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Immobilization of alendronate on titanium via its different functional groups and the subsequent effects on cell functions

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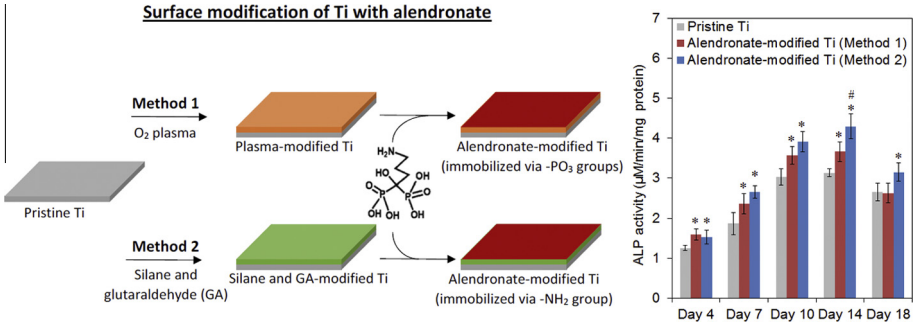
GRAPHICAL ABSTRACT



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ABSTRACT

Immobilization of alendronate on orthopedic implants offers the possibility of enhancing osteogenesis without potentially adverse effects associated with systemic administration of this drug. In this work, alendronate was immobilized on titanium (Ti) via either its phosphate (Method 1) or amino (Method 2) groups, and responses of osteoblasts and human mesenchymal stem cells (hMSCs) on these surfaces were investigated. These modified substrates have similar surface roughness and are negatively charged. With similar amounts of immobilized alendronate, these two types of modified substrates showed comparable osteogenic stimulating effects in enhancing osteoblasts' alkaline phosphatase (ALP) activity and calcium deposition for the first 10 days. However, alendronate immobilized via its phosphate groups was less stable, and gradually leached into the medium. As a result, its stimulating effect on osteoblast differentiation diminished with time. On the other hand, alendronate immobilized via its amino group stimulated osteoblast differentiation over 21 days, and with 1655 ng/cm^2 of immobilized alendronate on the Ti substrate, calcium deposition by osteoblasts and hMSCs increased by 30% and 69%, respectively, compared to pristine Ti after 21 days. The expressions of runt-related transcription factor 2, osterix, osteopontin and osteocalcin in hMSCs cultured on this substrate were monitored. The up-regulation of these genes is postulated to play a role in the acceleration of osteogenic differentiation of hMSCs cultured on the alendronate-modified substrate over those on pristine Ti.

1. Introduction

Titanium (Ti) and its alloys are currently the gold standard for orthopedic implants due to their excellent physical, chemical, biocompatible properties and resistance to corrosion [1,2]. However,

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Ti-based implants may fail to achieve complete integration with the host tissue, which may then lead to implant loosening over time and ultimately failure of the implant. The surface properties of the implant can be modified to improve its fixation with the host tissue. For instance, moieties such as drugs, collagen, growth factors, proteins and engineered peptides can be conjugated on the implant surface to direct cell adhesion, proliferation and differentiation [3–9].

The bisphosphonate (BP) family constitutes a class of drugs with a long history in treatment of bone diseases by promoting bone mineralization and inhibiting bone resorption [10]. BP has been reported to impair osteoclast function and induce osteoclast apoptosis by binding to the natural substrate-binding site of the enzyme farnesyl pyrophosphate synthase, and inhibiting and interrupting the intracellular mevalonate pathway of these cells [11,12]. Recent investigations suggest that BPs can also enhance osteoblast and stem cell functions [13,14]. For example, BPs are reported to prevent apoptosis of osteoblasts, and directly regulate the differentiation and expression of osteogenic gene markers of osteoblasts and stem cells [13,15–18]. It has also been shown that free alendronate, a member of BP family, binds to certain catalytic domain of phosphatases on osteoblast's membrane and the alendronate is internalized and inhibits the activity of protein tyrosine phosphatases in osteoblasts [19,20]. However, the binding domain and the detailed signal transduction pathway are not yet known.

BPs can be delivered via oral administration or intravenous injection (systemic delivery) or via BPs adsorbed or immobilized on implants (local delivery). Systemic delivery of BPs has potential adverse side effects. Osteonecrosis of the jaw, which is caused by over-suppression of bone metabolism, is closely associated with the intravenous and oral administration of BPs [21,22]. Local delivery of BPs via adsorption or covalent binding on implants requires a comparatively smaller dose of BPs [23], and the specificity for local biological responses minimizes the probability of adverse side effects. *In vivo* studies have shown that local delivery of BPs by adsorption on Ti or stainless steel implants results in better implant fixation than systemic delivery of BPs in rat models [24,25]. Although the adsorption of BPs on the implant is a simple procedure, it suffers from unavoidable release of BPs over long term. On the other hand, formation of chemical bonds between BPs and the implant via covalent immobilization may influence specific physiological response through exposure of the appropriate functional groups of BPs at tissue-implant interface [26].

In this work, alendronate, a FDA approved drug from the BPs family, was used to modify the surface of Ti. BPs with amino groups such as alendronate and pamidronate can be conjugated with substrates via this group. For instance, pamidronate has been conjugated by carbodiimide reaction to fibrinogen-functionalized Ti screws to improve mechanical fixation in rat tibia [27]. The immobilization of alendronate on silanized poly(ε-caprolactone) surface via glutaraldehyde linker promotes the differentiation of rat bone marrow stem cells [28]. The strong affinity between the phosphate groups in alendronate and hydroxyapatite also enables alendronate to be immobilized on hydroxyapatite-modified Ti surface, hydroxyapatite ceramics or calcium phosphate bone cements [29–32]. These alendronate-modified substrates inhibit osteoclast functions [29] and enhance osteoblast and stem cell functions [30–32]. However, the functionality within the surface-immobilized alendronate that is responsible for modulating cell functions has not been identified.

To the best of our knowledge, it is not known whether alendronate immobilized on surfaces via its different functional groups would achieve the same degree of enhancement on osteoblast functions. Herein, we immobilized alendronate on Ti via two different methods, and compared the response of osteoblasts on these surfaces to evaluate which strategy would be better for formulating alendronate-functionalized Ti surfaces. In Method 1, the phosphate groups of alendronate molecules were conjugated to surface hydroxyl groups of Ti, leaving the amino and unreacted phosphate groups of the alendronate free to interact with the cells and culture medium. In Method 2, alendronate was immobilized via its amino group on silane-modified Ti surface using a glutaraldehyde linker and only the phosphate groups of the alendronate were free to interact with the cells and culture medium. The osteogenic differentiation of human mesenchymal stem cells (hMSCs) on alendronate-modified substrates was also studied since such cells have high capacity to self-replicate and ability to differentiate into different cell lineages such as osteoblasts. These distinctive advantages thus make them a good candidate in bone tissue engineering applications [33].

2. Materials and methods

2.1. Materials

Ti foils were obtained from Goodfellow Inc (Cambridge, UK). (3-aminopropyl)triethoxysilane (APTES) and glutaraldehyde were purchased from Sigma-Aldrich (St. Louis, US). Alendronate sodium salt was purchased from Merck (Darmstadt, Germany). Mouse osteoblast cell line (MC3T3-E1) was purchased from American Type Culture Collection (Manassas, US). Poietics™ human mesenchymal stem cells (hMSCs) were purchased from Lonza (Walkersville, US). The primers for human osterix (OSX), runt-related transcription factor 2 (RUNX2), osteopontin (OPN), osteocalcin (OC) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Qiagen (Hilden, Germany). All other chemicals, unless specified, were purchased from Sigma-Aldrich.

2.2. Grafting of alendronate on plasma-treated Ti (Method 1)

Ti foils were cut into 1 cm × 1 cm coupons and sonicated twice with acetone, isopropanol and distilled water for 20 min each time. To further clean the surface, the Ti substrates were etched using Kroll's reagent (7.2% HNO_3, 4% HF and 88.8% H_2O acidic aqueous solution), sonicated three times with distilled water for 30 min each time, and dried under reduced pressure (denoted as Pristine-Ti). The Pristine-Ti substrates were treated in a barrel plasma etcher (Anatech, Union City, US) using oxygen at 30 W for 1, 5 or 10 min (denoted as Pls-Ti(t), where t = 1, 5 or 10 depending on the duration of the plasma treatment). In each case, only one surface of Pristine-Ti was plasma-treated. After plasma treatment, the substrates were immediately immersed in 0.5 mL of methanol-water (60:40) solution containing alendronate of different concentrations (0.5, 1 or 2.5 mg/mL of alendronate). The reaction medium was kept overnight (~12 h) at room temperature to facilitate conjugation of alendronate to the surface. Immobilization of alkyl phosphonates or bisphosphonates on metal surfaces has been carried out in anhydrous organic solvent such as tetrahydrofuran or ethanol [34–37], or in methanol-water solution [38,39] or in pure aqueous medium under stringent reaction condition [38,40]. Methanol-water mixture was chosen as the solvent for alendronate because of its low solubility in organic solvents and stringent reaction conditions are required in pure aqueous medium. The substrates after treatment with the alendronate solution were then rinsed with distilled water and annealed in an oven at 100 °C for 2 h. In this work, different experimental conditions (i.e. duration of oxygen plasma treatment and concentration of alendronate solution during the immobilization process) for surface functionalization of Pristine-Ti were used. The as-prepared substrates were denoted as ALN-P-Ti-x(t), where x = 0.5, 1, or 2.5, depending on the concentration of alendronate solution during the immobilization process and t = 1, 5 or 10 depending on the duration of the