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Polymeric smart coating strategy for titanium implants

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ABSTRACT Hyaluronan based hydrogel coatings can mimic extracellular matrix components and incorporate growth factors that can be released during a progressive degradation while new tissue regenerates. This paper describes a structural characterization of a hydrogel coating made of modified hyaluronan polymers and how these coatings interact with bone morphogenetic protein-2 (BMP-2). Quartz crystal microbalance and neutron reflectivity measurements were used for in-situ, real-time measurements of the adsorption properties of polymers and proteins on smooth titanium oxide surfaces that mimic implant products in orthopedics. The adsorption of BMP-2 on a bare titanium oxide surface is compared to that on titanium oxide coated with different chemically modified hyaluronan, the most important being hyaluronan with bisphosphonate groups (HA-BP). The subsequent release of the BMP-2 from these hydrogel coatings could be triggered by calcium ions. The amount of adsorbed protein on the surfaces as well as the amount of released protein both depend on the type of hyaluronan coating. We conclude that HA-BP coated titanium oxide surfaces provide an excellent material for growth factor delivery in-vivo.

KEYWORDS hyaluronan coating; bisphosphonate; BMP-2, adsorption, release

1. INTRODUCTION

Implants made of titanium and its alloys are well-established products in orthopedics.^[1] Titanium has high load bearing capacity and good biocompatibility. However, poor fastening of the implant to the surrounding tissue is one of the drawbacks of existing implants that shortens their lifetime.^[2] To improve the fastening, and hence increase the lifetime, a number of strategies have been proposed in the literature, such as roughening the surface,^[3] increasing the porosity,^[4] ion implantation,^[5] and use of coatings of biomolecules or ceramics.^[6] The main focus of the development of biomolecular/ceramic coatings is that they should be bioactive, i.e. they should be able to interact with the biological environment to enhance the response and bonding to tissue and surfaces. For example modification of the implant surface with adhesive proteins has been used to enhance the bioactivity as seen by cell adhesion.^[7] Binding or mineralization between the bone tissue and the implant is one of the most common processes today to increase bioactivity in bone repair and fixation applications.^[1]

Biodegradable polymers, both of synthetic and natural origin, have been extensively studied for many orthopedic applications. They are found on sutures, rods, screws, pins, and plates.^[8] These coatings are desirable as they can incorporate and deliver useful molecules, and be reabsorbed with a controlled chemical breakdown.^[1] The third and current generation of biomaterials combines the concept of bioactivity and biodegradability.^[9] These materials should be able to stimulate specific cellular responses at the molecular level, as well as undergo progressive degradation when new tissue regenerates and heals. Temporary three-dimensional scaffolds that mimic extra cellular matrix belong to this category. Biopolymers that slowly degrade are perfect as

scaffolds for tissue regeneration. Hyaluronan (HA), collagen and fibrin are examples of such polymers.

We report a study of a coating system based on HA hydrogel. Scaffolds using HA hydrogels are well known and have been optimized to mimic the extracellular matrix components to trigger cell response, as well as to deliver growth factors.^[10, 11] HA is a naturally occurring biopolymer and the main non-sulfated glycosaminoglycan of the extracellular matrix.^[12] Although HA is frequently used to engineer biomaterial surfaces,^[13] alone it is known to poorly support cell adhesion and usually needs additional chemical modifications.^[14] Optimally, these changes should make groups that enable biocompatible conjugation or cross-linking to form a three-dimensional hydrogel structure in-situ, i.e. under physiological conditions, and without the need for additional coupling agents. On the basis of previous studies, hydrogels were prepared by hydrazone chemoselective cross-linking between two HA derivatives, one modified with hydrazide groups (HA-hy) and another modified with aldehyde groups (HA-al). The multiple functionalization of the HA macromolecules with chemoselective groups results in formation of a hydrogel within a short time (1 min) on simple mixing of aqueous solutions of HA-hy and HA-al.^[15, 16] Preparation of the hydrogel is schematically illustrated in Figure 1.

Hydrogel deposition on titanium surfaces in this work was performed by alternating a coating with HA-hy and HA-al. Modified HA derivatives with both hydrazide and bisphosphonate groups (HA-BP-hy) were also used in this study.^[16] Bisphosphonates (BPs) are well known drugs for the treatment of osteoporosis and osteolytic bone diseases.^[17] BP alone has also been suggested as an adjuvant to anticancer agents for treatment of bone metastasis.^[18] The biological activity of BPs are related to their exceptionally high affinity to Ca²⁺ ions. Moreover, BPs can coordinate to metal ions located on the surface of

Figure 1. Illustration of the HA cross-linking reaction. The HA polymer backbone is shown in blue. The functional groups are shown in purple (hydrazide), green (aldehyde) and red (bisphosphonate). The hydrazide and the aldehyde groups enable cross-linking through hydrazone bond.

some metal oxides, such as titanium oxide and iron oxide. Several studies have utilized coating of titanium oxide surfaces with BPs.^[19] However, BP conjugated polymers have been very rarely considered for surface functionalization.^[20] In the present work we have applied the HA-BP-hy derivative to coat titanium oxide surfaces followed by hydrazone immobilization of an HA-al layer. The rationale of using the HA-BP-hy dual derivative as opposed to its HA-hy analog is its ability to anchor to the metal oxide surface through Ti-O-P bonds. We therefore expected a stronger attachment of the HA-BP hydrogel film to the substrate as compared to the HA polymer without BP groups. However, the amines on HA-hy and carboxylic acids on HA repeating units are also able to interact with titanium oxide.^[21]

Bone morphogenetic protein-2 (BMP-2) is an osteogenic factor that has been used to promote bone regeneration. It has a structure of a disulfide-linked homodimer, that has an overall molecular mass of 26 kDa.^[22] The osteoinductive effect of BMP-2 has been particularly used to improve bone growth on implant surfaces.^[23, 24] In previous work, the sustained delivery of BMP-2 from an HA scaffold was shown to have a promising osteoinductive effect and the degradation behaviour of the scaffold was well-controlled.^[10] The efficiency of BMP-2 nevertheless depends strongly on the method of delivery. Liu et al have shown that BMP-2, directly adsorbed onto the naked implant surface, did not have any osteogenic effects. However, BMP-2 incorporated into calcium phosphate coatings was a potent bone-inducer.^[23] This suggests that upon direct adsorption of BMP-2 on TiO₂ surfaces, the protein structure may change leading to its deactivation, while appropriate surface coating can have a protective effect on the protein. Although BMPs can promote robust bone formation, they also induce adverse clinical effects, including cyst-like bone formation and significant soft tissue swelling. Concentrations that are too low do not give any effect while those that are too high lead to abnormal tissue development.^[25] The optimal concentration is above 30 µg ml⁻¹. The mechanism for controlling release kinetics of BMP-2 from coatings is therefore of great importance.

Previous work has confirmed that HA mediated delivery of BMP-2 could induce local bone formation subcutaneously and subperiosteally.^[15, 26] In the present study, we have investigated the structure of two types of HA hydrogel coatings, with and without

matrix-attached BP groups. In addition, we investigated the interactions of these coatings with BMP-2 and compared them to the interaction of BMP-2 on a non-polymer-coated titanium oxide surface. We have also studied the structural changes of the surfaces with adsorbed protein on replacing the solvent with calcium chloride solutions. The two techniques used in this work were quartz crystal microbalance with dissipation monitoring (QCM-D), and neutron reflectometry. The quartz crystal microbalance is an ultrasensitive weighing device, based on the resonant frequency shifts of a sensor upon adsorption. It allows the mass of an adsorbed layer to be determined. Neutron reflectometry provides a measurement of the density profile perpendicular to an interface based on the difference in the refractive index for neutrons of the different adsorbed layers and the substrate. Both techniques are excellent for in-situ, real-time measurements of thin films at interfaces. Coating of the implant surfaces with polymers having different binding abilities to BMP-2 and study of desorption of the protein from these surfaces under the conditions mimicking in vivo environment are essential in improving the implants to integrate into the bone tissue and support its regeneration.

2. RESULTS AND DISCUSSION

2.1. Polymer and protein adsorption followed by QCM-D

QCM-D data for samples A, B, and C, described in section 4.3, are shown in Figure 2a-c with signals collected simultaneously for the first five resonance overtones, $n = 3, 5, 7, 9$ and 11 (the last overtone was removed for sample B due to instability). The changes in the normalized frequency ($\Delta f/n$) and the changes in dissipation (ΔD) during the polymer and protein adsorption processes, as well as the rinsing, were monitored at $25.0 \pm 0.1^\circ\text{C}$. For sample A, the first injection of HA-BP-hy gave an immediate decrease in frequency and increase in dissipation, with large differences for the overtone numbers ($\Delta f/n = -50$ to -75 Hz, $\Delta D = 10$ to 30×10^{-6}). This indicated an immediate and rapid adsorption of HA-BP-hy with viscoelastic behavior on the titanium oxide sensor. After rinsing with water, the region is marked with (1) in Figure 2a, only small shifts were observed for the different overtones ($\Delta f/n$ roughly -15 Hz for all the overtones). This shows that rinsing removed the bulk HA-BP-hy polymer that is not bound to the surface, leaving the thin and relatively rigid layer of attached polymer.

The conventional display of the QCM-D data is a plot of the frequency which has been normalized by the overtone number. It is therefore possible to compare the changes in the normalized frequency from different overtones, which provides evidence of the viscoelastic properties of the layer. The adsorption of a homogeneous, rigid layer will dampen the resonance overtones, and the value of $\Delta f/n$ will be the same for each overtone. This is shown in Figure 2a marked with (1), where we could observe a fairly thin and rigid layer formed by the HA-BP-hy adsorption. However for a viscoelastic, liquid-like layer, the resonance of the sensor will vary with the overtone number. Here the different overtones probe different depths of the layer, with the penetration depth decreasing with increasing overtone number.^[27] Before rinsing the first HA-BP-hy layer, $\Delta f/n$ and ΔD were largest for the first overtone number (darkest blue for frequency and darkest red for dissipation). This indicated a viscous structure that is more diffuse further away from the surface.

Figure 2. QCM-D data for samples A (a), B (b), and C (c). The curves in different shades of blue indicate the change in frequency for the overtones 3, 5, 7, 9 and 11 (the darkest shade for the lowest overtone number). The curves in different shades of red indicate the change in dissipation for the overtones 3, 5, 7, 9 and 11 (the darkest shade for the lowest overtone number). Each step of sequential adsorption of (1-8) HA (1 mg ml⁻¹), (9) dilute BMP-2 (7.5 μg ml⁻¹), (10) concentrated BMP-2 (37.5 μg ml⁻¹), is shown in the graph. The arrows point at times where the layer is stable after thorough rinsing with the solution used corresponding to the injections. Step (11) was a rinse with 0.2 M CaCl₂. The final step (12) was a rinse with pure water.

The first injection of HA-al for sample A, followed by a rinse (2) did not provide any further change. This injection of HA-BP-hy and HA-al in alternating manner followed by rinsing was repeated six more times (steps 3-8), without any significant changes in neither the frequency nor the dissipation. Steps (1) to (8) indicated relatively rigid polymer films where the saturation had already occurred after the first injection. Similar development was observed for sample B (deposition of HA-hy and HA-al layers), shown in Figure 2b. After the final injection and rinsing (8), the $\Delta f/n$ and ΔD were comparable for samples A and B. However, steps (1-7) for sample B showed minor fluctuations and a small gradual decrease in frequency throughout the injection. The first injection of HA-BP resulted in less surface associated material compared to HA-BP-hy.

A large drop in $\Delta f/n$ could be observed for all three samples when introducing the dilute (7.5 μg ml⁻¹) BMP-2 solution in phosphate buffered saline, between step (8) and (9) in Figure 2. After rinsing with the same solvent, $\Delta f/n$ stabilized at approximately -40 to -60 Hz, step (9) in Figure 2. The large differences in the overtones for samples A and B indicates a viscous material. Another interesting aspect was that the changes in frequency and dissipation after rinsing were no longer largest for the first overtone, which suggests that the density of the adsorbed layer was not simply decreasing away from the surface but rather had a non-uniform distribution. The BMP-2 layer for sample C appeared much more rigid as $\Delta f/n$ is the same for all overtones, which suggests that the protein layer is more densely packed. Introducing more BMP-2 in the form of a concentrated solution (37.5 μg ml⁻¹) did not cause any large changes for any of the samples (step 10). This suggests that the surface is essentially saturated at the lower concentration. The differences in the overtones were still large for samples A and B, but not for sample C, i.e. the BMP-2 layer appeared to be softer or less rigidly adsorbed on the samples with layers of polymer than on the bare oxide.

When changing the solvent from the phosphate buffer, containing about 0.14 M Na⁺ and K⁺, to a 0.2 M CaCl₂ solution (step 11), the frequency rose for samples A and B, which suggests a loss in mass. Hence rinsing the surfaces with solvent containing calcium ions seems to remove some of the BMP-2 adsorbed to the surface. This effect was more marked for sample A than for sample B. The only change observed for sample C is the spread in the overtones. This is an indication that there was no significant desorption of the BMP-2 layer but that some rearrangement of the surface bound BMP-2 giving a more diffuse structure occurs. When changing the solvent to pure water, as used for the equilibration of the baseline, the frequency rose for all samples. This further rinsing could cause protein that had been released from the surface by the calcium ions, to be rinsed away. For sample A both $\Delta f/n$ and ΔD returned to the same value as for the polymer coating before introduction of BMP-2. The data cannot completely rule out the possibility of the calcium ions causing a loss of material from the HA layer and that the remaining film is a mixture of the HA layer and BMP-2. However, previous work has indicated that calcium ions have very strong interactions with HA-BP-hy [16] and these ions could therefore replace BMP-2 bound to BP groups. The released protein was subsequently rinsed away.

The thickness of the HA layers was obtained by fitting the QCM-D data using equation (2) (see Figure 3). The thickness of the polymer layer is a reasonable estimate as only small overtone differences were observed. The thickness calculation for the BMP-2 layers should only be considered as a lower estimate as the larger differences in overtones indicated that there was a viscous layer. Sample A has the best desorption

properties: all the BMP-2 was released by rinsing with calcium ions and water. Samples B and C adsorbed roughly equal amounts of BMP-2 for both the dilute and concentrated injections. Both have adsorbed larger amounts compared to sample A. The BMP-2 was not desorbed from the bare titanium upon rinsing with the solution containing calcium ions.

The changes in ΔD after each step of rinsing were very close to the baseline. No conclusions about the layer viscosity could be drawn from these data. QCM-D provides a good overview of the polymer and protein adsorption properties. It is easy to follow the relative changes in mass and the technique provides some information regarding the adsorbed surface structures. For a more detailed analysis of the structure of each deposition we performed neutron reflectivity measurements following the same injection protocol as for the QCM-D.

Figure 3. Thicknesses from fits based on Δf for sample A (pink), B (green) and C (blue) determined from equation (2). The deposition steps (1-8) represent the repeated (8×) HA coating procedures performed in water. Steps (9) and (10) are the introduction of dilute BMP-2 solution, followed by an introduction of concentrated BMP-2 to the sensors; the BMP-2 was dissolved in phosphate buffered saline. Step (11) is the change of solvent from the phosphate buffered saline to 0.2 M CaCl_2 . Step (12) was a change of solvent back to pure water with an increased flow in the sample cell.

2.2 Interfaces for neutron reflection - sputtering and measurements of the bare substrates

Sputtering titanium on to polished silicon surfaces provided smooth surfaces suitable for reflectivity measurements as well as mimicking implant products in orthopedics. The bare substrates were characterized with neutron reflectivity at a solid-liquid interface. A three layer model was used for the fitting of the data. The thicknesses were 20 ± 4 Å, 116 ± 8 Å, and 31 ± 4 Å for the silicon oxide, the metallic titanium, and the titanium oxide layers respectively. These substrates were kept in water at 37°C for three days during the in-situ depositions of polymer and protein. During this time, an insignificant increase of the thickness of the TiO_2 of approximately 3 Å, as well as an equally insignificant decrease of the metallic titanium layer of 1–2 Å was also observed. This slight change in the metal and metal oxide thicknesses is caused by oxidation and is observed in literature.^[28] The neutron reflectivity data are shown in Figure S1a-c in the Supplementary information.

2.3 Neutron reflectivity results for the HA adsorption and BMP-2 interactions

Samples used for the neutron reflectivity measurements followed the same injection protocol as used in the QCM-D measurements, although all injections were performed manually. Two contrasts, D_2O and H_2O , were measured after the eight HA injections, the injection of dilute BMP-2, the concentrated BMP-2, and finally after rinsing with the CaCl_2 . Figure 4 shows the reflectivity data for sample A with all the subsequent measurements in D_2O as log reflectivity R against Q with the corresponding fits. However, the changes in the reflectivity data for the subsequent injections are clearer when the data are plotted as RQ^4 against Q . Figure 5a-c shows the data for all three samples over the Q -region where the changes are most noticeable. Figures S2-S5 in the Supplementary information show the data for two contrasts of water and models for all three samples for the full Q -range, both log R as a function of Q , and RQ^4 as a function of Q . The model parameters are given in Table 1.

Figure 4. Neutron reflectivity data for sample A, shown as log reflectivity R as a function of Q . All measurements are performed in D_2O (open circles) and H_2O (open squares) contrast. The bare titanium sputtered surface is indicated as (○) in D_2O and (□) in H_2O , the HA coating is indicated as (○) in D_2O and (□) in H_2O . The dilute BMP-2 coating is indicated as (○) in D_2O and (□) in H_2O and the concentrated BMP-2 is indicated as (○) in D_2O and (□) in H_2O . The rinse using 0.2 M CaCl_2 is indicated as (○) in D_2O and (□) in H_2O . The continuous lines through the data points are simultaneous fits with both contrasts.

The polymers on sample A could be modeled as a layer with a uniform thickness followed by layers with an exponential decay of polymer density towards the solvent. The uniform region had a thickness of 19 ± 4 Å and the exponential decay length, l , was 15 ± 7 Å. The polymers on sample B could be modeled simply as a uniform layer of thickness 10 ± 2 Å, with a roughness of 2 ± 1 Å. This data shows that the polymer layer on sample A has a more extended structure compared to that on sample B. Due to the favorable bond between the BP-groups and the titanium surface, more of HA-BP-hy was deposited leading to a thicker polymer layer. Both samples had a polymer volume fraction of 7% near the solid surface. The region of uniform density of sample A was larger than for sample B, and in addition it had a more extended profile, this leads to a total HA polymer surface coverage of $0.2 \pm 0.07 \text{ mg m}^{-2}$ for sample A which is doubled compared to sample B of $0.1 \pm 0.04 \text{ mg m}^{-2}$. These values are obtained from the model for data measured after eight polymer injections, with rinsing occurring between each injection. It was not possible to follow

the polymer injections step-by-step with neutron measurements in the same way as for the QCM-D experiments. Due to the high hydration level of the thin polymeric gel layer, only very small changes in the reflectivity data were observed compared to the measurement of the bare substrates as the thick metal layer dominates the signal. The thicknesses determined for the uniform layers near the titanium are lower than those obtained from the QCM-D data. This is due to the extended profile or the roughness contributing to the mass detected by the oscillating quartz sensors. The overall thickness profiles for sample A is however larger compared to the thicknesses obtained by QCM-D, indicating that at a certain distance from the sensor (>30 Å), one cannot detect any difference between the extended HA profile and bulk water. Nevertheless, considering the large uncertainties in the interpretation using the Sauerbrey relations and in the rigidity of the packing, small differences in the thickness for the QCM-D and reflectivity data are not unexpected. Due to this difference, one cannot draw any conclusion about whether the HA bound to the titanium oxide surface has cross-linked with the further HA added to the surface or whether only the first layer was attached.

The layer thicknesses of 19 and 10 Å are consistent with a previous neutron reflectivity study on adsorbed HA.^[29] The volume fraction profiles for samples A and B are shown in red in Figure 6a-b. These are calculated based on the scattering length density profiles in Figure S6 in the Supplementary information. The scattering length density in H₂O for the HA-BP layer of sample A is higher than expected. This is believed to be caused by the strong Ti-O-P bond that forces the polymer into the rough surface of the TiO₂ layer. The scattering length density in H₂O for the HA layer of sample B corresponds to the volume fraction measured in D₂O.

Although the HA layers are thin, they influence significantly the subsequent BMP-2 adsorption and desorption on the three surfaces measured. The adsorption of BMP-2 on both types of HA coated surfaces and on the bare titanium oxide rapidly reached equilibrium as seen in Figure 2. There are also large changes in the reflectivity data shown in Figure 5a-c on the introduction of the dilute BMP-2 solution to the surfaces. It is immediately apparent from inspection of the data that the BMP-2 interacts strongly with both HA coated surfaces and the bare titanium oxide (comparison of the red curves (a, b), and black curve (c) with the blue curves). However, the rinse with a solution of calcium ions has the biggest effect on the surface coated with HA containing BP groups, while the effect is not as clear for the samples B and C (comparison of the purple curves with the green curves).

BMP-2 is a dimer with dimensions of $70 \text{ Å} \times 35 \text{ Å} \times 30 \text{ Å}$. It has a butterfly-like shape where the middle, thinnest part is only about 10 Å thick.^[30] This non-uniform shape makes it difficult for the molecule to pack well. When modeling the BMP-2 layers on top of the HA or bare titanium surfaces, the BMP-2 layer could be modeled as two regions with different scattering length density ρ . These two regions are denoted as (1) and (2) in Table 1, where (1) is the region closer to the surface and (2) is the region further away towards the bulk solvent. These two regions contain different fractions of BMP-2 volume. The outer region also had a profile of decaying intensity towards the bulk solution. This structure can be understood as arising from the organization of the BMP-2 that cannot pack to create a homogeneous layer on the surface. In general, the volume fraction of the protein is lower close to the solid surface compared to the region slightly further away, see Table 1 and Figure 6a-c. The QCM-D measurements, as

mentioned previously, also indicated that the adsorbed BMP-2 had a rather inhomogeneous density distribution perpendicular to the surface as the first overtone number did not have the largest change in frequency. The BMP-2 surface coverage, Γ , is $2.3 \pm 0.6 \text{ mg m}^{-2}$ for sample A, $3.4 \pm 0.5 \text{ mg m}^{-2}$ for sample B, and $4.1 \pm 0.3 \text{ mg m}^{-2}$ for sample C (Table 1). When more BMP-2 was added, an increase in adsorption was observed for samples A and B to $3.7 \pm 0.4 \text{ mg m}^{-2}$ and $4.6 \pm 0.5 \text{ mg m}^{-2}$ respectively. However for sample C the amount adsorbed was unchanged. After rinsing the samples with calcium chloride, sample A lost a significant amount of the bound BMP-2, reducing to $\Gamma = 1.5 \pm 0.3 \text{ mg m}^{-2}$. The losses of BMP-2 for samples B was only marginal, $\Gamma = 4.2 \pm 0.4 \text{ mg m}^{-2}$, while again the total amount was unchanged for sample C.

Instead of two layers; one uniform and one extended, the HA-BP layer for sample A was modeled as a single uniform layer during the interaction with BMP-2 and calcium ions. This uniform layer had a total thickness of 24 Å and maintains the same overall surface coverage as before. The change is apparently a compression of the polymer caused by the protein molecules. The thickness of the HA layer on sample B remained unchanged during the adsorption and desorption of BMP-2. However, the scattering length density of HA with H₂O increased slightly after the introduction of the protein. This is again believed to be due to the compression towards the underlying TiO₂ layer, which have a higher scattering length density and high roughness. This was not observed for the D₂O contrast where the contrast is higher between the polymer layer and the solvent. It is not possible to rule out the possibility that the HA layer is depleted or altered by the proteins or the calcium ions. It has been shown previously that calcium chloride solutions can compress grafted HA if the layer has a diffuse structure.^[31] However only very extended structures are changed significantly.

For sample A, protein binding increased with the concentration of BMP-2. The rinse with calcium chloride removed most of the BMP-2 bound on the HA layer that contained BP groups, leading to a final surface coverage of BMP-2 lower than that found for the low protein concentration initially. As the amount of the polymer apparently remains unchanged, we can conclude that the calcium ions replaced most of the adsorbed BMP-2. Sample B has higher BMP-2 binding. One explanation for this is that the slightly thinner HA layer allowed some interactions between the protein molecules with the bare substrate beneath. Sample C showed the highest protein binding at the low concentration, however it did not change significantly when more protein was added or when the system was rinsed with calcium chloride. As previous work indicated that the BMP-2 bound to bare titanium oxide lacks biological activity,^[23] this comparison of adsorption suggests that the amount adsorbed to the surface is less important than the amount that can be released to promote growth of bone. In the case of sample A, the BP groups act as good anchors between the HA polymer backbone and titanium surfaces due to formation of Ti-O-P bonds. As sample B lacks BP groups, the binding with the titanium is poorer, this leads to a decreased thickness of HA. As calcium ions have much stronger affinity for HA containing BP groups, this can explain why the calcium ions could remove a much higher fraction of the bound BMP-2 on sample A. This calcium generated desorption effect of the BMP-2 is crucial for the activation of bone regeneration. The BP-containing coating system could be further optimized for clinical use.

Figure 5. Neutron reflectivity data for sample A (a), B (b), and C (c) in D_2O , shown as $RQ^4 \times 10^8$ as a function of Q . The graphs show the most visible change in the reflectivity data at the Q -region 0.015 – $0.035 \text{ \AA}^{-1}$, indicated with the yellow arrow. The bare substrates are shown in black; the surfaces coated with HA in red, the surfaces coated with HA and BMP-2 are drawn in blue for dilute BMP-2 solutions and in purple for concentrated BMP-2 solutions. The surfaces after final rinse with $CaCl_2$ are shown in green.

Figure 6. Volume fraction profiles for samples A (a), B (b), and C (c). The variable, z , is the distance from the titanium oxide surface. The volume fraction of the HA layer is shown in red; the effect of the introduction of the dilute BMP-2 is shown in blue. The total amount of both the dilute and the concentrated BMP-2 solution is shown in purple. The volume fraction of the BMP-2 after rinsing with $CaCl_2$ is shown in green. The inset illustrates how the surface may look like (not to scale) when dilute BMP-2 is introduced to the surface (shown with blue BMP-2 molecules), and the overall change when the concentrated BMP-2 solution was added (shown with purple BMP-2 molecules), and finally how the surface may look like after rinsing with $CaCl_2$ (shown with green BMP-2 molecules).

Table 1. Fitted parameters that describe the BMP-2 organization on top of sample A, B and C, before and after rinsing with calcium chloride.

Sample	Layer description	BMP-2 dilute (1)	BMP-2 dilute (2)	BMP-2 concentrated (1)	BMP-2 concentrated (2)	Calcium rinse (1)	Calcium rinse (2)
A	$t / \text{\AA}$	15±4	3±2	13±2	3±2	16±3	3±1
	ϕ BMP-2 / %	18±10	65±6	18±7	65±6	18±7	45±6
	$l / \text{\AA}$	--	16±3	--	30±2	--	13±4
	$r / \text{\AA}$	6±2	--	8±2	--	8±3	--
	$\Gamma / \text{mg m}^{-2}$	2.3±0.6		3.7±0.4		1.5±0.3	
B	$t / \text{\AA}$	5±2	3±2	7±2	34±3	3±2	30±4
	ϕ BMP-2 / %	18±7	57±3	18±8	40±5	18±10	40±5
	$l / \text{\AA}$	--	34±3	--	38±3	--	38±3
	$r / \text{\AA}$	2±1	--	2±1	--	1±1	--
	$\Gamma / \text{mg m}^{-2}$	3.4±0.5		4.6±0.5		4.2±0.5	
C	$t / \text{\AA}$	12±3	16±3	21±2	19±3	20±2	12±3
	ϕ BMP-2 / %	13±8	50±5	13±5	50±3	13±5	50±5
	$l / \text{\AA}$	--	35±2	--	29±3	--	39±4
	$r / \text{\AA}$	3±2	--	4±3	--	3±2	--
	$\Gamma / \text{mg m}^{-2}$	4.1±0.3		4.1±0.4		4.1±0.5	

3. CONCLUSIONS

This study describes three components for surface coating: HA, BP and BMP-2, all of which can contribute to improve the surface properties of bone implants. A general aim was to determine whether BP modified HA is advantageous as a coating material. The results show that HA-BP binds strongly to titanium oxide surfaces, can adsorb BMP-2, and can also release it under the influence of calcium ions. This implies that future titanium implants could be coated with polymers and proteins where the protein could be released in-situ once in contact with bodily fluids. Using QCM-D combined with neutron reflectivity, we were able to follow in-situ depositions of functionalized HA and BMP-2 and obtained detailed structural descriptions of the bound layers as well as the release properties of BMP-2 from these coatings.

Adsorption and calcium triggered desorption of BMP-2 was studied on HA-BP coated titanium oxide, and was compared with HA coated titanium oxide and bare titanium oxide surfaces. Both HA hydrogel coatings are relatively similar in terms of the surface coverage and solvent volume fraction. However, the coating with HA that contains BP provides better anchors to the titanium surface due to the formation of Ti-O-P bonds. This provides a HA layer that has a thicker and more extended structure. The adsorption of BMP-2 to the polymer substrate could be reversed by rinsing with calcium chloride solutions. The release is due to the strong interaction of calcium ions with BP groups on HA that in turn weakens the interaction of the BP groups with BMP-2. It allows the protein to re-dissolve in the bulk solvent. The effect of calcium triggered release of BMP-2 is much weaker for the

titanium surface coated with HA lacking BP groups. The calcium ions have no overall effect on the BMP-2 adsorbed to the bare titanium surface.

Calcium generated desorption of the BMP-2 is crucial for the activation of bone regeneration, and the present work indicates the value of BP modified HA hydrogel coatings to improve integration of current implants to bone. This study has provided a detailed structural description of the bioactive coating system, which can guide further development for clinical usage.

4. EXPERIMENTAL SECTION

4.1. Materials

All the modified HA used in this work was prepared according to previous procedures.^[16] BMP-2 was purchased from InductOs®, Wyeth Europe, Berkshire, UK. The surfaces used in the QCM-D study were piezoelectric quartz crystal sensors with a diameter of 14 mm, coated with titanium oxide (Q-Sense, Biolin Scientific AB, Västra Frölunda, Sweden). The substrates used for the neutron reflectivity study were silicon crystals (Crystran, Poole, Dorset, UK) of dimensions 50 mm × 50 mm × 10 mm. These were cut with the 111 orientation on the large face. Titanium was sputtered from a 99.9955 atomic% pure target on to the silicon oxide layer on these substrates. D₂O (99% purity) was supplied by EURISOTOP, CEA, Saclay, France. H₂O used in this study was passed through a purification system (Milli-Q, resistivity 18.2 MΩ cm). Physical properties of materials used in the study are listed in Table 2.

Table 2. Properties of materials used in the study at 25°C.

Name	Formula	Formula mass / g mol ⁻¹	Density / g cm ⁻³	ρ / 10 ⁻⁶ Å ⁻²
Silicon	Si	28	2.33	2.07
Silica	SiO ₂	60	2.20	3.41
Titanium	Ti	48	4.51	-1.95
Titanium oxide	TiO ₂	80	4.27	2.63
HA repeat unit	C ₁₄ O ₁₁ H ₂₀ NNa	401	1	1.43–2.20*
BMP-2	C ₁₁₄₂ H ₁₇₇₀ O ₃₂₇ N ₃₂₀ S ₁₈	25776	1.53	2.11–2.75*
Water	H ₂ O	18	0.997	-0.56
Heavy water	D ₂ O	20	1.10	6.35

* Values depend on level of deuteration of aqueous solvent.

4.2. Sputtering of the substrates

The titanium layers were grown at room temperature in an ultrahigh vacuum system by dc-magnetron sputtering. The sputtering gas was 99.9999% pure argon at a pressure of 0.13 Pa and the base pressure of the system was 3×10^{-7} Pa. No pre-sputtering treatments were performed on the surfaces. The substrates were rotated at 20 RPM to achieve a uniform surface. The nominal thickness of the titanium layer was 15 nm. Once the substrates were subjected to atmospheric oxygen, titanium oxide layer was formed on top.

4.3. Sample preparation

Three samples were prepared for the measurements with QCM-D and neutron reflection. Sample A refers to the HA-BP gel coating made by alternating the dual functionalized HA containing both bisphosphonate and hydrazide groups (HA-BP-hy), with HA with aldehyde groups (HA-al). Sample B refers to the HA gel coating made by alternating the HA with hydrazide groups (HA-hy) and HA with aldehyde groups (HA-al). All HA solutions used in this study had a concentration of 1 mg ml⁻¹. Sample C is the bare titanium oxide surface with no gel coating. These three samples were exposed to BMP-2 solutions, first as a dilute concentration of 7.5 µg ml⁻¹, and then at a higher concentration of 37.5 µg ml⁻¹. BMP-2 was dissolved in phosphate buffered saline and these concentrations are related to those used in other studies.^[25, 32] Finally, the surfaces were rinsed with a 0.2 M CaCl₂ solution in order to study the interaction with calcium ions.

For the QCM-D experiments, in the case of samples A and B, the polymer solutions were flushed 8 times on to the titanium oxide coated sensors at a flow rate of 0.1 ml min⁻¹. A rinse was performed between each injection. The dilute BMP-2 solution was injected after 160 min with a rinse at 200 min, followed by the concentrated BMP-2 solution (225 min) with a rinse (240 min). The calcium chloride solution rinse occurred at 255 min. Then an additional step was performed by rinsing with pure water at an increased flow rate of 0.55 ml min⁻¹ at 270 min. For sample C, the dilute BMP-2 was introduced at a time of 25 min with rinse at 60 min, followed by the concentrated BMP-2 at 80 min with a rinse at 100 min. The calcium chloride rinse occurred at 115 min, followed by a pure water rinse with increased flow at 135 min.

The neutron reflectivity measurements were made at the solid-liquid interface using 50 mm × 50 mm titanium oxide surfaces. The in-situ injections of polymers, proteins, and rinsing follow a similar

protocol to that of the QCM-D experiment. All measurements were made with both D₂O and H₂O after thorough rinsing to remove unbound material.

4.4. QCM-D measurements and data analysis

The measurements were performed on a QCM-D instrument that can simultaneously measure changes in frequency as well as energy dissipation of a freely oscillating sensor.^[27, 33] The QCM-D technique is thoroughly described by Höök;^[34] the underlying physical principle is that the changes in frequency, (Δf), depend on the difference in the total oscillating mass, both that directly adsorbed and the solvent associated with the bound molecules. If the film is thin and rigid one can relate the decrease in frequency to the mass of the film according to the Sauerbrey relation.^[34, 35] The mass m of the adhering layer is calculated as:

$$\Delta m = C / n \Delta f \quad (1)$$

where C is the mass sensitivity constant of the crystal (17.7 ng Hz⁻¹ cm⁻² for a 5 MHz quartz crystal), and n is the frequency overtone number (3, 5, 7, 9 and 11 were measured in this work). The overtones refer to the whole number multiples of the resonant frequency above the fundamental frequency, also known as harmonics. This equation is only valid when the film is rigid and one can disregard the viscoelastic coupling with the surrounding medium.^[27] It is also possible to estimate the effective thickness t_{eff} on the adhering layer:

$$t_{\text{eff}} = \Delta m / d \quad (2)$$

where d is the density of the adhering molecule.

If the film is soft and viscoelastic then the simple Sauerbrey relation needs to be extended due to incomplete coupling of the oscillation of the crystal to the bound layer as the equation would underestimate the adsorbed mass.^[36] The shift of resonant frequency and dissipation indicates the deposition of a soft layer on the sensor. In such situations where the molecules are associated with a lot of water, a Kelvin-Voigt model can be applied to describe the viscoelasticity and complex shear modulus of the layer.^[37] The viscoelasticity of the layer alters the damping of the oscillations or energy dissipation, D , defined as:

$$D = E_{\text{lost}} / 2\pi E_{\text{stored}} \quad (3)$$

where E_{lost} is the energy lost or dissipated during one oscillation cycle and E_{stored} is the total energy stored in the oscillator.

The QCM-D technique is widely used to study protein adsorption processes on solid surfaces.^[38]

4.5. Neutron reflection measurements and data analysis

The neutron reflection data were collected using the reflectometer D17 at the Institut Laue-Langevin, Grenoble, France.^[39] The basic principles, the measurement procedure, and the data analysis using the computer program cprof^[40] are described in an earlier publication.^[31] The adsorbed polymer or protein layers can be modeled either as well ordered layers with a uniform scattering length density, ρ , and thickness, t , or with a smooth exponential decay profile (with an additional decay length variable λ). The scattering length density of a layer ρ_{layer} can be described by the sum of the contributions from the adsorbed molecules and the solvent in the following way:

$$\rho_{\text{layer}} = \phi_{\text{mol}} \rho_{\text{mol}} + (1 - \phi_{\text{mol}}) \rho_{\text{sol}}, \quad (4)$$

where ϕ_{mol} is the volume fraction of the adsorbed molecule, ρ_{mol} and ρ_{sol} are the scattering length densities of the adsorbed molecules and solvent, respectively. From this equation the value of ϕ_{mol} is extracted. The surface coverage Γ_u for a uniform layer is calculated as

$$\Gamma_u = \phi_{\text{mol}} \times t \times d \quad (5)$$

where t is the thickness of the adsorbed layer and d is the density of the adsorbed molecule. This is the same definition of d as for the QCM-D. In a similar fashion, the surface coverage Γ_e for an exponentially decaying profile is calculated by integrating the volume fraction over the distance from the interface:

$$\Gamma_e = \phi_{\text{mol}} \times I \times d \quad (6)$$

where I is the exponential decay length. The total surface coverage Γ is calculated as the sum of Γ_u and Γ_e .

Measurement of samples in two solvent contrasts allows for the identification of the composition and the extent of hydration of the adsorbed layers.

ASSOCIATED CONTENT

Supporting information

Neutron reflectivity data and model fits for sample A, B and C.

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