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Titanium Implant Materials with Improved Biocompatibility through Coating with Phosphonate-Anchored Cyclic RGD Peptides

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One key point for improving osseous integration of implants is to render them osteopromotive by specifically favoring the adhesion of osteoblasts. Mimicking the physiological adhesion process of osteoblasts to the extracellular matrix improves cell adhesion in vitro and results in improved and earlier osseous integration of implants in vivo. Our approach involves coating titanium implants with a tailor-made cyclic-RGD peptide, thus allowing them to bind to specific integrin receptors on the cell surface through multimeric phosphonates. The advantages of this very stable, new type of anchoring for practical application are presented.

Introduction

Osteointegration of implants is known to be a biological process that occurs when new peri-implant bone forms in direct contact with the implant surface.[1,2] This process is osteoconductive, and numerous experimental studies have shown that surface modifications can enhance bone–implant contact in terms of both speed and intensity of bone formation. Accelerated and increased bone contact with the implant surface can be achieved by surface modifications, such as coating the implant with hydroxylapatite.[3] However, more advanced improvements of surfaces can be achieved by using RGD peptides for coating (for polymer coating see review by U. Hersel et al.[4]). Cellular binding sites for RGD peptides have been reported to play a major role in mediating cell adhesion through integrin receptors,[5,6] thereby transducing information to the nucleus through cytoplasmic signaling pathways. In this study, we used tailor-made cyclic-RGD peptides[7,8] with the general structure cyclo(-RGDfX-). X represents Lys or Glu, which can allow coupling of the peptide to anchors that were developed for the improvement of implant integration by biofunctionalization. These cyclic peptides specifically bind to $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins.[9] Both integrin receptors are known to adhere to vitronectin, but are differentially distributed on the cell surface. Only the $\alpha v\beta 3$ integrin is found in focal contacts and leads to the spreading and migration of cells onto vitronectin.[10,11] Adhesion studies have elucidated the binding specificities of the cyclic-RGD peptides towards osteoprogenitor cells and osteoblasts from different species. In vitro proliferation of osteoblasts on polymethylacrylate (PMMA) discs was achieved with cyclo(-RGDfK-) peptides (f = D-Phe) by using an acrylamide anchor.[12] In vivo effects of the same modified cyclo(-RGDfK-) peptides were investigated by implantation of peptide-coated PMMA granulate cylinders into the patella groove of rabbits. The newly formed bone stayed in direct contact with the modified implant; no fibrous layer between implant and bone was seen. Hence, coating the implants with the αv-specific RGD peptide accelerated their osteointegration compared with uncoated granulate cylinders.[13,14]

Enhanced cell attachment on Ti surfaces that have been modified with linear-RGD peptides has been achieved in vivo.[15,16] Ferris et al.[17] demonstrated significant improvement in bone formation in rats by using gold-covered Ti rods that were coated with linear RGD through the application of gold–thiol chemistry.

Schliephake et al. have coated titanium-implant surfaces with collagen type I and covalently bound cyclo(-RGDfK[mercaptopropionyl]-) onto the collagen. Three months after implantation into the alveola crest of beagle dogs, these implants displayed a bone contact rate that was twice as high as that observed with implants coated only with collagen.[18] Similar results were obtained when the thiol function of the peptide was immobilized directly onto the Ti surface. Fibrous tissue growth was only observed on two implants in the RGD group compared with five in the control (uncoated) implants. In the 0–100 μm zone from the interface, a significantly higher bone-volume percentage was found for the RGD-coated implants. There was also a moderate increase in the mechanical fixation.[19] Moreover, the potential of the peptide described here

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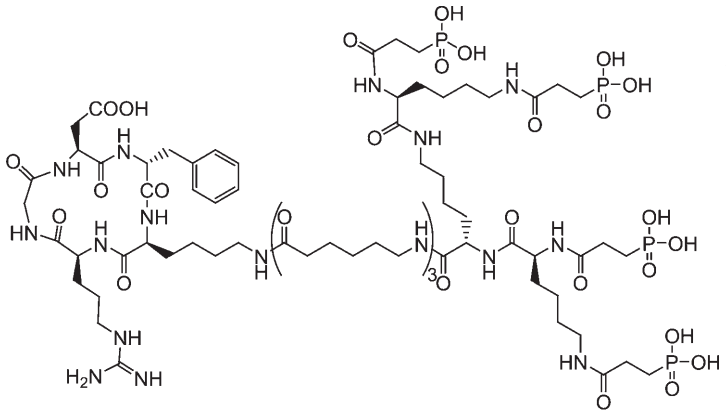
to improve osseous integration of Ti6Al4V implants has been shown in vivo in a sheep implant.[20]

The aim of this experimental study was to develop an easy and practical coating for Ti implants with the αv-specific cyclic-RGD peptide by using a new anchor system. It has already been shown that phosphonic acid groups bind strongly over a large pH range (pH 1–9) to TiO_2 and are then distributed on the Ti surface.[21] To improve binding to the Ti surface by the multimer effect, we synthesized an anchor block that consisted of four phosphonopropionic acids linked together by a branching unit that was made up of three Lys residues. The use of highly activated phosphonoacetic acid instead of the phosphonopropionic acid resulted in the formation of many by-products. The anchor blocks were conjugated with the cyclo (-RGDfK-) peptide.[8] They were bridged by a spacer that consisted of three aminohexanoic acids that provided sufficient distance between the peptide and the surface during integrin recognition. This conjugate (Scheme 1) allows a simple one-step coating of the Ti surface with the peptide.

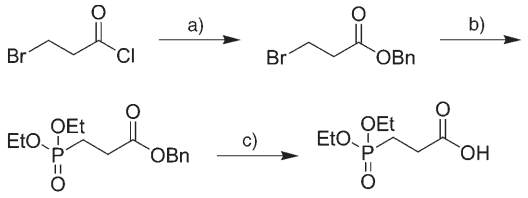


Scheme 1. Structure of the cyclic-RGD peptide with phosphonic acid anchors that was used for cell adhesion and coating of implants.

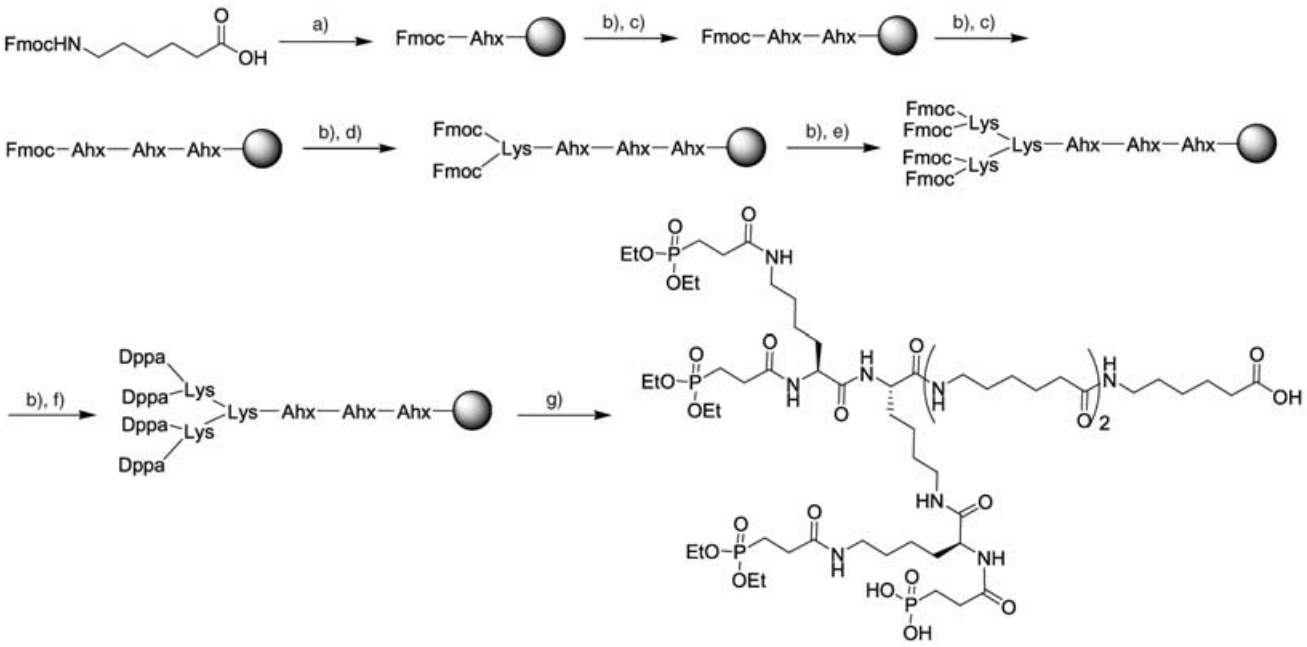
Results and Discussion

The cyclic RGD peptides cyclo(-RGDfX-), which bind to αvβ3 and αvβ5 integrin receptors were developed by our group previously.[7,8] These cyclic RGD pentapeptides, in which D-amino acids follow Asp, have a conformation that is best recognized by αv integrins and have a much reduced affinity to the platelet receptor, αIIbβ3. Additionally, a hydrophobic residue in this position, such as Phe, contributes to activity and selectivity.[22] Anchor and spacer structures were optimized in adhesion assays with osteoblast and osteoprogenitor cells.[12,13]

Phosphonate groups were chosen for anchoring in order to overcome the need to conduct excessive chemistry on the sur-



Scheme 2. Synthesis of Deppa. a) BnOH (1 equiv), CH_2Cl_2, 24 h; b) $P(OEt)_3$ (1.7 equiv), 4 h; c) H_2, Pd/C, *t*BuOH.



Scheme 3. Synthesis of the anchor-spacer unit. a) TCP-resin, DIEA (2.5 equiv), CH_2Cl_2; b) 20% piperidine in NMP; c) Fmoc-Ahx-OH (2 equiv), TBTU (2 equiv), HOBt (2 equiv), DIEA (5.6 equiv), NMP; d) Fmoc-Lys(Fmoc)-OH (2 equiv), TBTU (2 equiv), HOBt (2 equiv), DIEA (5.6 equiv), NMP; e) Fmoc-Lys(Fmoc)-OH (4 equiv), TBTU (4 equiv), HOBt (4 equiv), DIEA (11.2 equiv), NMP; f) 3-(diethoxy-phosphoryl)propionic acid (Deppa; 4 equiv), TBTU (4 equiv), HOBt (4 equiv), DIEA (11.2 equiv), NMP; g) CH_2Cl_2/HOAc/TFE (3:1:1).

face as with other coating procedures.[23] A Lys branching unit was used because its four phosphonate groups give the benefit of the multimeric effect (Schemes 2 and 3). D-Lys can also be used to avoid enzymatic degradation. Inhibition assays on the isolated integrin receptors showed that the peptide that contained the linker and anchor (Scheme 1) inhibited $\alpha v\beta 3$ slightly less than the reference peptide, cyclo(-RGDfV-). However, the IC_{50} was still in the low nanomolar range (Table 1). Hence, the addition of the linker and the phosphonic acids only had a minor effect on the apparent affinity.

Table 1. Inhibitory characteristics of cyclic RGD peptides with phosphonic acid anchors against soluble integrins.

Peptide	IC_{50} [nM][a] (inhibition at ligand concentration [nM])		
	$\alpha v\beta 3$	$\alpha v\beta 5$	$\alpha IIb\beta 3$
cyclo(-RGDfV-)[31]	2.5	320	8×10^3
cyclo(-RGDfK-)[8,32]	4.2	n.m.[b]	1.8×10^3
cyclo(-RGDfK[Ahx-Ahx-Ahx-K{K(3-phosphonopropionyl)$_2$}$_2$]-)	8.9	660	n.m.[b]

[a] The data shown represent the mean of at least two independent IC_{50} determinations. Typically, variation in such receptor–ligand inhibition measurements is routinely in the order of the measured value itself, as previously documented.[29] [b] not measured.

The peptide was attached to Ti surfaces in PBS or acetate buffer. Coated surfaces were washed several times with PBS before incubation with cells. Titanium surfaces coated with the peptide bound MC3T3-E1 mouse osteoblasts very efficiently (Figure 1). These cells express the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins; as shown by FACS analysis and real-time PCR.[24] The cell adhesion could be enhanced from 16% (uncoated Ti) to about 62% (100 μM peptide in coating solution). This represents the maximum binding that was achieved with 10 μM peptide solutions. Higher concentrations did not provide further improvement. No cell adhesion was observed on surfaces that were coated with a scrambled-sequence control peptide (RDG). This indicates that the specificity of the enhanced cell adhesion is probably due to the integrin–RGD interaction. Another proof for the specificity is the reduction of cell adhesion from 62 to 34% after incubating the cells with soluble cyclo(-RGDfV-) peptides (250 μM in media). Experiments with NIH/3T3 mouse fibroblasts showed that twice as many osteoblasts as fibroblasts adhered to the modified discs at a given concentration. As these experiments were performed in serum-free media, we also examined the effect of coating in the presence of fetal bovine serum (FBS). In medical applications other proteins will always be in contact with the implant surface. Figure 2 shows that cell adhesion onto uncoated Ti increases in the presence of 10% FBS. However, RGD coating further enhances cell adhesion by 27% compared with uncoated Ti in the presence of FBS. The coating was also monitored by a RGD-specific ELISA (Figure 3). The RGD-specific ELISA showed more immobilized peptide in the presence of a phosphonic acid anchor (0.95 ± 0.09 AU) com-

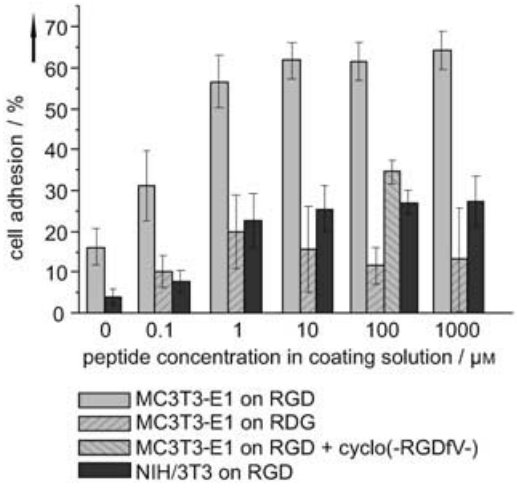


Figure 1. Adhesion of MC3T3-E1 mouse osteoblasts and NIH/3T3 mouse fibroblasts on RGD and RDG (as negative control) coated Ti discs (see text for details). To prove the specificity, in one experiment the cells were pretreated with the soluble peptide cyclo(-RGDfV-).

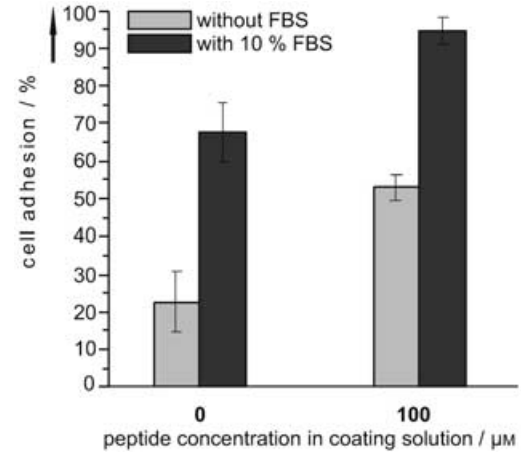


Figure 2. Adhesion of MC3T3-E1 mouse osteoblasts on RGD coated Ti discs. The media were supplied with and without 10% FBS.

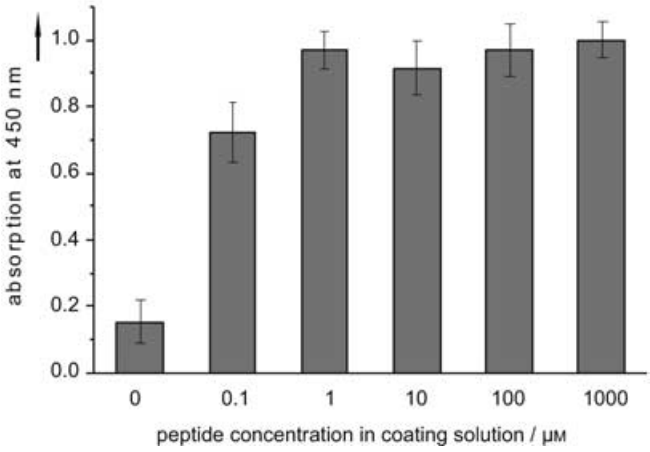


Figure 3. Detection of surface immobilized peptide on Ti after coating with RGD at various concentrations. The amount of attached peptide was quantified by a RGD-specific ELISA.

pared with the thiol functionalized peptide cyclo(-RGDfK [3-mercaptopropionyl]-) also known as EMD 73450 (0.71 ± 0.26 AU),[19] which can be directly immobilized on Ti.

The amount of attached peptide is assumed to be in the pmol cm^{-2} range asn PMMA and α silicon (unpublished data). This estimation was supported by a coating experiment in which the same solutions (10 or 100 μM) were used for the sequential coating of eight Ti discs; each disc was coated for 24 h followed by the next one. The amount of bound peptide was so low that even on the eighth disc no decrease in the signal intensity of a RGD-specific ELISA could be detected (Figure 4). The same coating solution could be used for the biofunctionalization of multiple Ti discs.

In order to determine the amount of surface-bound peptide accurately, radiolabeling was performed with a peptide analogue in which D-Phe was replaced by D-Tyr. The ^{125}I-labeled peptide was mixed with unlabeled peptide that contained D-3-iodo-Tyr, in a ratio of 83:17 (unlabeled/labeled) due to the limited metering range of the γ counter. Titanium discs were coated with a solution of this mixture in different concentrations in the same way as the RGD peptide. After coating and washing, the activity of the discs was measured and the amount of surface-bound peptide was calculated (Figure 5). The amount of bound peptide was 0.3–1.3% of the total amount of peptide in the coating solution on each disc.

For application of coated Ti as an implant material in vivo, it is necessary that the coating exhibits a strong stability against sterilization, thermal influences, and washing. To test the effects of sterilization, coated and uncoated Ti discs were γ irradiated with different doses and the cell-adhesion rate was compared with that on nonirradiated, coated and uncoated discs. Figure 6 shows that cell adhesion does not decrease after γ irradiation of the cyclo(-RGDfK-) peptide-coated discs even with the very high dose of 40 kGy. Hence, γ radiation does not affect the peptides or their attachment to the Ti surface. Further proof for the stability of the coating is provided in Figures 7 and 8. Application of dry heat at 70 °C for 7 days did not decrease cell adhesion relative to control samples stored at 18 °C (ANOVA analysis). The RGD coating even withstood a standard repassivation regime with subsequent exten-

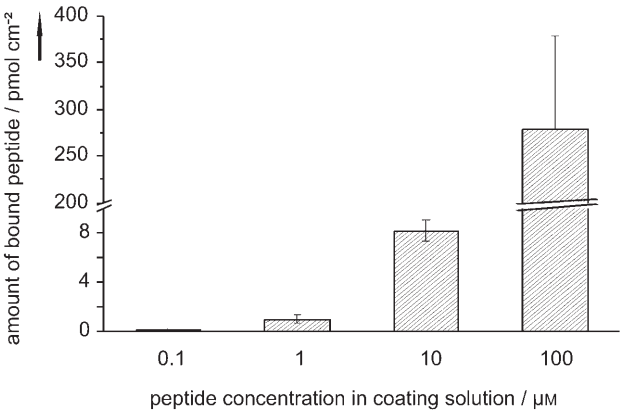


Figure 5. Detection of the amount of surface-bound peptide by radiolabeling of cyclo(-RGDyK-) with ^{125}I (where y = D-Tyr). It has previously been shown that after substitution of f by y and iodination, the peptide cyclo(-RGDyV-) still has considerable, although slightly reduced affinity for αvβ3 integrine binding.[30] Hence, we assume that attachment to the surface by Lys will not change this property.

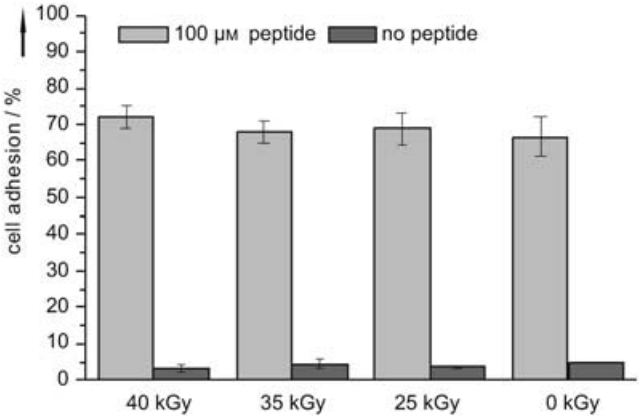


Figure 6. Testing the stability of cell adhesion against sterilization procedures. Adhesion of MC3T3-E1 mouse osteoblasts on RGD coated Ti discs. The discs were sterilized by γ irradiation after coating. The peptide concentration in the coating solution was 100 μM.

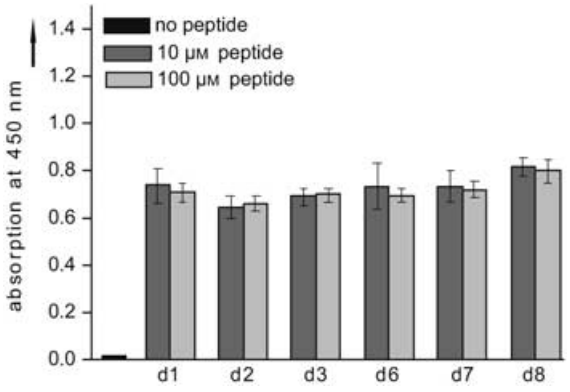


Figure 4. Consecutive coating of eight Ti discs with the same coating solution (10 or 100 μM) at RT; each disc was coated for 24 h. The amount of attached peptide was estimated by a RGD-specific ELISA; d = days.

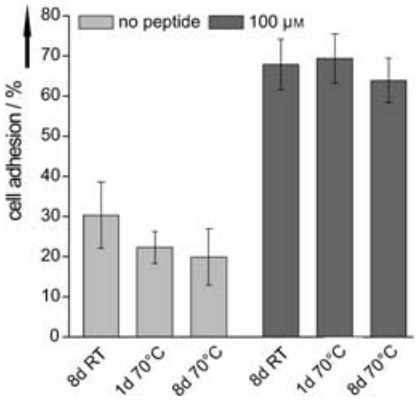


Figure 7. Testing the thermal stability of coated Ti surfaces. Adhesion of MC3T3-E1 mouse osteoblasts on RGD-coated Ti discs. The discs were stored at 70 °C for different lengths of times. The peptide concentration in the coating solution was 100 μM; d = days.

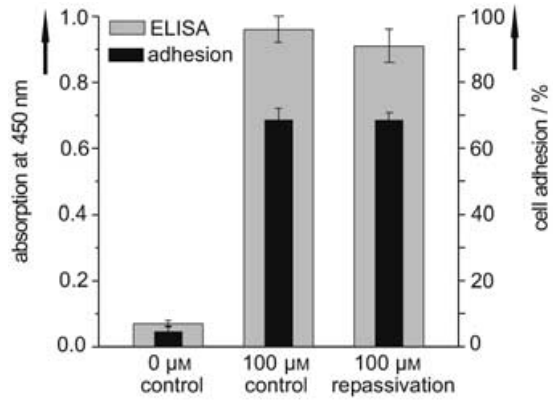


Figure 8. Testing the effect of repassivation on RGD-bound Ti surfaces. Results of a RGD-specific ELISA and cell-adhesion assay after repassivation of RGD-coated Ti discs are compared with freshly-coated specimens. The repassivation and cleaning protocols had no influence on either ELISA or cell adhesion of uncoated control samples (data not shown).

sive detergent and ultrasonic treatment (Figure 8). Neither the RGD-specific ELISA nor cell adhesion was significantly impaired in comparison to freshly coated samples.

Conclusion

A simple but efficient method for biofunctionalization of Ti has been developed. This technique can find application in the development of modern implants. In contrast to other techniques in which surfaces are coated with whole proteins, nonselective or enzymatically easy-to-cleave linear peptides, we used a small highly active and $\alpha v\beta 3$ selective cyclic-RGD peptide. The cyclic pentapeptide is completely stable against enzymatic degradation and as it is obtained by chemical synthesis it bears no risk of disease transmission. The chosen peptide exhibits high affinity for $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins. Due to its very low affinity to the $\alpha IIb\beta 3$ integrin an enhanced risk of thrombus formation as a result of platelet activation is not expected. The peptide was bound to Ti surfaces through branched phosphonic acid anchors. These anchors expose four groups to the implant surface, which then provide extremely tight binding; but, in comparison to a thiol anchor, the amount of immobilized peptide is higher. It is evident that the same coating compound can also be used to stimulate cell attachment to apatite or other potassium phosphates. Osteoblasts selectively bind to this peptide and consequently to the implant surface by their integrin receptors. These results demonstrate an attractive strategy for the development of cell-free and bioactive implants that carry the biological information for the selective activation of the target cells that are needed for selective bone regeneration.

Experimental Section

General: Amino acids and coupling reagents were purchased from Novabiochem (Schwalbach), and solid-phase resins from Pepchem (Tübingen). All other chemicals were purchased from Aldrich, Sigma, or Fluka. Semi-preparative HPLC was performed on a Beckmann instrument (system gold, solvent delivery module 126, UV detector 166) by using a YMC ODS 120–5C18 column (5 mm, 20× 250 mm), with a flow rate of 6 mL min^{-1}. The eluent was 0.1% TFA in various acetonitrile ± water gradients. HPLC-MS analyses were performed on a Hewlett Packard Series HP 1100. A YMC ODS-A 120–3C18 column (3 mm, 2×125 mm) with a flow rate of 0.3 mL min^{-1} was used. The eluent was 0.1% formic acid in an acetonitrile ± water gradient (10–50% acetonitrile in water over 15 min). ESI-MS measurements were performed on a Finnigan LCQ instrument. NMR spectra were recorded with a Bruker AC250 spectrometer.

Deppa synthesis: 3-Bromopropionyl chloride and benzylic alcohol (1 equiv) were stirred in dry CH_2Cl_2 (1.7 mL mmol^{-1}) for 24 h. The reaction mixture was diluted with $CHCl_3$ and extracted with saturated $NaHCO_3$ solution. The solvent was removed under reduced pressure after drying with $MgSO_4$. The resulting product was treated with triethylphosphite (1.7 equiv) at 140 °C under Ar without further purification. The resulting bromoethane was continuously distilled out of the reaction mixture. For cleavage of the benzyl ester, the product and Pd/C were suspended in *t*BuOH and stirred under hydrogen atmosphere at RT. After 2 h the catalyst was removed by filtration and the solvent was evaporated to yield the desired product as colorless oil (70.6%). ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 4.09$ (m, 4H, CH_2O), 2.61 (m, 2H, CH_2P), 2.07 (m, 2H, CH_2CO), 1.31 (t, $J = 7.1$ Hz, 6H, CH_3); ^{31}P NMR (101.26 MHz, $CDCl_3$): $\delta = 29.5$ (s, 1P); ESI-MS *m/z* 443.2 $[2M+Na]^+$, 459.1 $[2M+K]^+$.

***N*-Fmoc-D-3-iodo-tyrosine (Fmoc-y{I}-OH)**: was synthesized as described in the literature.[25] ^{1}H NMR (250 MHz, DMSO): $\delta = 10.11$ (s, 1H), 7.90 (d, 2H), 7.74–7.57 (m, 4H), 7.46–7.24 (m, 4H), 7.10 (dd, 1H), 6.78 (d, 1H), 4.20 (m, 3H), 4.10 (m, 1H), 2.94 (dd, 1H), 2.72 (dd, 1H); ESI-MS *m/z* 308.0 $[M+H]^+$.

Peptide synthesis: The RGD peptide cyclo(-RGDfK[Ahx-Ahx-Ahx-K{K(3-phosphonopropionyl)2}2]-) was synthesized by derivatization of the peptide cyclo(-R(Pbf)GD(O*t*Bu)fK-) as described before.[14] The other cyclic peptides cyclo(-R(Pbf)GD(O*t*Bu)y(*t*Bu)K-) and cyclo(-R(Pbf)GD(O*t*Bu)y{I}K-) were synthesized in a similar manner. The iodine-containing peptide was cyclized with PyBOP instead of diphenylphosphorylazide and the Lys side chain was protected with 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidine, which was removed with 2% hydrazine hydrate in DMF after cyclization.

The anchor-spacer unit was synthesized by SPPS by using TCP resin[26] and application of the Fmoc strategy[27] starting from *N*-Fmoc-6-aminohexanoic acid. Then two *N*-Fmoc-6-aminohexanoic acids followed by *N*-α,ε-di-Fmoc-L-Lys were coupled under standard peptide-coupling conditions with TBTU/HOBt/DIEA in NMP. After Fmoc-deprotection, two *N*-α,ε-di-Fmoc-L-Lys were coupled followed by four 3-(diethoxy-phosphoryl)propionic acids. Cleavage from the resin was accomplished with CH_2Cl_2/acetic acid/TFE (3:1:1).

Fragment coupling of the anchor construct with the partially protected peptides was carried out in dry DMF by using HATU/HOAt/collidine as coupling reagents. For deprotection the peptides were dissolved in a mixture of 95% TFA in H_2O and after 3 h the solvent was removed under reduced pressure and the residue was suspended in dry $CHCl_3$ and trimethylsilylbromide (10:1). After 3 h the solvent was removed under reduced pressure and the residue was lyophilized out of *t*BuOH/H_2O. RP-HPLC purification followed by lyophilization yielded a white hygroscopic powder.

For radiolabeling, peptide (300 μg), IodoGen® (150 μg) and $Na^{125}I$ (750 μCi) were dissolved in PBS buffer (350 μL). After 30 min, the labeled peptide was isolated by RP-HPLC purification.

cyclo(-RGDfK[Ahx-Ahx-Ahx-K{K(3-phosphonopropionyl)2}2]-): ^{1}H NMR (500 MHz, DMSO): $\delta = 8.37–8.10$ (m, 1H; H^N-Gly), 8,16–8,04 (m, 5H; H^N-Asp, 4 H^N-Lys), 8.03–7.87 (m, 5H; H^N-D-Phe, 4 $H^{\varepsilon N}$-Lys), 7.87–7.69 (m, 4H; 3 H^N-Ahx, $H^{\varepsilon N}$-Arg), 7.68–7.61 (m, 1H, H^N-Arg), 7.29–7.10 (m, 5H; $^{D\text{-}Phe}CH$), 4.62 (dd, $J = 8.0/6.0$ Hz, 1H; H^α-Asp), 4.49–4.40 (m, 1H; H^α-D-Phe), 4.20–4.11 (m, 1H; H^α-Arg), 4.11–3.98 (m, 4H; H^α-Gly, 3 H^α-Lys_{Spacer}), 3.98–3.85 (m, 1H; H^α-Lys), 3.24 (d, $J =$ 15.0 Hz, 1H; H^α-Gly), 3.15–2.87 (m 17H; 6 H^ε-Ahx, H^β-D-Phe, 8 H^ε-Ahx, 2 H^δ-Arg), 2.79 (dd, $J = 13.5/6.0$ Hz, 1H; H^β-D-Phe), 2.71 (dd, $J =$ 16.5/8.5 Hz, 1H; H^β-Asp), 2.47–2.18 (m, 7H; H^β-Asp, 6 H^α-Ahx), 2.10–1.93 (m, 8H; 8 CH_2CH_2P), 1.82–0.96 (m, 46H; 8 CH_2CH_2P, 2 H^β-Arg, 8 H^β-Lys, 6 H^β-Ahx, 2 H^γ-Arg, 6 H^δ-Ahx, 8 H^γ-Lys, 6 H^γ-Ahx, 8 H^δ-Lys); ^{31}P NMR (101.26 MHz, DMSO): $\delta = 29.57$ (s, 1P), 29.51 (s, 2P), 29.33 ppm (s, 1P); ESI-MS *m/z* 934.5 $[(M-2H)/2]^-$, 1869.9 $[M-H]^-$, 1891.9 $[M-2H+Na]^-$.

cyclo(-RGDyK[Ahx-Ahx-Ahx-K{K(3-phosphonopropionyl)2}2]-): ESI-MS *m/z* 942.9 $[(M-2H)/2]^-$, 954.8 $[(M-3H+Na)/2]^-$, 1885.9 $[M-H]^-$, 1909.9 $[M-2H+Na]^-$.

cyclo(-RGDy{I}K[Ahx-Ahx-Ahx-K{K(3-phosphonopropionyl)2}2]-): ESI-MS *m/z* 1005.6 $[(M-2H)/2]^-$.

Surface modification: Coatings with peptide solutions (100–0.1 μM) were performed in PBS (pH 7.4) or acetate buffer (pH 4). Titanium discs (Ti6Al4V) of 10 mm in diameter were placed in 48-well plates, coating solution (250 μL) was added to each well and the peptide was allowed to immobilize at RT, overnight. After washing three times with PBS, the discs were transferred to a new 48-well plate and blocked with 5% bovine serum albumin (BSA) in PBS for 2 h. Subsequently, the discs were tested for cell adhesion or submitted to a customized RGD-specific ELISA with colorimetric detection at 450 nm, measured with a microplate reader (SLT Rainbow).

Cell-adhesion assay: The cell-adhesion assays were performed as described by Landegren.[28] MC3T3-E1 mouse osteoblasts or NIH/3T3 mouse fibroblasts were suspended in Dulbecco's minimal medium (DMEM) containing BSA (1%, *w/v*). Cells were then seeded on the substrate at a density of 50000 cells per well. The cells were allowed to adhere for 1 h under standard tissue culture conditions (37°C, 5% CO_2). The wells were washed three times with PBS (pH 7.4) to remove nonadherent cells. Attached cells were quantified by a colorimetric test that detects the activity of the lysosomal enzyme hexosaminidase. *p*-Nitrophenol-*N*-acetyl-β-D-glucosaminide was cleaved by the enzyme and the amount of colored *p*-nitrophenol was measured with a microplate reader at 405 nm. To generate a calibration curve by linear regression, MC3T3-E1 cells were seeded at different concentrations in standard cell culture 48-well plates and treated similarly. Results are given as the percentage of the total number of cells seeded (considered as 100% of cell adhesion) and thus the cell adhesion rate defined. In all experiments, each data point given in the figures is the mean value of at least three identical but independent in vitro experiments; the error bars represent standard deviations. To test the effect of serum on the cell-adhesion assay BSA was replaced by different concentrations of FBS.

Sterilization: For sterilization coated and uncoated specimens were sealed into Tyvek bags (Dupont, Wilmington, DE, USA) and subjected to various doses of γ radiation (Willy Rüsch, Germany).

Repassivation: Repassivation of Ti specimens was performed in accordance with ASTM F86. Coated discs were subjected to nitric acid treatment (specific-gravity limits 1.1197–1.2527 g mL^{-1}) for 30 min at RT. Subsequently, discs were agitated briefly in dH_2O and soaked two times for 7 min in fresh dH_2O. Additionally, specimens were cleaned by ultrasonic treatment in common household detergent (Priel) solution for 30 min at 60°C. To remove any residual detergent, the Ti discs were thoroughly rinsed three times with dH_2O, subjected to acetone treatment (two times 15 min in an ultrasonic bath, RT), rinsed with dH_2O, washed in ethanol (two times 15 min in an ultrasonic bath, RT), and finally dried in a drying cabinet.

IC_{50} values: The ability of the peptides to inhibit ligand binding to immolilized purified integrins was measured as described previously.[29]

Abbreviations:

Ahx:	6-aminohexanoic acid
AU:	absorption unit
Deppa:	3-(diethoxy-phosphoryl)propionic acid
dH_2O:	deionised water
DIEA:	*N,N*-diisopropylethylamine
DMF:	*N,N*-dimethyl formamide
Fmoc:	9-fluorenylmethoxycarbonyl
HATU:	*O*-(7-azabenzotriazole-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate
HOAt:	1-hydroxy-7-azabenzotriazole
HOBt:	*N*-hydroxybenzotriazole
NMP:	*N*-methyl-2-pyrrolidinone
Pbf:	2,2,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl
PyBOP:	(benzotriazol-1-yloxy)-tris(pyrrolidino)phosphonium hexafluorophosphate
SPPS:	solid-phase peptide synthesis
TBTU:	2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TCP:	tritylchloride polystyrene
TFA:	triflouroacetic acid
TFE:	2,2,2-trifluoroethanol

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[1] T. Albrektsson, *J. Prosthet. Dent.* **1983**, *50*, 255–261.
[2] P. I. Branemark, *J. Prosthet. Dent.* **1983**, *50*, 399–410.
[3] M. Rocca, M. Fini, G. Giavaresi, N. Nicoli Aldini, R. Giardin, *Int. J. Artif. Organs* **2001**, *24*, 649–654.
[4] U. Hersel, C. Dahmen, H. Kessler, *Biomaterials* **2003**, *24*, 4385–4415.
[5] M. D. Pierschbacher, E. Ruoslahti, *J. Biol. Chem.* **1987**, *262*, 17294–17298.

[6] T. Matsuura, R. Hosokawa, K. Okamoto, T. Kimoto, Y. Akagawa, *Biomaterials* **2000**, *21*, 1121 – 1127.
[7] M. Aumailley, M. Gurrath, G. Müller, J. Calvete, R. Timpl, H. Kessler, *FEBS Lett.* **1991**, *291*, 50 – 54.
[8] R. Haubner, R. Gratias, B. Diefenbach, S. L. Goodman, A. Jonczyk, H. Kessler, *J. Am. Chem. Soc.* **1996**, *118*, 7461 – 7472.
[9] M. Pfaff, K. Tangemann, B. Müller, M. Gurrath, G. Müller, H. Kessler, R. Timpl, J. Engel, *J. Biol. Chem.* **1994**, *269*, 20 233 – 20 238.
[10] E. A. Wayner, R. A. Orlando, D. A. Cheresh, *J. Cell. Biol.* **1991**, *113*, 919 – 929.
[11] D. I. Leavesley, G. D. Ferguson, E. A. Wayner, D. A. Cheresh, *J. Cell. Biol.* **1992**, *117*, 1101 – 1107.
[12] M. Kantlehner, D. Finsinger, J. Meyer, P. Schaffner, A. Jonczyk, B. Diefenbach, B. Nies, H. Kessler, *Angew. Chem.* **1999**, *111*, 587 – 590; *Angew. Chem. Int. Ed.* **1999**, *38*, 560 – 562.
[13] P. Schaffner, J. Meyer, M. Dard, R. Wenz, B. Nies, S. Verrier, H. Kessler, M. Kantlehner, *J. Mater. Sci.* **1999**, *10*, 837 – 839.
[14] 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.
[15] T. A. Barber, S. L. Golledge, D. G. Castner, K. E. Healy, *J. Biomed. Mater. Res.* **2003**, *64A*, 38 – 47.
[16] S. Tosatti, Z. Schwartz, C. Campbell, D. L. Cochran, S. VandeVondele, J. A. Hubbell, A. Denzer, J. Simpson, M. Wieland, C. H. Lohmann, M. Textor, B. D. Boyan, *J. Biomed. Mater. Res.* **2004**, *68A*, 458 – 472.
[17] D. M. Ferris, G. D. Moodie, P. M. Dimond, C. W. D. Gioranni, M. G. Ehrlich, R. F. Valentini, *Biomaterials* **1999**, *20*, 2323 – 2331.
[18] H. Schliephake, D. Scharnweber, M. Dard, S. Rößler, A. Sewing, J. Meyer, D. Hoogestraat, *Clin. Oral Impl. Res.* **2002**, *13*, 312 – 319.
[19] B. Elmengaard, J. E. Bechtold, K. Søballe, *Biomaterials* **2005**, *26*, 3521 – 3526.
[20] E. Steinhauser, A. Liebendörfer, A. Enderle, R. Bader, S. Kerschbaumer, T. Brill, R. Busch, R. Gradinger, *Materialpruefung.* **2005**, *47*, 197 – 202.
[21] P. Péchy, F. P. Rotzinger, M. K. Nazeeruddin, O. Kohle, S. M. Zakeeruddin, R. Humphry-Baker, M. J. Grätzel, *J. Chem. Soc. Chem. Commun.* **1995**, 65 – 66.
[22] R. Haubner, D. Finsinger, H. Kessler, *Angew. Chem.* **1997**, *109*, 1440 – 1456; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 1374 – 1389.
[23] M. C. Porté-Durrieu, F. Guillemot, S. Pallu, C. Labrugère, B. Brouillaud, R. Bareille, J. Amédée, N. Barthe, M. Dardm, Ch. Baquey, *Biomaterials* **2004**, *25*, 4837 – 4846.
[24] P. Schaffner, Ph.D. thesis, TU Darmstadt (Germany), **2003**.
[25] R. Haubner, H.-J. Wester, U. Reuning, R. Senekowitsch-Schmidtke, B. Diefenbach, H. Kessler, G. Stöcklin, M. Schwaiger, *J. Nucl. Med.* **1999**, *40*, 1061 – 1071.
[26] K. Barlos, D. Gatos, J. Kallitsis, G. Papaphotiu, P. Sotiriu, Y. Weqing, W. Schäffer, *Tetrahedron Lett.* **1989**, *30*, 3943 – 3946.
[27] G. B. Fields, R. L. Noble, *Int. J. Pept. Protein Res.* **1990**, *35*, 161 – 214.
[28] U. Landegren, *J. Immunol. Methods* **1984**, *67*, 379 – 388.
[29] S. L. Goodman, G. Hölzemann, G. A. G. Sulyok, H. Kessler, *J. Med. Chem.* **2002**, *45*, 1045 – 1051.
[30] R. Haubner, H.-J. Wester, F. Burkhart, R. Senekowitsch-Schmidtke, W. Weber, S. L. Goodman, H. Kessler, M. Schwaiger, *J. Nucl. Med.* **2001**, *42*, 326 – 336.
[31] E. Lohof, E. Planker, C. Mang, F. Burkhart, M. A. Dechantsreiter, R. Haubner, H.-J. Wester, M. Schwaiger, G. Hölzemann, S. L. Goodman, H. Kessler, *Angew. Chem.* **2000**, *112*, 2868 – 2871; *Angew. Chem. Int. Ed.* **2000**, *39*, 2761 – 2764.
[32] R. Haubner, PhD Thesis, Technische Universität München (Germany), **1995**.

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