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Comparative Study of Collagen and Gelatin Coatings on Titanium Surfaces

Els Vanderleyden,[1] *Luc Van Hoorebeke,*[2] *Etienne Schacht,*[1] *Peter Dubruel**[1]

Summary: The vast majority of studies in the bone tissue engineering field are focused on the surface modification of titanium scaffolds to obtain integration of the scaffold in the surrounding bone tissue. Our approach consisted in benefiting from the advantages of the cell-interaction capabilities of collagen and gelatin. The biopolymers were immobilised onto the Ti surface through different methods and the stability of the obtained coatings was determined. The obtained results reveal that covalent immobilisation of collagen and gelatin is required to obtain stable surface coatings.

Keywords: biopolymers; surface modification; tissue engineering; titanium

Introduction

Until the present day, titanium (Ti) and its alloys are still the choice of interest for orthopaedic applications and dental implants. After all, these materials are known to posses a combination of properties which makes them particularly suitable for biomedical applications: (1) a naturally formed and chemically stable oxide layer which implies a high corrosion resistance, (2) a high specific strength, (3) a low specific weight, (4) good mechanical properties (low elastic modulus), (5) low metal ion release and most importantly (5) the biocompatibility.[1] The stable, natural oxide layer is, however, also responsible for the bio-inertness of Ti. This implies a lack of interaction between the implant and the surrounding tissue and thus a lack in integration of the implant. In combination with the stress shielding phenomenon, as a result of the high stiffness of Ti compared to bone, this can eventually lead to implant loosening. Surface modification of Ti to achieve better cell adhesion, better fixation and thus lesser micro-movements of the implant and formation of fibrotic tissue, can improve the integration of the implant in the surrounding bone tissue and accelerate bone repair.

Different methods to bio-activate the Ti surface are being explored world wide, including the deposition of calcium phosphates,[2–4] the adsorption of proteins like collagen[5–7] and fibronectin,[8–9] the attachment of peptides by the use of self-assembled monolayers (SAM's) and polymer coatings, etc. Binding of molecules to the surface can be physical, through adsorption or incorporation in the top surface layer, or chemical, through covalent bonds.[10–11] In the latter case, most of the strategies are based on the introduction of reactive groups onto the Ti surface by silanisation reactions.[12–16]

In a previous paper, we reported on the application of different silanisation procedures and silanisation reagents for the functionalisation of Ti surfaces.[17] We showed that a methacrylate modified Ti surface was an appropriate starting material for the subsequent bio-activation of Ti surfaces. In that study, we applied the covalent immobilisation of gelatin on the Ti surface as proof of concept. Gelatin is a water soluble, biodegradable polypeptide,

[1] Polymer Chemistry and Biomaterials Research Group, University of Ghent, Krijgslaan 281 S4, 9000 Ghent, Belgium
Fax: (+32)92644972; E-mail: Peter.dubruel@ugent.be
[2] Department of Physics and Astronomy, University of Ghent, Proeftuinstraat 86, 9000 Ghent, Belgium

derived from collagen by partial hydrolysis.[18] Altough it is used frequently in different areas of the biomedical world,[19–21] combinations with Ti are rather rare. Cai *et al*. deposited gelatin and chitosan onto Ti via a layer-by-layer technique which resulted in a increase in cell biocompatibility.[22] Heydari *et al.* immobilised a photo-reactive gelatin derivative onto a Ti surface in a micropattern fashion. Cell tests revealed that the seeded cells showed a pattern determined by the gelatin-micropattern on the surface.[23] A similar study was performed by Weng *et al*.[24] Kuangl *et al*. followed a different approach: they modified the Ti surface with 2-hydroxyethyl methacrylate (HEMA) and subsequently immobilised gelatin by gamma-irradiation.[25] In addition to gelatin, native collagen has also been applied on Ti. Collagen is one of the most abundant proteins in the human body as it is one of the main constituents of the extracellular matrix. Thanks to its enzymatic degradability, unique physico-chemical, mechanical and biological properties and its excellent biocompatibility, collagen is known to be one of the most useful biomaterials.[5,26–33] In case of Ti surfaces, researchers have shown that collagen coatings result in a better osteoblast adhesion[34] and differentiation *in vitro* [35] and in an increase in peri-implant bone growth and bon-implant contact *in vivo*.[5–6]

In the present paper, we aimed to perform a comparative study in which the immobilisation of gelatin and collagen are compared. For gelatin, the previously developed method was applied and investigated for its stability. For the collagen deposition, two different immobilisaton strategies were compared. The first one was developed by the group of Dupont-Gillain *et al*. and implies an adsorptive way of generating a collagen coating.[36] The second strategy includes the covalent attachment of collagen to an amine-functionalised Ti surface, developed by Müller *et al*.[37]

All applied coatings were studied by XPS to discriminate possible differences in chemical composition.

Materials and Methods

Materials

Ti foils (99.6% pure according to the data sheet) were obtained from Chempur. Ti plates (commercially pure grade 2, ASM 4902 F) were from Nippon Steel Corporation. Concentrated HCl (37%) was from Panreac Quimica S.A, H_2O_2 (30%) from Aldrich and NH_4OH (25%) from Acros. HPLC grade aceton, HPLC grade pentaan, acetic acid, 3-aminopropyltriethoxysilane (APTES), 3-(trimethoxysilyl)propyl methacrylate (TMSPMA) and collagen type I from New Zealand white rabbits were all purchased from Sigma. Cyclohexane (from Fiers) was dried with CaH_2 and distilled before use. Gelatin type B, isolated from bovine skins after an alkaline pretreatment, was a kind gift from Rousselot. Gelatin with an isoelectric point of ca. 5, gel strength of 257 Bloom and viscosity (6.67%, 60 °C) of 4.88 mPa.s was used. Gelatin type A, isolated from porcine skins after an acid pretreatment, was purchased from Rousselot. Gelatin with an isoelectric point of ca. 8, gel strength of 202 Bloom and viscosity (6.67%, 60 °C) of 2.97 mPa.s was used. The water used througout this study was milliQ (double distilled, >18 MegaOhm/cm).

Methods

Pretreatment of Ti

Cleaning of the Ti samples consisted of subsequent ultrasonic treatments in (1) cyclohexane, (2) 10 N HCl and (3) milliQ water. After cleaning, the samples were oxidised for 20 min in a 1/1/5 NH_4OH/H_2O_2/H_2O-mixture. These steps are described in more detail in a previous paper.[17]

Immobilisation of Gelatin on Ti

For the immobilisation of methacrylamide modified gelatin type A (Gel-mod Type A) oxidised Ti samples were dipcoated with a solution of Gel-mod Type A in milliQ water (0.1 to 5 w/v%) at 40 °C. For the immobilisation of Gel-mod Type B Ti samples were first silanised with a 10 v/v%

solution of TMSPMA in pentane and afterwards dipcoated with a solution of Gel-mod Type B in milliQ water (0.1 to 10 w/v%) at 40 °C. The degrees of modification of Gel-mod Type A and B were respectively 74% and 78% based on the lysine side chains. The synthesis and the characterisation of methacrylamide modified gelatin has been reported before.[38] After the dipcoating process the gelatin was radically crosslinked on the surface by e-beam treatment. The samples were irradiated with electrons from a 15 MeV linear electron accelerator. This accelerator delivered electron beams with a well-defined energy in the range 3–15 MeV and with an average power up to 5 kW. For electron irradiation, an 80 μA 10 MeV beam traversed a water-cooled vacuum window and 80 cm of air, so that the lateral dose distribution was flattened by scattering. The electrons that interacted with the samples had an energy of 8 MeV (that is 10 MeV minus the energy-loss in the vacuum window). The average duration for a 25 kGy irradiation was 400 s. Before every irradiation, a dosimetric calibration was performed.

In case of UV crosslinked coatings the photoinitiator Irgacure® 2959 was added to the Gel-mod solution (2 mol% relative to the amount of crosslinkable groups). After dipcoating the samples were irradiated with UV-C for 30 min.

Immobilisation of Collagen on Ti

Adsorption of Collagen. This method is based on a previous study.[36] Shortly, collagen Type I was dissolved in 0.2 M acetic acid (1.5 mg/ml) at 4 °C. The solution was diluted with (cold) phosphate buffered saline (PBS, pH = 5.8) untill 150 μg/ml and incubated at 37 °C during 15 min or 2 days. After incubation a part of the solution was further diluted untill 40 μg/ml. Cleaned and oxidised Ti samples were incubated in 2 ml of a collagen solution (40 or 150 μg/ml) during 1 h or 24 h at 37 °C. The samples were rinsed with milliQ water without exposing them to air. This was done by adding 2 ml of milliQ water, removing 3 ml of solution, adding again 2 ml of water and repeating these two last steps four times. Afterwards, the samples were gently blown dried with nitrogen.

Covalent Immobilisation of Collagen. The applied method is also described in a previous article.[37] Collagen Type I was dissolved in 0.2 M acetic acid (4 mg/ml) at 4 °C. The solution was diluted with PBS (pH = 5.5) untill 1 mg/ml. Oxidised Ti samples were silanised for 24 h with a 1 v/v% solution of APTES in pentane and subsequently incubated in 2 ml of the collagen solution for 4 h at room temperature. After 1 h incubation 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were added in such an amount that final concentrations of 2.5 mg/ml EDC and 0.63 mg/ml NHS were obtained. The samples were rinsed with milliQ water without exposing them to air. This was done by adding 2 ml of milliQ water, removing 3 ml of solution, adding again 2 ml of water and repeating these two last steps four times. Afterwards, the samples were gently blown dried with nitrogen.

Stability Study

Modified Ti samples were incubated in phospate buffer (50mM, pH = 7.4) at 37 °C during different incubation times. After incubation, the samples were gently rinsed with water and aceton and dried with pressurised air.

X-Ray Photo-Electron Spectroscopy

The chemical composition of the different Ti-surfaces was determined using "FISONS S-PROBE", a dedicated XPS (X-ray photoelectron spectroscopy) instrument designed to give the ultimate in performance, while providing a high sample throughput. The fine focus Al-Ka source with a quartz monochromator, developed by Fisons Instruments Surface Science ensures lower background and higher sensitivity than conventional twin anode sources. All measurements were performed in a vacuum of at least 10^{-9} Pa. Wide and narrow-scan spectra were acquired at pass

energy of 158 and 56 eV, respectively. The binding energy was calibrated by the C 1s peak at 284.6 eV. The spot size used was 250 μm on 1 mm. Data analysis was performed using S-PROBE software. The measured spectrum was displayed as a plot of the number of electrons (electron counts) versus electron binding energy in a fixed, small energy interval. Peak area and peak height sensitivity factors were used for the quantifications. All surface compositions reported in this work are expressed as atm%.

Results and Discussion

Immobilisation Strategies of Gelatin

In the present work, we aimed to compare different strategies for immobilising gelatin onto Ti surfaces. Both strategies, depicted in Figure 1, have the final goal to coat Ti surfaces with a homogeneous and stable layer of gelatin by applying a crosslinking reaction. The immobilised polymer layer should ideally enhance the cell-interactive properties of Ti surfaces since they are known to be biocompatible while lacking cell-interactive properties. Both methods developed are based on the crosslinking of methacrylamide modified gelatin on Ti surfaces.

The first strategy implies a physical interaction between Gel-mod Type A and the oxidised Ti surface. Gelatin Type A possesses an iso-electric point (IEP) of 7 to 9 while an oxidised Ti surface possesses an IEP of 5 to 6. Hence, at a pH of 7, the gelatin has a positive net charge and the Ti surface a negative one. Consequently, when an oxidised Ti surface is dipcoated in an aqueous solution of Gel-mod Type A, electrostatic interactions can be anticipated. Subsequent e-beam irradiation (25 kGy) initiates crosslinking of the Gel-mod chains which eventually leads to an insoluble gelatin layer.

In a second strategy, Gel-mod Type B was covalently coupled to Ti surfaces. For this approach, oxidised Ti surfaces were first silanised using TMSPMA. Gelatin Type B has an IEP of ca. 5 and is thus negatively charged at neutral pH, as the oxidised Ti surface. Electrostatic binding between the two is thus impossible and a functionalisation of the Ti surface through a silanisation reaction is required. In a next step, the silanised surfaces were dipcoated in an aqueous solution of Gel-mod Type B, followed by an e-beam treatment (25 kGy). This treatment led to mutual crosslinking of the Gel-mod chains and also to crosslinking between the Gel-mod chains and the methacrylates on the Ti surface. Using this approach, a covalent bond of the gelatin coating to the Ti surface in stead of a physical bond is obtained.

In our opinion, the main advantages of both approaches applied in the present work include (1) the speed of operation, (2) the low overall cost and (3) the combination of surface modification and sterilisation by the applied e-beam treatment.

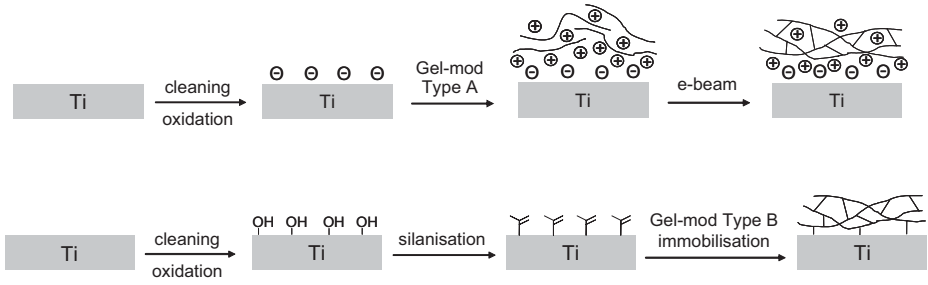

Figure 1.
Schematic representation of the different concepts of gelatin immobilisation: physical (upper) and covalent (lower) coupling to a Ti surface.

The gelatin modified surfaces were evaluated for their stability by determining the surface composition after different incubation times in a buffer solution at 37 °C.

XPS Analysis of the Applied Gelatin Coating

In the first strategy, Gel-mod Type A was immobilised onto the Ti surface by dipcoating an oxidised Ti surface in an aqueous solution of Gel-mod Type A, followed by a cross-linking of the polymer on the metal surface by e-beam irradiation. Atomic compositions, as determined by XPS, show a drastic change in surface composition upon gelatin immobilisation (Table 1). Compared to an oxidised Ti surface, the Ti signal disappears whereas the O signal decreased to half its original value when a gelatin concentration of 0.1 w/v% is applied. The variation in these two signals, that first characterised the Ti oxide layer covering the Ti surface, now indicates a polymer coverage of the metal surface. This was also reflected by an increase in the C signal and the appearance of a N signal upon polymer immobilisation. The absence of a Ti signal indicates a full coverage of the surface by the polymer so that both the C and N signal, as well as the O signal, originate from immobilised gelatin. Similar results were obtained for higher gelatin concentrations (0.5–5 w/v%, data not shown). The surface compositions measured merely reflect the composition of gelatin, since XPS analysis only yields information on the composition of the 1 to 10 nm thick top layer.

In case of Gel-mod Type B modified Ti, the surfaces were first functionalised by silanisation using TMSPMA. This already led to a fully covered surface (0% Ti, Table 1) with the appearance of a characteristic Si signal. Applying a subsequent gelatin layer was proven by a strong decrease or disappearance of this Si signal and the presence of a N signal. This effect was already observed from a concentration as low as 0.1 w/v % (Table 1) up to 5 w/v% (data not shown). As was the case for the Gel-mod Type A coating, the atomic composition of the Gel-mod Type B coated Ti surfaces match the composition of gelatin itself.

Table 1.
Atomic surface composition of Gel-mod Type A and B modified Ti samples, determined by XPS analysis. The surface compositions of an oxidised and a silanised Ti sample are added as references.

Modification	%Ti	%C	%O	%N	%Si
Oxidised Ti	13	38	49	–	–
0.1% Gel-mod Type A	–	64	20	14	2
Silanised Ti	–	60	32	–	7
0.1% Gel-mod Type B	–	65	23	8	4

Stability Study of the Applied Gelatin Coatings

An important aspect in the development of coatings on existing biomaterials, is the stability after application. As an example, when performing cell interaction studies, it is important that the applied coating at least remains stable during the adhesion of the cells to the surface. Depending on the type, cells usually need 10 min up to 3 h after seeding to adhere to a surface. Of course, the goal is to develop a coating with an even longer stability. The stability of the applied coatings was evaluated by incubating the coated Ti samples in phosphate buffer (pH = 7.4) at 37 °C for 1 to 24 h. In case of the crosslinked gelatins, polymer chains that are not chemically attached into the polymer network after the e-beam treatment will dissolve since the sol-gel temperature of gelatin is around 30 °C. The surface composition was evaluated by XPS and compared with the original samples.

In case of Gel-mod Type A, the results indicate that the coating thickness decreased rather quickly after incubation in phosphate buffer and that this occurred independent of the applied gelatin concentration. In view of the similar results for all selected concentrations, only the ones from a 3 w/v% Gel-mod solution are shown (Table 2) and further discussed here. This concentration is just below the gel point of gelatin at room temperature, implying that the initial deposited layer after dipcoating is bound to the Ti surface only through electrostatic interactions and no physical gelation occurred. The e-beam treatment

Table 2.
Atomic surface composition of 3 w/v% Gel-mod Type A coated Ti samples before and after incubation in phosphate buffer at 37 °C, determined by XPS analysis.

Incubation time	%Ti	%C	%O	%N	%Si
0 h	–	64	19	17	–
1 h	3	55	31	11	–
12 h	4	45	42	5	–
24 h	5	47	39	7	–
Ox Ti	13	38	49	–	–

Table 3.
Atomic surface composition of 10 w/v% Gel-mod Type B modified Ti samples (24 h of silanisation) before (0 h) and after incubation (24 h) in phosphate buffer at 37 °C, determined by XPS analysis.

Incubation time	%Ti	%C	%O	%N	%Si
0 h	–	63	21	16	–
24 h	3	54	34	5	3
Sil Ti	–	60	32	–	7

should enable further immobilisation of this initial, electrostatically bound layer. Nevertheless, after 1h incubation in phosphate buffer, there is already a reappearing Ti signal of 3%, showing the underlying Ti oxide surface. At the same time, the C and N signals, characteristic for the Gel-mod coating, decreased from respectively 64% and 17% down to 55% en 11%. This loss is mainly caused by the detachment of non-crosslinked gelatin chains and proceeded during the first 12 h of incubation. From then on, the surface composition remained stable for at least another 12 h (24 h of incubation in total). The remaining, stable N content (5 to 7%) indicates a thin gelatin layer is still present on the surface. This is probably the physically bound gelatin layer.

The same stability trend was observed for the Gel-mod Type B coated Ti samples. With a pretreatment of 1h silanisation with TMSPMA, with or without the addition of a catalyst (DMAP), there was only a fraction of the coating remaining after 24 h incubation (max. 3% N, data not shown). Besides varying the applied Gel-mod Type B concentration, we also prolonged the reaction time of the silanisation from 1 h to 24 h and evaluated the effect on the stability of the Gel-mod coating. A stable coating was achieved after a silanisation reaction of 24 h and subsequent dipcoating with a 10 w/v% Gel-mod Type B solution. The XPS results of this coating are presented in Table 3.

After 1 h incubation a loss of unbound gelatin chains was observed by a slight increase of the %Ti and O and a decrease of the %C and N. During further incubation in phosphate buffer (up to 24 h), the coating remained stable.

Alternatively, we also crosslinked Gel-mod coatings with UV irradiation in stead of e-beam. Therefore, a photo-initiator was added to the Gel-mod solutions. Based on earlier work on gelatin hydrogels in our research group, Irgacure® 2959 was selected as initiator.[38–39] Characterisation of the obtained coatings was already described in a previous paper.[40] XPS results from the present stability study of the coatings are summarised in Table 4. The results clearly indicate a very stable Gel-mod Type B coating which implies a more efficient crosslinking through UV irradiation compared to e-beam. Still, UV-irradiation is only applicable to 2D Ti samples.

Immobilisation Strategies of Collagen

As comparison to the gelatin coatings, we also immobilised collagen on the Ti surface. Two different immobilisation strategies were studied. The first method included an adsorptive immobilisation of fibrillar collagen. In this approach, a hydrophobic (cleaned) and a hydrophylic (oxidised) Ti surface were compared. In the second method the collagen was covalently attached to the Ti surface. To achieve covalent immobilisation, amine groups were introduced onto the Ti surface by silanisation using APTES. In a next step,

Table 4.
Atomic surface composition of 10 w/v% Gel-mod Type B modified Ti samples (UV crosslinked) before (0 h) and after incubation (24 h) in phosphate buffer, determined by XPS analysis.

Incubation time	%Ti	%C	%O	%N	%Si
0 h	–	65	21	11	3
24 h	–	58	28	6	6

Table 5.
Collagen adsorption, expressed as the N/C-ratio, on cleaned and oxidised Ti-surfaces, determined by XPS-analysis.

Incubation time of collagen	Collagen concentration	Adsorption time of collagen on Ti	N/C	
			cleaned Ti	oxidised Ti
0 h	40 μg/ml	1 h	0.09	0.09
		24 h	0.09	0.07
	150 μg/ml	1 h	0.19	0.10
		24 h	0.16	0.13
15 min	150 μg/ml	1 h	0.18	0.20
		24 h	0.16	0.13
2 days	40 μg/ml	1 h	0.17	0.21
		24 h	0.14	0.19
	150 μg/ml	1 h	0.22	0.19
		24 h	0.18	0.19

the amines reacted with the carboxylic acid groups of collagen (aspartic and glutamic acid) through a carbodiimide reaction.

Adsorptive Immobilisation of Collagen

Principle of Immobilisation. This method is based on the work of Dupont-Gillain *et al.*[36] They studied the influence of the aggregation state of collagen in solution on the supramolecular organisation of adsorbed collagen layers on polystyrene, as a hydrophobic surface, and on oxygen plasma treated polystyrene, as a hydrophylic surface. In this paper, cleaned Ti surfaces served as hydrophobic substrates and oxidised Ti surfaces as hydrophylic substrates. In case of the collagen solutions, two concentrations were used: 40 and 150 μg/ml. To achieve collagen aggregates, these solutions were incubated at 37 °C during 15 min and 2 days, before usage. According to Dupont-Gillain *et al.*, different degrees of aggregation of the collagen should be obtained depending on the incubation time. Collagen deposition was realised by incubating cleaned and oxidised Ti samples in the collagen solutions. Incubation times of 1 h and 24 h were compared. The efficiency of the collagen deposition was analysed through XPS measurements.

All of the obtained atomic surface compositions showed a Ti signal, pointing out a very thin and/or inhomogeneous collagen coating onto the Ti surface. Therefore, only the ratios of N/C are listed in Table 5. This ratio is a measure for the presence of collagen on the Ti surface and should be 0.22 in case of absence of the Ti signal. Looking at the obtained N/C values, it seems that a non fibrillar collagen solution (0 h incubation) of 150 μg/ml leads to a more extensive collagen adsorption on a cleaned Ti-surface (N/C $\approx$ 0.16-0.19) compared to an oxidised Ti-surface (N/C $\approx$ 0.10-0.13). Furthermore, the high collagen concentration of 150 μg/ml seems to be more effective then the one of 40 μg/ml (N/C $\approx$ 0.09). Other differences between the hydrophobic and the hydrophylic surfaces could not be determined. The XPS results of these samples also prooved that fibrillar collagen (15 min and 2 days of incubation) eventuated in a more efficient collagen deposition.

Stability Study. Additionally, the stability of the deposited collagen (fibres) coating was studied by incubation in a phosphate buffer (pH = 7.4) at 37 °C. XPS results indicate that the stability of the collagen layers, achieved with a low collagen concentration (40 μg/ml) and/or with a collagen solution with or without 15 min of aggregation, was rather low. Even after a few hours incubation in buffer increased Ti and O signals were detected together with decreasing C and N signals (data not shown). In case of other parameters similar results were only obtained after 24 h incubation. The results

Table 6.
Atomic surface composition of cleaned and oxidised Ti coated with collagen following the adsorptive method (150 μg/ml, 2 days incubation), before (0 h) and after incubation (24 h) in phosphate buffer at 37 °C, determined by XPS analysis.

	Incubation time		%Ti	%C	%O	%N	%Si	N/C
Cleaned Ti	1h incubation in COL I	0 h	5	53	27	12	–	0.23
		24 h	5	39	45	4	2	0.10
	24 h incubation in COL I	0 h	3	58	24	10	3	0.17
		24 h	11	38	45	2	–	0.05
Oxidized Ti	1 h incubation in COL I	0 h	2	55	27	10	1	0.18
		24 h	10	41	45	1	–	0.02
	24 h incubation in COL I	0 h	4	45	38	9	1	0.20
		24 h	6	39	45	4	2	0.10

of cleaned and oxidised Ti samples treated with a high collagen concentration (150 μg/ml) with 2 days of aggregation are summarised in Table 6.

Covalent Immobilisation

Principle of Immobilisation. The covalent attachment of collagen to a Ti surface was achieved by applying the method of Müller *et al*. Ti samples were first silanised with a 1 w/v% APTES solution during 1 or 24 h. The resulting amine functionalities on the Ti surface allow further reaction with the carboxylic acid groups from collagen through a carbodiimide reaction (EDC and NHS). Besides the formation of covalent bonds with the silanised surface, there is also a mutual crosslinking between the collagen chains due to the presence of amines and carboxylic acid groups in collagen. The reaction occured at room temperature for 4h with a collagen concentration of 1 mg/ml. The resulting surface composition (Table 7, 0 h incubation) indicated that a homogeneous collagen coating was obtained.

Stability Study. These coatings were also subjected to a stability study. Surface compositions of the coatings before and after incubation in buffer were determined through XPS measurements (Table 7). After 24 h incubation, the collagen coatings were still present, fully covering the Ti surfaces.

When comparing the two different immobilisation methods, it is clear that the covalent immobilisation method was much more efficient in providing a Ti surface with a stable collagen coating then the adsorptive one. Disadvantage however was the lack of a natural organisation of collagen. An ongoing study of cell-surface interactions will give information on the consequences thereof.

Table 7.
Atomic composition of APTES silanized (1 h) Ti coated with collagen using the covalent strategy, before (0 h) and after incubation (24 h) in phosphate buffer at 37 °C, as determined by XPS.

Incubation time	%Ti	%C	%O	%N	%Si	N/C
0 h	–	64	19	15	2	0.23
24 h	–	66	18	14	2	0.21

Conclusion

During this study it was shown that, although several polymer immobilisation strategies for metals are available, only the ones based on realising covalent bonds with the substrate lead to stable surface coatings. Physical interactions may have the advantage of maintaining the natural conformation of biomolecules or biopolymers, still the unstable character of such physically attached coatings is a limitation for their application.

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