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Peptide aptamers: Novel coatings for orthopaedic implants

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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.

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 recognised 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 allow for 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 are effectively 'melted' before being deposited onto 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

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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 processes for coating titanium, although less widespread are equally well established.

Dip coating is a process 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].

Similar 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-O^- and the negatively charged aspartic acid (D) and Ti-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 (Fig. 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.

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 (Gillingham, 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 (2×TBP-E8), 5(6)-fluorescein-RKLPDARKLPDA (2×TBP), 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 min. The resin was washed using 3× DMF, 3× DCM and 3× DMF. The next Fmoc protected amino acid (0.004 mol) was then preactivated by dissolving it in DMF in the presence of HBTU (0.40 g, 0.004 mol) and DIPEA (0.11 mL, 0.004 mol). 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 h. 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 × 21.2 C12 (2) 10 μm Jupiter Proteo 90 Å 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 peptide 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 h to allow the titanium oxide layer to form prior to any studies being carried out.

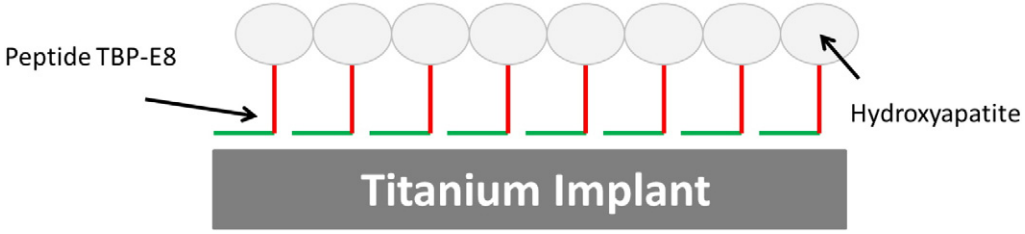

Fig. 1. a) Schematic showing the sandwich structure of the material with a titanium surface coated with a peptide which in turn binds hydroxyapatite.