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Abstract

Selective Laser Melting (SLM) is an additive manufacturing (AM) technology gaining more popularity in the biomedical field, because of its advantage with complex geometry and minimum post treatment requirement. In the biomedical sector, it become an apposite strategy for the development of customized metallic implants mimicking to native bone structure, medical surgical tools, and biomedical instruments etc. SLMed titanium (Ti) and Ti based alloys (majorly Ti6Al4V) are commonly used worldwide for orthopedic and dental applications. Even though they can mimic the innate bone structurally, their ability to integrate with the surrounding cells was ultimately determined by their surface properties such as surface composition, roughness, topography, and surface energy. These surface properties are the major candidates which trigger the initial biological responses of an implant like the binding of proteins, activation of signaling molecules and cell adhesion. The present study focuses on surface modification of SLMed Ti based porous scaffolds by acid treatment using a mixed acid solution (H_2O: HCl: H_2SO_4) followed by anodization. Subsequently electrodeposition of cerium was attempted over the scaffold surface. Thus modified surface was analysed by various characterization techniques and respective biological properties were also evaluated.

Keywords: Selective Laser Melting, Surface Modification, Acid Treatment, Anodization, Electrodeposition.

Introduction

The ability of biomedical implants to attain integrity on par with the host system along with stable and homogenous microstructure are the major requisite as per International Standards Organization (ISO). Biomedical implants are the need by medical practitioners to replace damaged organs, tissues and bone joints. Customization or patient specific implant development is also highly desired in the biomedical sector. This can be achieved by Additive manufacturing or Selective Laser Melting (SLM) which enables the development of biomimetic implants in the designed geometries using preferred metals. Metallic implants developed using SLM is a layered-by-layered printing process where the information for each layer is obtained from the slicing algorithm to the computer model of the part [1]. As per the design data, the laser beam selectively melts the metal powder and solidifies it layer by layer to finally fabricate complex metallic implants mimicking the damaged bone structure. Majorly metal based medical implants made of Titanium (Ti) and its alloys are used in orthopaedic and dental replacements due to their high mechanical strength, load bearing properties and biocompatibility. Titanium being a bio inert material, is not good enough to generate an active interaction with the surrounding environment [2].

Eventhough there is much evidence supporting the beneficial biomechanical properties of Ti and Ti alloy based implants, the interfacial bonding strength between bone and implant remains poor and also susceptible to antibacterial infections. This necessitated the need for different surface modifications to enhance the surface properties such as surface composition, roughness, topography and surface energy as they really contribute to the biological properties of the implant and make it more compatible [3]. Different surface modification approaches are there to improve

the implant properties where we can also incorporate biofunctional elements to give additional properties to the implant [4].

Electrochemical anodic oxidation is a well-known approach to fabricate TiO_2 nanotube arrays (TiNT) over Titanium substrate. There are reports that homogenous porous oxide layer can be generated and also by varying the process parameters, it is easy to vary the length, diameter and composition of nanotubes which are also highly reproducible [5, 6]. The porous oxide layer also improves the surface wettability nature and roughness which are the key factors for the improved biological properties. It also proved to have improved beneficial interactions with osteogenic cell types, with good adhesion of cells and no cytotoxicity [7, 8]. Along with anodization, surface properties can be further improved by the incorporation of biofunctional elements such as silver (Ag), Zinc (Zn), Strontium (Sr) etc [9, 10]. Among them ceria (CeO_2) is getting more attentions recently in the biomedical applications due to the physico-chemical properties and enhancement in the biocompatibility reported. It is one of the most reactive rare-earth metal oxides, which has enzymatic actions like superoxide dismutase, catalase and oxidase with distinguishable reactive oxygen species (ROS) scavenging capability [11]. The presence of mixed valence state is highly needed for these characteristic functionalities and they act in accordance with the immediate environment by their rapid switch between the two forms using the oxygen vacancies [12]. There are successful evaluation reports on the bone regenerative activity of ceria as a pure coating, integrated coating and nanoparticles by confirming the non-toxicity and enhancing the osteogenic actions of bone marrow mesenchymal stem cells (BMSCs) [13, 14].

In the present work, we aimed to develop coating of ceria onto anodized SLMed titanium substrates (TiNT) by a simple cost effective electrodeposition method, which gives good control over surface cerium content. Ceria incorporated samples were then further evaluated using

different surface characterization techniques. This additively manufactured (SLMed) Ti metal with the bioactive nanostructured surface layer proposed here is expected to have better and faster bone integration.

2. Material and Methods

2.1 Sample Preparation

Selective laser melted Commercially Pure Ti (CP-Ti) samples were polished with 400 Sic paper and cleaned with acetone, isopropanol and deionised water each with 15 minutes ultra sonication (Ti). To make the surface more rough the cleaned SLMed CP-Ti samples were subjected to acid etching using a mixed acid solution of HCl : H_2O : H_2SO_4 in a ratio of 3:2:5 at 70 ^{O}C in a water bath (MxTi) [15]. These acid etched samples were subjected to anodization (MxTiNT) using a two electrode system with DC power supply. Polished SLMed CP-Ti samples were used as anode as well as cathode under 45V for 45 minutes. The two electrodes were dipped into the electrolyte solution consist of 0.3 wt% (w/v) NH_4F, 2 vol% distilled water and 98 vol% ethylene glycol. After the anodization the SLMed Ti samples were washed and cleaned with deionised water and air dried. Ceria deposited samples (EdMxTiNT) were prepared by electrodeposition technique using a potentiostatic control, using anodized SLMed Ti samples (MxTiNT) as working electrode, platinum wire as counter electrode and saturated calomel electrode as reference electrode. The electrolyte consist of 1mM $Ce(NO_3)_3$ .6 H_2O (sigma aldrich) prepared in KCl solution of 100 times higher concentration at pH 9. It was carried out at a potential window of 2 to 0 V with a scan rate of 0.05 v/s for 10 cycles at a step size of 0.005 in room temperature. Later the samples were washed in deionised water and air dried. The different sample preparation and the work flow is clearly given in Figure 1.

Figure 1: Schematic workflow

2.2 Surface characterization

The change in surface morphologies at each chemical treatment was investigated using Field Emission Scanning Electron Microscope (FE-SEM; Carl Zeiss, SUPRA 55VP, Germany) at

an acceleration voltage of 15 kV. Atomic Force Microscopy (AFM) (Aglient Technology Inc., SPM 5500) imaging was performed to obtain the roughness parameters such as arithmetic mean deviation of roughness profile (R_a), total height of roughness profile (R_t), maximum height of roughness profile (Rz), 2 dimensional root mean square roughness (Rq), Skewness (Rsk) and Kurtosis (Rku) values of modified sample surfaces and were also evaluated using 3D topographical images. The different phases formed on the sample surface due to surface modification were investigated with Laser Raman spectroscopy (Horiba Jobin Yvon, LabRAM HR Evolution) using He-Ne Laser of wavelength 532 nm. X-ray diffraction (XRD; RINT-2500, RIGAKU Co., Japan) analysis was carried out to detect the changes in the surface compositions with a 1.5406 Å (λ) Cu Kα radiation source on the sample surface in the range of 30–80◦ in 2θ with a step size of 0.02^{O} per second. The water contact angle was measured using VCA Optima (Video Contact Angle System, AST Products, Inc) at room temperature by droping 10 µL of distilled water to evaluate the hydrophilic nature of surface modified samples.

2.3 Cell adhesion study

Mouse embryonic fibroblast cells 3T3 was used to evaluate the cell adhesion of cells over modified surface of SLMed CP-Ti samples. The culture of cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and penicillin-streptomycin (Pen-Strep) under 37°C in a humidified atmosphere of 5% CO_2 and 95% air in CO_2 incubator. As the cells attained 80 % confluence, they were subjected to trypsinization and a cell pellet was obtained by centrifugation. Cell suspension was made from the pellet by suspending it in the freshly prepared complete medium. Later the morphology and adherence of cells on different sample surfaces were observed under Fluorescent microscope (Nikon Eclipse TS100). Each sample surface of 10 x 10 x 1 mm in dimension was seeded with approximately 5000 cells without

overflowing and incubated in standard cell culture conditions (37 ˚C in 5% CO_2 and 95% air) for 48 h. Later the cells were fixed using 3.7% w/w formaldehyde in PBS and incubated for another 20 minutes at room temperature. Further, the cells were undergone a series of ethanol washing to dehydrate the samples and stained them with acridine orange. At last, Fluorescent microscope was used to observe the cell morphology over the surface modified SLMed CP-Ti samples.

3 Results

3.1 Evaluation of surface properties

The FE-SEM images given in Figure 2 displays the morphology developed at each level of surface modification of SLMed CP-Ti such as Ti, MxTi, MxTiNT and EdMxTiNT. Figure 2 clearly shows the acid treatment has made the surface more irregular and over that irregularities the nanotubes were formed after anodization and it remained even after the electrodeposition of ceria. The nanostructures formed over the surface were on par with the previous reports. The nanotubes were clean and formed throughout the acid etched surface which shows the evenness that could be achieved using this modification approach. The outer diameter of the nanotubes were measured around 40-90 nm. This irregular tube diameter is formed due to the irregularity generated by acid treatment. After electrodeposition increase in the outer wall thickness of the nanotubes without changing the tube diameter further confirmed the oxide layer growth after cerium oxide electrodeposition as reported earlier [16].

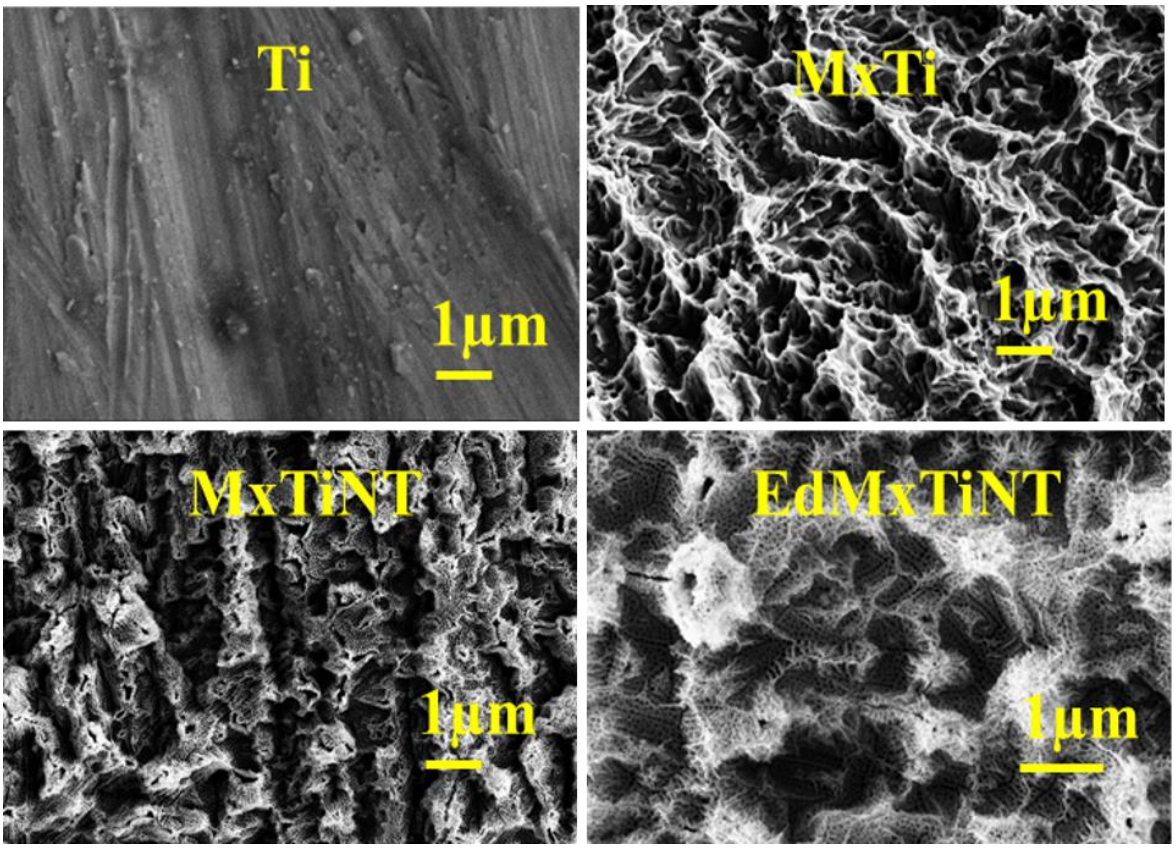

Figure 2: FE-SEM images of SLMed CP-Ti samples after different surface modification

A surface topographical analysis was carried out using AFM to get different parameters which is summarised in Table 1. This surface micro and nano roughness profile has their respective impact over bone cell interface. Acid etching has resulted in more a rough surface compared to bare polished SLMed CP-Ti sample. The arithmetic mean deviation and total height of roughness profile attained by acid etching has slightly varied by subsequent anodization and electrodeposition process. As the nanotubes generated over the acid etched surface, resulted in lowering the maximum roughness profile height attained before. Electrodeposition of cerium further thickened

these nanotube wall thickness and smoothened the sharp edges and thus lowered the Ra, Rt, Rz and Rq values.

Table 1: Values of different roughness parameters such as Ra, Ra, Rz, Rq, Rsk and Rku calculated from AFM analysis specific to Ti, MxTi, MxTiNT and EdMxTiNT.

Sample	Ra	Rt	Rz	Rq	Rsk	Rku
Ti	0.04	0.34	0.22	0.04	0.0003	0.003
MxTi	0.44	2.63	2.45	0.605	-0.29	2.58
MxTiNT	0.34	2.39	1.82	0.349	-0.35	3.27
EdMxTiNT	0.31	1.79	1.55	0.293	-0.40	3.40

This supports the nanotube wall thickening observed under FE-SEM. A relative proportion of peaks and valleys over the surface was represented by skewness parameter (Rsk) and kurtosis coefficient (Rku) describes the sharpness of the profile probability density, which reveals the relation of total number of peaks and the valleys on the surface. There are reports saying that the surfaces with negative skewness value will only generate very small endosseous tissue interface shear strength of bone knobs, which are into the pits over implants [17]. Negative Rsk values obtained from the samples after each chemical treatment is also corresponds to the gradual increase in the density of valleys. The presence of many low valleys on the surface can be revealed by a value higher than 3 for kurtosis coefficient. Cerium incorporation leads to a slight leverage in the

number of valleys as evidenced from the Rku values. 3D topographical imaging as given in figure 3 also displays the variations in the surface upon different treatments. FE-SEM and AFM analysis showed the visible influence of cerium incorporation over nanotube wall and its smoothening.

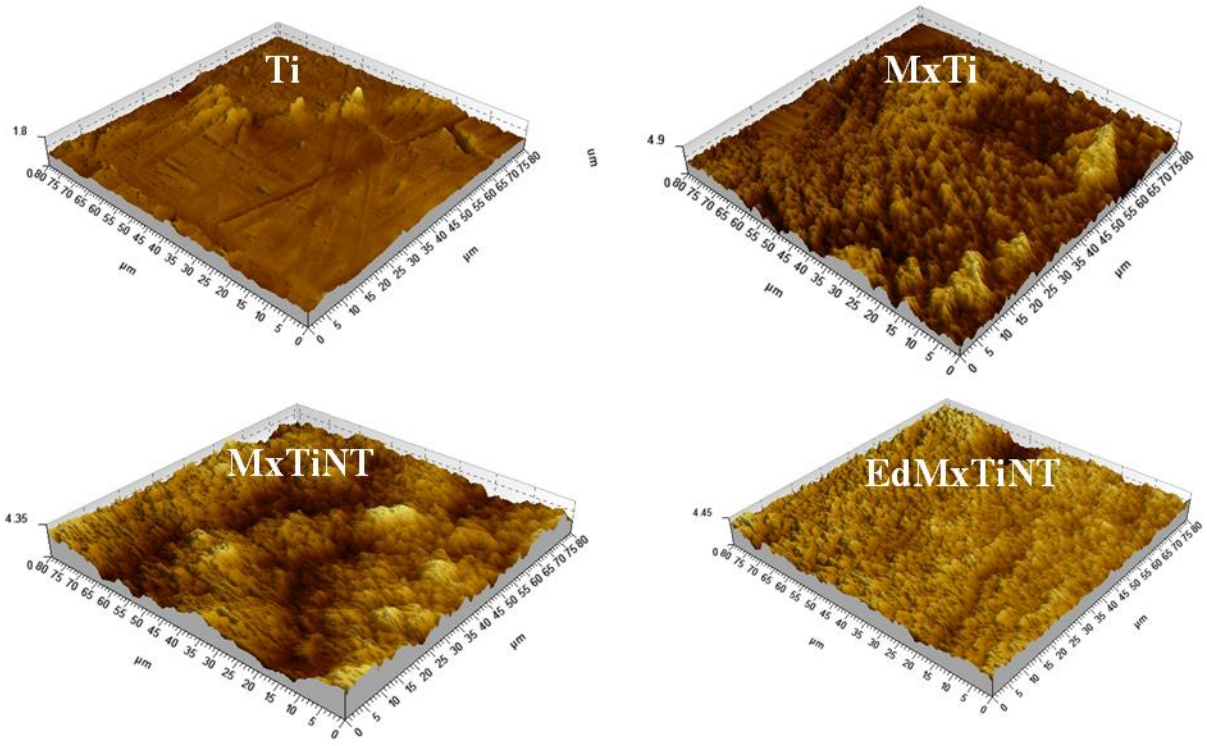

Figure 3: 3D topographical AFM images of SLMed CP-Ti samples after different treatments.

The phases formed over the SLMed CP-Ti samples at each stage of the surface modification approach were examined using XRD and the corresponding results are shown in Figure 4. Irrespective of all those treatments such as acid etching, anodization and electrodeposition the presence of Ti metal peaks were obvious. XRD patterns of Ti, MxTi and MxTiNT were only displayed the characteristic peaks for the highly ordered titanium metal alpha phase [18]. We were able to observe a characteristic peak at 2θ =28.309 corresponds to (1 1 1)

plane along with these Ti metal peaks in EdMxTiNT, shows the formation of crystalline cerium structures of CeO_2 [11]. Porous nature and micron level thickness of the nanostructured film over the SLMed CP-Ti samples can be the reason for low intensity of the CeO_2 peak. With this result the deposition of crystalline CeO_2 in EdMxTiNT could be confirmed.

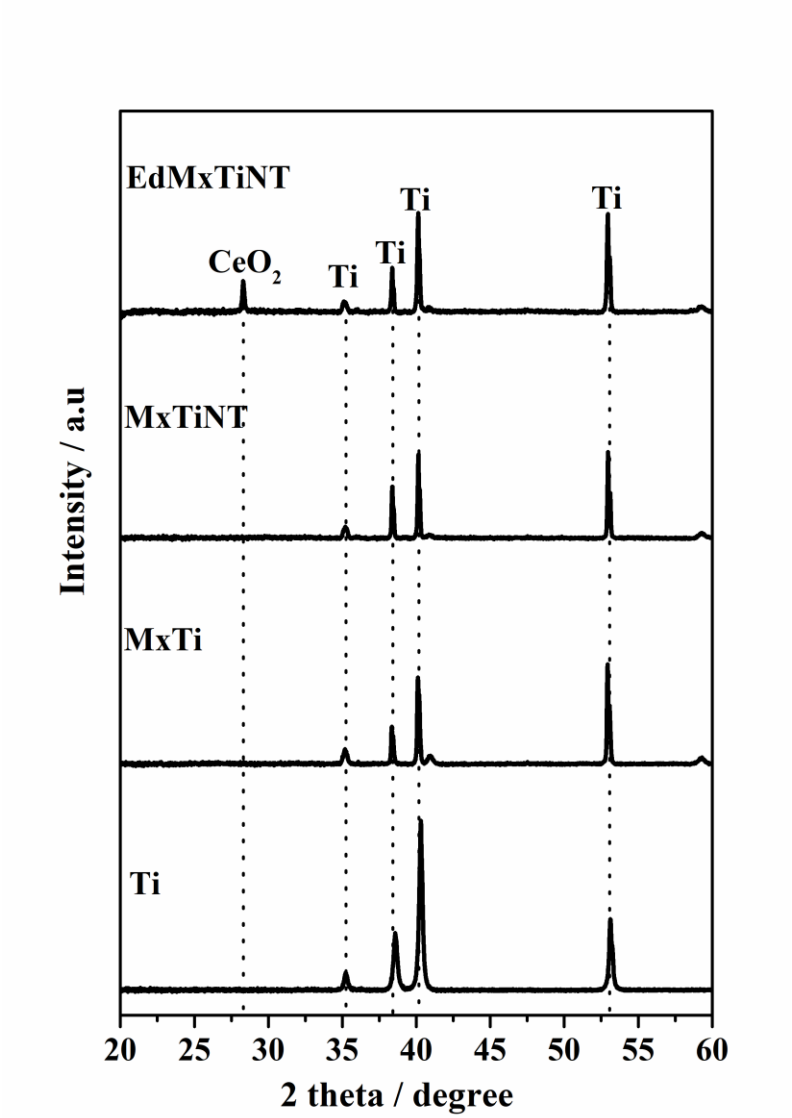

Figure 4: XRD spectra of SLMed CP-Ti samples after different treatments

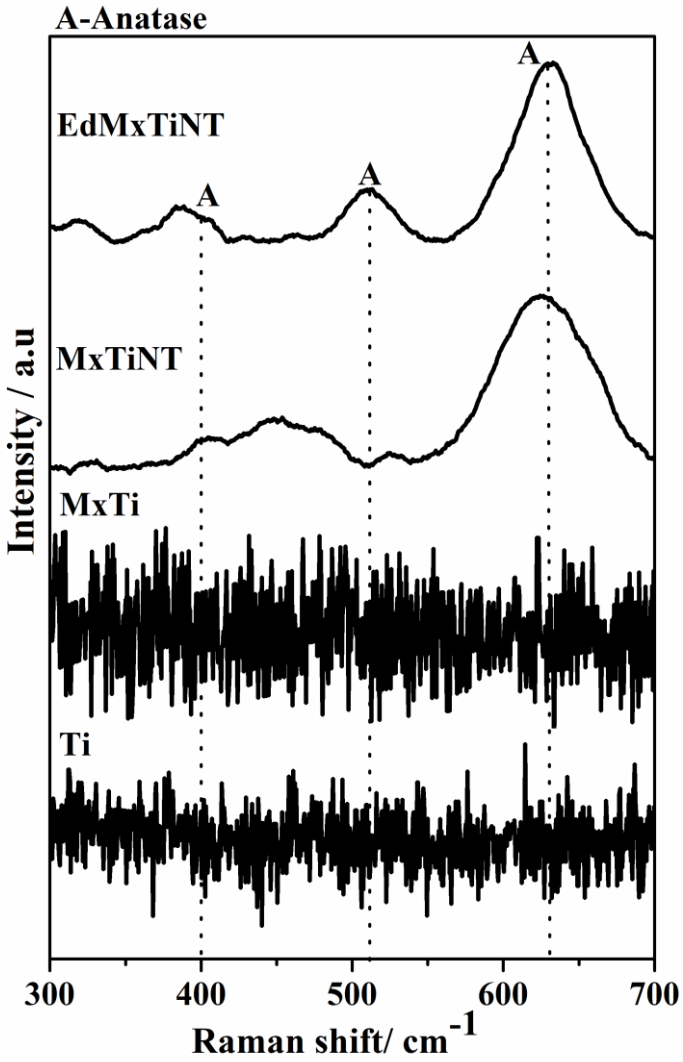

Figure 5: Raman spectra of SLMed CP-Ti samples after different treatments

Further the samples were examined using the Laser Raman spectroscopic technique and the spectra is given in Figure 5. Lack of peaks in Ti and MxTi confirms that there was no TiO_2 oxide layer formation during SLM and remaining in metallic state. Upon anodization and electrodeposition, three characteristic peaks were marked at 402 cm^{-1}, 513 cm^{-1} and 632 cm^{-1} corresponds to the anatase phase of TiO_2 [19]. Anatase TiO_2 is reported to have better biocompatibility compared to the rutile phase of TiO_2. Anodized MxTiNT has low intensity in the

peaks whereas EdMxTiNT has strong peaks at respective positions due to the improved wall thickness of nanotubes upon cerium incorporation.

3.2 Evaluation of cell compatibility

In vitro cell compatibility of samples Ti, MxTi, MxTiNT and EdMxTiNT were evaluated using Fluorescent microscope by analysing the morphology of cells upon attachment to the sample surface. Mouse embryonic fibroblast 3T3 cells were used for this study. Acridine orange stained cells were adhered and elongated into the valleys and peaks over the surface conditions as depicted in Figure 6.

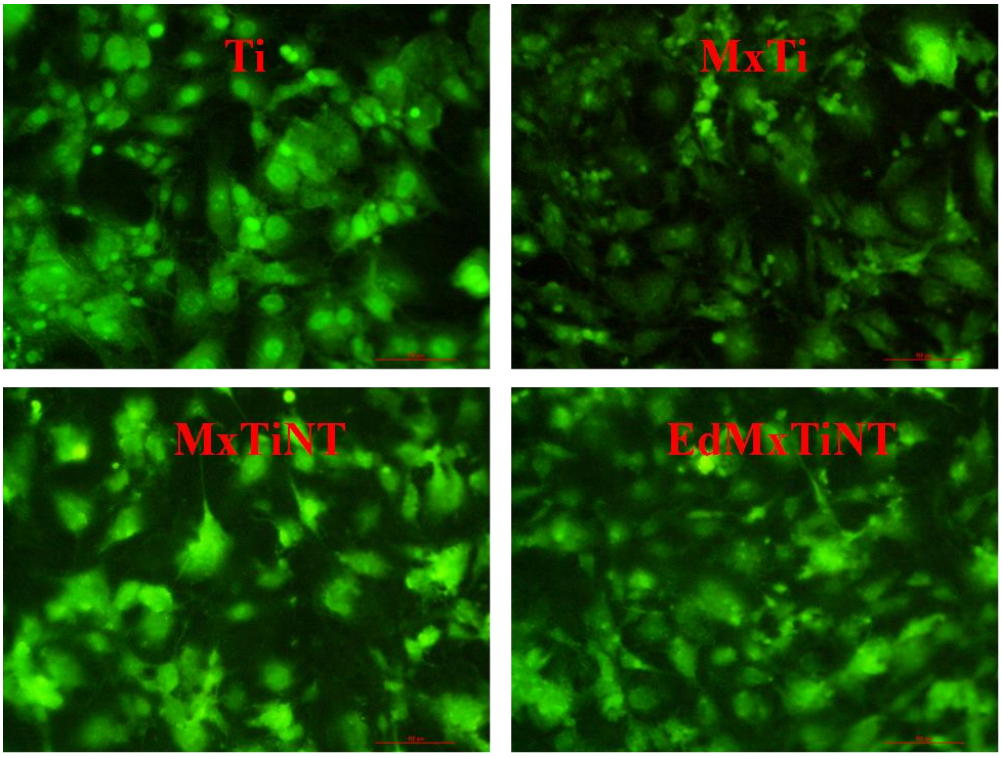

Figure 6: Flurescent microscopic images of 3T3 cells adhered over the surface of SLMed CP-Ti samples subjected to acid etching, anodization and electrodeposition

There was no significant difference in the appearance of adhered cells over Ti, MxTi, MxTiNT and EdMxTiNT. As per earlier reports, fate of cells after implantation could be determined after detailed in vitro analysis. Based on the cell-implant interactions are processed, it is possible to tune the cell fate. Non toxicity of Titania nanotubes were reported early, which also describes the ability of this nanotubes to induce rapid bone integration [20]. Cerium oxide is also reported to have good biocompatibility when used as coating material over titanium [21]. Cell compatibility of an implant can be improved when cerium oxide is electrodeposited to MxTiNT. Further *in vitro* analysis of EdMxTiNT in different cell lines needs to carry out to understand properties contributed to the SLMed CP-Ti by the coated CeO_2.

Conclusion

In summary, the valleys and peaks formed over SLMed CP-Ti metal surface by acid etching were covered by a layer of TiO_2 nanotubes upon anodization which further coated with cerium oxide by electrodeposition. These subsequent treatments resulted in improved surface roughness profile. Further analysis with XRD and laser Raman spectroscopc techniques confirmed crystalline nature of surface CeO_2 in EdMxTiNT and presence of anatase TiO_2 in both MxTiNT and EdMxTiNT. Irrespective of different treatments all sample surfaces had good platforms for cell adhesion and they were fully elongated and stretched over it.

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