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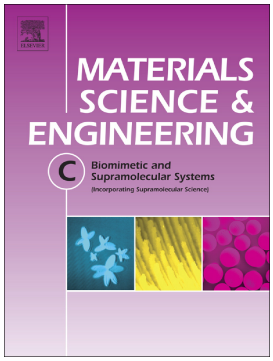

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Alkaline phosphatase binds tenaciously to titanium; implications for biological surface evaluation following bone implant retrieval.

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Abstract

Enhancing the performance and longevity of titanium (Ti) implants continues to be a significant developmental theme in contemporary biomaterials design. Our specific focus pertains to the surface functionalisation of Ti using the bioactive lipid, lysophosphatidic acid (LPA) and certain phosphatase-resistant analogues of LPA. Coating survivorship to a plethora of testing regimens is required to align with due regulatory process before novel biomaterials can enter clinical trials. One of the key acceptance criteria is coating retention to the physical stresses experienced during implantation. In assessing coating stability to insertion into porcine bone we found that a subsequent *in vitro* assessment to confirm coating persistence was masked by abundant alkaline phosphatase (ALP) contamination adsorbed to the metal surface. Herein we report that ALP can bind to Ti in a matter of minutes by simply immersing Ti samples in aqueous solutions of the enzyme. We strongly discourage the *in vitro* monitoring of osteoblast and stromal cell ALP expression when assessing bioactive coating survivorship following Ti implant retrieval form native bone tissue.

Key words: Titanium; functionalisation; bone; osteoblasts; alkaline phosphatase; contamination.

1. Introduction

Fabricating novel, biologically active, titanium (Ti) implants represents an active area of research and development within orthopaedics, dentistry and, more recently, veterinary medicine. An attempt at improving early osseointegration of implants includes coating devices with suitable biological agents that target bone forming osteoblasts. In addition, selected coatings that promote osteoblastogenesis from bone marrow stromal cells are also desirable features in fabricating next generation implantable devices. In searching for the most suitable Ti coating a plethora of tests need to be performed to satisfy the relevant regulatory pathways and acceptance criteria prior to any clinical trial or *in vivo* evaluation using the most appropriate animal models. These tests include exposing functionalised titanium specimens to γ-irradiation, autoclaving, irrigation, evaluation of coating survivorship to ambient storage for prolonged periods (12 months or more) and resistance to the rigors of physical stresses. With regard coating stability to physical forces the most pragmatic approach would be insertion into bone tissue, retrieval and surface analysis; recovered samples can subsequently be prepared, for example, for *in vitro* evaluation for molecular and biochemical assessment to confirm persistence of the biological coating for.

Some of the most important analyses are concerned with evaluating both osteoblast and stromal cell responses to implanted samples. Wherever possible it is generally agreed that tracking the expression of functional proteins from cells seeded onto functionalised surfaces is the most valuable. One widely studied protein produced by osteoblasts as they progress to a more mature phenotype is tissue nonspecific alkaline phosphatase (TNSALP), an

enzyme inextricably linked to bone matrix calcification, a loss of which is associated with hypophosphatasia [1]. In ascertaining the stability of our lysophosphatidate-functionalised Ti materials [2-3] to bone insertion, recovered and non-inserted functionalised controls were seeded with MG63 osteoblasts, cultured under conventional conditions for three days, and an assessment of cellular maturation determined via TNSALP activity assay. To our surprise we unexpectedly found that inserted, lysophosphatidate-functionalised dental implants were associated with the greatest TNSALP activity (~9 times greater) compared to irrigated, non-inserted, functionalised controls. The simplest explanation for this clear contamination would be TNSALP adsorbed to the metal surface during bone insertion and retrieval. Herein this short communication we provide compelling evidence for the facile binding of alkaline phosphatases to both dental and orthopaedic-grade Ti. We offer a cautionary note for the *in vitro* monitoring of osteoblast maturation responses via total TNSALP activity for Ti samples retrieved from native bone tissue.

2. Materials & Methods

2.1. General

Unless stated otherwise, all reagents were of analytical grade from Sigma-Aldrich (Poole, UK). Stocks of (3S)1-fluoro-3-hydroxy-4-(oleoyloxy)butyl-1-phosphonate (FHBP, 500μM, Tebubio, Peterborough, UK) were prepared in 1:1 ethanol:tissue culture grade water and stored at -20 °C. Likewise, 100 μM stocks of calcitriol (1,25D) were prepared in ethanol and stored at -20 °C. Orthopaedic-grade Ti discs (Ti6AL4V, 12mm diameter, thickness 2mm) were kindly provided by Corin (Cirencester, UK) whereas Ti dental screws (Advanced Implant 2 piece 4.5 x 8 mm) were provided by OsteoCare (Slough, UK). Bovine intestinal alkaline phosphatase (ALP) and porcine TNSALP (from kidney) were stored refrigerated and applied to Ti discs in cell culture-grade water. Porcine mandibles were provided by Brackley Butchers (Brackley, UK).

2.2. FHBP-functionalisation of dental implants and insertion into porcine mandibles

The surface functionalisation of Ti dental screws (Figure 1) using FHBP (2μM) was similar to that described by us recently [2]. Briefly a total of 18 screws were individually placed within polypropylene microcentrifuge tubes (1.5ml) and subjected to FHBP functionalisation under ambient conditions. Recovered screws were rinsed in cell culture grade water and allowed to dry before being split into three groups; one set remained “as is” another set was subjected to a tap water rinse and cleaning with an electric toothbrush (Oral-B Pro-Health) whilst the final set were inserted into pig mandibles (Figure 2), left for 10 minutes in bone, retrieved and then subjected to a tap water rinse and cleaning with an electric toothbrush. Both FHBP-Ti washed controls and FHBP-Ti bone inserted specimens were also given a triple

rinse in cell culture grade water followed by a triple rinse in 70% ethanol and stored in this solution for 24 hours prior to osteoblast cell culture. Six unmodified dental devices served as blanks for subsequent human osteoblast culture.

2.3. Human osteoblasts

Human osteoblast-like cells (MG63) were cultured in conventional tissue culture flasks (250 mL, Greiner, Frickenhausen, Germany) in a humidified atmosphere at 37 °C and 5 % CO2. Although osteosarcoma-derived, MG63 cells exhibit features in common with human osteoblast precursors or poorly differentiated osteoblasts. Specifically, these cells produce type I collagen with no or low basal osteocalcin (OC) and ALP. However, when MG63's are treated with 1,25D, OC expression increases [4-5] and, when the same cells are co-treated with 1,25D and selected growth factors, e.g. lysophosphatidic acid, the levels of ALP markedly increase [6], a feature of the mature osteoblast phenotype. Consequently, the application of these cells to assess the potential pro-maturation effects of selected factors is entirely appropriate. Cells were grown to confluence in Dulbecco's modified Eagle medium (DMEM)/F12 nutrient mix (Gibco, Paisley, Scotland) supplemented with sodium pyruvate (1 mM final concentration), L-glutamine (4 mM), streptomycin (100 ng/mL), penicillin (0.1 units/mL) and 10 % v/v foetal calf serum (Gibco, Paisley, Scotland). The growth media (500 mL final volume) was also supplemented with 5 mL of a 100x stock of non-essential amino acids. Once confluent, MG63s were subsequently dispensed onto control and FHBP-functionalised dental screws in the presence of 100nM 1,25D in serum-free growth medium. In each case, implants (placed within wells of a 24-well culture plate) were seeded with 1 mL of a 15 x 10^4 cells/mL suspension, as assessed by haemocytometry. Cells were then cultured

for 3 days, the screws transferred to clean microcentrifuge tubes and an assessment of total ALP activity determined to ascertain the extent of cellular maturation.

2.4. Total ALP activity

An assessment of ALP activity is reliably measured by the generation of p-nitrophenol (p-NP) from p-nitrophenylphosphate (p-NPP) under alkaline conditions. The treatment of cells to quantify ALP activity was similar to that described by us recently [7]. Cells on control and FHBP-functionalised Ti screws were lysed with 0.1 mL of 25 mM sodium carbonate (pH 10.3), 0.1 % (v/v) Triton X-100. After 2 min, each tube was treated with 0.2 mL of 15 mM p-NPP (di-Tris salt, Sigma, UK) in 250 mM sodium carbonate (pH 10.3), 1 mM $MgCl_2$. Samples were then incubated at 37°C. for 90 minutes. After the incubation period, 0.1 mL aliquots were transferred to 96-well microtitre plates and the absorbance read at 405 nm. An ascending series of p-NP (50-500 µM) prepared in the incubation buffer enabled quantification of product formation. Unless stated otherwise, total ALP activity is expressed as the mean micromolar concentration of p-NP ± SD.

2.5. Treatment of Ti with alkaline phosphatase

Dental screws were immersed in an aqueous solution of bovine intestinal ALP under ambient conditions for a maximum of 30 minutes. The ALP preparation in this instance generated p-NP at approximately 150nmols/ml/min^{-1}. Once incubated all samples were given 5 rinses using distilled water with moderate, manual shaking. Samples were placed within microcentrifuge tubes (1.5ml) and treated with 0.1 mL of 25 mM sodium carbonate (pH 10.3), 0.1 % (v/v) Triton X-100 followed by 0.2 mL of 15 mM p-NPP (di-Tris salt, Sigma, UK) in 250 mM sodium carbonate (pH 10.3), 1 mM $MgCl_2$. Samples were left to incubate for

5 minutes under ambient conditions prior to the quantification of p-NP as described above. To ensure that the specimens had been thoroughly washed a sample (0.1mL) of the final rinse was combined to 0.2mL of the p-NPP solution and assayed alongside control and ALP-treated Ti screws. Once analysed all screws were left o/n in their tubes and the following day rinsed under running tap water and cleaned with an electric toothbrush, rinsed in distilled water followed by 70% ethanol, allowed to dry and then reassessed for total ALP activity to ascertain whether some had remained on the metal surface. All other tests used the Ti discs for single use only within multiwell (24-well) plates and these were immersed in 0.7ml of either bovine or porcine ALPs for a maximum of 30 minutes followed by washing in distilled water only (five changes) prior to total ALP activity as described above. With regard to the porcine ALP this was sourced from kidney and the solution used to treat Ti discs generated p-NP at approximately 30nmols/ml/min^{-1}. Discs immersed in this ALP preparation were left, after rinsing, in the substrate for an hour prior to p-NP quantification.

2.6. Statistical analysis

All data were subject to an unpaired, two-tailed t-test (assuming unequal variances) to ascertain statistical significance. Data were deemed to be statistically significant when a *p* value of < 0.05 was found. All data are expressed as the mean together with the standard deviation.

3. Results

3.1. MG63 maturation at control and FHBP-modified Ti dental implants.

As anticipated cells exposed to FHBP-functionalised devices had the greater levels of total ALP activity compared to blank, control specimens (Table 1). Washing and brushing these dental screws resulted in a modest decrease in ALP activity which might be expected. However, to our surprise, FHBP-modified implants that had been inserted into bone were associated with the greatest total ALP activity; indeed the levels were approximately 10 fold greater than the non-washed FHBP-functionalised specimens. There was no evidence of bacterial/fungal contamination during the culture period; all growth media were clear and without odour. Also, the cells on the adjoining tissue culture plastic were of similar confluency and morphology to those neighbouring the non-inserted functionalised and control sample screws.

3.2. Alkaline phosphatase binds to Ti.

In considering Occam's razor, the most likely explanation for the (greatest) ALP activity associated with the bone-inserted implants is that the enzyme itself attaches to Ti. To explore this possibility, dental screws were simply steeped in an aqueous solution of bovine intestinal ALP (0.5ml/screw) and left for 30 minutes. We found clear evidence for ALP activity for samples incubated in the enzyme solution (Figure 3). These ALP-functionalised implants were left overnight in distilled water before being rinsed with tap water and scrubbing with a toothbrush. They also underwent 2 rinses in distilled water and a double rinse in 70% ethanol prior to reanalysis for total ALP. Despite these additional washing steps each of the screws exhibited ALP activity (275.4 – 1147.5μM p-NP versus 9.6-10.7μM p-NP

for blank screws) indicating a robust attachment of the enzyme. In a second series of tests we examined the rapidity to which ALP could bind to Ti discs; we could detect ALP activity for sample discs incubated for as little as 2 minutes and that more activity was associated with samples by increasing the incubation time for 15 and 30 minutes (data not shown). Following these initial pilot runs we set the incubation time at 30 minutes followed by distilled water washing (5 changes). It is clear from the data presented (Figure 4) that ALP binds to Ti, an interaction which most likely accounts for the highest enzyme activity for bone inserted implants.

4. Discussion

An obvious consideration in the development of biologically functionalised bone implants is that the coating(s) must withstand the physical rigors of arthroplasty or maxillofacial surgery. Evidence of coating survivorship to bone insertion should include *in vitro* assessment using osteoblasts and/or stromal cells seeded onto retrieved surfaces given that the intention of the surface modification is to enhance those cellular responses predicted to improve implant performance and longevity.

In fabricating our FHBP-functionalised dental implants we explored whether our surface treatment could indeed withstand insertion into bone tissue using similar procedures as would be expected for implanting prosthetic devices. Whilst this approach might be deemed entirely appropriate we herein provide evidence that this is not a desirable course of action and that alternatives to native bone be sought to evaluate coating stability to those physical insults experienced during implantation.

When we examined the MG63 maturation response to FHBP-functionalised dental screws and compared that event to the same modification but for implants inserted into porcine bone the latter were associated with demonstrably greater total ALP activity. Indeed the extent of enzyme activity was approximately 10-fold greater than non-implanted controls. There was no indication of any infection during the culture period and so we were confident that the alkaline phosphatase activity observed was not attributed to any widely recognised bacterial or fungal sources [8]. It was most likely that the titanium surface had been tainted by bone-derived TNSALP. To explore this possibility we simply steeped dental screws in a dilute aqueous solution of bovine intestinal ALP for 30 minutes at room temperature.

Recovered and rinsed samples were associated with abundant enzyme activity with the final sample rinse devoid of enzyme. When these same Ti samples were left to sit, overnight, in water and subsequently cleaned with an electric toothbrush under running tap water there was still evidence of ALP attachment, albeit much lower in amount. This finding supports a firm attachment of some ALP to the metal surface. Further tests were subsequently conducted with both intestinal ALP and porcine TNSALP using solid Ti discs and these surfaces could clearly be modified with the enzymes. Whilst groups have deliberately worked to covalently tether ALP to Ti surfaces [9-10] it is clear that this protein can attach to the metal by far more facile means.

In conclusion we provide evidence for the facile contamination of Ti surfaces with TNSALP and that an examination of osteoblast/stromal cell responses to implants retrieved from native bone should be avoided if the end point under investigation is TNSALP activity. We strongly recommend alternate approaches and materials in evaluating coating survivorship to the rigors experienced during implantation.

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Figure & table legends

Figure 1 – Depicted examples of the titanium dental screws used in this study. These Advanced Implant 2 piece devices (OsteoCare, Slough, UK) are 8mm in length and have a diameter of 4mm.

Figure 2 – Preparing porcine mandibles for dental screw implantation. **A –** Initially pilot holes are produced by drilling into the bone using an Ultra Pilot Drill (W&H Implantmed) followed by **B** - manual insertion of the implant using a 2mm ratchet connected hex driver.

Figure 3 - Compelling evidence for the adsorption of alkaline phosphatase (ALP) at titanium dental screw surfaces. Implants were either immersed in cell culture grade water (Blank Screw) or steeped in a dilute aqueous solution of ALP (ALP-Screw) for 30 minutes at room temperature. After 5 water rinses each screw was processed for total ALP activity using p-nitrophenylphosphate (p-NPP) as the enzyme substrate and subsequent quantification of p-nitrophenol (p-NP) at 405nm using a multiwell plate reader. Greater concentrations of p-NP reflect the presence of higher amounts of bound ALP at the implant surface. The data are the mean micromolar concentration of p-NP + SD of four replicate samples.

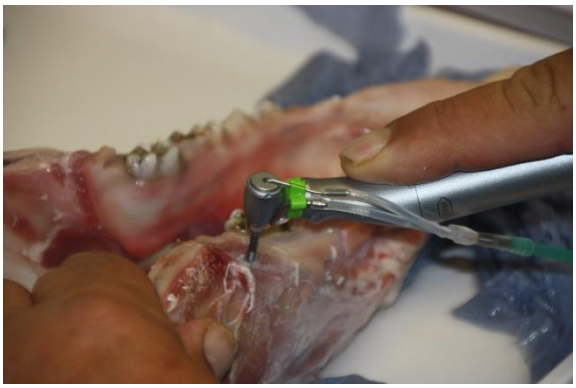
Figure 4– Alkaline phosphatase binds to titanium – Titanium (Ti) discs were either **A -** steeped in aqueous solutions of bovine intestinal alkaline phosphatase (iALP) or **B -** tissue non-specific alkaline phosphatase (TNSALP) sourced from porcine kidney. In each instance sample discs (n=6 per experiment) were left, at ambient temperature, for 30 minutes prior to 5 rinses with distilled water followed by an assessment of total enzyme activity associated with the Ti samples. Para-nitrophenylphosphate (p-NPP) was used as the enzyme substrate which is hydrolysed to p-nitrophenol (p-NP). Greater concentrations of p-NP reflect the presence of larger amounts of bound ALP at the metal surface ($^{*}p<0.001$ versus blank discs for both enzymes). The differences observed for the total Ti-ALP activity of iALP versus TNSALP is attributed to the initial specific activity of the enzymes used. Importantly it is clear that both enzymes can bind to the Ti surface. The data depicted are the combined results of two independent experiments, performed on different days and are presented as the mean (n=12) plus the standard deviation.

Table 1 – Post retrieval analysis of functionalised dental implants indicates evidence of surface contamination – Biologically (FHBP)-functionalised dental screws were divided into three groups of 6 implants; one set remained "as is", a second were given an electric toothbrush wash under running tap water and a third group implanted into porcine mandibles, retrieved and then given an electric toothbrush wash under running tap water. Washed implants were each given three rinses using cell culture-grade water followed by 3 changes of 70% ethanol. All specimens were allowed to dry before being exposed to human MG63 osteoblasts in the presence of 100nM calcitriol and the cells cultured on the implants for three days prior to an assessment of total alkaline phosphatase (ALP) activity. The production of p-nitrophenol (p-NP) from p-nitrophenylphosphate (p-NPP) was used to ascertain total ALP activity. As anticipated from previous studies, FHBP-modified screws were associated with greater ALP activity compared to blank, non-functionalised implants ($^{*}p<0.001$). Washing FHBP-functionalised samples led to a modest loss of the coating. Surprisingly modified screws implanted into bone exhibited the greatest activity ($^{**}p<0.001$ compared to FHBP-functionalised (and washed) controls) indicating that a substance(s) had bound to the metal surface during the implantation/retrieval procedure.

Figure 1

Figure 2

A



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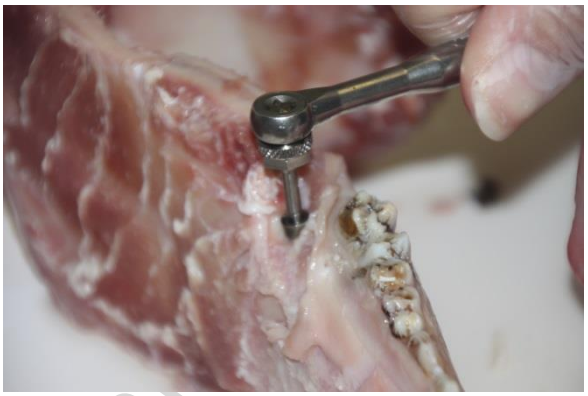


Figure 3

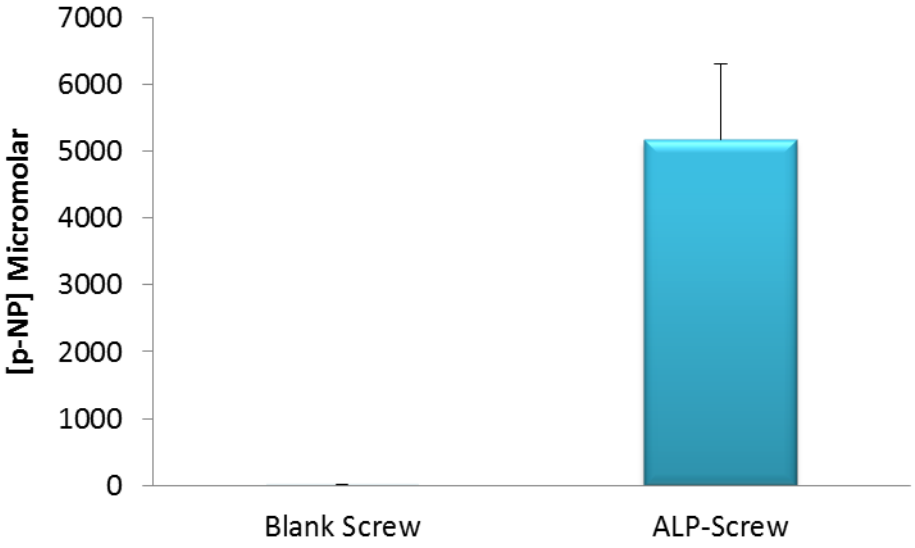
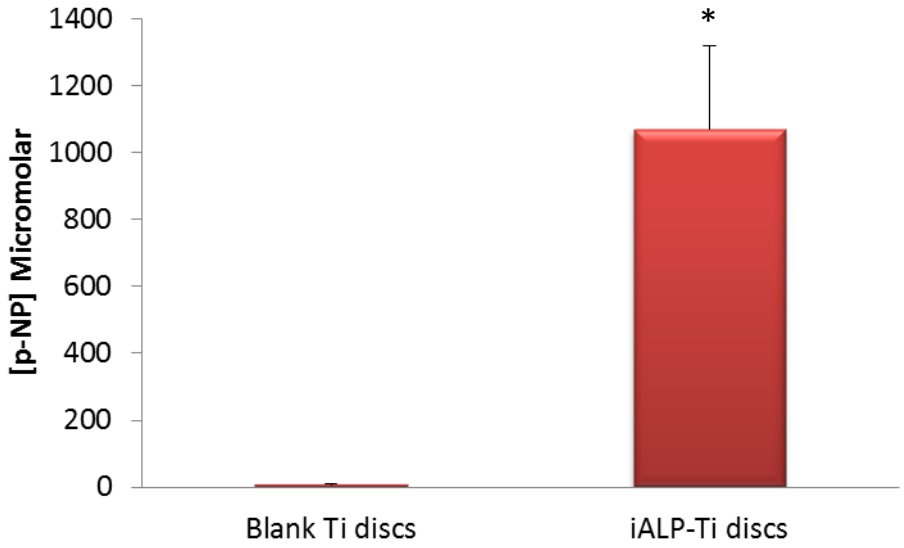


Figure 4

A



B

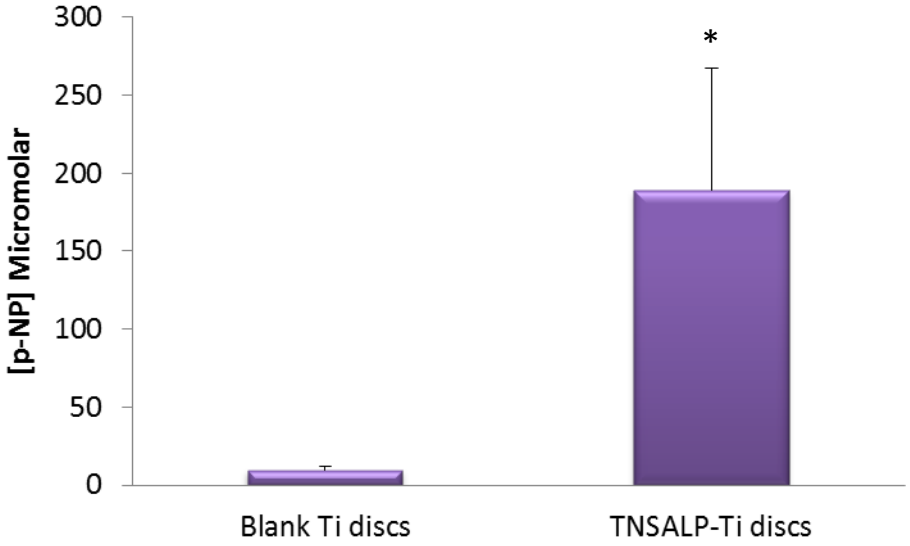


Table 1

Group	Screw 1	2	3	4	5	6	Mean [p-NP]μM	SD
Blanks	19.7	12.6	14.9	30	14.2	16.5	**18**	**6.3**
FHBP-functionalised controls	100.5	91	84.4	60.5	73.9	84.2	**82.4***	**13.9**
FHBP-functionalised & washed controls	48.1	43.1	48.6	53.4	62	59.7	**52.5**	**7.3**
FHBP-functionalised inserted into bone & washed	**652.6**	**562**	**480**	**505**	**372.5**	**147.2**	**452.9****	**176**

Highlights

- Titanium (Ti) is a widely used material for total joint replacements (TJR's)
- Approximately 10% of TJR's fail within the lifetime of the patient through loosening
- Enhancing the performance and longevity of TJR's will combat failures
- Biological coatings could realise improvements of existing TJR technology
- Coating survivorship to the rigors of implantation must be assessed prior to *in vivo* evaluation