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Full Length Article

Bio-functionalization of grade V titanium alloy with type I human collagen for enhancing and promoting human periodontal fibroblast cell adhesion – an in-vitro study

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ABSTRACT

Surface modification of medical grade V titanium alloy (Ti-6Al-4V) with biomolecules is an important and vital step for tailoring it for various biomedical applications. Present study investigates theinfluence of type I human collagen (T1HC) bio-conjugation through a three stage process. Polished grade V titanium alloy discs were functionalizedwith free —OH group by means of controlled heat and alkali treatment followed by coating of 3-aminopropyltriethoxy (APTES) silane couplingagent. T1HC were bio-conjugated through 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride *N*-hydroxysuccinimide (EDC—NHS)coupling reaction. At each stage, grade V titanium alloy surfaces were characterized by atomic force microscopy (AFM), scanning electronmicroscopy (SEM), Fourier transform infrared spectroscopy (FTIR) and Xrayphotoelectron spectroscopy (XPS). FTIR and XPS studies confirms thecovalent attachment of APTES with titanium alloy surface while terminalamine groups of APTES remained free for further attachment of T1HCthrough covalent bond. Aqueous stability of bio-conjugated titanium discsat various pH and time intervals (i.e. at pH of 5.5, 6.8 and 8.0 at timeinterval of 27 and 48 h) confirmed the stability of T1HC bioconjugated collagen on titanium surface. Further human periodontalfibroblast cell line (HPdlF) culture revealed enhanced adhesion on theT1HC bio-conjugated surface compared to the polystyrene and polishedgrade V titanium alloy surfaces.

1. Introduction

Interactions between medical and dental implant surfaces and their surrounding hard/soft tissues and body fluids are affected by physiochemical surface properties of the implant surface. Surface features of the implant material such as surface topography, chemistry, charge and interactions with the body fluids play an important role in biological system [1,2]. These surface features have a strong effect in determining the adsorption of proteins on implant surfaces [3–5].

Grade V titanium alloy is widely used for the fabrication of medical and dental implants due to its superior physical, mechanical and biological properties over other available biomedical metals and alloys [6–9]. Resistance to corrosion, excellent biocompatibility, light weight, superior tribological properties make the material far superior to other metals and alloys making it highly useful for cardiovascular, orthopaedic and dental applications. Titanium and its alloy surfaces in their native form are covered by oxide film on its outer surface which compromises its biological properties [10–12]. Also, the surface contamination and oxide layer prevents the adsorption of proteins, which is important for hard and soft tissue integration, thus compromising its biocompatibility and bio

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mimetic properties. Various surface modification techniques such as physical, chemical, biological and physicochemical have been designed and developed to improve bioactivity and biocompatibility of Ti alloy surfaces for biomedical applications [13].

With application of various surface modification techniques over past decades, it was observed that the best surface features can be obtained through biochemical approach. Various bio molecules (such as collagen, fibronectin, laminin, RGD peptide etc.) were used for this purpose [14–17]. But the problem was non affinity of the bio molecules with titanium alloy surfaces. In the native state, titanium and its alloys are covered with a thin layer of oxide layer [18] as well as surface contaminations. Ti alloys also poses a net negative charge at physiological pH, which furthers inhibits the cell adhesion and cell proliferation which necessitates the use of coupling. Coupling agents are specialised chemical motifs which bind with both titanium alloy surfaces as well as biomolecules through their functional groups. Chemical species such as silanes, carboxylates and phosphates are routinely used for this purpose. Out of these, silane is the most promising coupling agent for biochemical surface modification of titanium and its alloys [19,20]. It provides stable covalent linkage between the titanium alloy surfaces and bio molecules [21]. Oral environment is considered to be one of the most dynamic and tough environment because of pH and temperature fluctuation [22,23], thus the nature of bond between Ti alloy, coupling agent and the bio molecules should be strong and should stay stable in the oral environment.

Type I collagen is widely available protein in the biological system [24–27]. Adsorption of collagen on non-organic surfaces and its role in promoting and facilitating hard tissue growth is well documented [28]. Although, very limited information is available on how collagen bio-conjugated titanium alloy behaves when it is place in biological system, especially its aqueous stability at various pH ranges (acidic and alkaline pH) [29]. Stability of type I human collagen (T1HC) bio-conjugated titanium alloy discs in biological system needs to be studied as the pH in such system varies with time [22,23]. Current literature is deficient in variation in presence of functional groups (especially nitrogen) at various pH, which have a definite role to play as far as stability of titanium alloy is concerned. Therefore, it is interesting and important to investigate their relationship in present study. Collagen was considered and used as biomolecule in the present study as it is most common protein in the basal lamina of basement membrane, which allows various cells to adhere to it thus promoting cell adhesion, spread and growth.

The aim of the study was to modify the grade V titanium alloy (Ti-6Al–4 V) surfaces with T1HC for biomedical application. Various physical, chemical and biological tools were used to tailor the titanium alloy surfaces; this was followed by evaluation of cytotoxicity and cell proliferation to confirm effect of biochemical modification of the alloy surfaces on cell growth and differentiation within the limits of present study.

2. Experimental and methods

2.1. Sample preparation

2.1.1. Substrate, chemicals and cell line/culture media

Ti-6Al–4 V discs of 10 × 2 mm size were purchased from a certified vendor (Metalinox, Mumbai, India). 3-Aminopropyl triethoxysilane (APTES) and type I human collagen were obtained from Sigma Aldrich (Mumbai, India). Acetone, glacial acetic acid, NaOH, (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) i.e. EDC and N-hydroxysulfosuccinimide (NHS) were procured from Thermo Fischer Scientific (Mumbai, India). Human periodontal fibroblast cell line (HPdLF CC 28214 primary cells), culture media (SCGM Bullet Kit®), trypsin, EDTA and buffered saline solution were obtained from Lonza Walkersville Inc. (Walkersville, MD, USA). All chemical were used without any further purification.

2.1.2. Grade V titanium alloy surface preparation

Ti-6Al–4 V alloy discs were sonicated with acetone and alcohol separately for 30 min to remove the surface contaminations followed by drying under laminar air flow. This was followed by sequential polishing of discs with silicon carbide from grit size of 600 up to 2100 (3 M India) on a rotating machine. Diamond paste of particle size from 0.25 to 1.00 μm (Kemet Corp, UK) with oil was used on the rotating machine for further polishing of the alloy surfaces [30–32]. To remove the contamination and oil from surfaces, specimens were again sonicated for 30 min each with acetone, alcohol and double deionised water and dried under laminar air flow. Prepared samples were stored in airtight container until they were further use.

2.1.3. Surface functionalization

Polished Ti-6Al–4 V specimens were treated with 5 M sodium hydroxide (NaOH) at 60 °C for 24 h, followed by rinsing it with double deionised water ten times to remove alkali residue from the alloy surfaces. The alkali treated specimens were dipped in 1% aqueous solution of APTES at pH 10.5 for 15 min at room temperature [30,33,34]. Further Ti-6Al–4 V discs were removed from the solution and excess APTES on the surfaces were blown off using a stream of air. APTES coated discs were placed in electric furnace at 100 °C for 60 min, followed by cooling them to room temperature. APTES coated Ti-6Al–4 V discs were then dipped in 0.2% T1HC solution prepared in 1% glacial acetic acid for 2 h at 4 °C. This was followed by incubation of 0.25% EDC—NHS solution to T1HC solution with the Ti alloy discs overnight. Ti-6Al–4 V discs were rinsed 5 times each-with 1% glacial acetic acid solution and double deionised water and dried under laminar air flow [35,36]. Dried collagen bio-conjugate Ti-6Al–4 V discs plasma sterilized and stored at 4 °C.

2.2. Type I human collagen (T1HC) bio-conjugation on APTES coated Ti-6Al–4 V discs

T1HC solution (0.2% solution of T1HC in 1% acetic acid) was placed on APTES coated Ti-6Al–4 V discs in such a way that it completely covered the surface. The assembly was kept at 4 °C for 2 h followed by addition of 0.25% 1-ethyl-3-(3-dimethyl amino propyl) carbodiimide hydrochloride −*N*-hydroxysuccinimide (EDC—NHS) aqueous solution at 4 °C overnight. Ti-6Al–4 V discs were then washed 5 times each with 1% acetic acid solution and double de-ionised water and dried under laminar hood. Qualitative and quantitative evaluations were done using FTIR, SEM and XPS to evaluate the changes in the surface topography and functionality.

2.3. Atomic force microscopy (AFM)/Scanning electron microscopy (SEM)

Surface roughness of naive (as-received) and polished Ti-6Al–4 V discs was evaluated using AFM (Nanoscope Multimode AFM, Digital Instruments, USA) in tapping mode with standard non rotating AFM tips with tip radius below 10 nm and scan area of 100 μm^2. Surface topography of APTES coated and T1HC bio-conjugated discs was evaluated with scan area of 25 μm^2.

Surface topography of as-received, polished and collagen bio-conjugated Ti-6Al–4 V specimen was visualized using SEM (Zeiss EV) 50, Carl Zeiss Microscopy, GmbH, Germany) at a working distance of 11.5–13 mm and ETH of 20.00 KV at various magnifications.