Peptide aptamers

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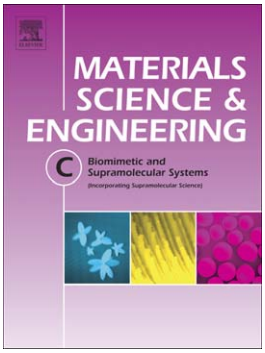

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Peptide Aptamers: Novel Coatings for Orthopaedic Implants

Micah Kelly[1], Richard Williams[2,6] Anuriti Aojula[1], Jenna O'Neill[1,6] Zuzanna Trzińska[3], Liam Grover[2] Robert Scott[4,6], Anna F.A. Peacock[5], Ann Logan[1,6] Artemis Stamboulis[3], Felicity de Cogan[1,6*]

1. School of Clinical and Experimental Medicine, University of Birmingham
2. School of Chemical Engineering, University of Birmingham
3. School of Metallurgy and Materials, University of Birmingham
4. Royal Centre for Defence Medicine, Queen Elizabeth Hospital, Birmingham
5. School of Chemistry, University of Birmingham
6. NIHR SRMRC Trauma Centre, Queen Elizabeth Hospital, Birmingham

Corresponding Author: Dr Felicity de Cogan, School of Clinical and Experimental Medicine, University of Birmingham, Vincent Drive, Birmingham, B15 2TT. f.decogan@bham.ac.uk, Tel +44 (0)121 414 8859, Fax +44 (0)121 414 48867

Abstract

Current processes for coating titanium implants with ceramics involve very high energy techniques with associated high cost and disadvantages such as heterogeneity of the coatings, phase transformations and inability to coat complex structures. In order to address the above problems, we propose a biomimetic hydroxyapatite coating process with the use of peptides that can bind both on titanium surfaces and hydroxyapatite. The peptides enabled homogeneous coating of a titanium surface with hydroxyapatite. The hydroxyapatite-peptide sandwich coating showed no adverse effects on cell number or collagen deposition. This makes the sandwich coated titanium a good candidate for titanium implants used in orthopaedics and dentistry.

Keywords: Biomimetic peptide, peptide coating, titanium coating, hydroxyapatite coating, implant coating,

Highlights

- Surface associating peptides bind to hydroxyapatite and titanium simultaneously
- The peptides interact with titanium to allow deposition of a hydroxyapatite coating
- This gives a low cost, low energy alternative method to implant coating manufacture
- The coating increases the osteoinductive properties of the titanium

1. Introduction

Titanium and its alloys are commonly used as implant materials for medical applications, including orthopaedic reconstruction and dental restoration [1]. The use of titanium for the manufacture of load-bearing implants is well established owing to the high strength and stiffness properties of the material, along with its corrosion resistance and excellent biocompatibility in bone replacement applications. Establishing a robust contact between the implant surface and the surrounding bone tissue is essential for enabling load distribution through the repaired limb and minimising the risk of implant loosening and, ultimately, implant failure. While this can be achieved in principle at the macroscale by bonding the surface of the implant to surrounding bone with a cement, there is a drive towards achieving a more intimate level of contact by encouraging new bone tissue to integrate with the surface structure of the implant [2]. This can be achieved by surface

modifications that improve the properties of the implant surface or by coating the implant surface with bioactive molecules or compounds such as peptide RGD which increases cell attachment [3-5].

The surface topology, chemistry and wettability all affect the initial interaction of implants with the biological environment. The surface roughness and morphology are recognized to improve cell attachment and promote osteointegration [4]. However, simply increasing the roughness with acid etching has shown little improvement in performance *in vivo* [6-7]. On the other hand, changing the surface chemistry using biocompatible coating materials such as hydroxyapatite (HA), which is similar in composition to the mineral component of bone, can improve osteointegration. HA can form a direct chemical bond with the bone tissue and allows extracellular matrix protein deposition that increases cell adhesion [4,8]. Currently HA coatings are being used clinically to coat cement-less implants [3,9-10].

An array of methods have been utilised to coat metal implants, which have previously been reviewed in detail [11]. High temperature plasma spraying is a common technique used for coating of titanium implants with HA. During the plasma spraying process, HA powders are introduced into the plasma and is effectively 'melted' before being deposited on to the titanium surface. There are two types of plasma arc; the transferred and the non-transferred arc. The latter is more suitable for HA coatings as there is no predominant thermal effect on the substrate. While it is possible to adjust coating composition and crystallinity by varying gas phase, anode current and atmospheric pressure, high energy coating conditions can result in a considerable reduction in coating crystallinity [12] and the co-deposition of impurity phases such as tetracalcium phosphate, calcium oxide, oxyapatite and amorphous calcium phosphate [13-14]. The combination of these phases makes the solubility of the deposited coating variable under physiological conditions. Consequently the coating has been shown to lead to accelerated localised dissolution of the ceramic coating [15]. The high temperature followed by rapid cooling can lead to dehydroxylation of HA, change of crystallinity and formation of an amorphous calcium phosphate as well as micro-cracks [4,9]. Despite these drawbacks, plasma spraying coating offers a high throughput method of depositing ceramic coatings on metal implants [4-9,16].

Other process for coating titanium, although less widespread are equally well established.
Dip coating, where the titanium is immersed in a concentrated solution of calcium and phosphate ions which forms hydroxyapatite on the surface [17]. While this is a low cost and simple approach to coating, maintaining the hydroxyapatite coating on the surface can require post-coating treatments such as sintering which can induce coating deformations [18]. Sol-gel deposition of hydroxyapatite onto titanium is also possible but has significant drawbacks depending on the organic precursor solutions which can cause problems in the thermal processing stage as phase changes in the coat are observed [10]. Other coating techniques such as hot isotactic pressing [19], thermal spraying [20], sputter coating [21] and electrophoretic deposition [22] have been used to coat hydroxyapatite on titanium.

Bone in the body undergoes a constant remodelling process which consists of the resorption of bone mineral by osteoclasts and the subsequent synthesis of new bone tissue by osteoblasts [23]. Calcium phosphate coatings (e.g. hydroxyapatite) on the surface of the titanium implant are conducive to protein deposition due to its similarity to bone this allows faster assimilation of the implant with bone. The bone forming cells are directed to attach, differentiate and spread on the implant surface by a layer of proteins such as bone sialoprotein [24]. In addition, bone sialoprotein, which is known for accumulating in large quantities in the bone-implant interface, has a high proportion of negatively charged residues that interact electrostatically with the positively charged calcium ions present and is involved in the nucleation of hydroxyapatite formation [25-26]. Similar high affinity

peptide-hydroxyapatite binding can be synthetically recreated when extracting negatively charged functional peptide sequences from a bone forming protein, for example octaglutamic acid (E8) extracted from bone sialoprotein [27].

Similarly to proteins, peptides have also been shown to interact with biological and non-biological inorganic materials. Sano *et al.* suggested that amino acid sequences such as RKLPDA (TBP) can interact electrostatically with the amphoteric titanium oxide layer formed in an aqueous solution on the surface of pure titanium nanoparticles[28]. The interactions occurred between both the positively charged Arginine (R) and $Ti\text{-}O^-$ and the negatively charged Aspartic acid (D) and $Ti\text{-}OH_2^+$ [21].

In this work, we propose a novel technology of applying a biomimetic, hydroxyapatite coating for titanium orthopaedic implants. This coating utilises short peptides to act as linking molecules between a hydroxyapatite deposit and a titanium surface. The peptides used consist of a combination of a hydroxyapatite binding peptide (E8) and a titanium binding peptide (TBP). Such linking molecule will be able to adhere on the titanium oxide surface of titanium at the TBP end due to its specific charge pattern and shape. The polyglutamic acid is then positioned to provide multiple negative charges to bind to the Ca^{2+} present in the hydroxyapatite (Figure 1). This results in the formation of a peptide-hydroxyapatite sandwich coating which presents an external hydroxyapatite layer using a low energy and cost effective manufacturing process.

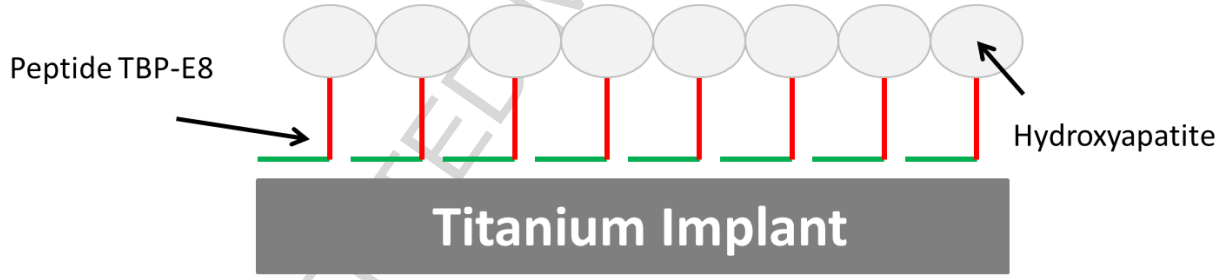

Figure 1 a) Schematic showing the sandwich structure of the material with a titanium surface coated with a peptide which in turn binds hydroxyapatite.

Table 1 Peptide sequences, abbreviations and the target surface of binding for each sequence.

Peptide Sequence	**Peptide Code**	**Binding Target**
5(6)-Carboxyfluorescein-RKLPDA	TBP	titanium
5(6)-carboxyfluorescein-RKLPDARKLPDA	2xTBP	titanium
5(6)-carboxyfluorescein-EEEEEEEE	E8	hydroxyapatite
5(6)-Carboxyfluorescein-RKLPDAEEEEEEEE	TBP-E8	titanium and hydroxyapatite
5(6)-Carboxyfluorescein-RKLPDARKLPDAEEEEEEEE	2xTBP-E8	titanium and hydroxyapatite

2. Materials and Methods

2.1 Materials

All fluorenylmethyloxycarbonylchloride (Fmoc) protected amino acids, preloaded Wang resins and *N,N,N',N'*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uranium hexafluorophosphate were purchased from NovaBiochem (UK). All other reagents were purchased from Sigma Aldrich (Gilingham, UK) unless otherwise stated. Titanium blocks and titanium orthopaedic screws were provided by the Maxillofacial Surgery Department, Queen Elizabeth Hospital, Birmingham. SAOS-2 cells were from ECACC and were used at passages 10-14.

2.2 Peptide Synthesis

Peptides [5(6)-Fluorescein-RKLPDARKLPDAGGGEEEEEEEE (2xTBP-E8), 5(6)-Fluorescein-RKLPDARKLPDA (2xTBP), 5(6)-Fluorescein-EEEEEEEE (E8), 5(6)-Fluorescein-RKLPDAGGGEEEEEEEE (TBP-E8), 5(6)-Fluorescein-RKLPDA (TBP)] were synthesised using standard solid phase peptide synthesis. Briefly, a preloaded Fmoc protected amino acid resin was swollen in DMF. The Fmoc group was deprotected using piperidine in DMF (20 %, v/v) for 30 minutes. The resin was washed using 3 x DMF, 3 x DCM and 3 x DMF. The next Fmoc protected amino acid (0.004 moles) was then preactivated by dissolving it in DMF in the presence of HBTU (0.40g, 0.004 moles) and DIPEA (0.11 mL, 0.004 moles). The amino acid solution was added to the resin and the mixture was shaken until completion of the coupling reaction. The completion of the reaction was monitored using the ninhydrin test. The excess reaction solution was then discarded and the resin was washed as described above. The Fmoc deprotection, coupling and washes were repeated until the desired sequence was achieved. For all sequences, 5/6-carboxyfluorescein was added to the N-terminus to allow the peptide to be tracked. The peptides were cleaved from the resin using a cleavage cocktail of TFA, deionised water and triisopropylsilane (95:2.5:2.5 v/v) stirred for 3 hours. The resin was then removed by filtration and the peptide precipitated from the TFA into ice cold diethyl ether and centrifuged to give a solid yellow powder. The purity of the peptides was monitored using a reverse phase high pressure liquid chromatography (HPLC) Phenomenex Luna C12 column (250 x 21.2 C12 (2) 10 micron Jupiter Proteo 90Å Axia Packed, (Phenomenex, Macclesfield, UK)) with a CH_3CN/H2O + 0.1 % TFA solvent mixture altered with a linear gradient from 0 to 100 % CH_3CN + 0.1 % TFA over 40 min. Peptides were purified to greater than 75 % purity. The peptides masses were identified by electrospray ionisation mass spectrometry.

2.3 Titanium Preparation

Pure titanium blocks were cleaned using 400 grit abrasive paper and acetone. The titanium was passivated in air for 24 hours to allow the titanium oxide layer to form prior to any studies being carried out.

2.4 Hydroxyapatite Synthesis

Aqueous solutions of calcium nitrate 1 M (Fisher Scientific UK Ltd., Loughborough, UK) and diammonium hydrogen phosphate dibasic 0.6 M (Sigma-Aldrich Ltd., Dorset, UK) were prepared and pH adjusted to 10 through drop wise addition of 37-39% ammonium hydroxide (Sigma-Aldrich Ltd., Dorset, UK). The phosphate salt solution was added drop wise to the calcium salt solution using a burette while stirring and maintaining a pH of 10 through further addition of ammonium hydroxide. After ageing overnight while under stirring, the nano crystals were washed by five cycles of centrifugation at 4000rpm and resuspension with deionised water before filtering the suspension using a vacuum Buchner funnel and filter paper (Fisher Scientific UK Ltd., Loughborough, UK). Typical yield of solid product was approximately 30 g, with 1 g resuspended at 1 mg per mL in deionised water adjusted to pH 7 and the remaining solid material dried overnight in an oven at 60°C.

2.5 Hydroxyapatite Disc Preparation

Dried hydroxyapatite powder was mixed with deionised water (830µL for each gram of HA) to form a thick paste. Cylindrical pellets were formed by manipulating the paste into the holes (6mm in diameter and 12mm in height) of a homebuilt PTFE mould using a spatula. 1 g of HA provided

enough solid material to make 2 pellets and a total of 90 pellets were made. During the filling process, the entire mould rig was on a vibrating plate to ensure that the paste filled the bottom of each hole before applying more paste. The filled mould was then placed in an oven at 60°C overnight before removing the formed pellets and transferring them to a furnace for sintering at 1100°C for 6 hours with a temperature ramp rate of 5°C/min. The pellets designated for surface modification were then cut into thirds across their length so they could fit inside the wells of a 96 well-plate. The calcium phosphate composition was tested using XRD (data not shown).

2.6 Dip Coating Hydroxyapatite Deposition on Titanium

Dip coated samples were prepared following literature protocols [16]. Briefly, cleaned titanium blocks were incubated in Ca^{2+} and HPO_4^{2-} for 14 days to allow deposition on the surface. The titanium blocks were washed with PBS ten times and used without any further modification.

2.7 Release Studies

All release studies were carried out in triplicate. The peptides were dissolved to a 0.01 mM solution using phosphate buffered saline (PBS). Titanium blocks were placed into wells of a 24 well plate (Fisher Scientific, Loughborough UK). The peptide solution (200 µL) was added to the surface of the titanium and incubated for one hour to allow attachment. The titanium blocks were then washed with PBS ten times to remove any unattached peptides. Surfaces were visualised using fluorescence microscopy to confirm the presence of the peptides on the surface and then immersed in fresh PBS (2 mL). Aliquots of the PBS (100 µL) were taken at specific timepoints and added to a 96 well plate (Fisher Scientific, Loughborough UK). The fluorescence was monitored at excitation/emission 495/525 nm on a Glomax multi detection system (Promega, Southampton, UK).

Hydroxyapatite pellets were placed into wells of a 24 well plate (Fisher Scientific, Loughborough UK). The TBP-E8 peptide solution (200 µL) was added to the surface of the titanium and incubated for one hour to allow attachment. The pellets were then washed with PBS ten times to remove any unattached peptides. Surfaces were visualised using fluorescence microscopy to confirm the presence of the peptides on the surface and then immersed in fresh PBS (2 mL). Aliquots of the PBS (100 µL) were taken at specific timepoints and added to a 96 well plate (Fisher Scientific, Loughborough UK). The fluorescence was monitored at excitation/emission 495/525 nm on a Glomax multi detection system.

Sandwich structures were prepared by coating the titanium blocks with TBP-E8 peptide as above. Hydroxyapatite suspensions were formed by suspending 10 mg of hydroxyapatite powder in PBS (1 mL). The sample was vortexed and added to the surface of the peptide coated titanium (200 µL). The samples were incubated at room temperature for one hour with shaking. The samples were then washed ten times with PBS. The samples were imaged at 488 nm to ensure the peptide remained on the surface through the HA addition. The samples were then placed in simulated body fluid (2 mL) for 14 days [29]. Aliquots were taken at each time point and the cumulative fluorescence in solution was measured excitation/emission 495/525 nm on a Glomax multi detection system.

2.8 Fluorescence microscopy studies

Studies were carried out in triplicate. The peptides were dissolved to a 0.01 mM solution using PBS. The peptide solution (200 µL) was added to the cleaned surface of the titanium blocks and incubated for one hour to allow attachment. The titanium blocks were then washed with PBS to remove any unattached peptides from the surface. The samples were then air dried in the dark to prevent photobleaching.

Sandwich coating samples were prepared as previously described. Samples were imaged with a fluorescence microscope at emission wavelength of 520 nm to visualise the peptide attachment to the surface. The images of the coverage were taken across the surface at random. Images were analysed and the number of green pixels counted, to give the percentage coverage of the total titanium block surface.

2.9 Scanning Electron Microscopy (SEM) Imaging and Raman Microscopy

Samples were prepared as detailed above. The samples were studied on a Philips XL30 FEG environmental scanning electron microscope. Energy-dispersive X-ray spectroscopy (EDX) was carried out on samples following SEM. Raman microscopy was carried out on a Renishaw InVia Raman Microscope with a 514 nm laser excitation source and a holographic Rayleigh filter (Renishaw InVia, Gloucestershire, UK).

2.10 Cell Studies

Cell culture studies were set up to test biocompatibility of the following devices and their coating and run in triplicate: a) titanium blocks with sandwich coating prepared as previously described prior to cell seeding, b) titanium blocks coated with peptide, as previously described, prior to cell seeding, c) titanium orthopaedic screws. The experiments were carried out in a 6 well tissue culture plate (Fisher Scientific, Loughborough, UK). Each treatment well was seeded with 10,000 SAOS-2 cells in McCoys 5A medium supplemented with Penicillin/Streptomycin (1 % v/v) and fetal Bovine Serum (10 %, v/v). The cells were incubated with the test agents and the alamar blue assay was run after 1, 2, 3, 7 days to measure cell number. At each time-point the media was removed and fresh media (1 mL) was added to each well. Resazurin salt solution (50 µL) was added to each well and the cells were incubated at 37 °C for 2 hours. The absorbance of the solution was then monitored at 570 nm. Cell number was determined against a standard curve of absorbance readings against known cell number. Following the assay the remaining media was removed from the cells and fresh media added prior to the next time point being taken.

2.11 Collagen Production

SAOS-2 cells were seeded on treated titanium as for the cell culture studies. At each time-point the cell media was removed from each sample and the cells washed with sterile PBS. The samples were then incubated in Bouin's fluid (Picric acid (15 mL, 70 % in H_2O), formaldehyde (5 mL, 35 % in H_2O), glacial acetic acid (1 mL)) for 60 minutes. The excess solution was removed and the cells incubated in distilled water for 15 minutes. Sirius Red (100 mg/mL in saturated picric acid) was added to the cells and the plate shaken at room temperature for 60 minutes, the excess dye was removed and the cells were washed with hydrochloric acid (0.01 M). Each well was incubated with sodium hydroxide (0.1 M) and shaken for 30 minutes at room temperature. The solution was transferred to a 96 well plate and the optical density measured at 550 nm on a Glomax multi detection system.

2.12 Statistics

All statistical analyses were carried out using SPSS 17.0 (IBM SPSS Inc., Chicago, IL) and data were presented as mean ± SEM. The Shapiro-Wilk test was used to ensure all data was normally distributed before parametric testing using a one-way ANOVA with Tukey post-hoc test or a t-test. The statistical significance threshold was $p<0.05$.

3. Results

3.1 Release of Peptide Coating from Titanium

Pure titanium blocks were coated with peptide and incubated in fresh PBS. Aliquots at different time points were used to measure the cumulative release of the peptides from the surface in order to ascertain the stability of the peptide coating. The four peptides 2xTBP-E8, TBP-E8, TBP and 2xTBP

were incubated on different surfaces and the cumulative release measured from each surface (Figure 2). This showed that TBP and 2xTBP showed minimal release over the time, 1.7 ± 0.1 % and 1.6 ±0.04 % with no statistical differences between the two peptides. TBP-E8 shows higher release values, 3.5 ± 0.7 % although this was not found to be statistically significant. However, 2xTBP-E8 showed the highest release at 9.4 ± 3.2 %. This was significantly higher than the peptides which did not include the E8 linkers, TBP and 2xTBP (p= 0.48 and 0.49 respectively).

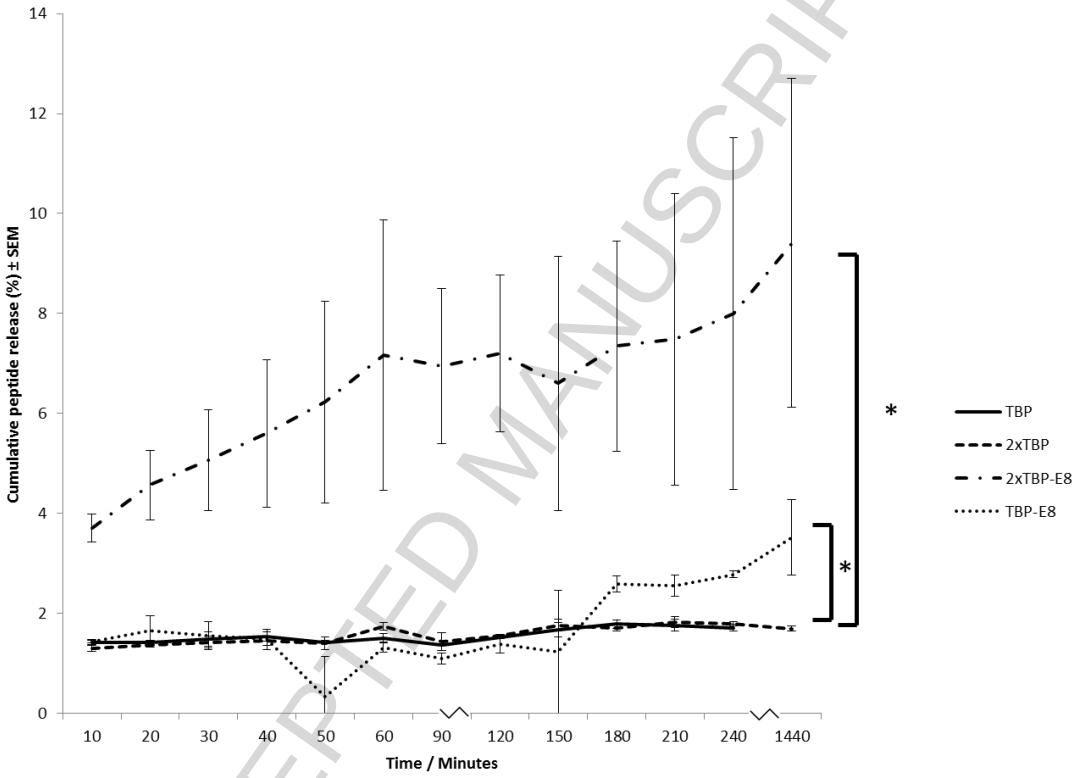

Figure 2 Cumulative release of the different peptide coatings from the surface of titanium into surrounding PBS solution over time. Release is an average of n=3, error bars show standard error of the mean, * denotes statistical significance <0.05.

3.2 Release of Peptide Coating of Titanium and Hydroxyapatite

TBP-E8 was selected for further studies as this peptide formulation displayed a lower cumulative release over time compared to 2xTBP-E8. Hydroxyapatite pellets were coated with the peptide solution and the cumulative release was measured (Figure 3). This showed that release of the peptide from the hydroxyapatite at 24 hours was 30.4 ± 4.5 %. Compared to the release already obtained from the titanium which was 3.5 ± 0.7 %. This demonstrates that the release from the hydroxyapatite is significantly higher than that from the titanium (p=0.004).

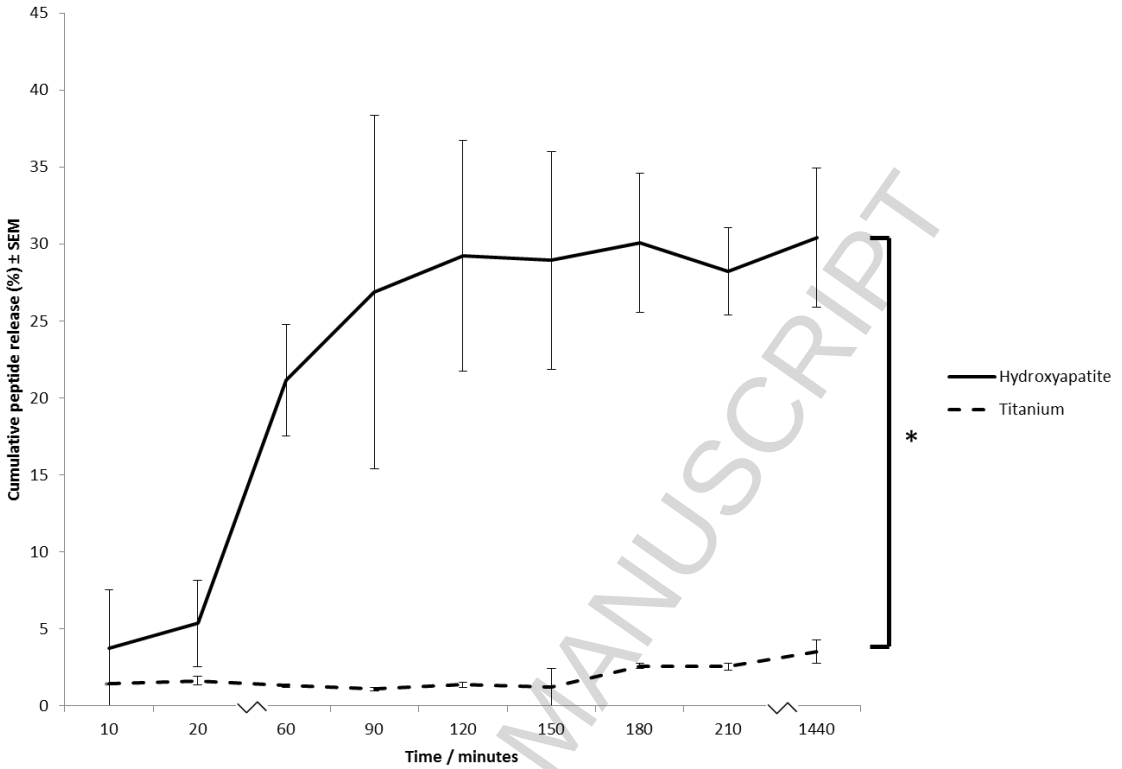

Figure 3 Cumulative release of TBP-E8 peptide coatings from titanium and hydroxyapatite surfaces into surrounding PBS solution over time. Release is an average of n=3, error bars show standard error of the mean, * denotes statistical significance <0.05.

3.3 Release of Peptide Coating from the Sandwich structure

The sandwich structure was set up by coating the titanium with TBP-E8 peptide and then exposing the peptide coated titanium surface to a hydroxyapatite powder suspension. The cumulative TBP-E8 peptide release from the sandwich structure was then measured (Figure 4a). Titanium with TBP-E8 peptide coating showed a standard release profile 3.5 ± 0.7 %. However, very little release was seen from the sandwich structure, $1.7 \times 10^{-3} \pm 0.1 \times 10^{-4}$ %. This showed that the addition of the hydroxyapatite layer significantly reduced the dissociation of the TBP-E8 peptide from the titanium surface ($p=0.01$). A long term study was conducted to ascertain the stability of the coating over an extended period of time (Figure 4b). It was found that there was a slow degradation of the structure and a slow release of the TBP-E8 peptide from the surface over 14 days, with 47.2 ± 3.8 % of the coating remaining on the surface at the 14 day timepoint. The surfaces were imaged using fluorescence microscopy to ensure the peptide was present in both cases prior to release studies being carried out (Figure 4c,d)

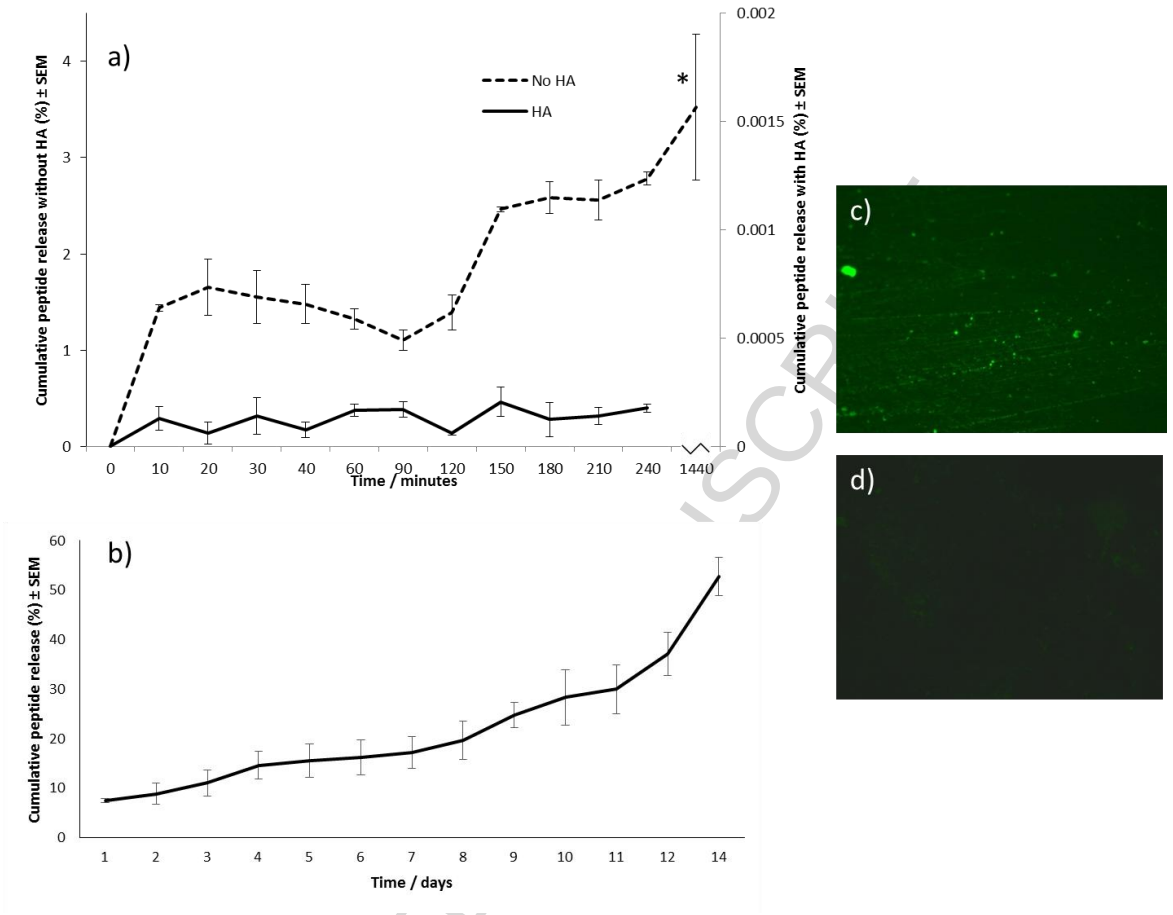

Figure 4 a) Cumulative release of peptide from titanium surface from titanium coated with peptide and titanium coated with peptide and hydroxyapatite to form the sandwich structure coating. Release is an average of n=3, error bars show standard error of the mean. b) Cumulative release of the peptide from the sandwich structure coating on titanium over a 14 day time period. Release is an average of n=3, error bars show standard error of the mean. c) fluorescence microscopy image of the surface after peptide coating prior to release study d) fluorescence microscopy of the sandwich structure coating prior to release study, * denotes statistical significance <0.05 between the two groups.

3.4 Raman Spectroscopic Analysis of Samples

Raman spectroscopy was carried out on uncoated titanium blocks, hydroxyapatite pellets, TBP-E8 peptide solution and the sandwich structure samples (Figure 5). The uncoated titanium blocks showed weak peaks in the 150-300 region consistent with the titanium dioxide layer[30]. The hydroxyapatite pellets showed characteristic peaks at 441 cm^{-1} which corresponds to the doubly degenerate bending mode of the PO_4 O-P-O bond, 588 cm^{-1} which is the triply degenerate bending mode of the PO_4 O-P-O bond, 959 cm^{-1} is the totally symmetric stretching mode of the tetrahedral PO_4 P-O bond and 1036 cm^{-1} is the triply degenerate stretching mode of the PO_4 P-O bond. The TBPE8 peptide spectra shows a broad peak which corresponds to the 5(6)-carboxyfluorescein tag built into the peptide. The spectra of the sandwich structure shows the peaks corresponding to hydroxyapatite and also a broad peak in the 450-550 region, demonstrating that both the TBP-E8 peptide and the hydroxyapatite are on the surface of the titanium.

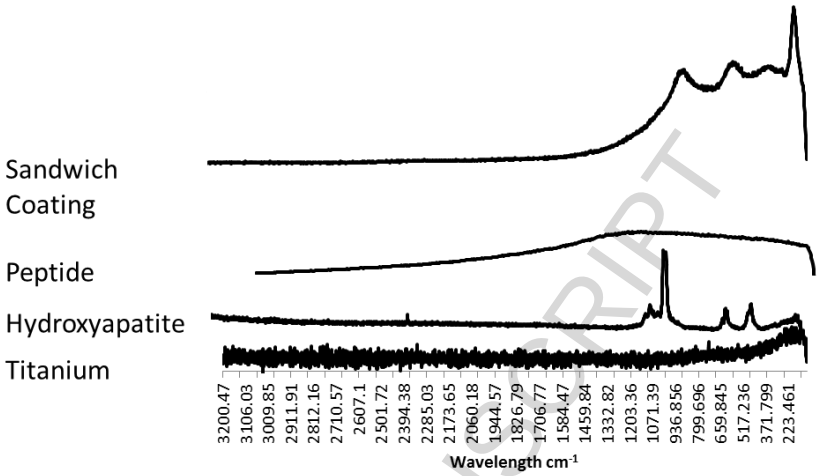

Figure 5 Raman Spectra of titanium without coating, hydroxyapatite without coating, peptide in a PBS solution and titanium with a sandwich structure coating.

Table 2 Peak assignment of hydroxyapatite raman spectra.

Peak Value (cm-1)	Assignment
441	Doubly degenerate bending mode (υ_2) of the PO_4 (O-P-O) bond
588	Triply degenerate bending mode (υ_4) of the PO_4 (O-P-O) bond
959	Totally symmetric stretching mode (υ_1) of the tetrahedral PO_4 (P-O) bond
1036	Triply degenerate stretching mode (υ_3) of the PO_4 (P-O) bond

3.5 Scanning Electron Microscopy

Titanium with the sandwich structure coating and uncoated titanium were studied using scanning electron microscopy. The uncoated samples show the surface of the metal with the marks from the abrasive cleaning visible on the surface (Figure 6a). Samples which had been coated with both TBPE8 peptide and hydroxyapatite to build the sandwich coating displayed a crystalline, homogenous coating across the surface of the titanium (Figure 6b). The sandwich structure coating was then compared to dip coating the titanium following literature protocols to form hydroxyapatite. The sandwich structure coating showed at low magnification a homogenous covering over the surface of the titanium block (Figure 6c). High resolution images of the same sample showed that the surface of the titanium was not visible between the crystals and the hydroxyapatite gave a complete coating on the surface (Figure 6d). The dip coated titanium at low resolution images showed a non-homogenous coating over the surface with large crystals in some areas and uncoated titanium in other areas (Figure 6e). The hydroxyapatite also showed a broad range in the size of the crystals compared to the sandwich structure which showed a narrower distribution. High resolution images of dip coated samples show the titanium surface clearly visible with hydroxyapatite crystals deposited in random areas (Figure 6f). EDX gave readings of 37 % – phosphate and 62 % calcium giving a ratio of 1.68 which is characteristic of hydroxyapatite.

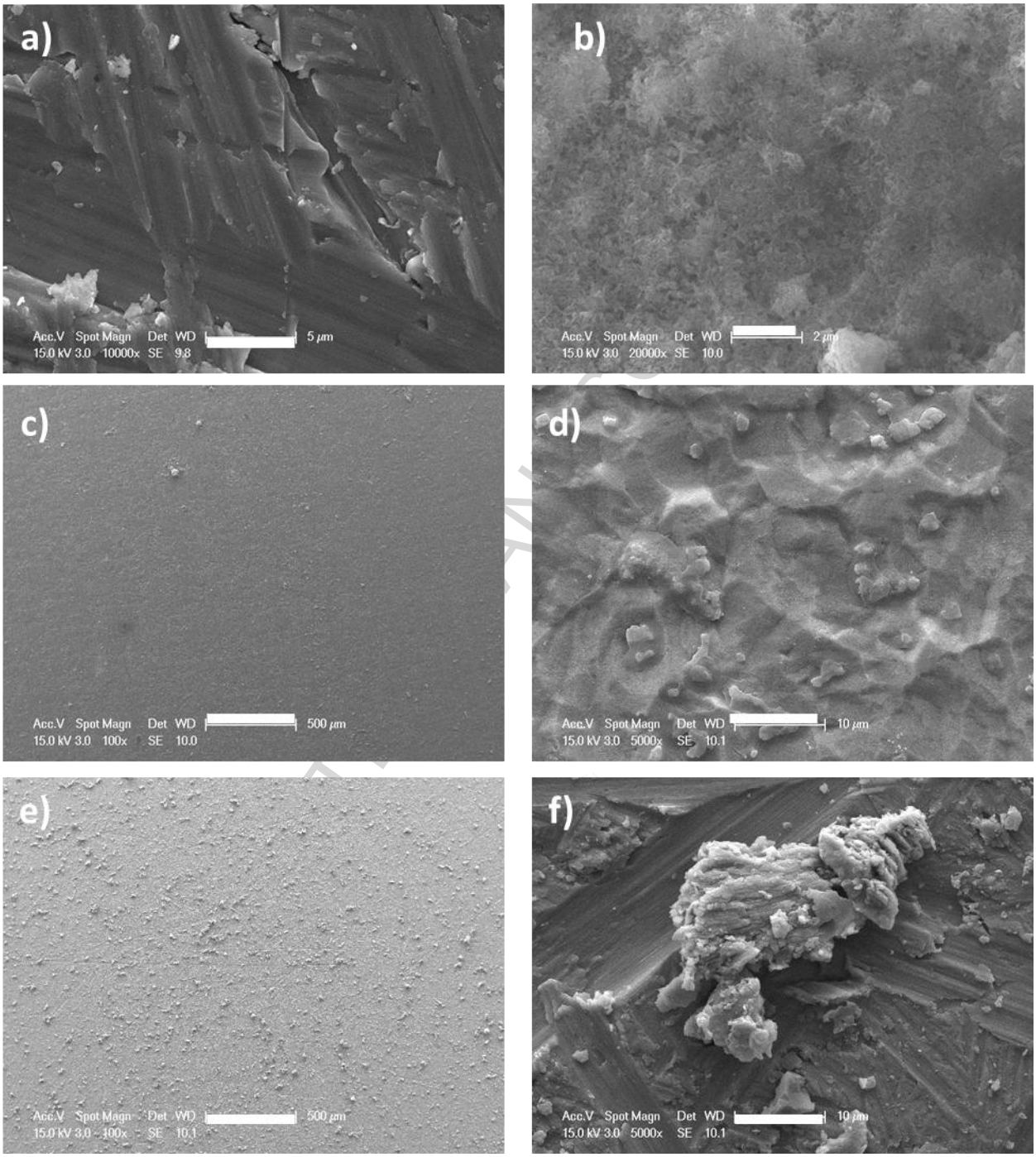

Figure 6 Scanning electron microscope image of a) titanium which has been cleaned and passivated to create a titanium oxide layer but is uncoated by peptide or hydroxyapatite, scale bar shows 5 µm. b) titanium block which has been coated with the sandwich structure coating, scale bar shows 2 µm. c) Titanium block which has sandwich structure coating, scale bar shows 500 µm. d) titanium block which has sandwich structure coating, scale bar shows 10 µm. e) dip coated titanium scale bar shows 500 µm. f) dip coated titanium, scale bar shows 10 µm. Images are representative of all samples.

3.6 Cell Studies

SAOS-2 cells were cultured on commercially available medical titanium screws used for orthopaedic implants with an undisclosed osteointegrative coating, titanium blocks with the sandwich coating and titanium blocks with the TBP-E8 peptide coating. Cell numbers were assessed at 7 and 14 days. The sandwich coated titanium showed higher cell numbers $2.5x10^4 \pm 1141$ than both the titanium screw $2.1x10^4 \pm 445$ and the titanium with TBP-E8 coating $2.1x10^4 \pm 527$, ($p=0.016$). There was no significant difference between the titanium coated with TBP-E8 and the titanium screw. This demonstrates the peptide coating and the sandwich coating had no immediate cytotoxic effect on the cells compared to current 'gold standard' (Figure 7a). The collagen deposited by the SAOS-2 cells at three days showed no significant differences between the titanium screw 0.039 ± 0.01 a.u. and the sandwich coated titanium 0.06 ± 0.01 a.u.(Figure 7b). However there was a significantly lower quantity of collagen deposited on the titanium coated with TBP-E8 0.03 ± 0.01 a.u.to both the sandwich coated titanium ($p=0.001$) and the titanium screw ($p=0.000$). However, by day 7 there were no significant differences between the groups.

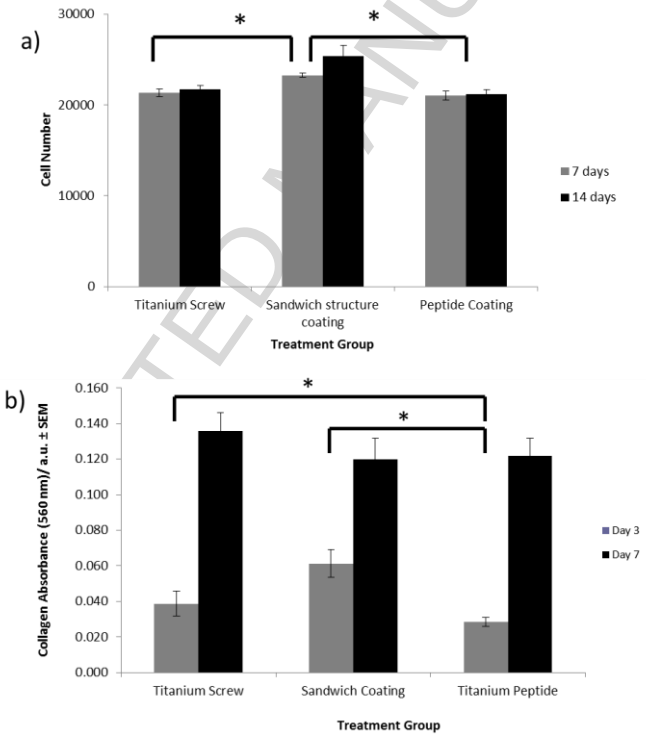

Figure 7 a) Cell viability of human osteosarcoma cells on commercially available orthopaedic screws (Titanium Screw), titanium block which has been coated with sandwich structure coating and titanium block coated with TBP-E8 peptide. Time points were taken at 7 and 14 days, error bars show standard error of the mean, n=3. b) Collagen deposition by osteosarcoma cells on commercially available orthopaedic screws (Titanium Screws), titanium block which has been coated with the sandwich structure coating and titanium block coated with TBP-E8. Error bars show standard error of the mean, n=3, * denotes statistical significance <0.05 between the two groups.

4 Discussion

The aim of this study was to use peptides to develop a cheap, energy efficient and fast solution phase directed coating of hydroxyapatite onto titanium surface that would be suitable for medical implants. Peptides have been designed with specific amino acid sequences will bind through electrostatic interactions to both titanium and hydroxyapatite. Peptides with multiple interaction sites can act as bridges between the two materials creating a unique linking molecule that will improve osteointegration of implants with bone.

The peptide TBP-E8 has two specific binding regions. The glutamic acid residue sequence EEEEEEEE is modelled on the repeat units found in bone sialoprotein and this sequence binds to positively charged calcium ions present in hydroxyapatite [31]. The TBP sequence has also been shown previously to interact electrostatically with titanium [32]. Hence, the inclusion of this sequence will allow the peptide to bind to the amphoteric titanium oxide layer present on the metal surface. The three GGG were included to give separation between the two units and build flexibility into the structure.

Regarding the stability of the peptide coating, the release data of peptide from coated titanium into PBS over time showed that the (TBP) sequences bounds to the titanium surface. The addition of a second (TBP) sequence in 2xTBP did not change the binding characteristics, with both TBP and 2xTBP peptides showing very low levels of release. Addition of E8 sequence increased the release of the peptide from the titanium surface of both TBP and 2xTBP. This increase is seen more strongly in the peptide with a double titanium binding sequence rather than the single. The addition of the hydroxyapatite binding sequence (E8) may interfere with the peptide-titanium interactions in several ways. Titanium is known to have a slightly negative character on the surface of an oxide layer this negative charge can cause repulsive interaction with the same charged E8 sequence of a peptide[32]. Glutamic acid residue could also exhibit some intramolecular electrostatic interactions interact with the positively charged Arginine and Lysine of the TBP sequence. This will block the interaction between the positive charges and the titanium oxide layer. There is also the possibility of steric effects such as steric hindrance or shielding. The long 2xTBP-E8 sequence has less flexibility which could prevent interactions between the TBP sequence and the titanium oxide layer both electrostatically and sterically. However, once the hydroxyapatite was included the interference from the polyglutamic acids was blocked. These observations led to the TBP-E8 sequence being chosen for cell studies investigation. This peptide sequence showed the strongest interactions with the titanium surface when glutamic acid residues were present in the sequence.

The ability of the TBP-E8 peptide sequence to interact with hydroxyapatite was studied next. The peptide bound electrostatically to the sintered hydroxyapatite discs despite the addition of the TBP sequence. The release studies have shown an initial burst of peptide release into PBS over the first 6 hours, but little further release was observed.

This gives rise to several possible explanations hydroxyapatite is a highly porous material with strong hydrophilicity enhancing the electrostatic binding process for the peptide. The hydroxyapatite can adsorb and bind peptide through the entire material structure, unlike titanium which can only complex peptide on the surface due to its low porosity.

The inclusion of the hydroxyapatite outer layer to form a sandwich coating structure inhibits the release of the peptide from the surface of the titanium. This could occur through a range of possible processes including the hydroxyapatite forming a physical barrier. The hydroxyapatite interacts with

the glutamic residue within the peptide which removes its interference with the titanium-TBP binding interaction and therefore enhancing the interaction strength. The interaction of the titanium surface material was studied over an extended period of time. The data showed that release of the sandwich coating material from the titanium blocks into the surrounding system was slow and steady, with ~50% released at 14 days. This demonstrates the relative stability of the new coating, an essential feature if the coating is to be suitable for *in vivo* implantation. Immature bone formation is established *in vivo* at 14 days [33]. This will allow the implant to give a structure to the healing process, but be quickly resorbable allowing the integration of the implant and the body to occur faster. The hydroxyapatite coating on the surface of titanium was analysed *in situ* using SEM and Raman spectroscopy. SEM showed small hydroxyapatite crystals indicative of a ceramic surface present all over the surface of the titanium, compared to the uncoated titanium control that showed the metallic surface with the pattern of the abrasive cleaning. The sandwich structure coating was also compared to dip coated samples. Low magnification images of the sandwich structure coating were taken which showed a uniform hydroxyapatite surface over the entire titanium sample. High magnification SEM of sandwich structure coating showed very fine crystals of hydroxyapatite which were of a similar size and shape and gave complete coverage of the titanium surface. By contrast, the dip coated sample showed an uneven surface coating of hydroxyapatite with large crystals apparent in clusters at specific sites and the surface was not reproducible as large areas of the titanium were uncoated. High magnification SEM of dip coated samples revealed irregular crystal formation with bare titanium visible between the crystals. The dip coated layer also gave a large range of crystal sizes at different sites across the surface. The comparison of the solution phase peptide coating technique to the solution phase dip coating technique, which is a common methodology for low temperature / pressure coating of titanium, ably demonstrates the superiority of the novel peptide-hydroxyapatite surface. The regularity of crystal size and the efficient surface coverage seen with this method occur due to the binding ability of the peptide. In dip coated samples the hydroxyapatite crystals form at physical nucleation sites and, as these occur in limited number, the crystals are not limited in size or shape. In nature, glutamic acid motif of bone sialoprotein is the sequence that provide nucleation sites for hydroxyapatite formation in bone [34]. By coating the titanium with this synthetic peptide, multiple binding sites are created for inducing the hydroxyapatite layer across the surface. The number of the binding sites present imposes a steric restrictions on crystal growth, which leads to fine, regular uniform crystal coating such as the one seen on the sandwich structure coated sample.

The structure of these crystals was confirmed as calcium phosphate structures by Raman spectroscopy which showed the stretching of the phosphate groups and by Energy Dispersive X-Ray Spectroscopy (EDX) analysis which showed the stoichiometric calcium to phosphate ratio of 1.68 which is only seen in hydroxyapatite. These analytic techniques confirmed the presence of a sandwich structure made by peptide and hydroxyapatite coating on the surface of the titanium.

Initial, preliminary biocompatibility tests of the surface coating on titanium sheets were tested by measuring effects on proliferation and metabolism of cultured human osteosarcoma cells over 14 days. Cell proliferation was seen in all treatment groups. There were no significant differences in the cell number on the titanium coated with peptide-hydroxyapatite and the commercial coated titanium screw. Both the screw and the sandwich structure showed better cell proliferation than the other treatment group. This demonstrates the need for an osteoinductive coating on titanium to maintain a strong proliferation profile of the osteosarcoma cells. The ability of the cells to produce

and deposit collagen was also examined. Osteoblast cells deposit a collagenous matrix which is then mineralized to form new bone tissue. In this context, collagen production can viewed as a template for new bone formation and hence is important for osteointegration. The SAOS-2 cells showed increasing collagen production over the period as they deposited extracellular matrix, but there was no difference observed between titanium covered with the sandwich structure coating and the commercially available coated titanium screws. Previous biocompatibility studies demonstrated that the cells need a coated titanium surface in order to integrate well with the sample[16]. However, this work demonstrates that the process used to deposit the hydroxyapatite layer is not an important factor with the peptide-hydroxyapatite coating inducing the same behaviour as the commercially available coating.

5 Conclusion

We have demonstrated that novel peptide sequences can be utilised to coat titanium with hydroxyapatite in a method which is cheaper, simpler and does not rely on a line-of-sight coating techniques unlike standard commercial methods. The use of the peptide does not adversely alter cell proliferation characteristics of the deposited hydroxyapatite. This novel process gives fast deposition onto the titanium surface and removes the need for high-tech, high cost equipment and can also works as a non line-of-sight method. The implants formed with the novel titanium coating showed no difference in performance in the initial biocompatability tests to standard commercially available alternatives and showed a significant enhancement in terms of energy and cost over the current methodologies for low temperature /pressure coating techniques.

6 Acknowledgments

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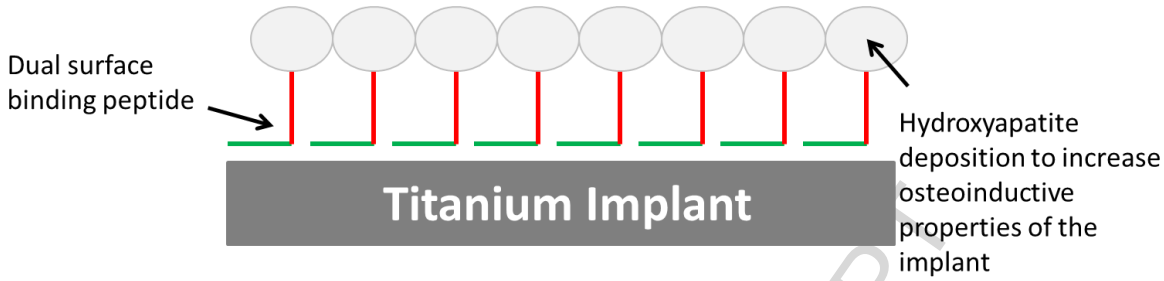

Graphical abstract

Highlights

- Surface associating peptides bind to hydroxyapatite and titanium simultaneously
- The peptides interact with titanium to allow deposition of a hydroxyapatite coating
- This gives a low cost, low energy alternative method to implant coating manufacture
- The coating increases the osteoinductive properties of the titanium