contacts of osteoblast-like cells.*

Titanium (Ti) is the material of choice for orthopedic and dental implants. However, undesired processes, such as inflammation, migration, or loosening of the implant can occur as a result of insufficient osseointegration and non-specific cell adhesion.[1] Peptide coatings derived from the extracellular matrix are suggested to optimize the biocompatibility of the Ti surface and to strengthen specific contacts with the surrounding tissue.[2] Cyclic Arg-Gly-Asp (RGD) pentapeptides have a high affinity and specificity towards certain integrins and can mediate strong cell adhesion.[3] Furthermore, the peptide sequence FHRRIKA, derived from the bone sialoprotein, is suggested to bind to heparan sulfate containing proteoglycans and thus promote cell attachment.[4] Since integrin-mediated cell adhesion is described to be supported by proteoglycan interactions, we suggest that a combination of c[RGDfK] and FHRRIKA adjacent to each other and within one molecule is desired to stimulate cell-surface interactions.[5] To anchor these cell attractive peptides to the Ti-surface, DOPA (L-3,4-dihydroxylphenylalanine) is used. This posttranslationally modified amino acid, which was found in proteins secreted by the blue mussel (*Mytilus edulis*), binds to the oxidized surface of Ti without further chemical treatment in a wet environment.[6]

Synthesizing artificial, DOPA-containing polymers or peptides by standard solid-phase peptide synthesis (SPPS) tremendously expands the possibilities for the introduction of several bioactive moieties compared to recombinant expression of mussel secreted proteins.[7]

A mussel-derived peptide (MP) was synthesized that combined two cell binding motifs and strong affinity for the Ti surface (TiO_2; Figure 1). Two orthogonal cycloadditions namely the Diels–Alder reaction with inverse electron demand (DAR_{inv}) and the Cu^{I} catalyzed azide–alkyne cycloaddition (CuAAC) were performed on-resin to functionalize the TiO_2-adhesive scaffold with c[RGDfK] and a heparin binding peptide (HBP with the sequence FHRRIKA; Figure 1). Peptides carrying more than one cell binding motif and strong surface affinity within one molecule have not been described until now. One major requirement is the stable and directed immobilization of bioactive molecules, preferably without additional surface functionalization, which is often complex and time consuming.[8] It is assumed that the adhesion of DOPA to TiO_2 is coordinative and shows higher affinity and stability under wet conditions than thiols or side chains of basic amino acids.[9] A modular scaffold peptide (MP) containing DOPA, functional groups, and spacers was synthesized. Polyethylene glycol (PEG) was integrated into the peptide backbone to increase solubility and to space functionalities. The binding capacity of MP was investigated by a previously described biotin-ELISA-like assay using N-terminally biotinylated derivatives (Figure 2a; **1**–**3**).[10] A positive control peptide (**2**), derived from the naturally occurring mefp-1 (*M. edulis* foot protein-1), was used to compare the adhesive properties.[11] Concentration response curves revealed that the artificial peptide MP (**1**; EC_{50} 23.6 nM, pEC_{50} 7.6 ± 0.1) binds with an affinity comparable to that of the natural peptide (**2**; EC_{50} 6.4 nM, pEC_{50} 8.2 ± 0.1)

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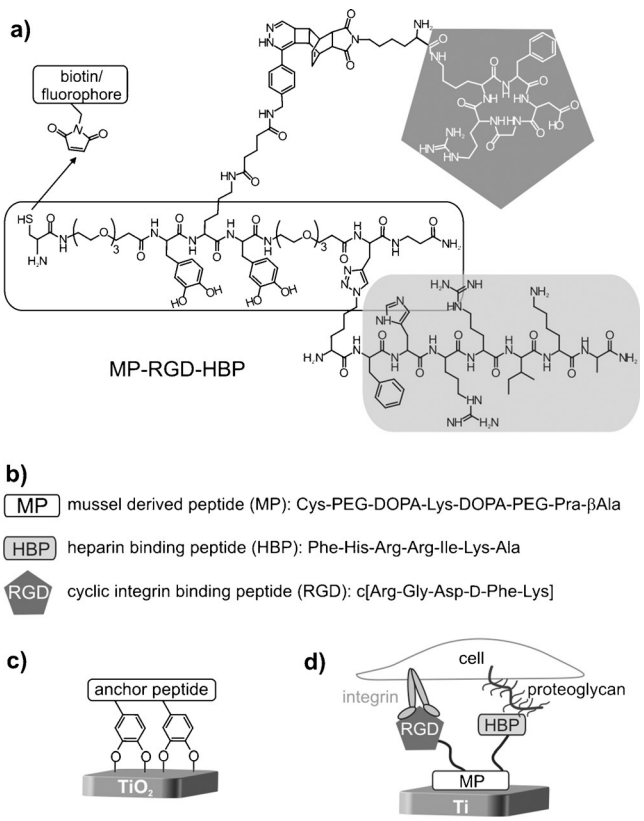

Figure 1. a) Chemical structure of the adhesive peptide MP-RGD-HBP. b) The construct consists of three functional peptides. The anchor peptide MP contains DOPA (L-3,4-dihydroxylphenylalanine) for Ti binding, PEG units as spacers, Pra, Cys, and Lys for modification, and β-alanine as a spacer. RGD and HBP are applied to enhance cell adhesion. The three peptides were modified for ligation by two orthogonal cycloadditions (CuAAC and DAR_{inv}), yielding the multifunctional peptide MP-RGD-HBP. The N-terminal Cys in MP was used to label the peptide construct with maleimide-coupled biotin and fluorophores. c) Coordinative binding of DOPA in MP (anchor peptide) to the naturally oxidized Ti surface. d) Integrin- and proteoglycan-mediated cell adhesion on Ti through MP-RGD-HBP.

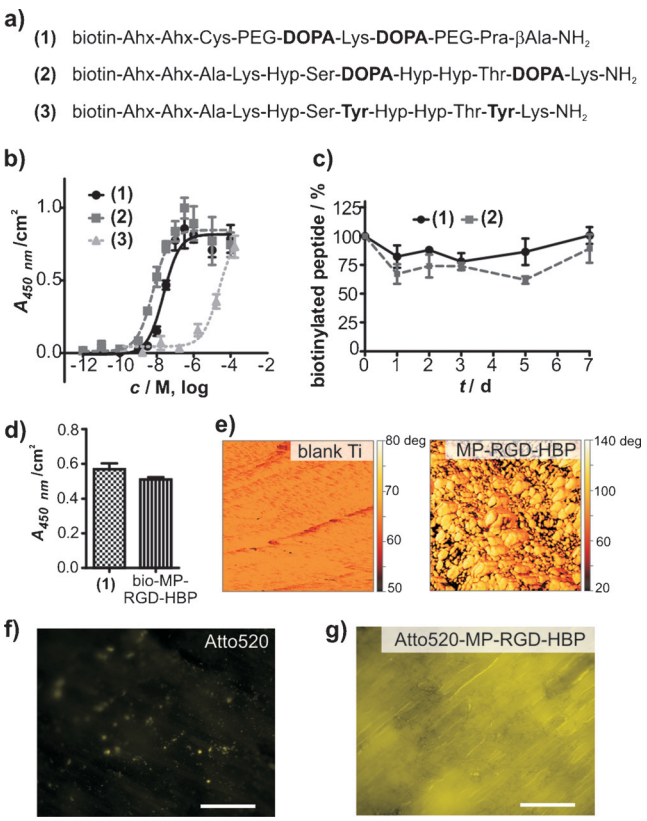

Figure 2. a) Sequences of natural derived (**2**,**3**) and artificial peptides (**1**); Hyp = 4-hydroxyproline, Ahx = aminohexanoic acid. b) Concentration response curves obtained by a biotin-ELISA-like assay. c) Stability of DOPA-containing peptides on Ti in cell supernatant of SaOS-2 cells at 37 °C for up to 7 days, evaluated by a biotin-ELISA-like assay. d) Biotin-ELISA with MP (**1**) and MP-RGD-HBP at $c = 1$ μM. e) AFM phase images (1×1 μm^2) of blank Ti and MP-RGD-HBP. f) Fluorescence microscopy picture of Atto520 on titanium and g) Atto520-MP-RGD-HBP on titanium. Data is presented as mean ± SEM of $n \geq 2$.

with a saturation phase starting in the nM range (Figure 2b). Dramatically decreased adhesive properties were determined by exchanging DOPA for tyrosine (**3**, $EC_{50} > 10000$ nM, $pEC_{50} > 4.6 \pm 0.1$), confirming the essential role of the catechol-unit. By using atomic force microscopy (AFM), the saturation of the titanium surface by MP in the nM range was confirmed (Figure S1 in the Supporting Information). In addition, the binding stability of the DOPA-containing peptides (**1**,**2**) was investigated. After incubation in cell supernatant of SaOS-2 cells at 37 °C, the peptide remaining on Ti was determined by the biotin-ELISA-like assay. During a period of 7 days, more than 80 % of the biotinylated peptide was constantly immobilized on the surface as a result of the strong and stable interaction of the catechol-unit with TiO_2 (Figure 2c).[12] Using biotinylated MP-RGD-HBP resulted in similar surface coverage as obtained for MP and was evaluated by the biotin-ELISA-like assay at a peptide concentration of 1 μM (Figure 2d). AFM images could additionally verify a complete peptide layer of MP-RGD-HBP, the single modified constructs, and MP (Figure 2e and Figures S2,S3). Incubation of the Ti surfaces with the peptides overnight, as performed for in vitro experiments, yielded a thicker peptide layer compared to an immersion over 2 h (Figure S2). Labeling of the peptides via Michael addition at the N-terminal Cys residue enabled the detection of the peptide-coated surface by fluorescence microscopy. A saturated surface was observed for di- and mono-functionalized constructs (Figure 2g and Figure S4). Furthermore, the fluorescent peptide was still detectable as a homogenous layer in presence of adhered cells (Figure S5).

Click-chemistry in combination with SPPS was used to decorate the anchor molecule MP with selective cell adhesive motifs in one peptide. Thus, peptides can be fully analyzed, sterile filtrated, and stored after lyophilization, prior to immobilization. A cyclic integrin ligand was specifically conjugated to the TiO_2 adhesive peptide by DAR_{inv} to generate a spacer between the anchor and the RGD-peptide, which is required for effective cell adhesion (Figure 3a).[13] Therefore, resin-bound MP-diene, modified at the Lys side-chain, was incubated with an aqueous solution of c[RGDfK(dienophile)] for 5 h yielding the expected conju-

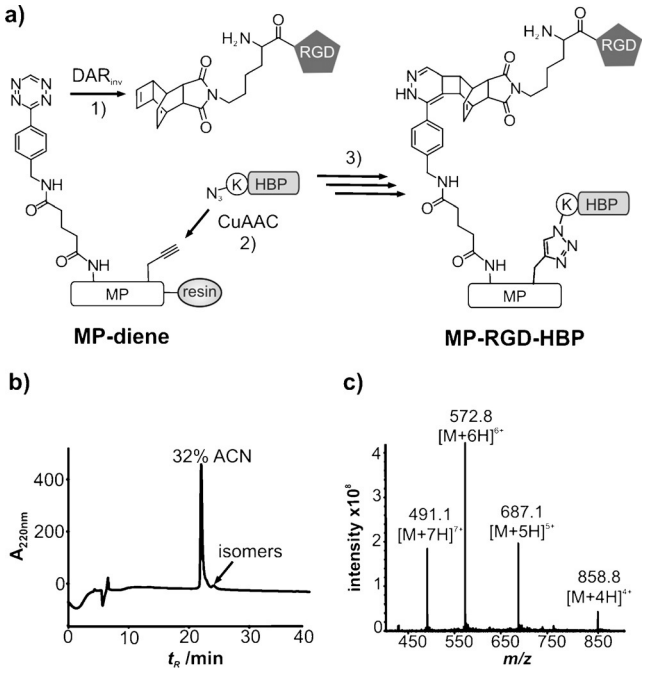

Figure 3. a) Reaction scheme of DAR$_{inv}$ and CuAAC; reagents and conditions: 1) water, room temperature, 5 h; 2) $CuSO_4$, THPTA, TCEP in water, room temperature, 24 h; 3) the peptide was cleaved from the resin, b) RP-HPLC chromatogram and c) ESI-ion trap-MS of purified MP-RGD-HBP.

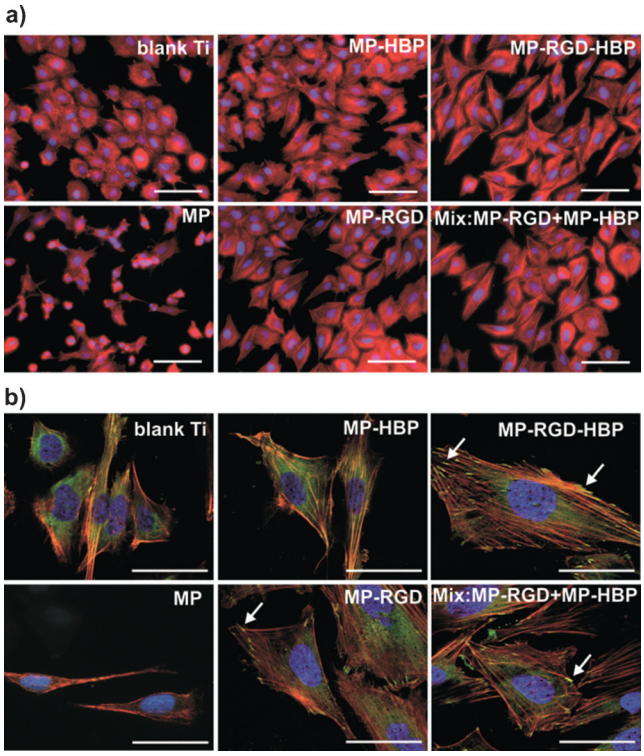

Figure 4. a) Adhesion of cells after 6 h on the synthesized peptides and untreated Ti (blank Ti), scale bar: 100 µm. b) Immunostaining of focal contacts indicated by arrows: fluorescence microscopy pictures of cells after 24 h adhesion, stained are actin cytoskeleton is in red, nuclei is in blue, and vinculin is in green. Scale bar: 50 µm.

gate MP-RGD and corresponding isomers. Orthogonality between the DAR$_{inv}$ and the CuAAC enabled further modification of MP-RGD with an azido-modified HBP which contains L-propargylglycine (Pra) as an alkyne functionality. Side-chain-protected DOPA on resin additionally avoids the oxidation of the catechol unit to a less-adhesive quinone.[14] Final cleavage of the ligated peptide revealed the desired product with a conversion of 75% (Figure S6). After purification giving a final yield of around 20%, the product was characterized by RP-HPLC as well as by MALDI-ToF (Figure S6) and ESI-ion trap mass spectrometry (Figure 3b,c). Performing the reactions on polymeric support facilitated the synthesis and increased the final yield.

To evaluate the capability of the DOPA-containing MP platform for immobilization, cell adhesion studies were performed. Osteoblast-like cells (SaOS-2) were seeded on Ti plates, which were coated by immersion in the peptide solution and monitored by fluorescence microscopy. Cell viability and proliferation were tested after 3 days of incubation of SaOS-2 cells. Attached cells on the scaffold peptide MP displayed a predominately spherical shape with weakly developed cytoskeleton (Figure 4a). Two short PEG units in MP induced a non-toxic, cell repellent effect shown by a decreased cell spreading and decreased number of adhered cells, yet simultaneously significantly higher cell survival and moderately improved proliferation than on blank Ti. To confirm this hypothesis, PEG was exchanged by an aminohexanoic acid spacer. In fact, a significant increase of cell size was observed, slightly higher than on uncoated Ti plates (Figure S7). This verifies the PEG-mediated cell repellent effect that could support selective cell adhesion.[15] The conjugation of HBP and RGD to MP gave rise to a stepwise improvement of cell adhesion (Figure 5). It has been shown, that grafting HBP (FHRRIKA) to different surfaces results in improved cell adhesion, probably induced by interaction of the peptides with cell surface proteoglycans.[4a,16] The herein demonstrated results underline this positive effect of HBP, since cell count, size, and survival are increased compared to the platform MP. Even better results could be achieved by coating Ti with MP-RGD since a further increase in cell spreading, number of adhered cells and viability was determined. Moreover, it could be shown that cells grow on a RGD coating in a fibroblast-like shape with well-developed stress fibers. Immunofluorescent staining of vinculin demonstrates accumulation of line-like focal contacts (green; Figure 4b), mainly at the periphery of the cells, which is crucial for signal- and mechanotransduction inside and outside the cell.[17] Notably, a combination of HBP and RGD in one peptide (MP-RGD-HBP) induced the highest average cell area, statistically significant to blank Ti, fibronectin, MP, MP-HBP, and MP-RGD, indicating a collaborative behavior. The additive effect was furthermore emphasized by improved viability, proliferation, cytoskeletal organization, and focal adhesion. This improvement could be explained by membrane bound proteoglycans acting as co-receptors for integrins as described for syndecans.[5] Even though it was shown that mixtures of bioactive molecules could improve cell adhesion, the random assembly of RGD and FHRRIKA on

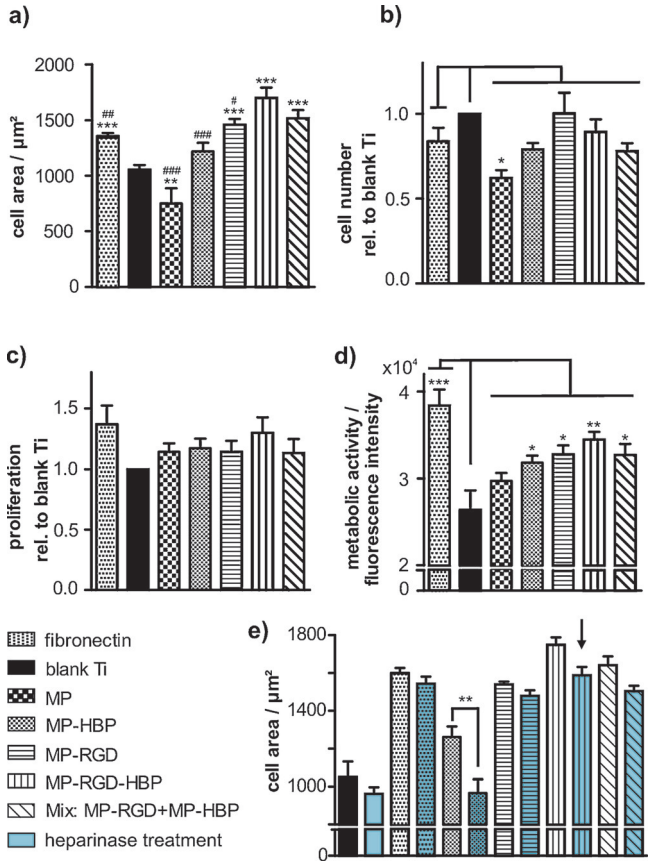

Figure 5. a) Average cell area (significant differences to blank Ti (*) and MP-RGD-HBP (#)) and b) cell number normalized to blank Ti = 1 after 6 h adhesion, evaluated by fluorescence microscopy. c) Proliferation (BrdU-assay) normalized to blank Ti = 1 and d) viability (metabolic activity determined by a resazurin assay) after 3 days of cell adhesion. e) Average cell area after 2 h adhesion of untreated and heparinase I treated cells (blue). Data is presented as mean ± SEM of $n \geq 3$ (significant differences were determined by one-way ANOVA (Newman-Keuls multiple comparison test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$)).

various surfaces led to diverse results probably because of inconsistent anchor strength, distance, and distribution of cell binding molecules.[4a,16,18] In contrast, the herein described strategy provides strong immobilization and presentation of both cell binding peptides adjacent to each other and suggests additive improvement of cell fate. Less impact on cell adhesion, viability, and proliferation was observed for a mixture of MP-RGD and MP-HBP (Figure 5). A mixture probably causes only a random distribution of bioactive molecules which results in irregular spacing of integrin and proteoglycan binding regions. Moreover, a decreased density of cell binding molecules on the surface is assumed, compared to a di-functionalized construct. Similar effects have been described for PHSRN in combination with RGD.[19] Proteins such as fibronectin or vitronectin, exhibit these sequences in a defined distance, which emphasizes the benefits of the herein presented one-compound approach.[20] Additive effects of RGD and HBP could also be observed testing the herein presented peptides with a premature osteoblast-like cell line (MG-63). Cell spreading and viability of MG-63 cells were increased on MP-RGD-HBP in an additive manner (Figure S8,9). To test whether this additive effect is specific, heparinase I was used to degrade the heparan sulfate of the transmembrane proteoglycans. Enzyme-treated cells showed slightly lowered cell size on all surfaces (Figure 5e and Figure S10). As anticipated, a further decrease in cell spreading was observed on all HBP-containing coatings. Heparinase-treated cells on MP-RGD-HBP showed an average cell size comparable to cells on MP-RGD since the positive influence of HBP is disturbed. Hence, a specific cooperative effect of HBP and RGD is suggested. Further analysis of the influence of the distance between HBP and RGD could help to optimize these additive effects since it is known that distinct spacing between cell adhesive ligands such as RGD is crucial for improved cell behavior.[21]

In conclusion the herein described data clearly underlines the benefits of multifunctional coatings that display two cell binding peptides with strong surface affinity in one molecule to support osseointegration of orthopedic and dental implants. This versatile method facilitates the cellular investigation of chemically well-characterized single and double-modified peptide constructs.

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[1] D. Schwartz-Arad, A. Laviv, L. Levin, *Implant Dent.* **2008**, *17*, 200–207.
[2] a) A. Shekaran, A. J. Garcia, *J. Biomed. Mater. Res. Part A* **2011**, *96*, 261–272; b) K. G. Sreejalekshmi, P. D. Nair, *J. Biomed. Mater. Res. Part A* **2011**, *96*, 477–491.
[3] a) R. Haubner, R. Gratias, B. Diefenbach, S. L. Goodman, A. Jonczyk, H. Kessler, *J. Am. Chem. Soc.* **1996**, *118*, 7461–7472; b) J. Auernheimer, D. Zukowski, C. Dahmen, M. Kantlehner, A. Enderle, S. L. Goodman, H. Kessler, *ChemBioChem* **2005**, *6*, 2034–2040.
[4] a) A. Rezania, K. E. Healy, *Biotechnol. Prog.* **1999**, *9*, 19–32; b) J. R. Klim, L. Li, P. J. Wrighton, M. S. Piekarczyk, L. L. Kiessling, *Nat. Methods* **2010**, *7*, 989–994.
[5] M. R. Morgan, M. J. Humphries, M. D. Bass, *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 957–969.
[6] H. Lee, N. F. Scherer, P. B. Messersmith, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 12999–13003.
[7] a) D. S. Hwang, S. B. Sim, H. J. Cha, *Biomaterials* **2007**, *28*, 4039–4046; b) Q. Wei, K. Achazi, H. Liebe, A. Schulz, P. L. M. Noeske, I. Grunwald, R. Haag, *Angew. Chem. Int. Ed.* **2014**, *53*, 11650–11655; *Angew. Chem.* **2014**, *126*, 11834–11840; c) W. Tang, G. M. Policastro, G. Hua, K. Guo, J. Zhou, C. Wesdemiotis,

G. L. Doll, M. L. Becker, *J. Am. Chem. Soc.* **2014**, *136*, 16357 – 16367.
[8] S. Sun, W. Yu, Y. Zhang, F. Zhang, *J. Mater. Sci. Mater. Med.* **2013**, *24*, 1079 – 1091.
[9] a) Y. Li, M. Qin, Y. Cao, W. Wang, *Langmuir* **2014**, *30*, 4358 – 4366; b) J. B. Schlenoff, M. Li, H. Ly, *J. Am. Chem. Soc.* **1995**, *117*, 12528 – 12536.
[10] R. Hassert, M. Pagel, Z. Ming, T. Häupl, B. Abel, K. Braun, M. Wiessler, A. G. Beck-Sickinger, *Bioconjugate Chem.* **2012**, *23*, 2129 – 2137.
[11] C. M. Taylor, C. A. Weir, *J. Org. Chem.* **2000**, *65*, 1414 – 1421.
[12] J. L. Dalsin, B. H. Hu, B. P. Lee, P. B. Messersmith, *J. Am. Chem. Soc.* **2003**, *125*, 4253 – 4258.
[13] a) M. Kantlehner, P. Schaffner, D. Finsinger, J. Meyer, A. Jonczyk, B. Diefenbach, B. Nies, G. Holzemann, S. L. Goodman, H. Kessler, *ChemBioChem* **2000**, *1*, 107 – 114; b) D. Pallarola, A. Bochen, H. Boehm, F. Rechenmacher, T. R. Sobahi, J. P. Spatz, H. Kessler, *Adv. Funct. Mater.* **2014**, *24*, 943 – 956.
[14] T. H. Anderson, J. Yu, A. Estrada, M. U. Hammer, J. H. Waite, J. N. Israelachvili, *Adv. Funct. Mater.* **2010**, *20*, 4196 – 4205.
[15] E. Wischerhoff, K. Uhlig, A. Lankenau, H. G. Börner, A. Laschewsky, C. Duschl, J. F. Lutz, *Angew. Chem. Int. Ed.* **2008**, *47*, 5666 – 5668; *Angew. Chem.* **2008**, *120*, 5749 – 5752.
[16] a) A. A. Sawyer, K. M. Hennessy, S. L. Bellis, *Biomaterials* **2007**, *28*, 383 – 392; b) M. Schuler, D. W. Hamilton, T. P. Kunzler, C. M. Sprecher, M. de Wild, D. M. Brunette, M. Textor, S. G. Tosatti, *J. Biomed. Mater. Res. Part B* **2009**, *91*, 517 – 527.
[17] W. H. Goldmann, *Cell Biol. Int.* **2012**, *36*, 649 – 652.
[18] X. Chen, P. Sevilla, C. Aparicio, *Colloids Surf. B* **2013**, *107*, 189 – 197.
[19] C. Mas-Moruno, R. Fraioli, F. Albericio, J. M. Manero, F. J. Gil, *ACS Appl. Mater. Interfaces* **2014**, *6*, 6525 – 6536.
[20] a) B. A. Dalton, C. D. McFarland, P. A. Underwood, J. G. Steele, *J. Cell Sci.* **1995**, *108*, 2083 – 2092; b) P. Brun, M. Scorzeto, S. Vassanelli, I. Castagliuolo, G. Palu, F. Ghezzo, G. M. Messina, G. Iucci, V. Battaglia, S. Sivolella, A. Bagno, G. Polzonetti, G. Marletta, M. Dettin, *Acta Biomater.* **2013**, *9*, 6105 – 6115.
[21] M. Arnold, E. A. Cavalcanti-Adam, R. Glass, J. Blummel, W. Eck, M. Kantlehner, H. Kessler, J. P. Spatz, *ChemPhysChem* **2004**, *5*, 383 – 388.

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