

Mechanistic insight on the combined effect of albumin and hydrogen peroxide on surface oxide composition and extent of metal release from Ti6Al4V

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Abstract: The titanium–aluminium (6 wt%)–vanadium (4 wt%) (Ti6Al4V) alloy is widely used as an orthopedic and dental implant material due to its high corrosion resistance in such environments. The corrosion resistance is usually determined by means of electrochemical methods, which may not be able to detect other chemical surface reactions. Literature findings report a synergistic effect of the combination of the abundant protein albumin and hydrogen peroxide (H₂O₂) on the extent of metal release and corrosion of Ti6Al4V. The objectives of this study were to gain further mechanistic insight on the interplay of H₂O₂ and albumin on the metal release process of Ti6Al4V with special focus on (1) kinetics and (2) H₂O₂ and albumin concentrations. This was accomplished mainly by metal release and surface oxide composition investigations, which confirmed the combined effect of H₂O₂ and albumin on the metal release process, although not detectable by electrochemical open circuit potential measurements. A concentration of 30 mM H₂O₂

induced substantial changes in the surface oxide characteristics, an oxide which became thicker and enriched in aluminum. Bovine serum albumin (BSA) seemed to be able to deplete this aluminum content from the outermost surface or at least to delay its surface enrichment. This effect increased with increased BSA concentration, and for time periods longer than 24 h. This study hence suggests that short-term (accelerated) corrosion resistance measurements are not sufficient to predict potential health effects of Ti6Al4V alloys since also chemical dissolution mechanisms play a large role for metal release, possibly in a synergistic way. © 2018 The Authors. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* published by Wiley Periodicals, Inc. *J Biomed Mater Res Part B: Appl Biomater* 107B: 858–867, 2019.

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INTRODUCTION

The titanium–aluminum (6 wt %)–vanadium (4 wt %) (Ti6Al4V) alloy is widely used as a metallic implant material, especially for parts that are not exposed to friction and that require good osseointegration.¹ It is generally considered as biocompatible and to have a high corrosion resistance.¹ The release of Ti, Al, and V (ions, corrosion products such as oxides, and/or metal particles) from implant materials such as Ti6Al4V can potentially induce adverse health effects. Ti ions and oxides, such as Ti dioxide (TiO₂) are generally considered to be nontoxic and biocompatible, however, recent

studies suggest that this is not always true.² Al release is generally considered more problematic since it has known neurotoxic effects, and wear debris of Al oxide has in a few reported cases resulted in soft tissue sarcoma.³ V, of which some compounds are used as pharmaceuticals, can accumulate in the bone, most probably due to the similarity between vanadate and phosphate, and induce some toxic effects.⁴ All three metals can cause hypersensitivity reactions, although very scarce observations have been made in the case of Ti,⁵ more common in the case of V⁶ and Al,⁷ but still less common compared to hypersensitivity reactions induced by

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metals such as cobalt, nickel, and chromium.⁸ Metal release processes can be governed both by corrosion (oxidation of metal) and dissolution of the surface oxide (electrochemical and chemical processes, for example, by chelation/complexation).⁹ For this reason, electrochemical corrosion tests may not solely be sufficient tools for hazard/risk assessment of alloys such as Ti6Al4V. Two recent studies show that there exists a synergistic effect of hydrogen peroxide (H₂O₂) and the protein albumin in the enhancement of corrosion and metal release from Ti6Al4V.^{10,11} It could be argued whether the relatively high concentration of H₂O₂ in those studies, 30 mM (0.1 wt %), is clinically relevant, as only 10 μM H₂O₂ has been suggested to be physiologically relevant at inflammatory conditions.¹² However, macrophages and other neutrophils can induce very oxidative conditions, even sufficient to dissolve gold.¹³ Studies on retrieved implant devices have also shown that there can be a very high oxidative potential *in vivo* under inflammatory conditions.^{14,15} This could imply that a high redox potential, similar to the potential induced by intermediate or high concentrations of H₂O₂ could have physiological relevance. The objective of this study was to gain further mechanistic insight on the combined interplay of H₂O₂ and albumin on the metal release process for Ti6Al4V. This was achieved by quantitatively assess metal release rates and changes in surface oxide composition via kinetic studies (up to 2 weeks) and investigations at different H₂O₂ and albumin concentrations.

MATERIALS AND METHODS

Ti6Al4V alloy disks and surface preparation

Ti6Al4V alloy disks (diameter-22 mm, thickness- 2 mm) were supplied by Ionbond, Switzerland. The material was annealed at 700–750°C and air cooled in conformation with standards ASTM F136, ASTM B348/10 GRADE 5, and ISO 5832-3. The chemical analysis (of batch: heat number 04111x) is given in Table I based on supplier information from measurements performed by Edelstahl Böhler, Germany. The ultimate tensile strength was 950.5–960.8 MPa.

The disks were ground with 1200 grit SiC article and water, consecutively cleaned by acetone for 5 min and ethanol for 5 min in an ultrasonic bath, dried with cold nitrogen gas and in all cases (if not indicated differently) stored in a desiccator (low relative humidity) for 24 ± 1 h at room temperature.

Simulated physiological fluids

Simulated physiological fluids of varying H₂O₂ and bovine serum albumin (BSA) content were based on phosphate buffered saline (PBS). BSA, article number A7906, and 30% puriss. p.a. H₂O₂ were purchased from Sigma Aldrich, Sweden. The salts of PBS were of analytical grade (8.77 g/L NaCl, 1.28 g/L Na₂HPO₄, 1.36 g/L KH₂PO₄, adjusted with 50% NaOH to

pH 7.2–7.4). The amount of BSA was either 1, 4, 10, or 40 g/L. Forty grams per liter is a relevant concentration for the human blood,¹⁶ and approximately 11 (normal) to 20 (at infection) g/L albumin are present in synovial fluid.^{17,18} The amount of H₂O₂ was either 10 μM, 300 μM, 3 mM, or 30 mM. An amount of 10 μM H₂O₂ has been found as a physiologically relevant concentration at inflammatory conditions.¹² The concentration of 30 mM corresponds to 0.1 wt % and was chosen as the upper limit based on earlier work showing synergistic effects of hydrogen peroxide and albumin on corrosion and metal release.^{10,11} Higher concentrations than 10 μM H₂O₂ might be relevant for the simulation of some specific physiological conditions, for example, oxidation by macrophages, see discussion section. PBS was used as buffer, as it has been shown that the pH in synovial fluid is buffered in a similar way as PBS in the presence of metal ions.¹⁹ Ultrapure water (18.2 MΩcm resistivity) was used as solvent for all solutions.

Metal release investigations and analysis

All equipment in contact with the solutions was acid-cleaned using 10% HNO₃ for 24 h and then rinsed four times with ultrapure water. Surface-prepared Ti6Al4V disks (c.f. section “Ti6Al4V alloy disks and surface preparation”) were immersed in closed centrifuge tubes at 37°C with a surface area to solution volume of ~1 cm²/mL (that is, 8 mL for one disk). The entire surface area of the disk was exposed to the solution and the tubes gently bi-linearly shaken (12°, 22 cycles per min) at dark conditions. Exposures were performed for 4 h, 24 h, 1 week and 2 weeks for solutions containing 40 g/L BSA and/or 30 mM H₂O₂, and 4 h and 2 weeks for the other solutions. For each solution and exposure duration, three disks were exposed in parallel together with one solution without any disk (blank). This resulted in 184 solution samples in total, of which 46 were blank samples. After exposure, the Ti6Al4V disk was removed from the solution. The solution pH was measured (<0.08 pH change in all cases), followed by acidification to pH <2 by ultrapure 65% HNO₃, and stored frozen. After solution unfreezing, the samples were digested. Between 1 (for highest BSA concentration) and 4 mL (for lowest BSA concentration) of the solution sample was diluted with ultrapure water to approximately 10 mL into which 1–2 mL 30% ultrapure H₂O₂ was added. An UV digester (90°C, Metrohm 705 UV digester) was used for complete digestion (at least 60 min) until the solution was transparent and odorless. After the digestion, the final volume was recorded, and the dilution factor DF calculated based on the final volume divided by the initial solution volume. Total concentrations of Ti, Al, and V were analyzed by means of graphite furnace atomic absorption spectroscopy (AAS) using optimized methods. Details on the methods, limits of detection (1.5,

TABLE I. Nominal Composition (wt %) of Ti6Al4V Disks (Average of Two Measurements, Relative Standard Deviation < 1.4% for All Elements, with the Exception for H < 11%), Based on Supplier Information

Ti	Al	V	Fe	O	N	C	H	Y
89.7	6.05	4.09	0.0605	0.119	0.0035	0.0101	0.0013	<0.0003

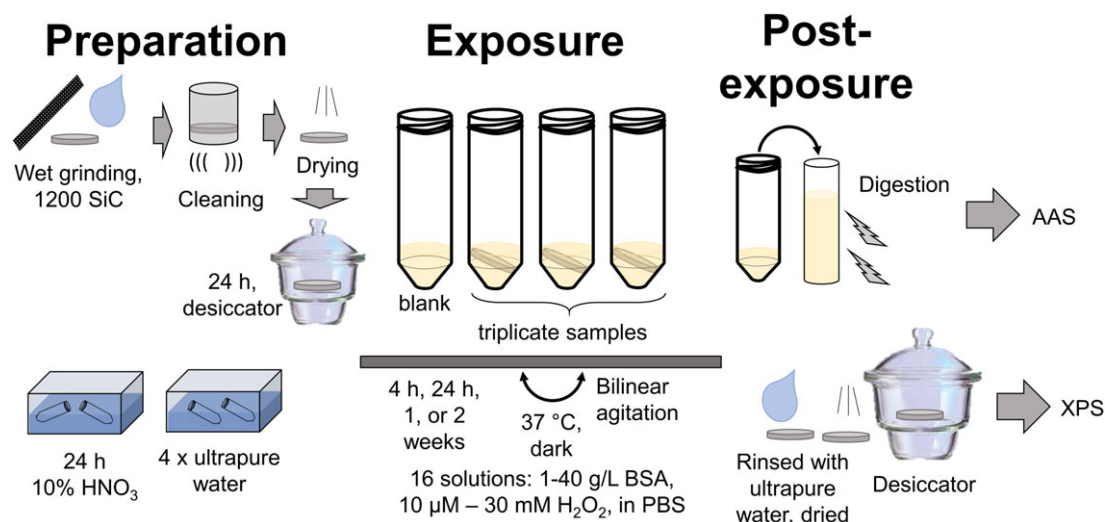


FIGURE 1. Schematic illustration of the preparation, exposure conditions, and post-exposure treatments prior to analyses with AAS and XPS.

1, 0.5 μg/L for Ti, Al and V, respectively), limits of quantification (3 μg/L for all elements), and % recovery are given in supporting information (Supporting Information Tables S1–S5). The released and nonprecipitated amount of metal (Me_{aq}) in solution in the unit of μg/cm² was then calculated as follows:

$$Me_{aq} \left(\frac{\mu\text{g}}{\text{cm}^2} \right) = \frac{(c_{\text{sample}} \left(\frac{\mu\text{g}}{\text{L}} \right) - c_{\text{blank}} \left(\frac{\mu\text{g}}{\text{L}} \right)) * V(\text{L})}{A(\text{cm}^2)} * DF$$

where c_{sample} is the measured sample concentration, c_{blank} is the corresponding blank concentration, V is the exposure volume (8 mL), A is the total surface area (approx. 8.5 cm²), and DF the dilution factor. If $c_{\text{blank}} > c_{\text{sample}}$, this is denoted as “<blank” in the figures.

The preparation, exposure conditions, and post-exposure treatments prior to analyses with AAS and X-ray photoelectron spectroscopy (XPS) are illustrated in Figure 1.

Electrochemical measurements

Open circuit potential measurements were conducted for at least two samples of each exposure condition for up to 72 h at (37 ± 1)°C in selected solutions using a PARSTAT multi-channel PMC Chassis instrument equipped with six PMC-1000 (AC/DC) channels. The reference electrode was Ag/AgCl saturated with KCl.

Surface compositional analysis

XPS (UltraDLD spectrometer, Kratos Analytical, Manchester, UK) measurements using a monochromatic Al K_α X-ray source (150 W) were performed on two separate surface areas approximately sized 700 μm × 300 μm for compositional analysis of the outermost surface composition (with information depth of 5–10 nm). Measurements were performed on non-exposed Ti6Al4V disks and on disks exposed for selected solutions and exposure periods. Elements of the outermost surface oxide were distinguished by running a wide spectrum. High resolution spectra

(pass energy of 20 eV) were acquired for Ti 2p, V 2p, Al 2p, N 1s, O 1s, and C 1s. All binding energies were corrected by the C 1s (C–C, C–H) peak at 285.0 eV. The results are presented as the relative mass content of oxidized Ti, Al, and V in the outermost surface oxide, for

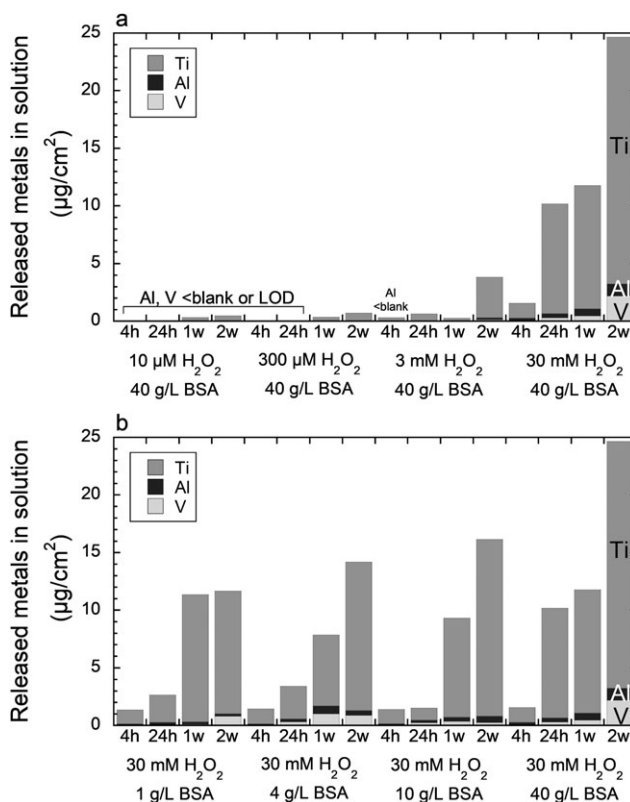


FIGURE 2. Total amount of released and non-precipitated metals in solution (Ti, Al, V) after 4 h, 24 h, 1 week, and 2 weeks of exposure of the Ti6Al4V disks in PBS containing 40 g/L BSA and varying amounts of H₂O₂ (a), and to PBS containing 30 mM H₂O₂ and varying amounts of BSA (b). < blank – released amounts lower than blank concentration; LOD – limit of detection.

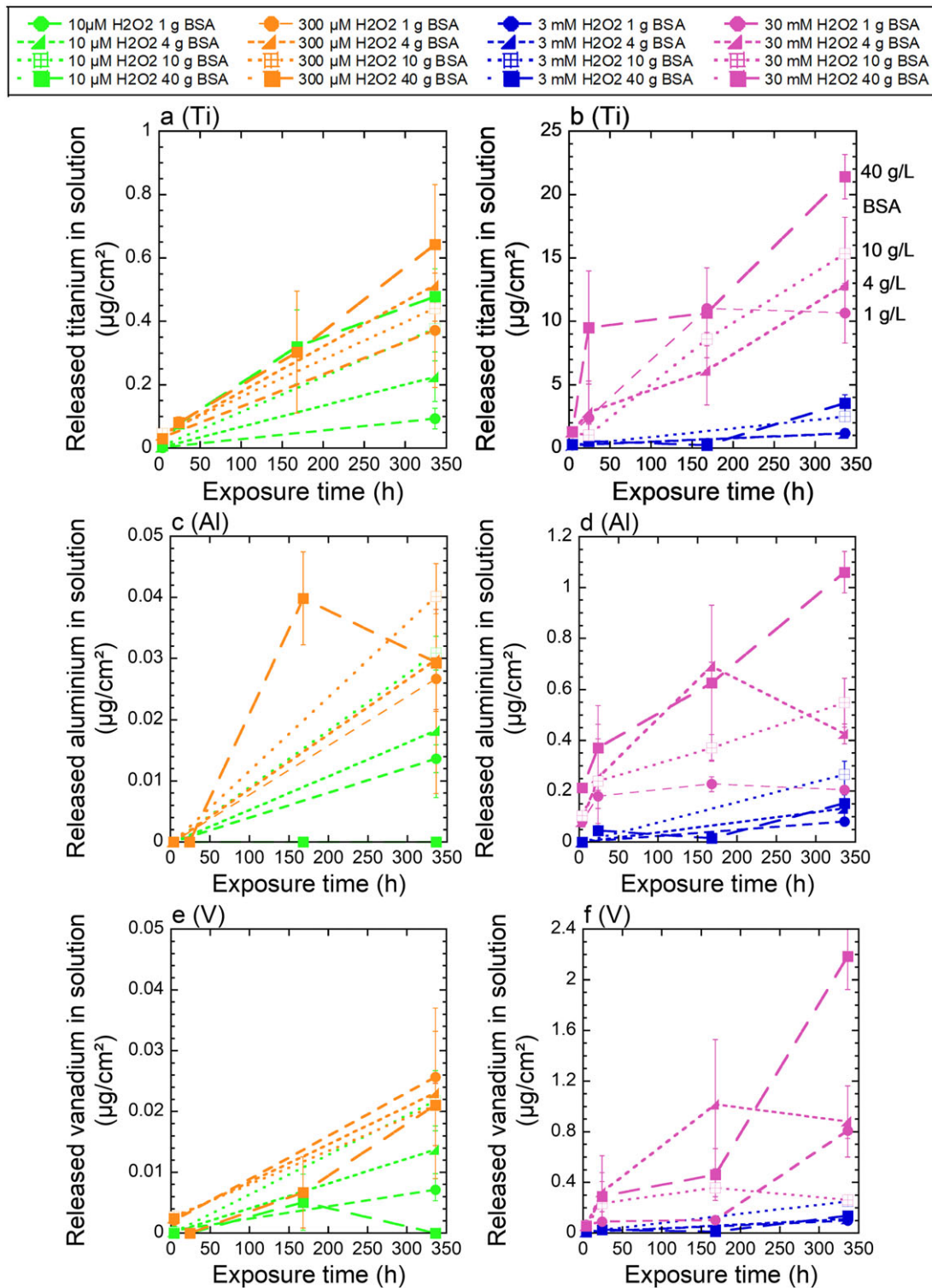


FIGURE 3. Total amounts of released and non-precipitated Ti (a,b), Al (c,d), and V (e,f) in solution after 4 h, 24 h, 1 week, and 2 weeks of exposure of the Ti6Al4V disks to different solutions. Twenty four hour and 1 week exposure data is only available for solutions containing 30 mM H_2O_2 and/or 40 g/L BSA. Note the different scales. The dotted lines are only intended for guidance.

example, $[\text{Ti}_{\text{ox}}/(\text{Ti}_{\text{ox}}+\text{Al}_{\text{ox}}+\text{V}_{\text{ox}})]\times 100$ wt %. Corresponding atomic ratios of N 1s (400.1 ± 0.5 eV) to the sum of the carbon peaks corresponding to C–N, C–O (286.6 ± 0.1 eV), C=C–O and O=C–N (288.4 ± 0.2 eV)

bonds, denoted as $\text{N}/(\text{C}2+\text{C}3)$, are presented. The C1 peak at 285.0 eV is not considered as it mainly derives from adventitious surface contamination. The Kratos software was used for peak deconvolution and analysis.

Statistical methods

An unpaired student's *t* test with unequal variance (KaleidaGraph v. 4.0) was used to test the probability of statistically significant difference between two independent sets of samples. When $p < 0.05$, the difference was considered statistically significant.

RESULTS

Metal release

A 3000-fold increase of the H_2O_2 concentration strongly increased the amount of released metals, Figure 2(a). The effect was nonlinear with the difference between 3 and 30 mM H_2O_2 mostly pronounced (six-fold, $p = 0.001$, for Ti), as compared to a non-significant difference between 10 and 300 μM H_2O_2 (1.3-fold, $p = 0.42$, for Ti) and a significant difference between 300 μM and 3 mM H_2O_2 (5.5-fold, $p = 0.01$, for Ti). A 40-fold increase in concentration of BSA (from 1 to 40 g/L BSA-physiologically relevant) generally resulted in increased amounts of all metals, Figure 2(b). The difference between 10 and 40 g/L BSA in PBS containing 30 mM H_2O_2 was mostly pronounced after 2 weeks (1.4-fold, $p = 0.046$, for Ti), as compared to a non-significant difference observed between 1 and 4 g/L BSA (1.2-fold, $p = 0.29$, for Ti) and a nonsignificant difference between 4 and 10 g/L BSA (1.2-fold, $p = 0.30$, for Ti). The same trend of increased metal release with increased BSA concentration was also observed for H_2O_2 concentrations lower than 30 mM [Figure 2(a)], that is, 10 μM , 300 μM , and 3 mM H_2O_2 , Figure 3. The absolute metal release decreased with decreasing BSA and H_2O_2

concentrations, Figure 2. The only exception was effects due to precipitation of released metals associated with protein aggregates (visible as reduced metal concentrations with time) in PBS containing 10 and 300 μM H_2O_2 for the highest (40 g/L) BSA concentration, Figure 3.

In all cases with detectable concentrations of Ti, Al and V, their released proportion was close to the bulk composition ratio (Table I), with V slightly preferentially released. The proportion between released metals was on average 88(Ti):6.0(Al):5.6(V) (by weight) with no apparent dependence on solutions or exposure time.

Surface oxide composition

XPS spectra were recorded for unexposed Ti6Al4V disks and disks exposed to PBS containing 40 g/L BSA and varying H_2O_2 concentrations, and disks exposed to 30 mM H_2O_2 containing varying amounts of BSA for 4 h, 24 h and 2 weeks. Representative spectra are given for Ti 2p, Al 2p, C 1 s, N 1 s and O 1 s for the lowest and highest concentrations of H_2O_2 (10 μM and 30 mM) at constant BSA concentration (40 g/L), and the lowest and highest concentrations of BSA (1 and 40 g/L) at constant H_2O_2 concentration (30 mM), Figures 4 and 5. Spectra for a medium concentration of BSA (10 g/L) and 30 mM H_2O_2 are provided in supporting information (Figure S1). Atomic concentrations and binding energies are compiled for all recorded peaks and measurements in Supporting Information Tables S6 and S7.

For the unexposed disks, both metal and oxide peaks were present for Ti (Ti 2p_{3/2}, metal peak at 454.6 ± 0.9 eV,

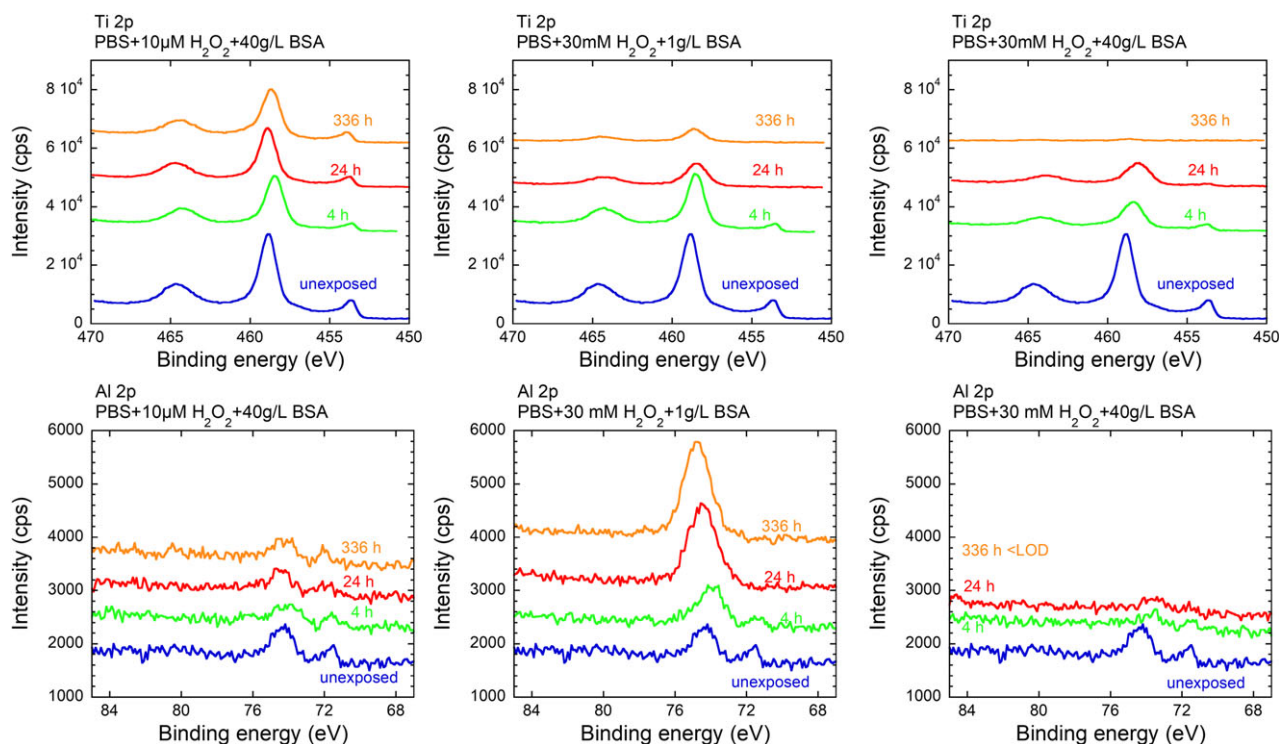


FIGURE 4. XPS spectra of Al 2p (bottom) and Ti 2p (top) of unexposed Ti6Al4V disks and exposed disks to PBS containing 10 μM H_2O_2 and 40 g/L BSA (left), 30 mM H_2O_2 and 1 g/L BSA (middle), and 30 mM H_2O_2 and 40 g/L BSA (right) after 4 h, 24 h, and 336 h (2 weeks) at 37°C. The spectra are off-set for clarity.

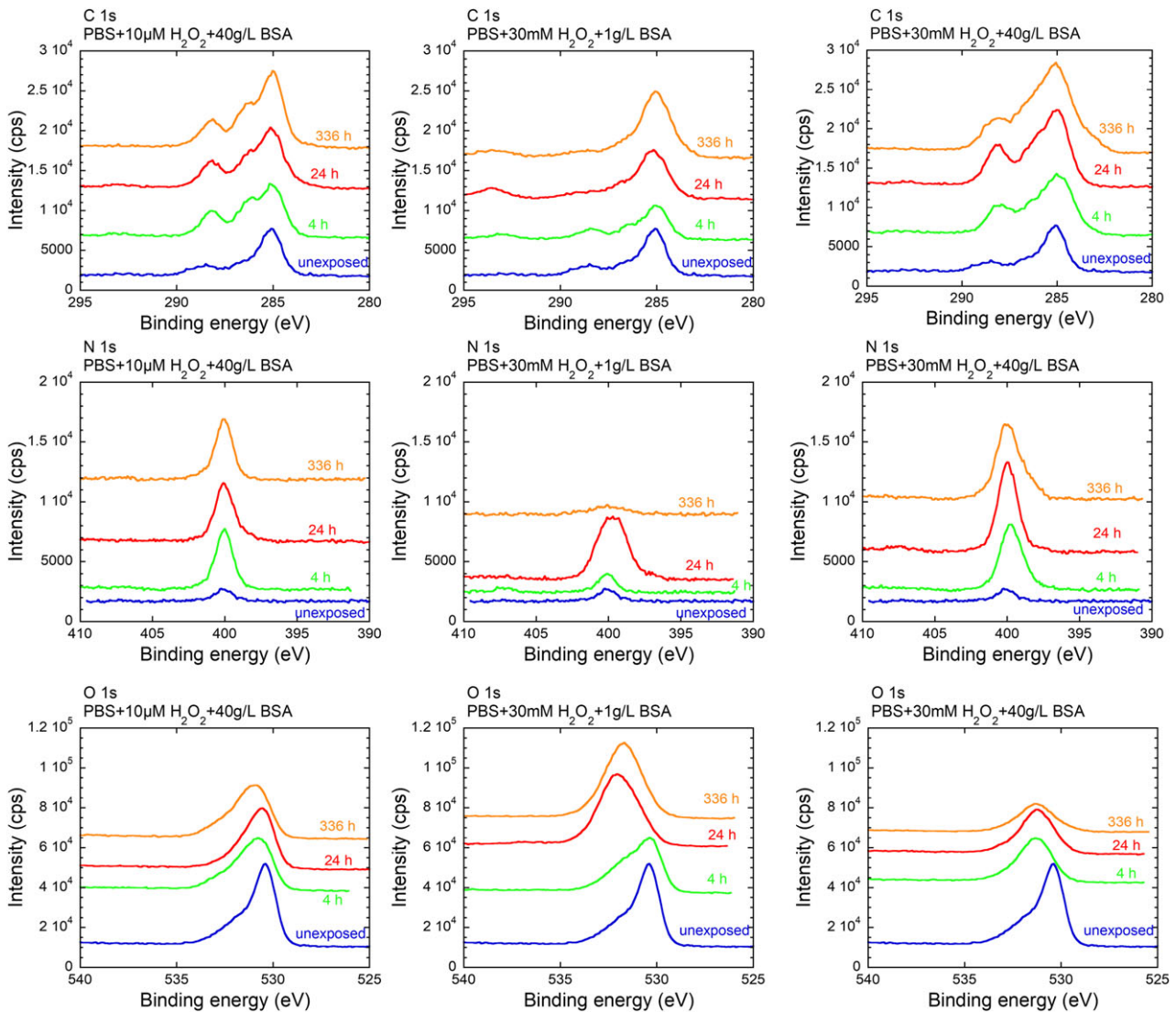


FIGURE 5. XPS spectra of C 1s (top), N 1s (middle row), and O 1s (bottom) of unexposed and exposed disks of Ti6Al4V to PBS (37°C) containing 10 μM H_2O_2 and 40 g/L BSA (left), 30 mM H_2O_2 and 1 g/L BSA (middle column), and 30 mM H_2O_2 and 40 g/L BSA (right) after 4 h, 24 h, and 336 h (2 weeks). The spectra are off-set for clarity.

oxide peak at 459.2 ± 0.5 eV), for Al (Al 2p, metal: 71.8 ± 0.6 eV; oxide: 74.9 ± 0.6 eV), and for V (V 2p_{3/2}, metal: 512.3 eV; oxide: 515.7 ± 0.01 eV), Figure 4 and Supporting Information Table S6. Oxygen was the predominant nonmetallic element, mainly attributed to metal oxides and to adventitious oxidized surface carbon. Nitrogen was present to a very low extent, Figure 5. No V (neither oxidized nor in metal form) was observed in the outermost surface oxide of any of the exposed disks, Supporting Information Table S6. No significant changes with time of the Ti and Al peaks were observed after exposure in PBS containing 40 g/L BSA and low H_2O_2 concentrations. However, at a H_2O_2 concentration of 30 mM, the oxide peaks gradually prevailed over the metal peaks. After two weeks of exposure, no metal peaks were discerned on the surface for any samples exposed in the solutions containing 30 mM H_2O_2 , Figure 4 and Supporting Information Figure S1 and Table S6. In the

presence of 40 g/L BSA, peaks originating from proteins were most evident, and increased generally with an increased BSA concentration. The N 1s peak (400.1 ± 0.2 eV) increased in intensity with increasing BSA concentration, the C 1s peak (285.0 eV, mainly related to C–C and C–H bonds) broadened with contributions of peaks at 286.6 ± 0.2 eV related to C α (peptidic residues), C–O, and C–N bonds and at 288.4 ± 0.2 eV related to N=C=O bonds.²⁰ Also the O 1s peak (530.3 ± 0.2 eV) broadened (531.5 ± 0.4 related to O=C in peptidic carbonyl groups, and 532.8 ± 0.4 eV related to C–OH bonds²⁰ due to the adsorption of proteins. However, a portion of the O 1s peak was also attributed to underlying oxides of Ti and Al. Compared to a theoretical atomic ratio of nitrogen to oxidized carbon $[\text{N}/(\text{C}_2+\text{C}_3)]$ of 0.48 for BSA,²¹ the observed ratio was lower for the lowest BSA concentration of 1 g/L (0.24 ± 0.10) and close to the theoretical ratio for the higher BSA

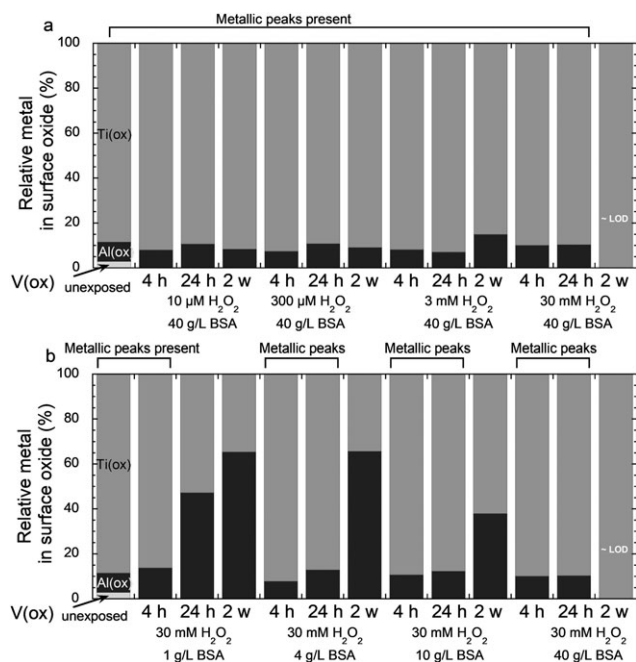


FIGURE 6. Relative metal content of oxidized metals (%) in the probed surface oxide by means of XPS (information depth 5–10 nm) after 4 h, 24 h, and 2 weeks of exposure of Ti6Al4V disks in PBS containing 40 g/L BSA and varying amounts of H₂O₂ (a), and to PBS containing 30 mM H₂O₂ and varying amounts of BSA (b). If metallic peaks were observed, hence the surface oxide was thinner than the XPS information depth, this is indicated in the figures. “≈LOD” denotes that only a weak peak assigned to oxidized titanium signal, and no aluminium peak were observed due to the presence of a thick layer of adsorbed BSA. Oxide composition is represented in wt %.

concentrations (4 g/L: 0.47 ± 0.011 ; 10 g/L: 0.49 ± 0.083 ; 40 g/L: 0.48 ± 0.067), indicative of relatively thicker layer of adsorbed BSA for the higher BSA concentrations compared with 1 g/L BSA.²² Despite the adsorption of proteins, clearly pronounced high-intensity peaks of Ti and Al were observed in all cases except for the disks exposed to the highest concentrations of H₂O₂ (30 mM) and BSA (40 g/L) after 2 weeks of exposure. In that case, the only detectable signal was a relatively weak Ti oxide signal, Figure 4.

Figure 6 shows the outermost surface oxide composition (oxidized peaks only) of the unexposed Ti6Al4V disks, disks exposed to PBS containing 40 g/L BSA and varying H₂O₂ concentrations [Fig 6(a)], and disks exposed to 30 mM H₂O₂ containing varying amounts of BSA [Figure 6(b)] for 4 and 24 h and 2 weeks. When the surface oxide was thinner than the information depth of XPS (5–10 nm), also metallic peaks were observed as indicated in Figure 6a,b (top). The surface oxide was thinner than the XPS information depth for the unexposed Ti6Al4V disks and disks exposed to PBS containing H₂O₂ at concentrations lower than 30 mM for up to 2 weeks, Figure 6(a). For PBS containing 30 mM H₂O₂, the surface oxide was thicker than information depth for the solution containing 1 g/L BSA already after 24 h, but for the solutions containing 4, 10, or 40 g/L BSA after 2 weeks of exposure, Figure 6(b). When the oxide was thicker than the XPS information depth, the fraction of Al in the oxide was

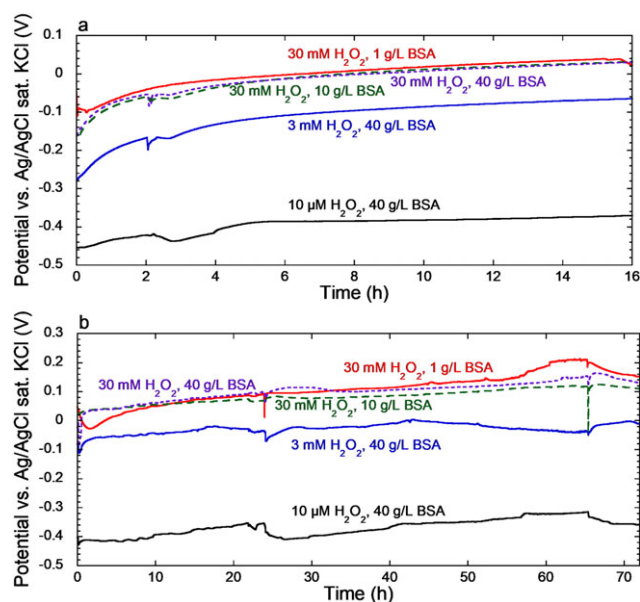


FIGURE 7. Changes in open circuit potential with time of Ti6Al4V disks exposed to PBS containing 10 μM, 3 mM or 30 mM H₂O₂ and 1, 10, or 40 g/L BSA. Two independent measurements are shown in (a) during 16 h and in (b) during 72 h. Measurements in each graph were run in parallel in different cells using the same water bath.

either significantly increased ($p < 0.05$ in all cases) or not observed (in the case of 30 mM H₂O₂ and 40 g/L BSA after 2 weeks of exposure), as compared to all other conditions for which the surface oxide was relatively thin. The fraction of Al in the oxide increased with exposure time for the PBS solutions containing 30 mM H₂O₂ and 1, 4, or 10 g/L BSA. For the disks exposed to PBS containing 30 mM H₂O₂ and 40 g/L BSA, no increase in Al content of the outermost surface oxide was observed up to 24 h. No Al was observed and only a weak oxidic Ti peak was present in the surface oxide after 2 weeks of exposure related to a dominant signal from adsorbed proteins. When comparing the Al fraction of the surface oxide after 2 weeks of exposure in the 30 mM H₂O₂ solutions of different BSA content, the fraction was approx. 65 wt % for 1 and 4 g/L BSA, 38 wt % for 10 g/L BSA, and non-detectable for 40 g/L BSA. A similar trend with reduced relative Al content in the outermost surface oxide with increased BSA concentration was observed after 24 h of exposure: 47 wt % for 1 g/L BSA, 13 wt % for 4 g/L BSA, 12 wt % for 10 g/L BSA, and 10 wt % for 40 g/L BSA.

Open circuit potential (OCP)

A positive slope of the OCP, indicative of surface passivation, and no pitting corrosion were observed for all tested conditions, Figure 7. The overall OCP increased with increasing H₂O₂ content in the solution. The effect of BSA concentration was investigated for the highest H₂O₂ concentration (30 mM) with only small differences observed, Figure 7. The features present in all measurements of each graph (in a) after ~ 2 h, and in (b) after approx. 24 and 66 h) are caused by a temperature change (for example, upon replenishment

of water in the water bath) or physical contact and can therefore be ignored.

DISCUSSION

This study shows a strong influence of a 3000-fold variation in H_2O_2 concentration and a combined (possibly synergistic) effect of H_2O_2 and BSA on the extent of metal release from a Ti6Al4V alloy exposed in PBS at pH 7.3. Generated results are in agreement with earlier studies.^{10,11,23} Compared with findings from one of these investigations,¹⁰ we measured up to five times higher released amounts of metals per surface area ($\mu\text{g}/\text{cm}^2$) in similar solutions. This could partially be explained by a higher surface area to solution volume ratio in that study¹⁰ causing a solution saturation effect, and partially by their use of syringe membrane filtration, which possibly removes some metal-rich protein aggregates. Our kinetic data indicates that aggregation and subsequent metal precipitation take place in at least some cases (highest BSA concentration and lowest H_2O_2 concentration), which suggests that the actual amount of released metals may be higher than reported. Compared to another study investigating the release of metals from Ti6Al4V in 100 mM H_2O_2 and unbuffered 0.9% NaCl after 1 week,²⁴ our release data is about 20-fold higher, possibly related to the presence of BSA or possibly also a pH effect. From the XPS measurements in this study, it is evident that significant changes in the surface oxide composition take place after a certain time period (between 24 h and 2 weeks) in the case of the highest H_2O_2 concentration (30 mM), seemingly more rapidly in solutions of the lower concentrations of BSA (1 and 4 g/L BSA) as compared with higher (10 and 40 g/L) BSA concentrations in PBS containing 30 mM H_2O_2 . No active or metastable corrosion was detected by the OCP measurements up to 72 h, and the influence of BSA concentration was small. A recent study investigating a similar solution (30 mM H_2O_2 , 10 g/L BSA, in minimum essential media at pH 7.4) found metastable pitting events when commercially pure titanium was polarized at 0.1 V vs. SCE (saturated calomel electrode),²³ effects not observed in this study on Ti6Al4V at OCP. Our OCP measurements are in agreement with previous observations in other studies on Ti metal²⁵ and Ti6Al4V¹¹ with a seemingly increased passivation with time (increased OCP) with increased H_2O_2 concentration due to its oxidative action.^{23,25} For short term (1–2 h) investigations of polarized commercial Ti metal at 0.4 V vs. SCE, the presence of H_2O_2 in PBS has been reported to result in the thinning of the surface oxide.²⁵ However, a recent study on Ti6Al4V at similar exposure conditions as in this study for up to 2 weeks, observed a thicker oxide caused by H_2O_2 , which however seemed to become thinner in the presence of albumin.¹¹

XPS measurements of this study indicate that Al is enriched in the surface oxide for Ti6Al4V disks exposed to the 30 mM H_2O_2 concentration, which probably indicates preferential dissolution of Ti. The formation of complexes and a defective non-stoichiometric TiOOH-rich oxide binding superoxide have been reported for H_2O_2 -TiO₂ interactions.^{25–27} Our XPS results further indicate a reduced

enrichment of Al in the outermost surface oxide with increased BSA concentration in PBS containing 30 mM H_2O_2 after 24 h and 2 weeks of exposure when comparing the higher and the lower BSA concentration. This could indicate that an increased BSA concentration results in a delay of the enrichment of Al in the surface oxide (coupled to a thicker surface oxide). This could possibly be explained by the initial suppression of cathodic currents due to adsorbed BSA, as previously suggested from electrochemical measurements.^{10,11} However, this delay in Al enrichment and oxide thickening was not associated with any reduction in released amounts of metals, instead, most metals were released into solution for the highest BSA concentration (40 g/L) after 2 weeks of exposure as compared to lower concentrations in PBS containing 30 mM H_2O_2 . We speculate that this observation can be explained by a preferential binding of BSA to Al, possibly facilitated by the H_2O_2 -induced defective TiOOH, and a relatively slow detachment process. Albumin is known to bind to Al.²⁸ The detachment process of Al bound to albumin depends on many factors, such as albumin concentration in solution,²⁹ ionic strength,³⁰ agitation,²⁹ and temperature.³¹ At the conditions of the human body, and of this study, this detachment process could take place.³²

No drastic changes in surface oxide composition (except for the depletion of V) or thickness and reduced amounts of released metals were observed for conditions with H_2O_2 concentrations lower than 30 mM. Findings of an earlier study suggest that H_2O_2 increases both the cathodic and the anodic reaction rate for Ti6Al4V at pH 6.7–7.¹⁰ Our study suggests that this results in a significantly thicker oxide, however, that this process initially is delayed if BSA to a large extent is adsorbed at the surface. After some time (24 h – 2 weeks), albumin seems able to bind to Al in this thicker oxide and deplete it from the surface. Our study does not suggest that albumin lowers the potential toward active ranges and thereby accelerates the corrosion process as claimed in reference.¹⁰ It seems rather to be a combined electrochemical and chemical (complexation) process. This interpretation is further supported by findings of a recent study¹¹ that shows that the induction of an “active” region by albumin may take place though that its role is minor. The same article suggests instead that albumin-induced dissolution of the surface oxide, which has mainly been formed by interactions with H_2O_2 , is the predominant and dominating mechanism after approximately 24–120 h, findings in agreement with our observations. The XPS results of our study further suggest that the combined effect of H_2O_2 and albumin results in the preferential complexation of H_2O_2 with Ti, and a preferential complexation of albumin with Al.

H_2O_2 treatments of Ti6Al4V alloys, resulting in similar changes in the surface oxide composition as observed in this study,³³ have been suggested beneficial for osseointegration and biocompatibility based on electrochemical investigations and apatite layer growth studies.^{33,34} However, the role of metal release, especially protein-induced non-electrochemical processes, has not been a target of investigation. It has previously been suggested that resistance toward dissolution of Ti may be reduced by H_2O_2 treatments.³⁵

Macrophages and other neutrophils may induce a high redox potential,¹³ which according to this study in the combination with albumin (or other Al-binding proteins) can result in an enhanced metal release process. An *in vivo* model suggests increased inflammatory response to Ti6Al4V particles and an increased number of macrophages,³⁶ and further a synergistic effect on inflammation by different biomaterials. It remains to be investigated whether this enhanced metal release declines or not after several weeks due to surface passivation. An investigation in rat tibia tissue suggests that released Ti concentrations increase up to 12 weeks, thereafter they remain constant up to 48 weeks.³⁷ This process was reported faster for released Al with increased concentrations up to 6 weeks followed by constant levels.³⁷ These observations could agree with our observations with Al depletion from the surface oxide.

The released proportion of Ti, Al, and V in this study was found to be close to the proportion expected from the bulk alloy composition. Generated results are in agreement with studies in 1% lactic acid, 1.2% L-Cysteine, and 0.01% HCl for 1 week,³⁸ with studies in rat tibia tissue for 48 weeks,³⁷ and with observations in tissue after failed Ti-alloy implants.³⁹ Preferential release of Al and V has been reported for less aggressive solutions (artificial saliva, 0.9% NaCl, and calf serum) than investigated in this study.³⁸ This suggests that our study might be clinically relevant. We recommend that future *in-vitro* investigations should make use of a complexing agent and H₂O₂ in sufficiently long (> 1 week) investigations to enable the relatively slow complexation/detachment reactions. The use of short-term electrochemical investigations to accelerate the corrosion process is believed to be insufficient to explore potential adverse effects induced by Ti6Al4V alloys in implant applications. A similar suggestion was raised in a recent electrochemical investigation.¹¹

CONCLUSIONS

The objective of this study was to gain further mechanistic insight from a surface composition and metal release perspective on the combined interplay of H₂O₂ and albumin from Ti6Al4V addressing aspects of kinetics (up to 2 weeks) and effects of different concentrations of H₂O₂ and albumin. The following main conclusions were drawn:

1. BSA and H₂O₂ enhanced in a combined way the extent of released metals from Ti6Al4V at all investigated conditions (10 μM–30 mM H₂O₂; 1–40 g/L BSA; PBS at pH 7.3, 37°C, 4 h – 2 weeks). The effect was strongest at the highest H₂O₂ concentration (30 mM) and the highest BSA concentration (40 g/L).
2. V was completely depleted from the outermost surface oxide for all conditions. The surface oxide remained thinner than the XPS information depth (5–10 nm) for all investigated conditions at H₂O₂ concentrations below 30 mM, but became substantially enriched in Al, and increased in thickness, when exposed in PBS containing 30 mM H₂O₂. This

enrichment of Al was however reduced with increasing BSA concentration, which may indicate a delay of surface reactions due to albumin adsorption and/or a complexation between BSA and Al.

3. The amount of released metals (Ti, Al, V) was approximately proportional to the nominal alloy composition (with V slightly preferentially released). These findings agree with worst case *in vitro* and *in vivo* observations reported in the literature.
4. OCP measurements up to 3 days indicated an improved passivation and the lack of metastable or active corrosion, and they were therefore unable to observe the substantial changes in surface oxide composition/thickness and amounts of released metals.
5. We propose a complexation-induced metal release process and a metal depletion process from the surface oxide, most probably induced by H₂O₂-Ti and/or albumin-Al interactions.
6. This study suggests that traditional short-term corrosion resistance measurements alone are not sufficient to predict potential adverse health effects induced by released metal species from Ti6Al4V alloys since also chemical dissolution mechanisms may be of large importance, possibly in a synergistic way and after relatively long-time periods (24 h or longer) of exposure.

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