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Graphical Abstract

3D-Nanoflowers of Rutile TiO$_2$ as Film Grown on Conducting and Non-conducting Glass Substrates for *In-Vitro* Biocompatibility Studies with Mouse MC3T3 Osteoblast and Human HS-5 Cells

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3D-Nanoflowers of Rutile TiO₂ as Film Grown on Conducting and Non-conducting Glass Substrates for In-Vitro Biocompatibility Studies with Mouse MC3T3 Osteoblast and Human HS-5 Cells

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Abstract

Thin films of 3D-nanoflowers of rutile TiO₂ on conducting (FTO and ITO) and non-conducting (glass) substrates were grown in a surfactant free one-step hydrothermal process. Field emission scanning electron microscope (FE-SEM) observations confirmed transformation of TiO₂ nanostructures from mesh like to 3D-nanoflowers with an increase in hydrolysis rate during the growth of TiO₂ films. X-ray diffraction (XRD) pattern of TiO₂ nanostructures as films grown on different substrates at various conditions had phase pure rutile crystallite structure. High resolution transmission electron microscopy (HR-TEM) diffraction pattern of the TiO₂ nanostructures showed tightly packed assemblies of titanium atoms and 0.23 nm lattice spacing along the longitudinal axis direction of rutile TiO₂. X-ray photoelectrons spectroscopic (XPS) analysis of TiO₂ nanostructures grown as films on glass substrates showed a spectra shift of 0.53 eV in binding energy which confirms the charge accumulation on non-conducting substrate whereas there was no spectral shift observed for TiO₂ films of similar structures grown on...
conducting substrates. The accumulated charge on conducting surfaces can be easily neutralized whereas non-conducting surfaces may retain these accumulated charges. Adhesion, viability and proliferation response of mouse osteoblast (MC3T3) and human stromal (HS-5) cells on 3D-nanoflower of TiO$_2$ as films grown on conducting and non-conducting substrates was assessed. Adhesion and proliferation of both the cells showed better response on non-conducting surfaces as compare to conducting surfaces despite of having similar crystallite structures and nanomorphology of TiO$_2$. Stromal cells had potential to prepare extra cellular matrix scaffolds for ex-vivo expansion/differentiation of stem cells [1,2]. Hence the current findings can be used to prepare 3D TiO$_2$ nanostructure supported cellular scaffolds for regenerative medicine in the future.

**Keywords:** One step process, 3D nano-structures, charge distribution, bio-active surface

1. **INTRODUCTION**

During the past two decades, substantial research work has been carried out to understand the correlation between different process parameters of various synthesis methods and their effects on tailoring structural and functional properties of metal and metal oxides [3-8]. In the past the use of metal oxides such as titanium dioxides with tunable nano-structural and chemical properties has been extensively explored for catalytic, energy and biomedical applications [9-13]. Different synthesis methods were employed to tailor structural and functional properties of metal oxides by manipulating processing conditions to have desired arrangements of nanocrystallites [13-18]. For each synthesis method detailed studies were carried out to understand the effects of processing parameters on modification of basic structure properties but
still it’s a challenge to obtain the material with higher degree of phase purity, tunable surface morphologies with desired surface chemical functionalities [19-21].

A number of researchers have addressed the challenges associated with tunable surface morphology and synthesis of micro/nanostructures of titanium oxides with highly oriented rod-like crystals [22-27]. The control over crystallite phase and morphologies were achieved either by surface treatment or metal ion doping [28-31]. Thin films of titanium oxides were deposited with controllable crystallite phases by choosing appropriate metal ion doping and were used for different applications [32,33]. Well ordered 1D TiO₂ as nanorods, nanowires, nanocorals, hierarchical microspheres and nanotubes were synthesized and extensive studies were performed to use these for energy and biosensor applications [23-26]. Previously biocompatibility of titanium oxides as thin film coatings deposited by plasma sputtering and dip coating were studied with different types of cells [9,12,28]. The simultaneous growth of 1D/3D nanorods/nanoflowers is advantageous for better adhesion at interface of these 1D/3D nanostructures [34]. However, the use of 1D and 3D nano-assembles of TiO₂ in biomedical field is not fully explored yet.

Here we report a surfactant free one-step hydrothermal process for growth of 1D/3D nano-morphologies of TiO₂ at various conditions. The simultaneous growth of 3D nanoflowers like structures on 1D nanorods provides better adhesion at interface of 1D/3D nanoroads. The other advantage of the method used in this study was to achieve uniform coatings on all sides of the substrates in a single step. Physicochemical properties of these nanostructures of TiO₂ grown on different substrates (non-conducting glass and FTO/ITO coated conducting glass) were investigated. The effects of molar ratio of precursor material and reaction time were optimized to grow 1D/3D nanostructures of rutile TiO₂ as films on different types of substrates. In this study, first time we have reported cellular response of osteoblast and HS-5 cells with TiO₂ without
alteration in 3D nano-structures by changing conducting and non-conducting nature of the substrates. The use of osteoblast cell for assessing the biocompatibility of metal oxide and polymeric materials is a well accepted in-vitro model system [35-38]. To further assess the usefulness of 1D/3D nanostructures, human stromal cells which has potential for preparing extracellular matrix scaffold were cultured on different types of nanoassemblies of TiO$_2$. In-vitro cell adhesion, viability and proliferation assays with human HS-5 cells were performed. The outcomes of this study may have potential in future to use these substrates to prepare 3D nano-structures supported cellular scaffolds for application in regenerative medicine.

2. EXPERIMENTAL

2.1 Materials

Titanium isopropoxide (Ti[OCH(CH$_3$)$_2$]$_4$, 97%), hydrochloric acid (HCl, 37 wt%), and isopropyl alcohol were purchased from Sigma-Aldrich (Sigma-Aldrich) and used as received. The plastic wares used for cell culture in this study were from Nunc, USA. Culture media ($\alpha$-MEM) was obtained from Invitrogen. All other tissue culture reagents and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) were purchased from Sigma. All other analytical grade chemicals and solvents purchased from local companies.

2.2 Hydrothermal growth of TiO$_2$ nanostructures as films

For the growth of nanostructures of TiO$_2$ as films titanium isopropoxide solution was prepared in HCl. Six different dilution ratio of HCl with water as (i) 20:80, (ii) 30:70, (iii) 40:60, (iv) 50:50, (v) 60:40 and (vi) 70:30 were prepared and used to control the hydrolysis rate of titanium isopropoxide. In the final step, 1ml of titanium isopropoxide was added to 40 ml of HCl solutions described above (of (i)-(vi)). Titanium isopropoxide mixed HCl solutions were placed in a teflon-lined stainless steel autoclave. The temperature of the autoclave was set at 180°C and the
growth of nanostructures achieved at 3 h on FTO substrates for the conditions (i) to (vi). After the reaction was completed, the autoclave was allowed to cool to room temperature and the TiO$_2$ nanostructures grown on FTO substrates at different conditions as described above were collected and then dried in vacuum at 70°C for 12h before use.

**Fig. 1** shows FE-SEM images of nanostructures of TiO$_2$ grown on FTO at various dilution conditions of HCl ((i)-(vi)). TiO$_2$ nanostructures obtained at conditions ((i)-(iii)) having higher water content in HCl showed round ball like 3D TiO$_2$ nanostructures (**Fig. 1 a,b&c**). A very significant change in the surface structure morphology was observed with an increase in dilution of HCl. FE-SEM images of TiO$_2$ nanostructures obtained at higher concentration of HCl (at condition vi) shows mesh like nanostructure. Mesh like nanostructures of TiO$_2$ were changed to 3D-nanoflower like structure for the use of equal volume to volume ratio of water and HCl. TiO$_2$ nanostructures obtained at equal volume to volume ratio of water and HCl (condition iv) showed uniform distribution of 3D-nanoflowers of TiO$_2$ on FTO. Hence, this optimum condition (iv) was used for further investigation of the effects of time on TiO$_2$ nanostructure growth as films on FTO. In this study, three different types of substrates (i) non-conducting glass (glass), (ii) ITO coated conducting glass (ITO) and (iii) FTO coated conducting glass (FTO) were used.

The TiO$_2$ films grown at these four different types of conditions as (a) 1 h on FTO, (b) 3 h on FTO, (c) 3 h on ITO and (d) 3 h on glass substrates were used for quantification of detailed crystallite structure by X-ray diffraction (XRD), surface chemical analysis by X-ray photoelectron spectroscopy (XPS) and cell cultures experiments to assess the biocompatibility.

**2.3 Cell Culture**

The MC3T3 and HS-5 cells were maintained in α-MEM with 10% Fetal Bovine Serum (FBS) and 100 U/mL penicillin and incubated at 37 °C in an atmosphere of 5% CO$_2$. Trypsin-EDTA
was used to detach the cells. Three samples were used for in-vitro cell adhesion, viability and proliferation assay and all the experiments were repeated for three times. For statistical analysis pair wise comparison of inter-experiment variation was done. MS Excel software was used for statistical analysis. Standard deviation values were calculated separately for all assays. The significance of the statistical analysis values was determined using Student’s t-test where the p values of < 0.05 were considered significant. The cell culture experiments were performed on TiO$_2$ 3D-nanoflower structures grown on FTO, ITO and glass substrates at the condition (iv) for 3 h. Additionally TiO$_2$ nanostructures grown at early stage (1h) on FTO substrate for condition (iv) was selected to perform cell culture experiments and tissue culture plastic surface were used as control condition.

2.4 Characterizations

The crystallite structure of TiO$_2$ 1D and 3D nanostructures grown on different substrates was characterized from XRD pattern obtained by using a XRD-6000 (Japan) X-ray diffractometer in the diffraction angle range 5–80° with Cu-K$\alpha$ radiation (\(\lambda = 1.54060 \text{ Å}\)). Transmission electron microscopy (TEM) and high-resolution transmission electron microscopy (HRTEM) were performed using JEM 2010F microscope. TEM, selected area electron diffraction (SAED) patterns and HRTEM images were obtained by TECNAI F20 Philips operated at 200 kV. The surface morphology of the samples was recorded by a field emission scanning electron microscope (FESEM; S-4700, Hitachi). XPS spectra were obtained on a MultiLab200 with standard MgK$\alpha$ radiation to quantify elemental composition and surface states of TiO$_2$ nanostructures. All spectra were taken at a working pressure of 10$^{-9}$ mbar. An XPS survey and high-resolution spectra of the C1s, O1s, and Ti2p were collected. The different surface states were obtained in the high resolution Ti2p, O1s and C1s spectra of the samples by specifying a
line shape, relative sensitivity factor (RSF), peak position, full width at half maxima (FWHM), and area constraints.

2.5 In-vitro Assays

Cell adhesion and viability assays: These assays were performed by plating $3 \times 10^4$ cells on nanostructures surfaces of $1 \text{ cm}^2$ placed in tissue culture grade plates. After 4 h, non-adherent cells were washed off and $100 \mu\text{L}$ of $2 \text{ mg/mL MTT}$ was added to the remaining cells of each condition followed incubation at $37 \degree\text{C}$ and $5\% \text{ CO}_2$ for 4 h. After removing culture medial, the reaction was stopped by adding dimethylsulfoxide and read at 595 nm in ELISA reader. In cell viability assay, after 24 h of incubation, MTT assay was performed as described above to calculate the percentage of cell viability.

Cell proliferation assay: The assay was performed by plating similar number of cells of all conditions in triplicates and incubated at three different time periods namely 24, 48 and 72 h. At each time periods cell number was calculated by MTT assay as described above.

3 RESULTS AND DISCUSSION

3.1 Surface Morphology and Crystallite Structure Characterization

Fig. 2 shows FE-SEM images of time dependent growth of TiO$_2$ nanostructure on FTO grown at 1 h and 3 h. The high resolution FE-SEM images of TiO$_2$ nanostructures grown at 1 h showed two distinct regions as 1D-nanorod and 3D-nanoflower nanostructures. The top view in FE-SEM image of the TiO$_2$ nanostructures surface morphologies grown at 3 h showed uniform coverage with 3D-nanoflowers. From these observations it appears that 1D-nanorods of TiO$_2$ grows on the substrates as films on which simultaneous growth of 3D-nanoflowers were initiated. Cross sections view of FE-SEM images of nanostructures obtained at 1h and 3h growth of TiO$_2$
demonstrates the formation of 3D-nanoflowers on vertically aligned 1D-nanorods of TiO$_2$. TiO$_2$ nanostructure surfaces obtained at 1 h of growth had both 1D-nanorods and 3D-nanoflowers whereas at 3 h of the growth the surfaces were completely covered by 3D-nanoflowers. The effect of different types of substrates on the growth of TiO$_2$ nanostructures as films was quantified by FE-SEM images of nanostructures grown on glass, ITO and FTO substrates at 3 h. A uniform distribution of 3D-nanoflowers of TiO$_2$ as films were seen for all three different types of substrates in FE-SEM images shown in Fig. 3 (a,b &c). Higher resolution FE-SEM images of 3D nano-flower structures shows about 300-500 nm tips of nano-rode at the surface which were very tightly packed as shown in Fig. 3 (d,e&f). Cross sectional FE-SEM image view showed formation of stable interface of 3D nano-flower like structures on these substrates.

TEM image of the tip of TiO$_2$ 3D-nanoflowers on FTO substrates was obtained at different magnification and results are shown in Fig. 4. Presence of densely packed TiO$_2$ as nanorods was seen in TEM images. Diffraction pattern of TiO$_2$ was obtained and lattice spacing (d) of TiO$_2$ was calculated. The results showed $d_{001} = 0.23$ nm along the longitudinal axis direction pertains to the d-spacing of rutile TiO$_2$ (001) crystal planes.

Fig. 5 (A) shows XRD pattern of TiO$_2$ nanostructures grown on FTO substrates at 1 h and 3 h. FTO substrate shows several strong peaks of Sn at (110), (002), (112), (202) and (113) planes in XRD spectra (Fig. 5 A(a)) as measured previously [39]. XRD pattern of FTO substrates and TiO$_2$ nano-structures as films grown on FTO at 1 h was similar. This may be due to smaller amount of TiO$_2$ growth, hence TiO$_2$ content was not detected by XRD in the presence of strong background of FTO substrate. TiO$_2$ nanostructures obtained at higher time points (at 3 h) showed TiO$_2$ XRD peaks appears at 2 theta values of 27.62, 36.28, 41.42, 44.16, 54.62, 56.72, 63.02, 69.28. These peaks were assigned (hkl) values (110), (101), (111), (210), (211), (220), (002) and
(301), respectively. All the diffraction peaks were marked to pure tetragonal rutile phase of TiO$_2$ (JCPDS no. 21–1276). XRD patterns of TiO$_2$ nanostructures grown on FTO, ITO and glass substrates are shown in Fig. 5 B. The XRD peaks appear at 2 theta values of 27.62, 36.28, 41.42, 44.16, 54.62, 56.72, 63.02, 69.28. XRD pattern of TiO$_2$ nano-structures as films grown on three substrates at 3 h seem to be identical. These peaks were assigned (hkl) values (110), (101), (111), (210), (211), (220), (002) and (301), respectively.

3.2 Characterization of Surface Chemical Functionalities

XPS spectra of TiO$_2$ nanostructure surfaces were obtained at 1h and 3h on FTO for quantification of surface elemental analysis. The % proportions of oxygen (O) to titanium (Ti) atoms were obtained from wide scan XPS spectra and the results are shown in Fig. 6A. The O/Ti value was 3.1 for the TiO$_2$ nanostructure surfaces obtained at 1h which was reduced to 2.7 for the nanostructure obtained at 3 h. The reduction in O/Ti values at higher time may correspond to decrease in % proportion of oxygen atom at the surface. This observation also indicates that titania atoms are tightly packed at the surface which is also confirmed by FE-SEM and TEM observations.

High resolution Ti2p and O1s XPS spectra were shown in Fig. 6 B & C. Details of peak fitting parameters for Ti2p and O1s are given in Fig. 6 D [16]. A small increase in Ti$^{4+}$ surface states of Ti2p was observed at the surface of TiO$_2$ obtained at 3h. The relative increase in the proportion of Ti2p surface states indicates relative decrease in number of oxygen atoms surrounding to Ti atoms in these nanostructure assemblies. Relative proportions of different oxidation states of oxygen in O1s XPS spectra at the surface of TiO$_2$ obtained after 1 h and 3 h and results are shown in Fig. 6 (C). Oxygen atoms as O= (O$^{2-}$) surface states were increased with increasing the
reaction time. These observations are consistent with the atomic % proportion obtained from wide scan XPS spectra.

XPS spectra of nanostructures grown as films on ITO, FTO and glass substrates were obtained to further quantify the chemical nature of nano-structures. The wide scan XPS spectra of TiO$_2$ on non-conducting (glass), conducting (ITO and FTO) substrates are shown in Fig. 7. Detailed elemental analysis and peak fitting for surface state quantification from C1s and Ti2p was done as described in our previous studies [27] and results are shown in Fig 8 & 9. The higher resolution C1s XPS spectra of TiO$_2$ film surface prepared on glass and ITO substrates was fitted with six peaks of different carbon environments as: hydrocarbon (C-H/C-C) at 284.6± 0.2 eV, (C-C(=O)OX) at 285± 0.1 eV, (C-OX) at 286.1± 0.2 eV, (C=O/O-C-O) at 287.6± 0.2eV, (C(=O)OX) at 289.2± 0.2 eV and C-Ti at 283± 0.2eV. TiO$_2$ film surface prepared on FTO coated glass substrates was fitted with five peaks of different carbon environments as: hydrocarbon (C-H/C-C) at 284.6± 0.2 eV, (C-C(=O)OX) at 285± 0.1 eV, (C-OX) at 286.1± 0.2 eV, (C=O/O-C-O) at 287.6± 0.2eV and (C(=O)OX) at 289.2± 0.2 eV. TiO$_2$ films on glass showed a spectra shift of 0.53 eV towards lower binding energy side which was adjusted to its original position. The bombardment of X-ray photons during XPS analysis causes a positive charge to accumulate at the non-conducting surface. This charge accumulation corresponds to 0.53 eV energy shift in the XPS spectra for the TiO$_2$ films grown on non-conducting substrates. There was no spectral shift observed for TiO$_2$ nano-structures grown on conducting (ITO and FTO) glass substrates.

Different titanium oxidation states were quantified in high resolution Ti2p XPS spectra of TiO$_2$ nanostructures grown on ITO, FTO and glass substrates. The spectra were fitted with four peaks as Ti$^{3+}$2p$_{3/2}$ at 453.2± 0.4 eV, Ti$^{4+}$2p$_{3/2}$ at 454.6± 0.2 eV, Ti$^{3+}$2p$_{1/2}$ at 455.8± 0.4 eV and Ti$^{4+}$2p$_{1/2}$ at 460.3± 0.2 eV. There was not much change in surface states of Ti2p obtained for TiO$_2$ nano-
structures grown on glass and FTO substrates. TiO$_2$ nanostructures grown on ITO substrates showed significant increase in Ti$^{3+}$ surface state of Ti2p.

A relative variation in the % proportion of the different surfaces states of C1s and Ti2p with different types of nano-structures film prepared on ITO, FTO and glass substrates are shown in Fig. 10. Mainly five different types of carbon surface states as C/C-H, C-C(=O)OX, C-OX, C=O and C(=O)OX were observed in TiO$_2$ films on all three substrates. Here we are interested to see the relative variation for three active functional groups C-OX, C=O and C(=O)OX present at the nano-surface obtained on ITO, FTO and glass substrates. The % proportion of hydroxyl functional group at the surface in TiO$_2$ nano-structures was significantly increased while the substrates were changed to glass, FTO and ITO, respectively for growth of TiO$_2$. There was no significant change in the % proportion of carbon atoms as C=O in C1s at the surface of TiO$_2$ nano-structures grown on different types of substrates. TiO$_2$ nano-structures grown on glass substrates showed highest level of carboxylic functional group. There was not much difference in % proportion of carboxylic functional of TiO$_2$ nanostructured surfaces on ITO and FTO substrates. An increase in the Ti$^{3+}$ surface states of Ti2p was obtained from TiO$_2$ nanostructures grown on ITO substrates. There was not much different in Ti$^{3+}$ surface states of Ti2p of TiO$_2$ nanostructures obtained on glass substrates and FTO coated conducting glass substrates.

### 3.3 Biocompatibility of TiO$_2$ nanostructures

Cell adhesion, viability and proliferation assays using MC3T3 osteoblast and HS-5 Cells were done on TiO$_2$ nanostructure surfaces obtained at 1h and 3h, respectively on FTO and the results are shown in Fig. 11. An increase in cell adhesion % on both types of nano-structures was obtained as compared to control condition (on tissue culture plastic surfaces). Combination of 1D-nanorode and 3D-nanoflower like structures of TiO$_2$ provide better entrapping of cells hence
highest % of cell adhesion was obtained. The cell viability did not show significant variation on these two different types of nano-structures. The cell proliferation was also a little less on these as compared to the control condition. The HS-5 cell adhesion % was similar on both types of nanostructures of TiO$_2$. The HS-5 cells viability was increased on TiO$_2$ nanostructure of 1D-nanorode/3D-nanoflower as compared to control condition. However, the cell proliferation was reduced on these two types of nano-structures.

MC3T3 cell adhesion, viability and proliferation assays were done on TiO$_2$ nanostructures film grown on FTO, ITO and glass substrates and results are shown in Fig. 12. An overall increase in % cell adhesion on TiO$_2$ nano-structures grown on all three different types of substrates was observed as compare to tissue culture plastic surface. The highest % cell adhesion was obtained on 3D nano-flower structures of TiO$_2$ grown on glass surface. There was a relative decrease in % of cell adhesion on TiO$_2$ 3D-nanoflowers grown on ITO and FTO, respectively as compared to the response observed on nano-structures on glass. A similar response was observed for % cell viability. Osteoblast cell proliferation on TiO$_2$ 3D flowers grown on ITO and FTO was less as compared to control conditions despite of having higher % cell adhesion. XPS analysis of TiO$_2$ films on non-conducting glass substrates showed a spectra shift towards lower binding energy side. This observation indicates that TiO$_2$ films on non-conducting glass surface can retain the charge whereas TiO$_2$ films grown on conducting (ITO and FTO) substrates do not show charge accumulation. TiO$_2$ 3D-nanoflowers grown on non-conducting glass substrate had showed higher cell proliferation rate as compared to the control conditions whereas decrease in cell proliferation was observed on TiO$_2$ 3D flowers grown on ITO and FTO. Hence, the charge nature of substrate play an important role in determining cellular respond despite of similar nanostructure of same material.
HS-5 cell adhesion, viability and proliferation assays were done on TiO$_2$ nanostructures film grown on FTO, ITO and glass substrates and results are shown in Fig. 13. The HS-5 cell adhesion assay showed less % cell adhesion on TiO$_2$ nano-structures grown on FTO substrates as compare to nanostructures grown on ITO and glass substrates. TiO$_2$ nanostructures on non-conducting glass substrates showed highest % cell adhesion and proliferation for HS-5 cells. Despite of similar nanostructure and crystallite structures of TiO$_2$ grown on conducting and non-conducting surfaces, the improved adhesion and proliferation response of both the cells on non-conducting surfaces confirms that the cells prefer non-conducting surfaces. This may enable these cells to create and sustain the local surface charge environment induced during cell-surface interactions for better cell growth on non-conducting surfaces which is not possible on the conducting surfaces.

4 CONCLUSIONS

Ordered growth of nanorod (1D) / nanoflower (3D) structures of TiO$_2$ was achieved on non-conducting and conducting (FTO and ITO coated) glass substrates. The method we have used was a surfactant free process for simultaneous growth of 1D/3D nanoroads and nanoflowers structure with better adhesion at the interface. TiO$_2$ nanostructures obtained at higher time points (at 3 h) on FTO, ITO and glass substrates are of rutile phase. The finding showed that mixed 1D-nanorode / 3D-nanoflower like structures showed better cell adhesion as compared to only 3D-nanoflower like structures. Cell viability and proliferation rate was higher on TiO$_2$ 3D-nanoflower grown on non-conducting surface as compare to conducting substrates.
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References


Figure Captions

Figure 1: FE-SEM images of rutile TiO$_2$ nanostructures grown on FTO substrates. (a), (b), (c), (d), (e) and (f) represents nano-structures grown at different dilution of HCl. HCl to water ratio (a) 20:80; (b) 30:70; (c) 40:60; (d) 50:50; (e) 60:40; (f) 70:30 at 180°C.

Figure 2: FE-SEM images of rutile TiO$_2$ nanostructures grown on FTO substrates at two different times (1 h and 3 h) for HCl to water ratio 50:50 at 180 °C.

Figure 3: FE-SEM images of rutile TiO$_2$ nanostructures grown on glass (a), FTO (b) and ITO (c) substrates at 50:50 dilution of HCl with water and reaction time at 3 h and 180 °C.

Figure 4: (A) FE-SEM image of TiO$_2$ nanostructures grown on FTO substrates at 50:50 volume to volume ratio of HCl with water and 180 °C for 3 h. TEM images (B & C) of the tip-view of TiO$_2$ nanostructures as seen in high resolution FE-SEM image (A). (D) Diffraction pattern of TiO$_2$ nanostructures grown at similar conditions.

Figure 5: (A) XRD pattern of TiO$_2$ crystallite growth on FTO at 50:50 volume to volume ratio of HCl with water and 180°C for two different times (1 h and 3 h). Prominent diffraction peaks in the XRD pattern of FTO substrate are marked with ‘*’. (B) XRD pattern of TiO$_2$ crystallite
growth on glass (a), FTO (b) and ITO (c) substrates at 50:50 volume to volume ratio of HCl with water and 180 °C for 3 h.

**Figure 6**: Wide scan XPS spectra of TiO₂ nanostructure growth on FTO substrates at 50:50 volume to volume ratio of HCl with water and 180°C for two different reaction times. of (a) 1 h and (b) 3h.

**Figure 7**: (A) high resolution peak fitted Ti2p XPS spectra and (C) high resolution peak fitted O1s XPS spectra of TiO₂ nanosurfaces prepared at 50:50 volume to volume ratio of HCl with water and 180°C for two different reaction times. (B) peak fitting parameter for Ti2p high resolution XPS spectra and (D) peak fitting parameter for O1s high resolution XPS spectra. (a) and (b) represents 1 h and 3h of TiO₂ nanostructure growth on FTO substrates, respectively.

**Figure 8**: Wide scan XPS spectra of TiO₂ nanosurfaces prepared at 50:50 volume to volume ratio of HCl with water and 180 °C for 3 h of reaction time on glass (a), FTO (b) and ITO (c) substrates.

**Figure 9**: High resolution C1s peak fitted XPS spectra of TiO₂ nanosurfaces prepared at 50:50 volume to volume ratio of HCl with water and 180 °C for 3 h of reaction time on glass (a), FTO (b) and ITO (c) substrates.
Figure 10: High resolution Ti2p XPS spectra of TiO$_2$ nanosurfaces prepared at 50:50 volume to volume ratio of HCl with water and 180 °C for 3 h of reaction time on glass (a), FTO (b) and ITO (c) substrates.

Figure 11: Relative variation in the % proportion of (A) COX in C1s, (B) CO in C1s, (C) C(=O)OX in C1s and (D) Ti$^{3+}$ in Ti2p XPS spectra of TiO$_2$ nanosurfaces prepared at 50:50 volume to volume ratio of HCl with water and 180 °C for 3 h of reaction time on glass (a), FTO (b) and ITO (c) substrates.

Figure 12: Cell adhesion assay done by using MTT on TiO$_2$ nanostructures grown at (a) 1 h and (b) 3 h on FTO substrates and (c) tissue culture plastic surfaces. (A) The percentage of osteoblast cell adhesion. The significance values between the groups were determined: ♦ represents p < 0.05 for conditions (a) and (b) with respect to condition (c); ◊ represents p < 0.05 for condition (a) with respect to condition (b). (B) The percentage of osteoblast cell viability. ♦ Represents p < 0.05 for condition (a) with respect to condition (c); ◊ represents p < 0.05 for condition (a) with respect to condition (b). (C) Osteoblast cell proliferation. ♦ Represents p < 0.05 for conditions (a), (b) and (c) at different times of cell proliferation. ◊ Represents p < 0.05 for condition (a) and conditions (b) with respect to conditions (c) at the same cell proliferation time points. (D) The percentage of HS-5 cell adhesion. ♦ Represents p < 0.05 for condition (a) and condition (b) with respect to condition (c); ◊ represents p < 0.05 for condition (a) with respect to condition (b). (E)
The percentage of HS-5 viability. ♦ Represents p < 0.05 for condition (a) with respect to condition (c); ◊ represents p < 0.05 for condition (a) with respect to condition (b). (F) HS-5 cell proliferation. ♦ Represents p < 0.05 for conditions (a), (b) and (c) at different cell proliferation times. ◊ Represents p < 0.05 for conditions (a) and (b) with respect to condition (c) at same cell proliferation time.

**Figure 13:** Osteoblast cell response were observed on (a) tissue culture plastic surfaces and 3D nanoflower like TiO$_2$ nanostructures grown on (b) FTO, (c) ITO and (d) glass substrates at 3 h. (A) adhesion %, the significance values between the groups were determined: ♦ represents p < 0.05 for conditions (b), (c) and (d) with respect to condition (a); ◊ represents p < 0.05 for condition (c) and (d) with respect to condition (b); ● represents p < 0.05 for condition (d) with respect to condition (c). (B) viability %, ♦ represents p < 0.05 for conditions (b), (c) and (d) with respect to condition (a); ◊ represents p < 0.05 for condition (c) and (d) with respect to condition (b); ● represents p < 0.05 for condition (d) with respect to condition (c). (C) Cell proliferation, ♦ Represents p < 0.05 for conditions (a), (b), (c) and (d) with respect to change in cell proliferation time. ◊ Represents p < 0.05 for conditions (b), (c) and (d) with respect to condition (a) at same cell proliferation time.

**Figure 14:** HS-5 cell response were observed on (a) tissue culture plastic surfaces and 3D nanoflower like TiO$_2$ nanostructures grown on (b) FTO, (c) ITO and (d) glass substrates at 3 h. (A) adhesion %, the significance values between the groups were determined: ♦ represents p <
0.05 for conditions (b), (c) and (d) with respect to condition (a); ◊ represents $p < 0.05$ for condition (c) and (d) with respect to condition (b); ● represents $p < 0.05$ for condition (d) with respect to condition (c). (B) Viability %, ♦ represents $p < 0.05$ for conditions (b), (c) and (d) with respect to condition (a); ◊ represents $p < 0.05$ for conditions (c) and (d) with respect to condition (b); ● represents $p < 0.05$ for condition (d) with respect to condition (c). (C) Cell proliferation, ♦ Represents $p < 0.05$ for conditions (a), (b), (c) and (d) with respect to change in cell proliferation time. ◊ Represents $p < 0.05$ for conditions (b), (c) and (d) with respect to condition (a) at same cell proliferation time.
Figure 1
Figure 2
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8

Table:

<table>
<thead>
<tr>
<th></th>
<th>%At Conc (a)</th>
<th>%At Conc (b)</th>
<th>%At Conc (c)</th>
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<tr>
<td>Si 2p</td>
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<tr>
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<td>Ti 2p</td>
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<tr>
<td>O 1s</td>
<td>36.8</td>
<td>50.9</td>
<td>60</td>
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Graph:

- Graph (a) shows peaks at O 1s and Ti 2p.
- Graph (b) shows a peak at C 1s.
- Graph (c) shows a peak at Si 2p.

CPS (a.u.) vs. Binding Energy (eV)
Figure 9
Figure 10

(a) Environment | Position / eV | FWHM / eV | Ti/ at %
--- | --- | --- | ---
Ti$^{4+}$ 2p$_{3/2}$ | 454.6 | 1.33 | 62.9
Ti$^{4+}$ 2p$_{1/2}$ | 480.3 | 2.1 | 31.4
Ti$^{2+}$ 2p$_{3/2}$ | 463.4 | 1.2 | 4.6
Ti$^{2+}$ 2p$_{1/2}$ | 465.8 | 1.1 | 0.9

(b) Environment | Position / eV | FWHM / eV | Ti/ at %
--- | --- | --- | ---
Ti$^{4+}$ 2p$_{3/2}$ | 454.6 | 1.4 | 62.4
Ti$^{4+}$ 2p$_{1/2}$ | 480.3 | 2.2 | 31.2
Ti$^{2+}$ 2p$_{3/2}$ | 463.4 | 1.2 | 4.3
Ti$^{2+}$ 2p$_{1/2}$ | 465.8 | 1.1 | 2.1

(c) Environment | Position / eV | FWHM / eV | Ti/ at %
--- | --- | --- | ---
Ti$^{4+}$ 2p$_{3/2}$ | 454.6 | 5.2 | 90.7
Ti$^{4+}$ 2p$_{1/2}$ | 480 | 3.05 | 90.3
Ti$^{2+}$ 2p$_{3/2}$ | 453.4 | 1.5 | 6
Ti$^{2+}$ 2p$_{1/2}$ | 465.8 | 1.4 | 3
Figure 11
Figure 12
Figure 13
Figure 14