45S5 bioactive glass coating on Ti6Al4V alloy using pulsed laser deposition technique

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Keywords: bioactive glass, pulsed laser deposition, Ti6Al4V

Abstract
Bioactive glass 45S5 (BG) has been proposed as a biomaterial and extensively studied on account of its superior bioactive behavior. In this paper, we report our results on thin film deposition of BG on Ti6Al4V alloy using pulsed laser deposition (PLD) technique. A Nd:YAG laser (532 nm) based pulsed laser deposition system has been used for this work. Post deposition, surface morphology, and chemical composition of the obtained films on Ti-alloy surface have been examined using scanning electron microscopy (SEM) and energy dispersive x-ray analysis (EDX). In vitro bioactivity of these PLD coated Ti-alloy samples was evaluated by immersing the BG coated Ti-alloy samples in simulated body fluid (SBF) for 10 days. SBF immersed samples have been analyzed using SEM, EDX, and micro Raman spectroscopic techniques. Superior growth of bone like apatite i.e. hydroxyapatite (HAP) have been observed on such BG coated Ti-alloy samples in comparison to uncoated Ti-alloy in our investigations. Also, biocompatibility tests carried out by us measuring tolerance of U2OS osteosarcoma cells to these PLD coated Ti6Al4V samples have shown positive results.

Introduction
Several studies on a plethora of biomaterials and associated technologies aimed towards increasing acceptance of implants inside human body have been reported of late. Biomaterials are classified into categories: bioinert, bioactive and bioreabsorbable on their basis of their response to host or surrounding environment. Materials which can be simulated for specific response are used for medical application [1]. Among all presently available biomaterials, bioactive materials are proven to be of potential interest [2]. This is on account of their ability to react with biological fluids, subsequently promoting successful bonding with bones, as well as, soft tissues by forming bone like apatite at the interface. Since invention, bioactive glass 45S5 (BG) with composition of SiO2 (45 wt%), Na2O (24.5 wt%), CaO (24.5 wt%) and P2O5 (6 wt%) has gained a lot of research interest as implant material in biomedical fields. BG has been commonly used in cranial repair, dental implants and many other low load bearing clinical applications. However, BG is brittle in nature and exhibits poor mechanical properties. These drawbacks limit applications of BG as bulk material in high load bearing implant sites [3]. For high load bearing implant applications metals have often been used. Due to the high strength to weight ratio, good corrosion resistance, low density and relatively low young’s modulus, and high biocompatibility, titanium and its alloys (namely Ti6Al4V) have been widely used as a bio-implant in dental and orthopaedic fields. However, titanium and its alloys have been reported to be largely under bioinert biomaterials [4, 5]. Due to their inert nature, no chemical or biological bond can form easily at the interface between the implant and host tissues. As a consequence, relative movements of such implants are likely to cause inflammatory reactions and implant failure [6].
Coating a metallic biomaterial (namely Ti6Al4V) with BG could be a possible route towards combining superior mechanical properties of bulk with good biological properties of films in the form of surface coating [7]. Over last decades, several techniques have been developed to coat BG on Ti6Al4V alloy (Ti-alloy) surface to improve the bone bonding capability of Ti-alloy. These techniques include plasma spraying [8], sol-gel method [9], enamelling [10], electrophotochemcal deposition [11], laser cladding [13], thermal spraying [14], and pulsed laser deposition (PLD) [15], to name a few. Studies in the past have also altered the compositions of BG to achieve good bonding capability between Ti-alloy and BG. However, changing BG compositions could also result in modifying the bioactivity of BG.

Among all techniques employed for surface coating, PLD is a feasible method for producing BG films having controlled stoichiometry on Ti-alloy surface. PLD is based on the ablation of bulk material by a pulsed laser beam and generation of a vapor plume. This vapor plume contains laser ablated products which get deposited on the substrate thus forming a thin film. It is well known that characteristics of films deposited by PLD method can be varied by varying parameters such as, substrate temperature, ambient pressure, target to substrate distance, and laser parameters such as wavelength and laser fluence [16]. Previous studies have shown that substrate temperature, the type of ambient gas and pressure, can greatly influence the quality of the deposited thin film [17, 18]. In particular, reports on PLD coatings of BG on Ti6Al4V have suggested optimum bonding configuration, morphology and mechanical properties of such coatings when deposited with substrate held at 200 °C during the PLD process [19].

We report here our study demonstrating advantage of surface coating of BG on Ti6Al4V bio–alloy combining good mechanical properties of Ti6Al4V bio–alloy and excellent bioactivity of BG. For this work, thin films of BG have been deposited using a Nd:YAG laser based pulsed laser deposition system. Surface morphology and chemical composition of deposited coating have been examined using scanning electron microscope (SEM) and energy dispersive x-ray analysis (EDX). In–vitro bioactivity of the BG coated samples of Ti6Al4V bio–alloy have been tested by immersing these samples in simulated body fluid (SBF). SBF immersed samples have been analyzed using SEM, EDX, and micro Raman spectroscopic techniques. Superior growth of bone like apatite (i.e. hydroxyapatite (HAP) has been observed on such BG coated Ti-alloy samples in comparison to uncoated Ti-alloy, in our investigations. Biocompatibility tests measuring tolerance of U2OS osteosarcoma cells to these PLD coated Ti6Al4V samples have also shown positive results.

**Experimental**

**Pulsed laser deposition**

BG was prepared by melt–quenched method as described in our previous publication [16]. These prepared BG discs served as a target material for deposition of coating on Ti-alloy substrates via PLD technique. Prior to PLD the Ti-alloy substrates and BG targets were ultrasonically cleaned in detergent soap and ethanol. In order to obtain uniform ablation from target, the BG sample was mounted on a rotating target holder, while the substrate was kept fixed on a substrate holder. PLD was performed employing a Q-switched pulsed Nd:YAG laser operating at a pulse repetition rate of 10 Hz delivering laser pulses of 6 ns duration at a second harmonic wavelength of 532 nm. In order to minimize spatial overlap between the incident laser beam and the laser generated vapor plume, the target was held inclined at an angle of 45 degrees with respect to the incident laser beam. All films were deposited under vacuum condition, at an ambient pressure of 2 \( \times 10^{-3} \) mbar. The substrate to target distance was maintained at 5 cm. In our PLD setup substrates could be heated to fixed chosen temperatures (room temperature (RT) to 800 °C) and once the required temperature was reached the temperature was kept constant during deposition. Each PLD run was performed for 1 h. Several sets of PLD coated samples were prepared. Typical samples chosen for subsequent bioactivity and biocompatibility tests were named as follows: (a) uncoated: T-1, (b) PLD coated on substrate at RT: T-2, (c) PLD coated on substrate at 200 °C: T-3. Substrate temperature was restricted to 200 °C for our PLD runs on the basis of available data on BG coating on Ti6Al4V alloy that reported best quality of BG coating in terms of bonding configuration, morphology and mechanical properties when PLD was done with substrate held at the elevated temperature of 200 °C [18, 19].

**Surface characterization**

The surface morphology of deposited samples has been investigated using field emission scanning electron microscopy (SEM) (Carl Zeiss EVO 40 SEM, 20 KeV beam energy, Tungsten filament). Elemental composition of deposited BG films on Ti-alloy surfaces was determined employing an energy dispersive x-ray analyser (EDX) (Bruker Quanta EDS), which was directly connected to a SEM system. Using micro–Raman analysis, Raman spectral characterization of BG coated and uncoated Ti-alloy samples was performed with excitation wavelength of 532 nm, laser power of 8.5 mW, over a spectral range of 100–3000 cm\(^{-1}\) with spectral resolution of 1 cm\(^{-1}\).
Bioactivity test
To check for bioactivity post PLD, the substrates were immersed in SBF for 10 days. In these tests, formation of bone like apatite i.e. hydroxyapatite (HAP) on BG coated and uncoated Ti6Al4V alloy surface was examined by immersing the samples in SBF. For preparation of SBF, the same procedure was followed, as described in our previous publication [20]. In brief, chemicals such as NaCl, KCl, NaHCO₃, MgSO₄·12H₂O, CaCl₂, and KH₂PO₄, were dissolved in double distilled water and pH of SBF was maintained at 7.4. All samples were immersed in SBF at a temperature of 37 C for 10 days. Freshly prepared SBF solution was used each day to ensure availability of fresh reactive ions for the reaction to occur with samples. At the end of 10 days, samples were taken out of SBF and rinsed in double distilled water to prevent further reaction and taken for surface characterization. Growth of HAP was evaluated and confirmed using SEM, EDX, and micro-Raman spectroscopic measurements. Using an energy-dispersive x-ray analyzer (EDX) [AZTEC microanalysis software (software version 3.1) manufactured by M/s Oxford Instruments Ltd, U.K., which was directly connected to the SEM system, elemental distribution analysis of the surface layer was carried out to identify the ionic substitution route taken for growth of HAP.

Biocompatibility test
Effect of BG coating on biocompatibility of Ti6Al4V was tested by examining adhesion and proliferation of U2OS osteosarcoma cells on samples T-1, T-2, and T-3.

Reagents
Cell culture media and antibiotic solution were procured from Himedia Labs, India; fetal bovine serum and FITC conjugated phalloidin from Invitrogen, USA; MTT reagent, Hoechst 33328 and crystal violet stain from Sigma, USA.

Cell culture
U2OS osteosarcoma cells were procured from the National Cell Repository (NCCS, India), and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotic solution (Penicillin-Streptomycin-Amphotericin B). Cells were maintained at 37 C under 95% relative humidity and 5% carbon dioxide.

Assay to determine tolerance of U2OS osteosarcoma cells to uncoated and PLD BG coated surfaces
The effect of uncoated and BG coated titanium alloy surfaces on cellular viability and growth rate was evaluated using MTT assay technique. Briefly, 0.1 × 10⁶ cells were seeded per well in 6-well plates, in the presence or absence of titanium alloy surfaces. MTT assay was performed 48 and 72 h post seeding. Briefly, cells were incubated for 4 h in the presence of 0.5 mg ml⁻¹ MTT reagent, followed by solubilisation of formazan crystals in DMSO. Thereafter, absorbance of DMSO-dissolved formazan crystals was acquired at 570 nm.

Microscopic analysis of cellular morphology
To determine the effects (if any) of titanium alloy surfaces on the gross cellular morphology of U2OS cells, 0.1 × 10⁶ cells were seeded per well in 6-well plates, in the presence or absence of titanium alloy surfaces. 48 h post seeding, the titanium alloy samples were removed, and cells growing in the wells in the vicinity of the respective surfaces were stained using a 0.5% crystal violet staining solution. Post staining, the gross morphology of the cells was analysed under a light microscope.

Analysis of cellular attachment using FITC-Phalloidin
The degree of cellular attachment on the various sample surfaces was analysed by staining the cells with FITC conjugated phalloidin, and analysing images captured on a Zeiss LSM 780 confocal microscope. Briefly, 0.1 × 10⁶ cells were seeded per well in 6-well plates, in the presence or absence of titanium alloy surfaces. 48 h post seeding, the titanium alloy samples were removed from the wells, and their surfaces were treated with 3.5% paraformaldehyde solution for 10 min to fix cells. Cells were then permeabilized with PBS containing 0.1% Triton X-100 (PBST), and treated with 1% BSA (prepared in PBST) for 1 h to preclude non-specific binding. Cells were then treated with FITC-conjugated phalloidin (supplemented with 1% BSA) and Hoechst 33328 (to stain nuclei) for 1 h. Surfaces were washed in PBS, dried, and then analysed under the microscope. Cellular attachment on the surface of each sample was evaluated by measuring the number of cells per field in 10 randomly selected fields.
Results and Discussion

Surface characterization

The surface morphology of BG films deposited on a Ti6Al4V alloy substrate via PLD technique was examined using SEM. In figure 1(a) is shown SEM image of an uncoated Ti6Al4V sample. Figures 1(b) and (c) show BG films deposited on a Ti6Al4V alloy substrate held at room temperature, and at 200°C substrate temperature, respectively. Spherical particles were observed in figures 1(b) and 1(c) on Ti-alloy surface post deposition, which is typical in case of PLD thin films [21]. In comparison, a smoother surface has been observed in case of uncoated Ti-alloy sample T-1 as depicted by SEM image in figure 1(a).

Typical EDX spectrum of an uncoated and two PLD coated samples are shown in Figures 2(a)–(c), displaying their elemental compositions. The spectrum for sample T-1, as expected, shows no elements belonging to BG, as can be seen in Figure 2(a). The major elements detected in this case were Ti, V, and Al. Presence of oxygen suggests possible contamination adsorbed from environment. PLD coated samples T-2, and T-3, showed presence of BG elements on their surfaces, namely Na, Si, and Ca (Figures 2(b), (c)). Presence of these elements indicates deposition of BG on Ti-alloy samples. Past studies have reported enhanced deposition of HAP layer on surfaces having BG elements, when immersed in SBF [22]. Hence, from our EDX results we can expect higher growth of HAP on samples T-2 and T-3 in comparison to sample T-1 when these samples are subjected to bioactivity tests in SBF.

In vitro bioactivity study in simulated body fluid

In vitro hydroxyapatite (HAP) formation during exposure to SBF is generally used as an indicator of bioactivity of a material surface. Therefore, the in-vitro bioactivity of all samples was evaluated, immersing the samples in SBF for 10 days. Characterization of the layer which got deposited on the surface of samples T-1, T-2, and T-3 when dipped in SBF for 10 days was carried out using SEM, EDX, and micro Raman spectroscopy techniques.

Figure 3 shows SEM images of uncoated and BG coated Ti-alloy samples post immersion in SBF for 10 days. Randomly distributed spheres of hydroxyapatite have been observed on all surfaces as can be seen in figures 3(a)–(c). A dense growth of HAP was observed on samples T-2 and T-3 as seen in figures 3(b) and 3(c). On the other hand, comparably less growth of HAP was observed on surface of sample T-1.

To confirm the composition of the layer which formed on the samples post immersion in SBF, EDX analysis was performed.
Figure 4 shows the EDX spectra of all the samples: uncoated and PLD coated with BG at various temperatures and subsequently immersed in SBF for 10 days. Ca/P ratio for the BG coated samples was calculated to range between ~1.3 and 1.6, similar to typical Ca/P ratio of HAP of human bone [23]. Comparison of EDX spectra in Figures 2(b), (c) and 4(b), (c) indicates a decrease in concentrations of Na and Si, and an increase in P in case of samples T-2 and T-3 post immersion in SBF. These changes can be attributed to the formation of HAP on the surface of the BG coated Ti-alloy samples when immersed in SBF.

To further confirm the growth of HAP on all samples after being immersed in SBF for 10 days, micro-Raman spectroscopy was performed, results of which are shown in figure 5. Raman spectra for all the samples showed a band at 960 cm\(^{-1}\) which corresponds to symmetric stretching of P-O bond, which is a characteristic peak associated with HAP [24]. Additional peaks around 435 cm\(^{-1}\), 586 cm\(^{-1}\), and 1070 cm\(^{-1}\) for sample T-2 and T-3 can be attributed to bending vibration of PO\(_4\)\(^{3-}\) tetrahedra, degenerate mode of O–P–O bending, and asymmetric P–O stretching mode or CO\(_3\)\(^{2-}\) impurity, respectively [25]. Detection of even these weaker modes of
HAP in case of Raman spectra of samples that had been BG coated denotes superior growth of HAP on these in comparison to the uncoated samples of Ti6Al4V alloy.

Coating of BG on Ti-alloy samples is expected to facilitate apatite formation. On being immersed in SBF, alkali and alkaline earth ions (Na\(^+\) and Ca\(^{2+}\)) from the BG coating on sample surface are released into the SBF solution via ion exchange with H\(^+\) or H\(_3\)O\(^+\) present in SBF, resulting in a negatively charged surface. This is followed by hydroxyl ions (OH\(^-\)) breaking the Si–O–Si bonds in BG and forming hydrated Silica (SiOH) groups and a silica rich layer. Thereafter, a calcium phosphate (Ca-P) layer is formed on the surface of this silica rich layer. Subsequently, the Ca-P layer crystallizes into the HAP layer, also referred to as the bonding layer since its composition is chemically and structurally similar to the contents of natural bone [26]. Our EDX results in Figure 2 have shown presence of higher atomic percentage of Na and Ca on samples T-2 and T-3 than on T-1. Higher concentration of Ca and Na on sample surface would enable more ion exchange from SBF hence, more HAP formation on such BG coated surfaces [27]. Therefore, our EDX data suggested that superior growth of HAP should occur on samples T-2 and T-3 when dipped in SBF. This was confirmed by our in-vitro bioactivity results in terms of EDX and micro-Raman spectroscopy which established higher growth of HAP on samples T-2 and T-3 in comparison to sample T-1.

**In vitro biocompatibility study using osteosarcoma cell lines**

Biocompatibility of PLD deposited samples was studied via in-vitro tests with U2OS osteosarcoma cell lines using MTT assay. Adherence of cells to such surfaces and their morphology were examined under a confocal microscope.

In figure 6(a) are shown absorption at 570 nm measured for MTT assay post seeding with U2OS osteosarcoma cells for samples T-1, T-2, and T-3. Post 48 h and 72 h of incubation we have observed comparable absorption at 570 nm (shown in figure 6(a)) for all three samples T-1, T-2 and T-3. This indicates that presence of BG coated Ti-alloy samples did not negatively impact cell viability of U2OS osteosarcoma cells. In figure 6(b) are shown results of morphological examination of crystal violet stained cells which had been exposed to samples T-1, T-2, and T-3. No significant changes in cell morphology were observed in these images. Our results
suggest that U2OS osteosarcoma cells showed comparable tolerance for all three sample types, whether uncoated or coated with BG.

In figure 7(a) are shown confocal microscopy images of samples T-1, T-2, and T-3 post cell culturing for 48 h and fixing cells on sample surfaces using Phalloidin-FITC to visualize actin filament and DAPI to visualize nuclei of cells. Figure 7(b) quantified the number of cells attached per field on the three samples T-1, T-2, and T-3. A minimum of 10 fields were analysed per sample, and the number of nuclei per field was plotted with a box-whisker plot. As indicated by both figures 7(a) and (b) attachment of osteosarcoma cells was higher on BG coated samples of Ti-alloy than in the case of bare uncoated Ti-alloy surface T-1.

**Conclusion**

Bioactive glass 45S5 coating was deposited on Ti-alloy surface using pulsed laser deposition technique. The effect of bioglass PLD coating on apatite forming ability of these samples has been examined. Investigation of bioactivity involving in-vitro tests examining HAP formation on these samples when immersed in simulated body fluid for 10 days, has been carried out. Superior growth of HAP has been observed for sample T-2 and T-3 in comparison to sample T-1 as evident by SEM, EDX, and micro Raman spectroscopy. Growth of HAP was enhanced for samples which had been coated with bioglass as compared to uncoated samples of Ti-alloy. Also, biocompatibility tests carried out by us measuring tolerance of U2OS osteosarcoma cells to these PLD coated Ti6Al4V samples have shown positive results.

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