Lipopolysaccharide inhibits or accelerates biomedical titanium corrosion depending on environmental acidity

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Titanium and its alloys are routinely used as biomedical implants and are usually considered to be corrosion resistant under physiological conditions. However, during inflammation, chemical modifications of the peri-implant environment including acidification occur. In addition certain biomolecules including lipopolysaccharide (LPS), a component of Gram-negative bacterial cell walls and driver of inflammation have been shown to interact strongly with Ti and modify its corrosion resistance. Gram-negative microbes are abundant in biofilms which form on dental implants. The objective was to investigate the influence of LPS on the corrosion properties of relevant biomedical Ti substrates as a function of environmental acidity. Inductively coupled plasma mass spectrometry was used to quantify Ti dissolution following immersion testing in physiological saline for three common biomedical grades of Ti (ASTM Grade 2, Grade 4 and Grade 5). Complementary electrochemical tests including anodic and cathodic polarisation experiments and potentiostatic measurements were also conducted. All three Ti alloys were observed to behave similarly and ion release was sensitive to pH of the immersion solution. However, LPS significantly inhibited Ti release under the most acidic conditions (pH 2), which may develop in localized corrosion sites, but promoted dissolution at pH 4–7, which would be more commonly encountered physiologically. The observed pattern of sensitivity to environmental acidity of the effect of LPS on Ti corrosion has not previously been reported. LPS is found extensively on the surfaces of skin and mucosal penetrating Ti implants and the findings are therefore relevant when considering the chemical stability of Ti implant surfaces in vivo.

Keywords: corrosion; electrochemistry; inductively coupled plasma mass spectrometry; lipopolysaccharide; titanium

INTRODUCTION

Titanium (Ti) and Ti alloys are routinely used for biomedical applications such as dental implants and bone anchorage of extra-oral prostheses and devices. Ti and its alloys are employed because of their perceived biocompatibility, suitable mechanical properties and corrosion resistance.1 The favourable cellular and bimolecular responses to Ti that are reported are conferred by the presence on the Ti surface of a thin passive oxide film which provides chemical stability.1–3 It has been established in in vitro experiments that Ti and its biomedical alloys are highly resistant to pitting corrosion and crevice corrosion at temperatures below 65 °C.4–6 Therefore, corrosion of Ti leading to the release of particles and ions into surrounding tissues is rarely considered by surgeons and patients as a likely event.

However, corrosion-related failures of implanted Ti devices and prostheses have been regularly reported within the medical literature7–8 and Ti corrosion products have been detected in significant quantities in the tissues and fluids adjacent to implants that have not been subjected to obvious tribological processes.9–11 In addition, using mass spectrometry, investigators have also shown that Ti levels can be significantly elevated in compartments remote from the implant site such as the serum of patients with indwelling Ti fixation devices.12 Characterisation of the systemically distributed Ti has demonstrated ionic associations with native biomolecules rather than a presence in particulate form which further supports that corrosion processes have been operative.12

The biological consequences of the release of Ti corrosion products and accumulation in tissues are significant, with pain,8 changes in the peri-implant tissue phenotype,13 genotoxicity14 and chronic inflammation leading to osteolysis and implant loss,15 being reported. The release of Ti under physiological conditions can be accounted for by both mechanically assisted crevice corrosion and biologically mediated processes. However, to date, our mechanistic understanding of the processes remains incomplete.7–8,16

The physiological environment into which a Ti implant is placed varies with site and with the device application, but is always a complex system containing various inorganic and organic components such as ions, amino acids, proteins and fatty acids, as well as living cells. The specific composition of this environment can also change in either health or disease due to physiological processes or as a direct consequence of the deterioration of the implant itself.8 Physiological fluids are buffered solutions and normally the pH remains close to 7.17 It has been demonstrated that in the presence of inflammation and/or in conditions associated with mechanically assisted crevice corrosion, the physiological environment can become locally acidified...
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Measurement of fluids in contact with Ti alloy orthopaedic implants retrieved during revision surgery has shown that the local acidity can reach as low as pH 2.5. For implants that become associated with a microbial biofilm such as dental implants, the presence of acidogenic bacteria has been shown to be capable of reducing the local pH to below 4.5 over sustained periods of time. It is evident that acidic environments can be generated in the body and are a potential hazard for metallic implants. More recently, there has been a focus on the contribution that organic molecules can have in altering the corrosion resistance of biomedical alloys in vivo. For a number of organic molecules relevant to the peri-implant environment the data reported have been inconsistent and it remains uncertain as to what effects they may have on the corrosion resistance of Ti and other biomedical alloys. It is, however, well known that many of these organic molecules strongly adsorb onto implant surfaces (including Ti and its alloys) and therefore, their potential role in modifying corrosion resistance is clinically relevant.

One organic molecule, lipopolysaccharide (LPS) also known as endotoxin, has received considerable attention because of its abundance and its crucial role as a mediator in peri-implant inflammation. Significant levels of adherent LPS are present on Ti implant surfaces and as LPS is a constituent of the cell wall of Gram-negative bacteria, any implant with a biofilm is likely to be associated with elevated LPS in the immediate environment. Although the effect of acidity on Ti corrosion is well reported, the influence of LPS has received much less attention and the results reported to date are inconsistent. Using electrochemical impedance spectroscopy and cyclic polarisation curves, it has been reported that the corrosion of ASTM Grade 2 commercially pure (CP) Ti was increased in LPS containing media. However, in the reported impedance spectroscopy results, the polarisation resistance \( R_p \) of Ti in the presence of LPS was increased when compared with that in the absence of LPS, indicating a lower corrosion rate and therefore the results appear to be inconsistent. The objective of the current study was to investigate the influence of LPS on the corrosion properties of relevant biomedical Ti substrates as a function of environmental acidity. Given the inconclusive data that have been previously reported, the null hypothesis that there is no impact of LPS on the corrosion resistance biomedical Ti was tested.

**MATERIALS AND METHODS**

**Ti sample preparation**

Commercial purity ASTM Grade 2 Ti (lower Fe content, CP-Ti-G2); commercial purity ASTM Grade 4 Ti (higher Fe content, CP-Ti-G4) and ASTM Grade 5 Ti (Ti6Al4V) were sourced (Titanium Products Ltd, Solihull, UK)—all grades of Ti were cold-rolled and annealed at 600–700 °C for 20 min (Grades 2 and 4) or 1 h (Grade 5) and compositional analysis acquired (Table 1). Nominally identical disc-shaped specimens of CP-Ti-G2 and CP-Ti-G4 (14 mm diameter and 1 mm thickness) were machined by the supplier. The surfaces were subsequently ground to a consistent finish using sequential grades of SiC abrasive paper from P400, through P800, P1200 and P2400 to P4000 grit with deionized water as a lubricant. The surfaces of Ti6Al4V disc-shaped specimens (14 mm diameter and 1.2 mm thickness) were prepared using abrasive cloths MD-Piano (Stuers, Ballerup, Denmark) and deionized water as a lubricant followed by MD-Largo (Stuers) with a 9 μm diamond suspension as a lubricant (Stuers). Finally all three grades of Ti were polished with a MD-Chem polishing cloth (Stuers) using OP-S Colloidal Silica suspension (Stuers) to produce a consistent mirror finish on both sides. All samples were thoroughly cleaned sequentially in acetone, ethanol, and deionized water using ultrasonic agitation for 10 min at each stage. Specimens were finally dried in a nitrogen stream and used in further experiments.

**Immersion testing**

Physiological saline (0.9% NaCl) was used as a representative immersion medium (ASTM F1801-97(2009)e1), enabling comparison of findings with similar studies. It also avoids the use of P, which causes potential polyatomic interferences in inductively coupled plasma mass spectrometry (ICP-MS) measurements on solutions containing Ti. Each solution was modified according to two experimental factors, pH (at four levels) and LPS (with or without the addition of LPS). The pH value of the media was measured with a calibrated O40 pH meter (Beckman Instruments, Brea, CA, USA) and modified to pH 7, 5.5, 4 or 2 by adding lactic acid (Sigma-Aldrich, Dorset, UK). To investigate the effect of LPS, a further series of test solutions at each pH were prepared containing 150 μg·mL⁻¹ of LPS (Escherichia coli LPS 055:B5; Sigma-Aldrich, Dorset, UK) with the pH re-measured immediately after LPS addition. To minimize contamination with elements of interest for subsequent mass spectrometry measurements, ultrapure water (Gibco, Life Technologies, Paisley, UK) and mass spectrometry grade reagents were used throughout.

All prepared Ti samples were immersed in 2 mL of test solution in a 30 mL container sealed to prevent evaporation. For each experimental condition, three samples of each Ti grade (CP-Ti-G2, CP-Ti-G4 and Ti6Al4V) were immersed and incubated at 37 °C. To maintain the majority of the surface area to be exposed to the immersion medium. Samples were gently agitated on an A500 orbital mixer (Thermo Denley, Colchester, UK) for 1 h per day. For each sample, the test media was retrieved and replaced with new solutions after 7 days. The pH values of the prepared test media were measured immediately before use on each occasion. Following 4 weeks of immersion, the retrieved media for each sample were pooled together to form 8 mL, which was used for ICP-MS measurement. Prior to the ICP-MS measurement, the pH values of all the collected immersion solutions were re-measured again to identify any modification in acidity over the immersion period. The exposed surface area of Grade 5 disc shaped specimens was ~2% greater than Grade 2 and 4 specimens, and this was taken into account in the interpretation of the ICP-MS data.

![Table 1 The composition of CP-Ti-G2, CP-Ti-G4 (manufacturers compositional certificate, weight-%) and Ti6Al4V (ASTM standard F1108-97A, weight-%)](image_url)
Determination of released ion concentration

Test solutions from each sample were syringe-filtered (pore size: 0.45 μm) and refrigerated at 4 °C prior to ICP-MS measurement. Elemental Ti concentration in the immersion solutions was quantified using ICP-MS (Agilent 7500ce ICP-MS) where the detectable limit for Ti was 0.2 ng·mL⁻¹. Initially two-way analyses of variance (ANOVA) were used to identify significant differences between ICP-MS measurements (p=0.05) where the factors were Ti grade (at three levels) and pH (at four levels) with tests run independently for samples with and without LPS. Subsequently, further two-way ANOVAs were conducted for each Ti grade where the factors were LPS (at two levels) and pH (at four levels). Post-hoc Tukey’s tests (p=0.05) were used to identify significant differences between individual groups.

Surface characterisation

The surfaces of the Ti discs were characterized before and after immersion testing to identify surface modifications. Scanning electron microscopy (SEM) was undertaken with a JEOL 7000 SEM (Jeol, Tokyo, Japan) using an accelerating voltage of 20 kV, a beam current of ~70 μA, an original magnification of ×5 000 and using both secondary electron mode and backscatter electron mode. The elemental composition was analysed using energy dispersive X-ray spectroscopy (detector model: 7558; collecting window: ATW 2; acquisition time: 60 s; quantification method: standardless; Oxford Instruments, Abingdon, UK).

Electrochemical testing

A standard three-electrode cell with reference electrode, counter electrode and working electrode was used in a potentiostat (ACM Instruments, Cumbria, UK). The counter electrode was a Pt mesh and the reference electrode was a commercial saturated calomel electrode (SCE). All potentials are quoted against this reference scale. Ti discs were mounted in VARI-SET cold mounting acrylic (MetPrep, Coventry, UK) and used as the working electrode. The electrode was polished to a mirror surface using identical sample preparation methods, and the electrode was used in a potentiostat (ACM Instruments, Cumbria, UK). The counter electrode was a Pt mesh electrode (SCE). All potentials are quoted against this reference scale. Ti was 0.2 ng·mL⁻¹. Initially two-way analyses of variance (ANOVA) were used to identify significant differences between ICP-MS measurements (p=0.05) where the factors were Ti grade (at three levels) and pH (at four levels) with tests run independently for samples with and without LPS. Subsequently, further two-way ANOVAs were conducted for each Ti grade where the factors were LPS (at two levels) and pH (at four levels). Post-hoc Tukey’s tests (p=0.05) were used to identify significant differences between individual groups.

RESULTS

Quantification of Ti release

Two-way ANOVAs demonstrated no significant effect of Ti grade on the magnitude of Ti release into physiological saline following immersion in LPS-free and LPS-containing media (p>0.05), whereas pH significantly influenced the detected Ti elemental concentration (p=0.01). Post-hoc Tukey’s tests demonstrated that Ti release was significantly increased at pH 2 for all Ti grades in comparison with pH ≥4 (p<0.01) (Figure 1 and Table 2). Further Two-way

![Figure 1](image-url)  
**Figure 1** Mean concentrations of Ti ions released from mirror polished CP-Ti-G2, CP-Ti-G4 and Ti6Al4V following immersion in physiological saline with and without LPS for 4 weeks at 37 °C. The final immersion solution pH values measured after the immersion period are provided below the initial values. Error bars refer to 1 standard deviation (n=3). CP, commercially pure; LPS, lipopolysaccharide.
ANOVAs run independently for each Ti Grades, demonstrated that LPS significantly modified the mean Ti concentration quantified by ICP-MS of the immersion solution (P < 0.01). At pH 2, the addition of LPS caused a significant decrease in the mean Ti concentration in the solution (P < 0.01), whereas for pH of 4, 5.5 and 7, LPS addition significantly increased the detected levels of Ti (P < 0.01) (Figure 1). Ti levels in the control immersion media with and without LPS were negligible. The action of LPS as a potential buffer was tested with pH measurements during immersion media preparation before and after LPS addition. pH was recorded again following the collection of the test solutions after the immersion period, prior to elemental quantification (Figure 1). A significant change in the pH of the media prepared at pH 7 was observed following the immersion period where a reduction in pH to 5.8 was recorded. All remaining immersion solutions were not significantly affected (Figure 1).

### Anodic polarization

The anodic polarization curves of polished Ti6Al4V and CP-Ti-G4 measured in physiological saline at pH 2 and 4 in the absence and presence of LPS at 37 °C are shown in Figure 2. The values of OCP were

| Condition   | Control group | CP-Ti-G2 group | CP-Ti-G4 group | Ti6Al4V group |
|-------------|---------------|----------------|----------------|---------------|
| pH 2        | 0.5±0.3       | 381±37         | 341±16         | 356±27        |
| pH 2+LPS    | 0.4±0.3       | 70±3           | 56±4           | 67±7          |
| pH 4        | 0.2±0.01      | 1±1            | 0.5±0.01       | 1±1           |
| pH 4+LPS    | 0.4±0.4       | 43±2           | 46±4           | 56±6          |
| pH 5.5      | 0.4±0.3       | 3±1            | 0.5±0.4        | 0.4±0.2       |
| pH 5.5+LPS  | <0.2          | 31±1           | 43±2           | 43±10         |
| pH 7        | <0.2          | 2±1            | 1±0.2          | 1±0.1         |
| pH 7+LPS    | 0.4±0.3       | 29±5           | 34±2           | 28±9          |

CP, commercially pure; LPS, lipopolysaccharide.

**Table 2** Mean concentrations and standard deviations of Ti ions released from mirror polished CP-Ti-G2, CP-Ti-G4 and Ti6Al4V following immersion in physiological saline with and without LPS for 4 weeks at 37 °C (n=3)

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**Figure 2** Anodic polarization curves. (a) Ti6Al4V; (b) enlarged image of the region outlined in (a); (c) CP-Ti-G4; (d) enlarged image of the region outlined in (c). Ti6Al4V and CP-Ti-G4 in physiological saline solution adjusted to pH 2 and pH 4 in the absence and presence of LPS at 37 °C (n=3—all data shown). CP, commercially pure; LPS, lipopolysaccharide.
also listed in Table 3. A higher anodic current was measured for both Ti6Al4V and CP-Ti-G4 at pH 2 compared with measurements at pH 4 (Figure 2). For media adjusted to pH 2 containing LPS, the anodic current decreased for both Ti6Al4V and CP-Ti-G4 when compared with the LPS free physiological saline. In contrast at pH 4, an increased anodic current was observed for both Ti6Al4V and CP-Ti-G4 in the LPS containing physiological saline when compared with the LPS-free media (Figure 2).

Cathodic polarisation
Cathodic polarisation curves of Ti6Al4V and CP-Ti-G4 were measured separately from the anodic measurements (Figure 3). A higher cathodic current was observed for Ti6Al4V and CP-Ti-G4 at pH 2 when compared to that at pH 4 (Figure 3). In the presence of LPS, the cathodic current decreased for both Ti6Al4V and CP-Ti-G4 at pH 2, but slightly increased at pH 4 when compared to the media without LPS.

Potentiostatic study
The potentiostatic measurements of polished Ti6Al4V and CP-Ti-G4 in physiological saline at pH 2 and 4 with and without the addition of LPS at 600 mV(SCE) at 37°C are shown in Figure 4. The solution was stirred at intervals to ensure mixing. LPS was added just prior to stirring at ~1 000 s for some of the measurements. It is clear that the passive current density of both grades of Ti is greater at pH 2 than at pH 4 (Figure 4). On addition of LPS, the current density measured for samples immersed at pH 2 decreases rapidly (within 1 000 s) to the

| Condition | CP-Ti-G4 group | Ti6Al4V group |
|-----------|----------------|---------------|
| pH 2      | -374±16        | -336±20       |
| pH 2 + LPS| -390±33        | -355±17       |
| pH 4      | -327±27        | -315±20       |
| pH 4 + LPS| -394±55        | -378±30       |

CP, commercially pure; LPS, lipopolysaccharide; OCP, open circuit potential; SCE, saturated calomel electrode.
value found for samples immersed at pH 4. However, LPS had no effect on the passive current density at pH 4. It should be noted that the addition of LPS had no detectable effect on the pH of the solution.

Surface morphology and composition
Figure 5 shows the surface morphology of all three alloys in the as polished condition, following immersion for 4 weeks in physiological saline at pH 7 and pH 2. CP-Ti-G2 shows no significant second phase particles, whereas CP-Ti-G4 (higher Fe level) shows TiFe\textsubscript{x} intermetallic particles, and Ti6Al4V shows the characteristic two-phase (α/β) microstructure. No significant difference in the surface morphology before and after immersion was observed.

DISCUSSION
In the presence of inflammation, chemical modifications of the peri-implant environment occur alongside changes in the cellular composition of the tissues. Local acidification and the presence of inorganic and organic species, which may modify the corrosion resistance of the implant itself, have been established.\textsuperscript{5-9,20} The current study demonstrated higher Ti ion release at pH 2 compared with pH 4 or

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Figure 4  Potentiostatic study (600 mV vs SCE). (a) Ti6Al4V and (b) CP-Ti-G4 in physiological saline adjusted to pH 2 or pH 4 at 37 °C; the solution was stirred for \~100 s at \~1 000 s intervals to ensure mixing; for two experiments, LPS (in physiological saline) was added at 1 000 s immediately before stirring to give a final concentration of 150 μg·mL^{-1} \(n=2\)—all data shown). CP, commercially pure; LPS, lipopolysaccharide; SCE, saturated calomel electrode.

Figure 5  SEM images of mirror polished surfaces of Ti and its alloy. CP-Ti-G2, CP-Ti-G4 and Ti6Al4V before and after immersion for 4 weeks at 37 °C in physiological saline adjusted to pH 7 or pH 2. BSE, backscatter electron mode SEM image; CP, commercially pure; PS, physiological saline; SE, secondary electron mode SEM image; SEM, scanning electron microscopy.
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7, consistent with previous work showing the observation of distinctly reduced ion release at and above pH 4. However, the addition of LPS to the immersion solution significantly modified this pattern of Ti release, promoting corrosion at pH ≥ 4 but inhibiting it at pH 2, a finding which has not previously been reported. The null hypothesis that there is no impact of LPS on the corrosion resistance biomedical Ti was therefore rejected. In addition, it has been previously demonstrated that the solubility of the Ti oxide surface layer is largely independent of acidity at pH ≥ 4 and therefore, the direct effect of LPS in lowering the solution pH is not considered to provide an explanation for the increased Ti release at pH ≥ 4.

Despite the differences in microstructure and composition between the three grades of Ti studied, no differences in Ti ion release were observed in the immersion conditions used, and accordingly, only CP-Ti-G4 (lower purity commercially pure Ti) and the alloy Ti6Al4V were studied in electrochemical tests. However, the presence of localized impurities (CP-Ti-G4) and intergranular zones (Ti6Al4V) is known to modify the morphology and potential rate of corrosion attack, and the current findings cannot exclude that differences would be observed with modified environmental conditions and measurement procedures.

The increased solubility of the passive oxide film on Ti alloys in highly acidic conditions was confirmed using anodic polarisation and potentiostatic measurements of CP-Ti-G4 and Ti6Al4V specimens. When the saline immersion solution was maintained at pH 2, a shift towards both higher anodic currents, cathodic currents and passive currents was observed when compared with pH 4 (Figures 2–4). Following addition of LPS to the pH 2 solution, the anodic, cathodic and passive currents were all decreased (Figures 2–4). In contrast, addition of LPS to the pH 4 solution increased the anodic and cathodic currents when compared with an LPS free environment (Figures 2 and 3). There was no significant change for the passive current at pH 4 with and without the addition of LPS for both Ti6Al4V and CP-Ti-G4 (Figure 4).

It is generally established that organic molecules can affect the corrosion behaviour of metals mediated by adsorption onto the surfaces and/or chelation with the released dissolved ions. Studies on serum protein interactions with biomedical Ti surfaces, using potential sweep techniques, have established that surface adsorption processes are potential dependent. Further electrochemical tests have demonstrated that serum proteins such as albumin and fibrinogen can act to decrease the cathodic current, but also increase the anodic component. It is possible that complexation of Ti ions in the component is implicit, suggesting that where the metal is exposed to a neutral environment of the surface of acidogenic bacteria may be considerably lower approaching pH 2. Importantly, however, in the body, the electrochemical actions will take place through the biofilm developed on the implant surface and therefore, the observed behaviours may differ from the experimental outcome. Ti corrosion products have been demonstrated in the peri-implant tissues and the release of Ti ions and accumulation of Ti has been shown to be pro-inflammatory and have the potential to affect clinical outcomes.

CONCLUSIONS

LPS is a component of the cell walls of Gram-negative bacteria, which may colonize the surfaces of Ti implants in the body. Under the experimental conditions of the current study, LPS inhibits corrosion of Ti (Grades 2, 4 and 5) in highly acidic environments representative of sites where mechanically assisted crevice corrosion may be occurring, whereas in slightly acidic and neutral environments, representative of sites of peri-implant inflammation LPS accelerates corrosion. Clinically, these findings are highly significant for skin and mucosa penetrating implants where the presence of a surface biofilm formation is implicit, suggesting that where the metal is exposed to a neutral pH, LPS may cause a slight increase in the passive current density.

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