IMMOBILISATION OF LINEAR AND CYCLIC RGD-PEPTIDES ON TITANIUM SURFACES AND THEIR IMPACT ON ENDOTHELIAL CELL ADHESION AND PROLIFERATION

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Abstract

Functional coatings on titanium vascular stents and endosseous dental implants could probably enhance endothelial cell (EC) adhesion and activity with a shortening of the wound healing time and an increase of peri-implant angiogenesis during early bone formation. Therefore, the role of the structure of linear and cyclic cell adhesive peptides Arg-Gly-Asp (l-RGD and c-RGD) on differently pre-treated titanium (Ti) surfaces (untreated, silanised *vs*. functionalised with l- and c-RGD peptides) on EC cell coverage and proliferation was evaluated. After 24 h and after 3 d, surface coverage of adherent cells was quantified and an alamarBlue® proliferation assay was conducted. After 24 h, l-RGD modified surfaces showed a significantly better coverage of adhered cells than untreated titanium (p=0.01). Differences between l-RGD surfaces and silanised Ti (p=0.066) as well as between l-RGD and c-RGD surfaces (p=0.191) were not significant. After 3 d, c-RGD surfaces showed a significantly higher cell coverage than untreated Ti, silanised and l-RGD titanium surfaces (all p<0.0001). After 24 h, c-RGD modified surfaces showed significant higher cell proliferation compared to untreated Ti (p=0.003). However, there were no differences in proliferation between c-RGD and l-RGD (p=0.126) or c-RGD and silanised titanium (p=0.196). After 3 d, proliferation on c-RGD surfaces outranged significantly untreated titanium (p=0.004), silanised (p=0.001) and l-RGD surfaces (p=0.023), whereas no significant difference could be found between untreated Ti and l-RGD surfaces (p=0.54). According to these results, the biomimetic coating of c-RGD peptides on conventional titanium surfaces showed a positive effect on EC cell coverage and proliferation. We were able to show that modifications of titanium surfaces with c-RGD are a promising approach in promoting endothelial cell growth.

Keywords: RGD modification, titanium, immobilisation, linear, cyclic, endothelial cells.

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Introduction

Stable and uneventful healing of endosseous implants and vascular stents is an essential precondition for long-term success of the implant. Implant surface characteristics have a direct influence on the functional integration into the surrounding tissue. In certain subpopulations, and depending on a particular material use, an increased implant and prosthesis failure can be seen (Davies and Hagen, 1993; Balaji, 2008; Huynh-Ba *et al.*, 2008; Fine *et al.*, 2009; Javed and Romanos, 2009; Linsen *et al.*, 2009; Mellado-Valero *et al.*, 2009). This underlines the importance of developing strategies to enhance endothelialisation (Fine *et al.*, 2009) as well as peri-implant new bone formation (Schliephake *et al.*, 2005) with faster wound healing around the biomaterial to increase short- and long-term implant stability.

Accordingly, for vascular stents, the creation of a uniform healthy endothelium on the inner surface would be favourable in order to mask the underlying titanium material from inflammatory cell interference (Fine *et al.*, 2009) and to play a regulatory role in haemostasis and thrombosis (Wu *et al.*, 1988). It has been observed that conventional metal stents can trigger an inflammatory response which can even result in scar formation (James *et al.*, 1990). This amplifies the possibility of ongoing obliteration, re-stenosis, especially in small diameter prostheses.

For dental titanium implants, survival length is determined by interactions between implant surface and hard and soft tissues. Especially in the early stages of wound healing these interactions play a determining role. Endothelial cells (ECs) are crucially involved in angiogenesis and, due to increased nutrition (Davies and Hagen, 1993), in further wound healing processes (An *et al.*, 2009). As a conclusion, timely and sufficient angiogenesis at the dental implant surface is essential for a successful bone healing. Accordingly, an association between inadequate bone vascularity and decreased bone formation/ mass has been shown (Burkhardt *et al.*, 1987; Glowacki, 1998). An inhibition of angiogenesis during fracture healing in rats *in vitro* resulted in formation of fibrous tissue only (Hausman *et al.*, 2001). Furthermore, poor blood supply has been identified as a risk factor for the failure of osteogenesis (Huang *et al.*, 2005; Kanczler and Oreffo, 2008) and functional osseointegration of dental endosseous implants (Carano and Filvaroff, 2003). In addition, a supplemental close and reciprocal interaction and stimulation between ECs and bone cells has been

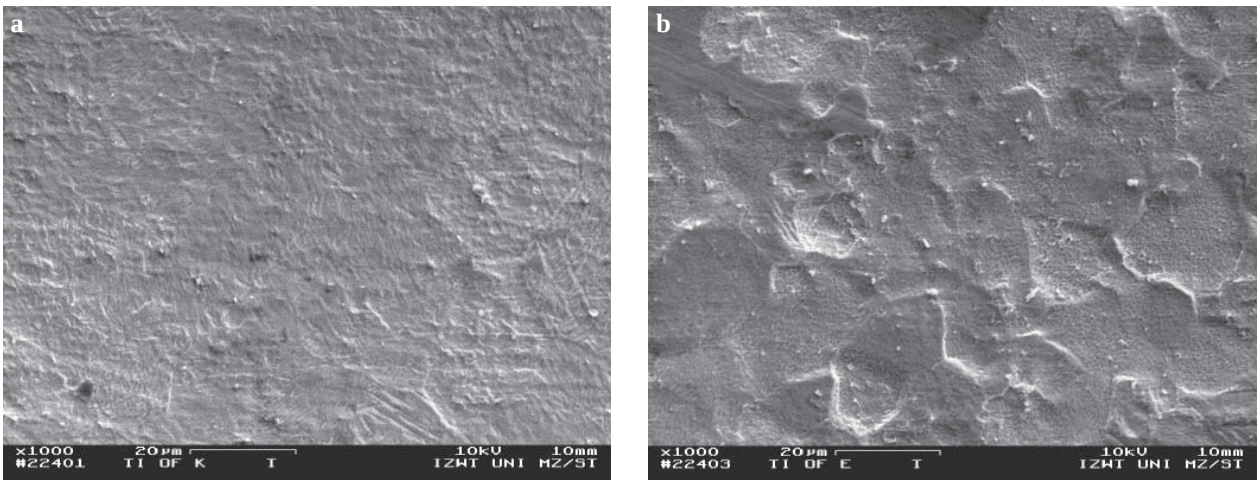


Fig. 1. (a) Scanning electron microscopy (SEM; DSM 962 scanning microscope, Carl Zeiss, Oberkochen, Germany) of the surface of a titanium disc (working distance: 10 mm; accelerating voltage: 10 kV) after grinding (mean roughness (R_a) 1.31 µm). **(b)** SEM of the surface of a titanium disc (working distance: 10 mm; accelerating voltage: 10 kV) after grinding and 3 min of etching (mean roughness (R_a) 1.31 µm).

described (Wang *et al.*, 1997; Bouletreau *et al.*, 2002; Carano and Filvaroff, 2003).

For the biomaterials described above, an increased activation of ECs on the surface with subsequent increased EC growth has been identified as a strategy to enhance implant healing (Choi *et al.*, 2008; Kanczler and Oreffo, 2008; Fine *et al.*, 2009). A promising approach to enhance endothelial cell affinity (Choi *et al.*, 2008), as well as further peri-implant bone regeneration (Schliephake *et al.*, 2002), is the use of certain peptides such as the three-amino acid motif Arg-Gly-Asp (RGD) that is a primary recognition site in extracellular matrix proteins (Kim *et al.*, 2005). This motif can enhance cellular integrin-mediated cell adhesion to different surfaces and may thus help to increase the biocompatibility of the material (Bhadriraju and Hansen, 2000; Holland *et al.*, 1998; Pallu *et al.*, 2009; Schliephake *et al.*, 2005). RGD-peptides have been shown to regulate cell adhesion of osteoprogenitor cells (Verrier *et al.*, 2002) and osteoblasts on titanium surfaces (Secchi *et al.*, 2007). An increased bone formation *in vivo* employing RGD-peptides on titanium surfaces has been reported (Ferris *et al.*, 1999; Kroese-Deutman *et al.*, 2005).

Activation of ECs by RGD motifs mediated by integrin receptors (Matsuura *et al.*, 2000) has been reported in various studies (Lin *et al.*, 1992; Lin *et al.*, 2001; Massia and Stark, 2001) and has been examined for RGD-coatings on polymer surfaces (Hirano *et al.*, 1993). To our knowledge, an examination of interactions between ECs and RGD-peptides on titanium surfaces has not been conducted to date.

Differences in the biological features of linear and cyclic RGD-peptides have been described earlier (Kim *et al.*, 2005; Kostidis *et al.*, 2004; Verrier *et al.*, 2002; Xiao and Truskey, 1996). It has been observed that cyclic RGD-peptides influence platelet aggregation more efficiently than linear ones (Kim *et al.*, 2005). After a short incubation time, bovine aortic endothelial cells have shown higher rates of adhesion on glass surfaces, functionalised with immobilised cyclic RGD peptide, than with linear RGD peptide (Xiao and Truskey, 1996). On the other hand, delivery of sphingosine 1-phosphate by linear RGD peptide showed an increased formation and better long-term-adhesion of endothelial cells than with cyclic RGD peptide, though adhesion strengths were higher on cyclic RGD (Wacker *et al.*, 2008). These results indicate the importance of sequence, structure and conformation of the peptide. Differences in cellular interactions with RGD peptides of different conformation and orientation of the amino acids (Verrier *et al.*, 2002) require a better understanding. The specification of the most appropriate RDG-form for EC activation may have big impact on further modification development techniques. To our knowledge, this interesting question has also not been examined in prior studies either.

Therefore, the aim of this study was to determine the role of the structure of linear and cyclic RGD-containing peptides immobilised onto titanium surfaces for EC cell coverage and proliferation.

Materials and Methods

Preparation of titanium discs

A titanium sheet (Ti; 99.7% alloy; Alfa Aesar, Karlsruhe, Germany) with a thickness of 2 mm was cut into 10 x 10 mm^2 pieces. Grinding of the Ti-plates was accomplished in a series of steps using wet grinding paper (400, 800 1200 and 2000 grid). Samples were etched in a solution of 9% NH_4FxHF, 8.5% concentrated sulphuric acid and 0.5% urotropin in water for 3 min at room temperature. After rinsing with water, the specimens were oxidised in Piranha-solution (30 % H_2O_2, H_2SO_4 concentration (1:1)) for 1 h at room temperature followed by washing in water.

Roughness measurements and scanning electron microscopy

In order to show the influence of etching and oxidation on Ti surfaces, the roughness of samples was analysed by dynamical focusing (Marsurf XR 20, Mahr GmbG,

Göttingen, Germany). Calculation was performed with the software Marsurf XR 20 V 1.21-1 (Mahr GmbG). Additionally, before and after etching and after oxidizing procedures, scanning electron microscopy (SEM; DSM 962 scanning microscope, Carl Zeiss, Oberkochen, Germany) of the surfaces was conducted to verify the results optically. An example of the results is shown in Fig. 1a,b. The roughness of these samples can be compared to machined or double etched titanium dental implant surfaces (Al-Nawas and Götz, 2003).

Silanisation and RGD-immobilisation of Ti samples

Silanisation of oxidised samples was performed in 5% Aminopropyl-triethoxysilane (APTES; Sigma-Aldrich, Deisenhofen, Germany) in toluene at room temperature overnight. After cleaning in toluene and ethanol, samples were cured at 80 °C for 1 h. Subsequently, amino-silanised Ti-plates were immersed in a 5% solution of diethyleneglycol diglycidylether (diepoxide) in 50 mM carbonate-buffer (pH 9) for 2 h. The excess solution was removed by repeated rinsing with water. RGD-immobilisation was then immediately performed by incubating the activated samples in 0.5 mg/mL RGD-peptide (l-RGD: Sigma-Aldrich; c-RGD: Anaspec, Fremont, CA, USA, respectively) in carbonate-buffer overnight in a wet chamber. The samples were stored dry after being thoroughly rinsed with water. Although theoretically other methods of sterilisation would have been possible (Hersel *et al.*, 2003) for the cell experiments, chemical sterilisation with 70 % ethanol for 30 min has shown to be sufficient.

Distribution and surface density of primary amino groups on Ti discs

Amino-silanised Ti-samples were subjected to fluorescent labelling. Fluorescein isothiocyanate (FITC; 0.5 mg/mL) dissolved in buffer was given to the APTES-modified Ti for 1 h and non-reacted dye was removed with ethanol. Micrographs were taken on an Olympus X-70 fluorescence microscope (Olympus, Hamburg, Germany; Fig. 2). Successful coating was analysed by means of the sulpho-SDTB-assay (Interchim, Mannheim, Germany) according to the manufacturer's instructions.

Static contact angle microscopy

Before experimental use, the hydrophilic properties of untreated and RGD modified Ti samples were examined by static water contact angle microscopy (Krüss DSA 10-MK2; Krüss Optronic, Hamburg, Germany) in order to quantify the amount of RGD peptide (Hersel *et al*., 2003). Smaller contact angles (CA) indicate an increased hydrophilicity of a solid surface and are usually detected when the zwitterionic RGD moieties are immobilised (Lin *et al*., 1992; Lin *et al.*, 1994). All measurements were done in triplicate. Mean values as well as standard deviations (SD) are given.

Cell culture

50,000 ECs (25,000/mL; Human Aortic Endothelial Cells, PromoCell, Heidelberg, Germany) from three donors (male n=1, female n=2; 28-31 years (mean: 29)) were cultivated separately in 24-well plates in Endothelial Cell Growth Medium (ECGM, PromoCell) including manufacturer's supplement. According to the manufacturer's information, ECGM with supplement (0.004 mL/mL) contains 1 ng/mL basic Fibroblast Growth Factor, 0.004 mL/mL Endothelial Cell Growth Factor, 0.1 ng/mL Epidermal Growth Factor, 22.5 µg/mL heparin, 1 µg/mL hydrocortisone, 0.62 ng/mL phenol red and 0.02 mL/mL foetal bovine serum. Cells were cultured at 37 °C, 5 % CO_2 and 95 % rel. humidity. The medium was changed every two days. To detach the cells, Accutase/PBS (phosphate buffered saline) (PAA, Pasching, Austria) was used.

Interactions between titanium discs and ECs

Initial cell-substrate interactions were tested by incubating the untreated, APTES and RGD-modified Ti-specimen with 5 x 10^4 cells in 2 mL of medium per sample. Behaviour of cells (cell coverage, proliferation) was evaluated at fixed time points (24 h and 3 d after incubation).

Cell coverage

Cell coverage was quantified by microscopic measurement of covered surface. The total area the cells were plated onto was 10 x 10 mm^2. Cells can adhere differently on different areas of the examined surface. Therefore, a single photograph cannot reflect the ratio of cell distribution on the whole surface. To minimise this problem, examinations of the percentage of colonised surfaces were done in three randomly chosen areas for each group of donor cells. To visualise attached cells, the Ti-plates were rinsed with PBS and subsequently incubated in foetal calf serum (FCS)-free medium containing 1 µg/mL Calcein-acetoxymethylester. Calcein specifically stains vital cells. After 30 min of incubation at 37 °C, samples were again washed with medium. Micrographs were obtained using standard fluorescein isothiocyanate (FITC) filters. Surface coverage of attached cells was quantified with ImageJ software (NIH, Bethesda, MD, USA) and expressed as percentage of total area.

Proliferation

Proliferation of cells was assayed with the alamarBlue® substrate (Invitrogen, Karlsruhe, Germany). For this purpose, Ti-plates were rinsed with PBS and immersed in 1 mL FCS-free medium containing 100 µL alamarBlue® solution (concentration 10%). Controls (pure medium without EC with alamarBlue® substrate) were processed in parallel. After 3 h, relative fluorescence units, indicating cellular metabolism, corresponding to the cellular proliferative activity (Ahmed *et al.*, 1994), were determined by the use of a Fluoroscan Ascent - reader (Thermo Scientific, Schwerte, Germany). Wavelengths were 538 nm for excitation and 600 nm for emission. Control values were subtracted; measurements were done in triplicate for each EC donor sample.

Statistics

A one-way analysis of variance (ANOVA) with Tukey simultaneous post-hoc test was conducted to compare groups. Altogether, the interactions of three commercial available EC samples from different donors with different

surfaces were evaluated. Each sample was measured in triplicate. The nature of this experiment was exploratory; therefore, we report descriptive *p*-values of tests. *P* values <0.05 were termed significant. The analyses were conducted using SPSS version 15.0 (SPSS, Chicago, IL, USA).

Results

Distribution and surface density of primary amino-groups on Ti-discs

The amount of accessible amino functions was determined using a colorimetric sulpho-SDTB assay (Fig. 2). The presented silanisation protocol generated a mean of 7 NH_2-groups per nm^2.

Static water contact angle microscopy

The untreated samples showed a mean contact angle (CA) of 90.8° (Standard Deviation (SD) 3.1). After silanisation and coating with RGD peptides, a mean CA of 18.1° (SD 4.5) was measured. Therefore, together with clinical observations, a successful immobilisation was assumed. No significant difference in water contact angle measurements could be seen between l- and c-RGD surfaces.

Surface coverage

Surface coverage of ECs was evaluated on untreated and on modified (APTES, l-RGD, c-RGD) titanium surfaces. On each surface, three randomly chosen different areas (3 x 3) were examined. These data were used to generate mean values and to calculate the statistical tests.

After 24 h, only marginal surface coverage was observed on untreated surfaces. On silanised surfaces as well as on both RGD-surfaces, EC adhesion was increased (Fig. 3). Accordingly, the untreated surface showed a mean colonisation of 7.1% (SD 1.04), followed by APTES surface (13%; SD 5.08), c-RGD (16.4%; SD 3.35) and l-RGD (26.1%; SD 8.69; Fig. 4a).

A statistically significant difference was found between untreated and l-RGD surfaces (p=0.01). Between c-RGD surfaces and untreated titanium, the difference was not statistically significant (p=0.223). Between APTES and RGD-modified surfaces, no significant difference was seen after 24 h (l-RGD: p=0.066; c-RGD: p=0.868). In addition, the difference between l-RGD and c-RGD surfaces was not significant (p=0.191; Fig. 4a).

After 3 days, coverage of ECs was less on surfaces with l-RGD-peptide, whereas the highest coverage of adhered cells was seen on c-RGD surfaces. The coverage of untreated and APTES surfaces ranged between the coverage values of l- and c-RGD-peptide. Accordingly, the untreated surface exhibited increased cell coverage (9.1%; SD 1.43; +2%), the APTES surface had a cell coverage of 4.6% (SD 0.36; -8.4%), the c-RGD surface gained 3.9% (20.3%; SD 1.68), l-RGD surface lost 16% of cell coverage (10.1%; SD 0.17) (Fig. 5). The difference between untreated and APTES surfaces was seen to be statistically significant (p=0.005) after 3 d. Differences between APTES and RGD-modified surfaces were significant as well (l-RGD: p=0.001; c-RGD: p<0.0001).

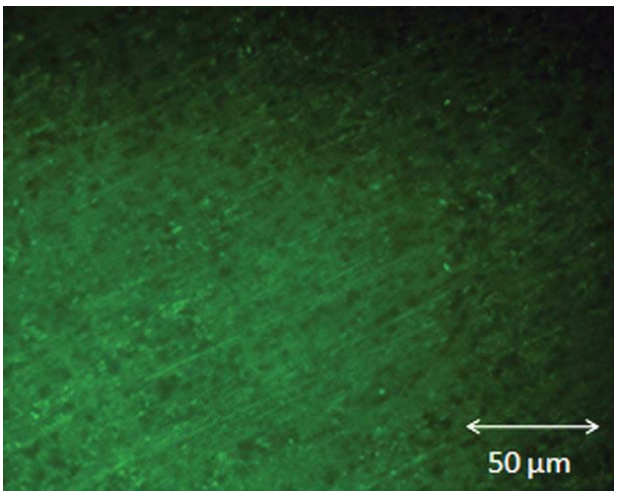


Fig. 2. FITC stain of titanium surface after silanisation with 5% APTES in toluene. Amino groups are distributed equally.

Colonisation on c-RGD surfaces differed significantly from untreated titanium (p=0.001), though no significant difference could be found between untreated and l-RGD surfaces (p=0.722). The comparison between l-RGD and c-RGD surfaces showed a significant difference in favour of c-RGD (p<0.0001; Fig. 4b).

Proliferation

The proliferative activity of ECs was analyzed with the alamarBlue® assay and measured as fluorescence units. Additionally, data are calculated as a percentage of controls (PC). After 24 h, the lowest mean activity was observed in ECs cultured on untreated titanium (7.26 Fluorescence Units (FU); SD 1.05; PC 100%), followed by APTES (24.58 FU; SD 5.29; PC 311%) and l-RGD surfaces (22.46 FU; SD 1.09; PC 279%). The highest proliferative activity could be seen in cultures on c-RGD surfaces (36.56 FU; SD 11.99; PC 463%; Fig. 6a). Accordingly, the difference between untreated and APTES surfaces was seen to be statistically significant (p=0.049). As between untreated and RGD modified surfaces, the differences were significant for c-RGD only (l-RGD: p=0.077; c-RGD: p=0.003). Neither between APTES and RGD modified (l-RGD: p=0.988; c-RGD: p=0.196) nor between the two RGD modifications (p=0.126) were significant differences detected (Fig. 6).

After 3 d on APTES surfaces, a decrease of proliferative activity was observed (-3.48 FU) whereas an increase was seen on untreated (+40.12 FU) and RGD-modified surfaces (l-RGD +49.84; c-RGD +82.43). Altogether, after 3 d of incubation, minimum activity could be observed on APTES surfaces (21.1 FU; SD 4.83; PC 45%), followed by untreated (47.41 FU; SD 4.71; PC 100%), l-RGD (72.3 FU; SD 21.5; PC 153%) and c-RGD surfaces (119 FU; SD 23.6; PC 251%; Fig. 6b). No significant difference between untreated and APTES surfaces (p=0.305) was seen. After 3 d, the difference in proliferation was significant between APTES and RGD modified surfaces (l-RGD: p=0.045; c-RGD: 0.001). Proliferation on c-RGD surfaces differed significant from untreated titanium (p=0.004), though no

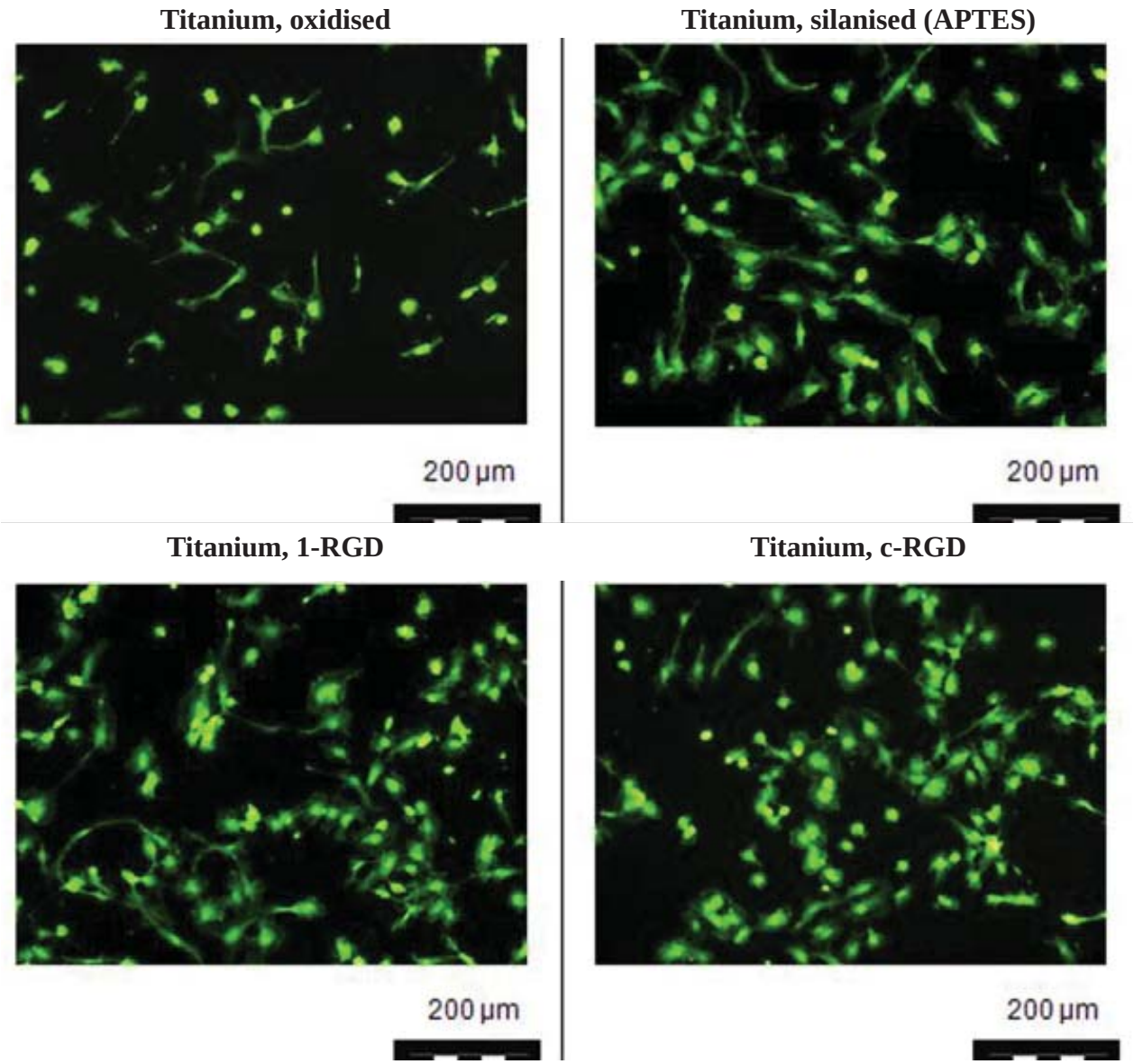


Fig. 3. ECs on pre-treated surfaces after 24 h of incubation

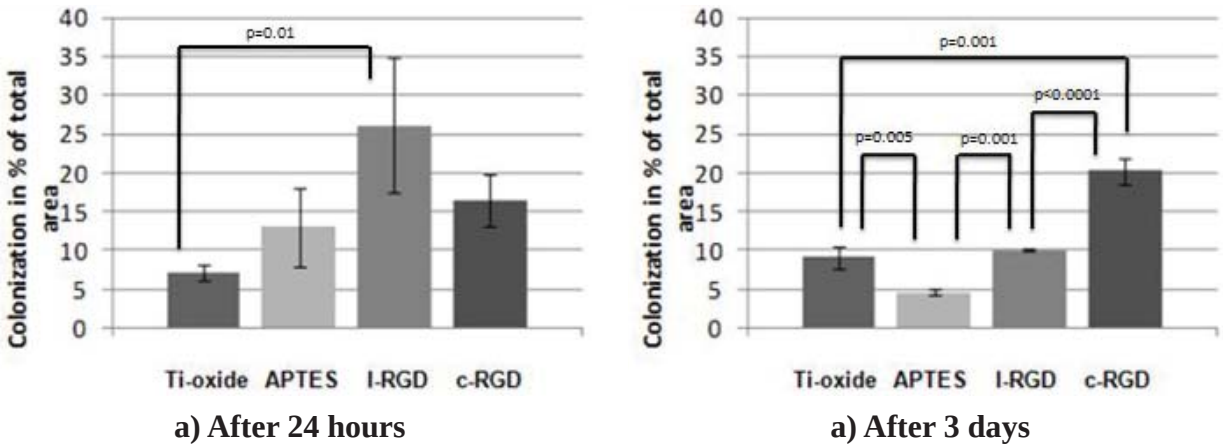


Fig. 4. Percentage of EC-colonisation on pre-treated titanium surfaces after **(a)** 24 h and **(b)** 3 d. Statistically significant differences are given.

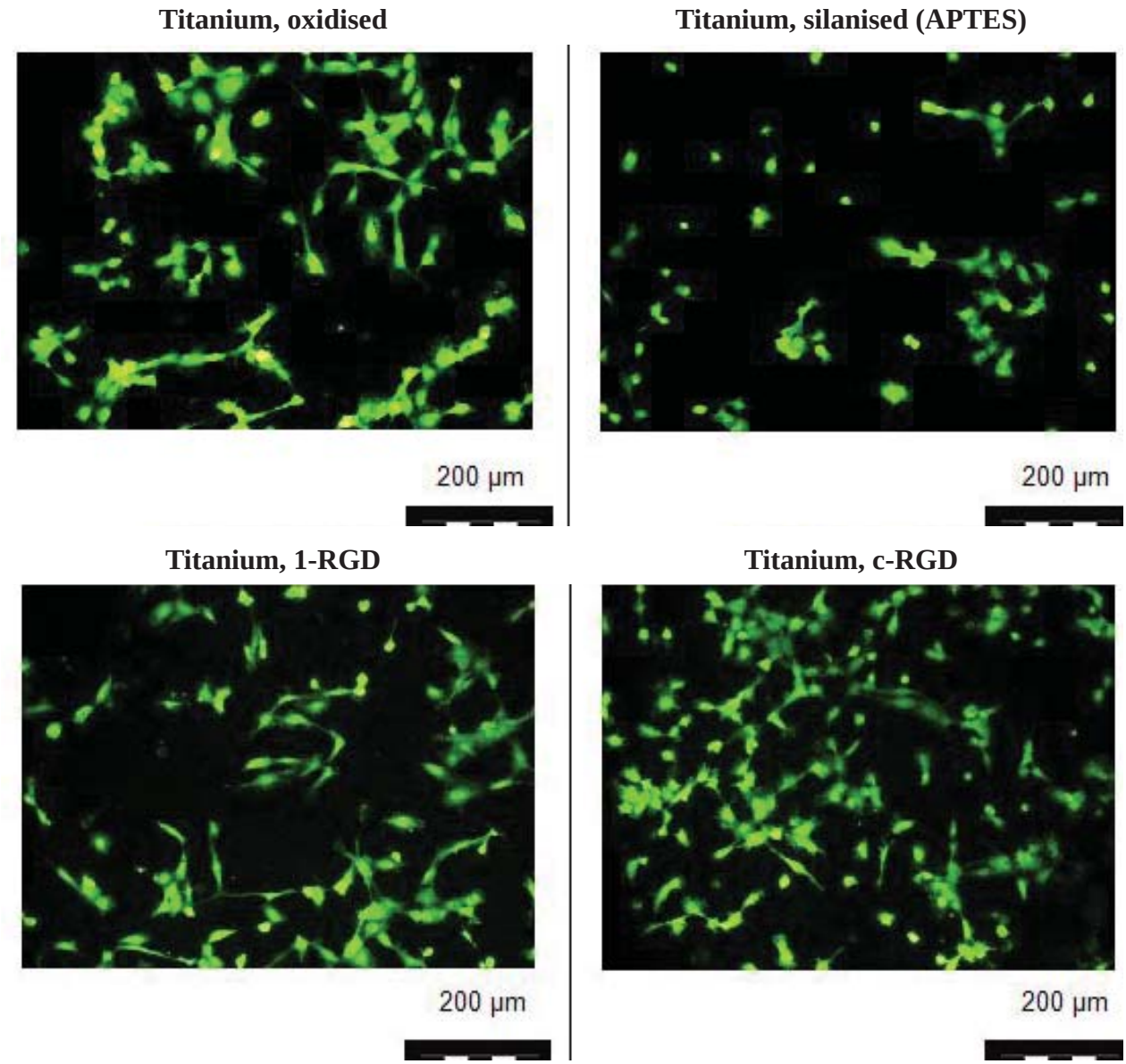


Fig. 5. ECs on pre-treated surfaces after 3 d of incubation.

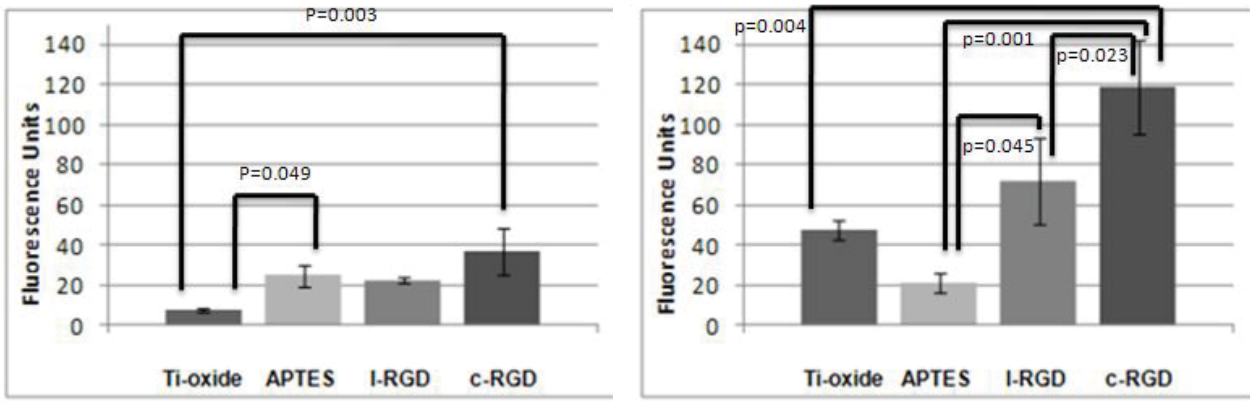
significant difference could be found between untreated and l-RGD surfaces (p=0.54). The comparison between l-RGD and c-RGD surfaces showed a significant difference (p=0.023) in favour of the c-RGD coated titanium (Fig. 6b).

Discussion

The RGD-sequence (Arg-Gly-Asp) is a central element of the integrin-mediated signal transmitting cascade of endothelial cells (outside-in signalling). It has been demonstrated that the RGD-peptide motif alone is sufficient to stimulate cell activity. Accordingly, an improvement of cell adhesion and further cell activity on surfaces of RGD-coated biomaterials has been reported (Rezania and Healy, 1999, Germanier *et al.*, 2006). For RGD-coated synthetic membranes, an enhanced viability of endothelial cells, in accordance with our results, was already proven (Choi *et al.*, 2008; De Mel *et al.*, 2009; Huang *et al.*, 2010). Also, for RGD-coated dental implants, an improved osseointegration has been shown (Schliephake *et al.*, 2002; Schliephake *et al.*, 2005).

RGD-peptides can be manufactured synthetically; there is no potential for the transmission of diseases. Additionally, the peptides are heat-resistant and can be sterilised without damage (Hersel *et al.*, 2003). Therefore, their use might be beneficial in later clinical settings. However, examination of RGD-stability after immobilisation in implant handling needs further *in vitro* and *in vivo* examination.

The RGD-peptide can be synthesised in a linear and in a cyclic form. Between the two forms, functional differences in the interaction between cells and surfaces are described (Verrier *et al.*, 2002). It is of much interest to examine the conformation of the RGD-peptide, which once immobilised to titanium surfaces, will be able to improve biointegration of an implant by stimulation of ECs. The cyclic peptide seems to enhance the binding



a) 24 hours **b) 3 days**

Fig. 6. Intensity of fluorescence/proliferation activity of ECs on pretreated titanium surfaces after **(a)** 24 h and **(b)** 3 d. Proliferative activity was calculated as fluorescence changes based on blank controls. Statistical significant differences are given.

capacity of osteogenic cells on surfaces (Delforge *et al.*, 1998; Hsiong *et al.*, 2008; Zhu *et al.*, 2009). A difference in EC cell coverage and proliferation on titanium surfaces by means of the structure of RGD peptides, which could be shown in our study, has not been investigated.

The analysis of EC coverage consisted of a quantitative interpretation of fluorescent microscopic photographs. Thereby, a visual assessment of EC characteristics (cell count and cell shape) on the different surfaces was conducted. For proliferation, the alamarBlue® assay was used. The resultant change of fluorescence activity can be used as marker of proliferation and cell activity (De Mel *et al*., 2009).

ECs seem to respond to RGD modified surfaces with an increased cell coverage and proliferation. This activity might be contributed the expression of the RGD binding integrin receptor αVβ3 on EC surfaces (Tagada *et al.*, 2007). After 24 h, EC coverage and proliferation was facilitated on silanised and RGD modified surfaces. After 3 d, the strongest cell coverage and proliferation was seen on the surfaces coated with cyclic RGD peptide. For EC coverage on oxidised titanium surfaces after 24 h, l-RGD was superior to c-RGD but not after 3 d. This suggests that non-covalent attachment of l-RGD is more efficient but less stable compared to c-RGD. However, this positive effect of l-RGD was not observed for EC proliferation.

The prolonged and more consistent effect of c-RGD may be due to one of the major advantages of small cyclo-peptides, which is their resistance to proteolysis (Bogdanowich-Knipp *et al.*, 1999; Haubner *et al.*, 1996). The stronger effect of cyclic RGD after 3 d can be further explained by its potentially higher activity due to its specific binding capacity to the integrins αVβ3 and αVβ5 (Dechantsreiter *et al.*, 1999) and its fibronectin-like sterical conformation (Leahy *et al.*, 1996). The ability of cyclo-peptides to bind with higher affinities, as linear peptides to integrin receptors, have been described to be their second major advantage (Bogdanowich-Knipp *et al*., 1999; Haubner *et al*., 1996).

Conclusion

For the l-RGD and c-RGD titanium surface coatings, a positive influence on cell coverage and proliferation of ECs was demonstrated. After 3 d, c-RGD-modified surfaces showed a significantly increased influence on EC coverage and proliferation compared to the l-RGD modified surfaces and compared to the untreated and silanised samples. For EC surface coverage and proliferation, the superiority of c-RGD- compared to l-RGD-coated titanium could be shown. A coating of titanium surfaces with c-RGD peptide may help to direct longer-lasting selective tissue responses. The positive results of c-RGD-coatings on EC stimulation needs to be confirmed in follow-up *in vivo* experiments.

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Discussion with Reviewers

Reviewer I: Have the authors any idea as to the stability of their coatings i.e., can they be handled without damaging etc.?"

Authors: This important issue has not been examined in our study. This topic is under evaluation and will be part of our next paper.

Reviewer II: Normalisation of cell proliferation assay – the method used (normalised to control surface) is a bit vague. For instance, a 279% increase may sound like a lot but without any indication of the cell number being normalised to, this is misleading. What was the actual cell number?

Authors: Generally, nearly 100% of seeded cells adhere after 24 h. We found in our experiments no evidence for lowered adhesion or increased dead cell counts. Therefore, we assumed to measure proliferation and adhesion from at least 50,000 cells after 24 h, which should be sufficient for a reliable calculation.