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# Immobilization of the cell-adhesive peptide Arg–Gly–Asp–Cys (RGDC) on titanium surfaces by covalent chemical attachment

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Surface modification of acid-pretreated titanium with 3-aminopropyltriethoxylsilane (APTES) in dry toluene resulted in covalently bonded siloxane films with surface coverage that was relatively controllable by regulating the reaction conditions. A hetero-bifunctional cross-linker, *N*-succinimidyl-3-maleimidopropionate (SMP), reacted with the terminal amino groups, forming the exposed maleimide groups. Finally, a model cell-binding peptide, Arg–Gly–Asp–Cys (RGDC), was immobilized on the surface through covalent addition of the cysteine thiol groups to the maleimide groups. X-ray photoelectron spectroscopy, radiolabelling techniques, and ellipsometry were used to quantify and characterize the modified surfaces.

## 1. Introduction

Titanium is a successful biocompatible material that is extensively used today for manufacturing bone-anchoring devices, such as dental implants or hip-joint fixation and replacement, as well as for pacemakers, heart-valves or ear-drum drainage tube coatings. It has advantageous bulk and surface properties: in particular, a low modulus of elasticity, a high strength-to-specific-weight ratio, excellent resistance to corrosion and a biocompatible surface oxide film [1]. The surface chemistry and structure are prime factors governing bone integration [2] and there is – from the view point of both surgeon and patient – considerable interest in increasing both speed of formation (healing time) and degree (long-term success) of close bone apposition for cement-free implantation.

Recent *in vitro* and *in vivo* studies on polymer and model oxide surfaces have demonstrated that specific surface–cell interactions and further cell organization can be mediated by surface-immobilized cell-adhesive peptides [3–7]. In our study we have immobilized the peptide Arg–Gly–Asp–Cys (RGDC) through silanization of a titanium metal surface, followed by reaction with a maleimide coupler – a technique adapted from procedures used for the covalent attachment of thiolipids to optical waveguides [8]. Fig. 1 shows the modification steps used in this work: first, silanization of a pretreated titanium surface (surface A) with 3-aminopropyltriethoxylsilane (APTES) in toluene, resulting in surface B with terminal amino groups; secondly, reaction of surface B with a hetero-bifunctional cross-linker, *N*-succinimidyl-3-maleimidopropionate (SMP), in *N*,*N*-dimethyl-formamide (DMF), resulting in surface C with exposed maleimide groups; finally, immobilization of a model cell-binding peptide Arg–Gly–Asp–Cys (RGDC) through covalent addition of the cysteine thiol (–SH) group to the maleimide group (surface D). This functionalization technique has a high flexibility, in the sense that it allows the attachment of any suitable biomolecule having a chemically accessible thiol group.

X-ray photoelectron spectroscopy (XPS), $^{14}C$ radiolabelling techniques, and ellipsometry were used to characterize and quantify the modified surfaces.

## 2. Materials and methods

### 2.1. Substrate and chemicals

Titanium coatings, 100 nm thick, on one side of commercial float glass samples (48 mm × 12 mm × 2 mm)

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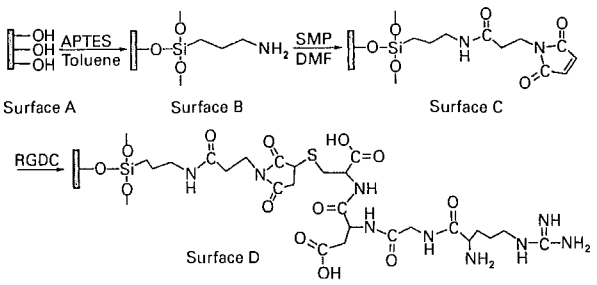


*Figure 1* Sequence of the titanium surface modification procedure. Surface A corresponds to the pretreated titanium surface.

were produced in a Leybold Z600 DC-magnetron sputtering facility [9]. Pure water was obtained from an EASYpure™ device, Barnstead/USA. [$^{14}$C]-formaldehyde for radioactive labelling (specific activity 54 mCi mmol$^{-1}$) was purchased from Amersham, Buckinghamshire/UK. 3-aminopropyltriethoxylsilane (APTES) was obtained from Fluka, Buchs/Switzerland, distilled, stored, and used under a nitrogen atmosphere. RGDC was purchased from Bachem AG, Bubendorf/Switzerland, and all other chemicals from Fluka.

## 2.2. Surface analysis

XPS spectra were recorded using a Specs SAGE 100 system with unmonochromatized Mg$K_\alpha$ radiation at 300 W (12 kV). Measurements were taken at a take-off angle of 90° with respect to the sample surface. The analysed area is typically 9 × 9 mm$^2$. Survey scans over a binding energy range of 0–1150 eV were taken for each sample with a constant detector pass energy range of 50 eV, followed by high-resolution XPS measurement (pass energy 14 eV) for quantitative measurements of binding energy and atomic concentration. Background subtraction, peak integration and fitting were carried out using SpecsLab software. Electron binding energies were calibrated to the hydrocarbon C 1s at 284.6 eV on pure titanium surfaces. To convert peak areas to surface concentration, sensitivity factors published by Evans *et al.* were used [10].

Surface film thickness was obtained using a Gaertner L-116C ellipsometer and software. The angle of incidence was set as 70°. The optical constants of the pretreated titanium surfaces were determined in at least four different areas on each individual substrate. The characterized substrates were then silanized. The thickness of the silanized organic film was calculated using the software for a single organic thin film and a refractive index of 1.44 [11].

## 2.3. Chemical functionalization

### *2.3.1. Pretreatment*

Prior to silanization, the titanium-coated substrates were first pretreated by incubating them in a solution of 1 : 1 (vol/vol) methanol/HCl (37%) at room temperature for 30 min, followed by rinsing five times with water. The substrates were then treated in concentrated sulfuric acid at room temperature for 15 min, and again washed extensively with water. After treatment in boiling water for 10 min, they were washed five times with water, rinsed with acetone, and dried under vacuum for 12 h. The pretreatment resulted in a slight decrease of titanium coating thickness; XPS measurements, however, showed no glass to be exposed on the surfaces.

### *2.3.2. Silanization*

Silanization was performed by incubating single substrate samples at 120 °C for 3 h in 30 ml dry toluene containing 0.5 ml (2.15 mmol) APTES. After reaction, the substrate was ultrasonically washed with chloroform (five times), acetone (twice), methanol (five times), and extensively with water. The APTES-modified substrates were stored in argon for XPS measurements and in water or organic solvents for further reactions as detailed below.

### *2.3.3. [$^{14}$C]-formaldehyde labelling of APTES-grafted surface*

This was performed with the APTES-grafted samples mounted in a sample holder with four holes (diameter 7 mm), titanium metal surface facing upwards; 50 µl 10.0 mM $NaBH_3CN$ and 2.0 mM [$^{14}$C]-formaldehyde in $CH_3CN$ were injected into each hole. After incubation for 12 h at 20 °C, the excess radioactive solution was removed and the exposed surface washed with $CH_3CN$ ten times and water ten times. The sample was dried in a nitrogen jet and then cut into four pieces. The radioactivity of each piece was measured by scintillation counting in 5 ml scintillation fluid (1080 ml toluene, 920 ml Triton X-100, 5.4 g 2,5-diphenyloxazole, 0.2 g 1,4-*bis*-2-(5-phenyloxazolyl) benzene, and 40 ml acetic acid) on a Betamatic V liquid scintillation counter.

### *2.3.4. Preparation of maleimide-(surface C) and RGDC-grafted (surface D) substrates*

A 24 mm × 12 mm silanized titanium substrate was placed in a small bottle with 400 µl *N*,*N*-dimethylformamide (DMF) containing 3 mg SMP (28 mM) with only the titanium surfaces contacting the solution. After incubation at room temperature for 1 h (with 2 min sonication every 10 min), the substrate was washed with DMF (five times) and water (ten times). To prevent hydrolysis, the substrates were immediately subject to the next chemical step: a 12 mm × 12 mm maleimide-grafted substrate was incubated at room temperature for 2 h (with 1 min sonication every 10 min) in 300 µl pure water containing 1.35 mg RGDC (10 mM). The pH had to be adjusted to 7 with 0.1 M NaOH because of the presence of $CF_3COOH$ in the peptide solutions. The RGDC-grafted substrates were washed thoroughly with water, dried with nitrogen and stored in argon.

## 3. Results and discussion

### 3.1. Pretreated surface

The XPS data in Table I show that the pretreated surface A was relatively clean. The surface carbon concentration of 18% is typical for organic contaminant levels on "clean" oxide surfaces. No further contaminants were detected by XPS. Special precautions were taken to exclude silicon contamination (such as PDMS), which is a frequent problem as regards surface cleanliness and functionalization.

### 3.2. Silanization of the surface

The thickness of the organic siloxane layer and the number of reactive $NH_2$ groups introduced after silanization with APTES were quantified by XPS, ellipsometry, and [$^{14}$C]-formaldehyde radiology measurements (Table I). XPS survey spectra show signals of carbon, oxygen, titanium, nitrogen and silicon with intensities depending on the degree of silanization. After 3 h reflux silanization in dry toluene, only small signals of nitrogen and silicon in the range of 1%–3% surface concentration were detected, estimated to correspond to a submonolayer to monolayer coverage (approximately 0.5 nm from ellipsometric measurements). A surface coverage of 0.22 nmol $NH_2$-groups per cm$^2$ was calculated from radioactivity values of [$^{14}$C]-methylated surfaces [12]. Further incubation for another 12 h at room temperature (12 h RT in Table I) led to multilayer formation with a thickness of 2.6 nm and a surface coverage of 0.57 nmol $NH_2$-groups per cm$^2$, supported by the XPS results in Table I. If samples were washed after 3 h reflux silanization and returned to the same reaction solution for another 12 h at room temperature (wash + 12 h RT in Table I), a thicker siloxane film (ca. 5.2 nm) with a higher surface coverage of 1.54 nmol $NH_2$-groups per cm$^2$ was formed. Only a very weak titanium signal was detected by XPS in this case, confirming that the surface layer thickness is of the order of the XPS information depth (ca. 6 nm). The XPS N/Si atomic ratios in Table I show a gradual decrease with further increasing siloxane film thickness. This is likely to be the result of increasing lateral polymerization and network formation reducing the surface concentration of freely accessible amino groups.

### 3.3. Covalent attachment of peptide

For the further reactions of surface B with the cross-linker SMP and subsequently with RGDC, multilayer siloxane films (APTES 3 h reflux + 12 h RT) were chosen because both the attached siloxane and the underlying titanium can be obviously detected with XPS. The subsequent surface chemical changes were followed by XPS at each stage.

### 3.4. XPS chemical shifts and spectra deconvolution

The individual XPS spectra of C 1s, O 1s, and N 1s on surfaces A, B, C, and D are compared in Figs 2–4. Table II lists the proportion of the individual chemical states after deconvolution.

#### *3.4.1. C 1s spectra*

The major hydrocarbon peaks at 285.2 eV on surfaces B, C, and D (Fig. 2) appear at 0.6 eV higher energy compared to the hydrocarbon contaminants on the titanium metal surface A (284.6 eV). This is expected if the physically adsorbed hydrocarbons are replaced by the covalent-bonded APTES. The binding energy at 286.8 eV is assigned to amines ($CH_2N$) and alkoxy groups ($CH_2$–O). From surface B to C, an additional peak at much higher binding energy 289.0 eV attributed to carbon atoms in imide groups [$C$(=O)–N–$C$(=O)] strongly confirms the covalent bound maleimide groups. The increase at this position from C to D is attributed partly to carboxyl (*C*OOH) and guanidine [NH–$C$(=NH)–$NH_2$] carbons. C 1s energies of the amide functions (O=$C$–N) are expected at around 288.0 eV, but are difficult to be deconvoluted from the adjacent peaks. They contribute to both of the fitting peaks at 286.8 and 289.0 eV. The increase of intensity in the energy range 286.8–289.0 eV and the decrease at 285.2 eV from surface B to D reflect the results of the surface modification process.

#### *3.4.2. O 1s spectra*

Fig. 3 shows the evolution of O 1s peaks from surface A to B, C, and D. The main O 1s contribution in the case of titanium metal/oxide surface A is at a binding energy of 530.0 eV, typical for the natural $TiO_2$ surface layer [13]. The high-energy tail is attributed to surface hydroxides (and possibly organic contaminants). For surface B, a prominent siloxane O 1s peak at 532.5 eV is present; this peak increases with the siloxane surface coverage and is the only peak at high siloxane coverage of ca. 50 nm (not shown here). The O 1s position of the new amide functions ($O$=C–N) on surfaces C and D is expected at a position of 532.0 eV and overlaps with the siloxane signals. The carboxyl groups (*C*O*O*H) from RGDC sequence (surface D) cannot be separately deconvoluted and most likely also appear at around 532.0–532.5 eV. It is expected that the two carboxylic O 1s energies are not different if intraion or interion bonds are formed with amino moieties within the functionalized layer. By deconvolution of the two peaks at 530.0 and 532.5 eV (Table II), the intensity increase at 532.5 eV and decrease at 530.0 eV indicate that the surface chemistry changes from surface A to D.

#### *3.4.3. N 1s spectra*

Surface B shows two peaks at 399.7 eV (75%) (free amino groups) and 401.7 eV (25%, H-bonded and/or positively charged amines), also observed by other groups for APTES-modified titanium surfaces [14]. On surface C the imide [C(=O)–$N$–C(=O)] (N 1s at 400.6 eV) is introduced and part of the amine functions on surface B are converted to amides (O=C–$N$, N 1s at 400.0 eV). This can explain the shift of the peak

TABLE I Pretreated and APTES silanized titanium surfaces characterized with XPS for elemental composition, ellipsometry for surface layer thickness, and radioactivity measurements for surface coverage. The standard deviations refer to five samples from different batches

| Sample | Elemental composition | | | | | | Thickness (nm + S.D.) | Radio activity (nCi cm$^{-2}$ ± S.D.) | Surface coverage[b] (nmol cm$^{-2}$ ± S.D.) |
|---|---|---|---|---|---|---|---|---|---|
| | C (% ± S.D.) | O (% ± S.D.) | Ti (% ± S.D.) | N (% ± S.D.) | Si (% ± S.D.) | N/Si[a] | | | |
| Pretreated titanium | 18.3 ± 1.4 | 58.4 ± 2.6 | 23.3 ± 1.3 | | | | | | |
| APTES 3 h reflux | 18.5 ± 1.3 | 54.0 ± 2.3 | 22.7 ± 1.7 | 2.2 ± 0.8 | 2.6 ± 0.8 | 0.85 | 0.4 ± 0.2 | 11.7 ± 3.1 | 0.22 ± 0.06 |
| APTES 3 h reflux + 12 h RT | 24.3 ± 2.8 | 50.0 ± 3.4 | 16.1 ± 2.2 | 4.1 ± 1.3 | 5.4 ± 1.3 | 0.76 | 2.6 ± 0.4 | 30.4 ± 4.6 | 0.57 ± 0.09 |
| APTES 3 h reflux + wash + 12 h RT | 48.4 ± 4.6 | 28.2 ± 2.7 | 1.5 ± 0.8 | 8.7 ± 1.9 | 13.3 ± 2.7 | 0.66 | 5.2 ± 0.6 | 81.9 ± 11.7 | 1.54 ± 0.22 |

[a]Mean atomic ratio of N/Si.
[b]Assuming a molar ratio [H$^{14}$CHO]/[NH$_2$] = 1, refer to [12].

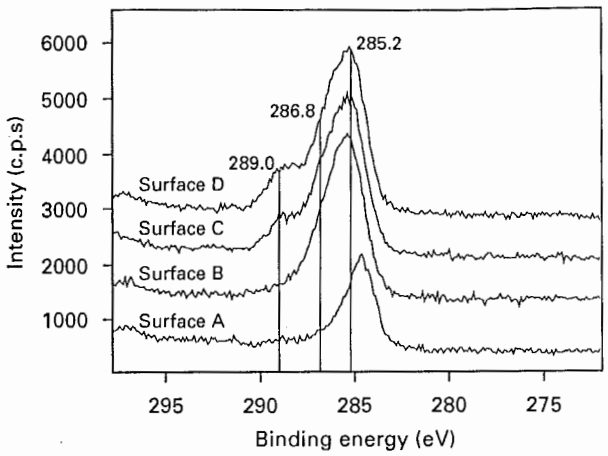


*Figure 2* XPS O 1s spectra of surfaces A, B, C, and D.

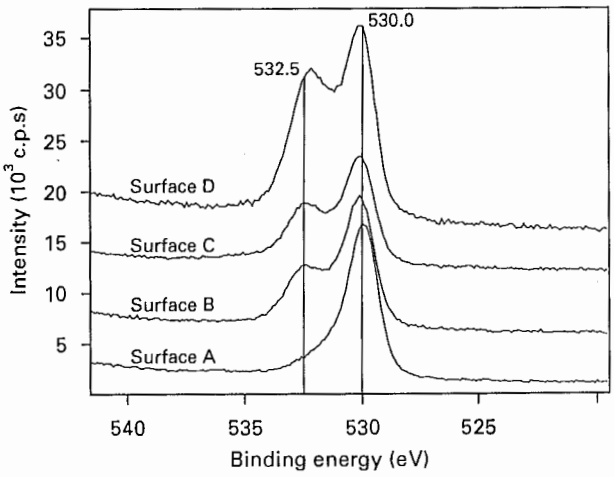


*Figure 3* XPS C 1s spectra of surfaces A, B, C, and D.

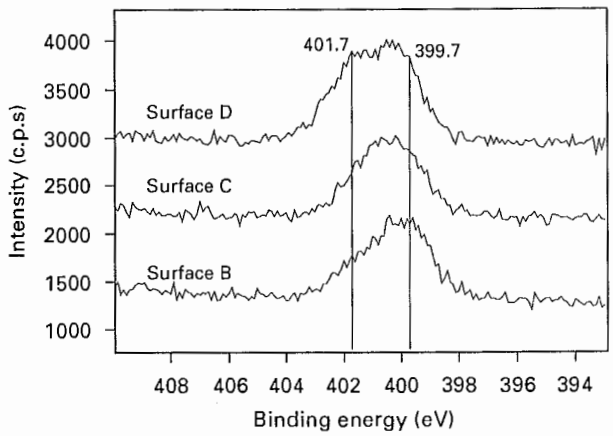


*Figure 4* XPS N 1s spectra of surfaces B, C, and D.

maximum to 400.6 eV. On surface D, the increase of high binding energy peaks at 401.7 eV is clearly due to the more positively charged nitrogen species in guanidine and amino moieties forming intraion or interion bonds with carboxyl groups.

## 3.5. Estimation of reaction yields

Because of the inevitable carbon contamination and oxygen concentration on the starting surface A,

TABLE II Results of deconvolution of the XPS spectra in Figs 2, 3, and 4: binding energies (eV) and relative ($\Sigma = 100\%$) intensities of the deconvolution peaks

| Surface | C 1s | | | O 1s | | N 1s | | N/Si[b] |
|---|---|---|---|---|---|---|---|---|
| | 285.2 eV | 286.8 eV | 289.0 eV | 530.0 eV | 532.5 eV | 399.7 eV | 401.7 eV | |
| A | 71.1%[a] | 21.2%[a] | 7.7%[a] | 87.3% | 12.7% | – | – | – |
| B | 68.1% | 27.2% | 4.7% | 62.2% | 37.8% | 75.2% | 24.8% | 0.70 |
| C | 57.1% | 31.3% | 11.7% | 55.0% | 45.0% | 61.4% | 38.6% | 0.89 |
| D | 50.3% | 34.5% | 15.2% | 51.2% | 48.8% | 44.2% | 55.8% | 1.13 |

[a]The C 1s binding energies of the pretreated titanium surface A are 284.6, 286.1, and 288.5 eV.
[b]Mean atomic ratio of N/Si.

quantitative statements as regards reaction yields for the subsequent modification reactions, are difficult or impossible. The N 1s signal has been used by Ratner as a quantitative measure of the amount of proteins on a surface [15]. Here the N 1s signals are used to calculate the reaction yields. However, the absolute atomic concentrations depend on the measured volume and the chemical components in this volume. Changes of the chemical components and the film thickness on surfaces B, C, and D make the estimation difficult. Fortunately, silicon, which is not present on the starting surface A, can be used as an internal reference. The absolute silicon surface concentration remains constant on surfaces B, C, and D (no hydrolytic decomposition of the siloxane layer during the subsequent modification steps was detected by XPS). Because the whole modified layer thickness is below the information depth of XPS, the reaction yield can be deduced from the following simple formula

$$\text{Reaction yield (B} \rightarrow \text{C)} = \frac{\text{(number of imide-N on surface C)}}{\text{(number of N on surface B)}} = \frac{n_{N(C)} - n_{N(B)}}{n_{N(B)}} = \frac{(N/Si)_C - (N/Si)_B}{(N/Si)_B} = \frac{0.89 - 0.70}{0.70} = 0.27 \quad (1)$$

$$\text{Reaction yield (C} \rightarrow \text{D)} = \frac{\text{[(number of N in RGDC)/7]}}{\text{(number of imide-N)}} = \frac{[n_{N(D)} - n_{N(C)}]/7}{n_{N(C)} - n_{n(B)}} = \frac{[(N/Si)_D - (N/Si)_C]/7}{(N/Si)_C - (N/Si)_B} = \frac{(1.13 - 0.89)/7}{0.89 - 0.70} = 0.18 \quad (2)$$

where $n_{N(i)}$ is the absolute nitrogen atomic concentration of surface $i$, and $(N/Si)_i$ is the atomic ratio of nitrogen to silicon of surface $i$ from Table II. The divisor 7 takes account of the seven nitrogen atoms in one RGDC molecule. The conclusion from the semiquantitative coverage estimations is that under the chosen experimental conditions, only about one-fourth of amines on surface B were converted to maleimide functions and one-fifth of maleimides converted to a RGDC-grafted surface. The optimum reaction conditions are still under investigation. Combining the $NH_2$ surface coverage of 0.57 nmol cm$^{-2}$ (Table I) and the reaction yields, the peptide surface coverage was calculated to be approximately 0.03 nmol cm$^{-2}$. This corresponds to submonolayer surface coverage regardless of the "lying-down" conformation of peptides. However, it is not likely that a full surface coverage of peptides is optimum for specific cell recognition and the currently achieved coverage can be expected to be sufficiently high to influence cell-surface adhesion [3].

## 4. Conclusion

The present results show that the model cell-adhesive peptide, Arg–Gly–Asp–Cys (RGDC), can be covalently bound to titanium (oxide) surfaces by the described procedure using silanization, cross-linking, and peptide attachment through the cysteine thiol group. The individual reaction steps were semiquantitatively characterized by X-ray photoelectron spectroscopy (XPS), radiolabelling techniques, and ellipsometry. The RGDC surface coverage is estimated to be 0.03 nmol cm$^{-2}$, expected to be high enough for specific surface–cell interactions. Future perspectives of this study include the investigation of protein adsorption and osteoblast interaction with peptide-functionalized titanium surfaces.

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