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Original Article

Biologically Modified Titanium Substrates for Improved Surface Bioactivity

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Abstract

Post-surgery infections and not effective integration represent a serious issue in the Titanium (Ti) based implants function for a long term stability. To reduce such issue various surface functionalization method including surface coating has been explored. Here we successfully coated Ti substrates with Graphene Oxide (GO), Chitosan (Cs), and nanocomposite of GO and Cs (GO/Cs) via spin coated method to evaluate the osteogenic properties of each coatings. Uncoated Ti substrates were used as control. Scanning electron microscopy was used to investigate the coating morphology. Surface roughness measurements were achieved from atomic force microscopy. To measure surface wettability, contact angels method was performed. Ti substrates coated with Cs (Ti_{Cs}) and Cs/GO ($Ti_{Cs/GO}$) showed the highest surface wettability compared to Ti substrates coated with GO (Ti_{GO}) and the control. The highest surface roughness was also observed in $Ti_{Cs/GO}$. To test cellular attachment and proliferation the samples were exposed to human osteoblast-like MG63 cells after 2 hours, 4 hours, 6 hours, 1 day, 3 days, and 1 week. MTT [3-4,5 dimethylthiazol-2yl (2,5diphenyl-2H-tetrazoliumbromide)] assay was performed to measure the percentage of cellular attachment and proliferation for each coatings. Cell adhesion and cell proliferation was most improved in Ti_{Cs} followed by $Ti_{Cs/GO}$. Corrosion resistance of the coatings was investigated using potentiodynamic polarization test in simulated body fluid. The result indicated that the nanocomposite coating could provide effective protection of Ti substrates from corrosion.

Keywords: Titanium, Chitosan, Graphene Oxide, Nanocomposite, Surface Modification

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Introduction

As a very promising biomaterial for the fabrication of dental implants, Titanium (Ti) and its alloys have attracted increasing scientific interest for the decades. The success of Ti-based implants is particularly due to their desirable mechanical strength, corrosion resistance and biocompatibility [1, 2]. However the main problems associated with Ti-based dental implants are a poor and delayed osseointegration [3]and implant-related infections [4]. In fact, for a long-term stability this class of biomaterials still cannot satisfy all the criteria. Generally, it is well known that the response of a biomaterial to human tissue highly depends on its surface properties and biocompatibility [4]. In other words, the surface characteristics of biomaterial implants play a crucial role on the osseointegration process since the surface is the only part that is in contact with surrounding biological environment [3, 5]. Thus, to enhance surface properties and accelerate osseointegration of implants various surface modification approaches have been developed so far. These approaches can be classified into three major categories including the mechanical, chemical and physical methods. These methods are used either for morphological modifications such as increasing surface roughness, enhancing wettability, improving corrosion resistance and switching topography from the micro to the nanoscale, or for acquiring different coatings on the implant surface. To achieve a mixed synergic effect, a combination of morphological changes and coatings could be applied. The primary aim of surface coating is to achieve a better osseointegration as well as to reduce bacterial infection of Ti substrates. It is known that after bone-implant interface, the host tissues begin to create an intermediate layer of collagenous fibrous tissue in between the implant surface and the bone tissue as a biological response to a foreign body [6-8]. The formation of this soft fibrous tissue is one of the main reasons of failure of implant function [9]. Thus, surface coating main purpose is to avoid the soft tissue formation and increase the functional surface area of implants by creating a firmly integrated and interlocked transition between tissue and the implant surface [5, 9].

Up to now, various new materials have been employed to enhance surface properties of dental implants. The most common such materials are carbon and carbon-based materials, bisphosphonates, bone stimulating factors, bioactive ceramics and polymers, hydroxyapatite, and calcium phosphate[10]. Among these, carbon-based materials and bioactive polymers in particular have demonstrated promising application potentials for use in biomedical implant coatings area. As one of the most important bioactive polymers, chitosan is a chitin derivatives with unique characteristics such as biocompatibility, biodegradability, and antibacterial properties [11]. It has been reported that chitosan also can play a positive role in bone repair and regeneration by triggering osteoprogenitor cells differentiation. Bumgardner *et al*., [12], investigated the potential role of chitosan coating on morphological changes of Ti

substrates, and observed that surface wettability decreased compared to uncoated samples, but protein adsorption and cell attachment on coated Ti substrates increased. Pavitra R *et al*., [13], investigated the biocompatibility and antimicrobial effect of chitosan coated Ti substrates and concluded that the coating increases biocompatibility and reduces microbial invasion.

One of the most considered carbon-based materials which has been proposed as promising candidate for use in biomedical fields is graphene [3]. Graphene oxide (GO) and reduced graphene oxide (rGO) are two derivatives of graphene. GO is prepared by oxidation of graphite. By taking advantage of the presence of several functional groups in its structure, GO has the potential to combine with several materials. This ability offers great opportunity to make bio-composites with desirable properties for achieving a more synergic effect [3]. Besides, various excellent properties such as desirable biocompatibility, proper mechanical strength, and high conductivity have made GO a desirable candidate for use in dental implants coating application. Moreover, some studies also reported that GO could show antibacterial properties. Li *et al*., (2017) [3] fabricated reduced graphene oxide coatings on Ti6Al4V alloy substrates to evaluate the osteogenic properties of coated and uncoated substrates, and reported that the coating has a positive influence on biocompatibility and oseoinductive performance of Ti6Al4V alloy substrates. To accelerate bone regeneration Ho *et al*., [14] modified commercially pure Ti surfaces with nanoscale reduced graphene oxide coating, and according to the results, pre-osteoblast cells cultured on the coated samples showed higher cell viability and cell attachment than those on pure samples. Zhao [4] investigated the potential application of GO in bone repair, and observed that GO-coated samples show excellent biocompatibility with MC3T3-E1 cells, the differentiation of the cells was also enhanced

Although, these studies hold great promise for demonstrating the positive effects of surface coating on enhancing surface characteristics of Ti substrates, but in terms of long term replacement there is not a general solution to meet the requirements for an ideal implant surface. For example, although combination of polymer-coated or modified implant surfaces with therapeutic agents showed great potentials to improve implant-tissue integration and reduce foreign body infection [15, 16], but the main problem remains with their poor wear resistance and some degradation products such as lactic acid which has a negative influence on cell proliferation by generating acidic conditions [17]. Thus, there is an urgent need to improve both mechanical properties and coatings adhesion to the implant surfaces simultaneously [18]. So, development in surface modification techniques and proper surface coating materials candidates would require for more advances in this field.

In this study, first we coated Ti substrates with chitosan, GO, and the combination of chitosan and GO to achieve a more synergistic biological function using spin coating method. After surface characterization of coated substrates with atomic force microscopy (AFM) and contact-angle measurements, cell substrate interactions were investigated using human osteoblast-like MG63 cells through evaluation of cell attachment, proliferation, morphology, corrosion resistance, and antibacterial activity.

Materials and Methods

Materials

The titanium substrates (sheets of Ti–6Al–4V) were commercially available from McMaster-Carr Company (Los Angeles, CA, USA). Chitosan, $C_2H_4O_2$, H_2SO_4, HNO_3, K_2MnO_4, H_2O_2 and HCl were purchased from Merck. Dulbecco's modified eagle medium (DMEM) and tripsin were purchased from Gibco BRL, France. Graphene Oxide (GO), MTT [3-4,5-dimethylthiazol-2yl (2,5diphenyl-2H-tetrazoliumbromide], and fetal bovine serum (FBS), PBS and penicillin/streptomycin (PS) were purchased from Sigma-Aldrich (USA).

Synthesis of Graphene Oxide

The modified Hummer's method was used to prepare the GO sheets. In a typical experiment, natural graphite powder (1 g) was suspended in 120 ml of sulfuric acid (98%). The mixture was cooled in ice bath and under moderate stirring (200 RPM) $NaNO_3$ (500 mg) was added. $KMnO_4$ (6 g) was added over 60 min. The temperature of the mixture was then allowed to warm to 35°C with constant stirring. After 48 h the brownish green solution becomes too viscose to stir. 400 ml double distilled water (DDW) was slowly added to the reaction, keeping the temperature at 70°C for one hour. Finally, 30% H_2O_2 (10 ml) was added until the color of mixture changed to brilliant yellow. The mixture was rested for 2 days to allow precipitation of graphene oxide nanosheets. The supernatant was decanted away and the residuals was then rewashed again with 0.5 M aqueous HCl for 10 times to remove metal ions and then washed with DDW to remove the acid residue. To obtain nano-sized mono layer graphene oxide sheet, a probe-typed ultrasonic treatment (200W, 2H) was used to disperse GO suspension. The resultant brown solution was dried under freeze drying to produce a fine nanographene oxide powder.

Preparation of nanocomposite solution

Chitosan solution (1%) was produced by adding Cs powder to the acetic aside solution (1%). The resulting mixture was stirred at medium speed for 24 hours. Then, GO powder was mixed with distilled water (1% weight ratio) and placed on a steady magnet for 1 hour. The mixture was sonicated for 1 hour by a probing ultrasonic device to separate the GO nanosheets and to obtain a homogenous solution. The Cs and GO solutions was then mixed with an equal volume and sonicated for 1 hour. In all the above steps, the ultrasonic device was set at 40 Hz and 0.5 rpm.

Titanium sample preparation

Titanium (Ti) discs 13 mm in diameter and 2 mm in thickness, were mechanically polished with P1500 silicon carbide paper and Grinder/Polisher machine (MP-28) to achieve smooth surfaces before coating. Subsequently, after placing the Ti discs in an ultrasound bath for 1 hour, they were cleaned ultrasonically in ethanol, acetone, isopropanol and distilled water for 20 min separately to remove the surface contaminants. The cleaned Ti discs were then autoclaved at 121°C for 30 min and dried at 70°C in a

conventional oven for 12 hours. Spin coating method was performed to create Ti discs surface coating. The titanium discs were spin-coated with composite solution for 60s at 2500 rpm at the laboratory temperature by a spin coater device (2M.T.D.92). The samples were kept in the substrate holder of the spin coater and subjected to coating treatment 5 times in a row. Then, by placing the samples in a conventional oven at 50°C for 24 hours, the heating was applied to connect the coating to the discs surface. The samples were then washed twice with distilled water and BPS for further experiments. All samples were dried at room temperature then exposed to UV-light for 20 minutes to sterilize surfaces prior to MG-63 cell culturing experiments.

Surface characterization of Ti substrates

The coating morphology was observed by scanning electron microscope (SEM, AMRY 1-1910FE). The sessile-drop contact angle method (OCA 15 plus; Data physics) was used to determine surface wettability of the uncoated and coated Ti samples at room temperature. A small deionized water droplet (0.4 µl) was placed on the sample surface, then after placing the drop at 37°C, the angle between the droplet and the surface was measured during five seconds. Samples in triplicate were tested for each composite coating. Surface roughness of the samples was analyzed by atomic force microscope (AFM, Veeco Instruments Inc., Woodbury, NY, USA). AFM image provides quantitative three-dimensional topographical structure with the calculation of dimensional roughness parameters with sharp probing tip at room temperature. NanoScope Analysis software was used to process the images.

Cell culture

Human osteoblast-like MG63 cells were used for this study, the cells were cultured in Dulbeccos modied essential medium (DMEM) (Gibco BRL, France) containing 10% of fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin (PS) (Gibco). Cell suspension was plated in a cell culture dish and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air and the culture medium was refreshed every 2 day. Cultured cells were detached by trypsinization, suspended in new culture medium and used for the designed experiments.

I- Cell morphology on Ti substrates

The samples were placed with the coated side downwards into a 24-well plate and cells were seeded on to the titanium discs with a final density of 15,000 cells per well. After three days, media was removed and washed with PBS, then, all samples were fixed in 2.5% glutaraldehyde (w/v) for 24 h at 4°C. After three rinses with PBS, the samples were then dehydrated in a sequential series of ethanol solutions (30, 40, 50, 60 70, 80, 90, 100%) for 15 min each and air-dried at room temperature for 24h. The samples were then sputter-coated with gold. The surface of the specimens and the cell morphology on different samples was observed by scanning electron microscopy (SEM; EM3200).

II- Cell attachment and viability

An MTT test was used to determine the number of viable adherent cells and the rate of cell viability. MG63 cells were seeded (10000 cells for cell viability, 50000 cells per ml for adhesion tests) on of each titanium sample in 24-well tissue culture polystyrene plates. After each time point, the samples were transferred to new cell culture plates to analyze only those cells on the samples surface. Non-adherent cells were removed by washing the surface gently with PBS (pH 7.4). One ml of fresh medium and 0.5 mg/ml MTT was added to each well and then the plates were incubated for another 3 h to form formazan. After 24 h incubation the formazan crystals were dissolved in solubilizing solution and transferred to a 96-well plate. Absorbance of each solution was measured at a wavelength of 570 nm, with subtraction of the 650 nm background, using a UV–Vis spectrophotometer. The viable adherent cells and cell proliferation rate in samples surface was compared with the control and repeated three more times.

Electrochemical characterization

The corrosion resistance behavior of the samples was evaluated by potentiodynamic polarization studies using conventional three electrode cell (reference electrode: saturated calomel, counter electrode: graphite rod, working electrode: test material). The polarization experiments were carried out using a potentiostat (model PG State 30, Auto Lab the Netherland B.V) controlled by personal computer and the GPES Version 6.0 soft-ware. To achieve a steady circuit potential (OCP), all samples were immersed into simulated body fluid (SBF) and stabilized for 60 min. The potential was applied on the working electrode at a scan rate of 1 mV/s from cathodic to anodic direction. The corrosion parameters were investigated using Tafel extrapolation method. To achieve the test reproducibility, the experiments were carried out in triplicate.

Statistical analyses

Differences in contact angle, protein absorption and cell attachment between the Ti and chitosan coated-Ti surfaces were analyzed using ANOVA and were statistically significant $P< 0:05$.

Results and Discussion

Surface roughness and wettability

The hydrophilicity of coated and pure titanium samples was investigated by the contact angles measurement and the results are shown in Figure 1. The contact angle of Ti, $Ti_{CS,}$ Ti_{GO} ,$Ti_{CS/GO}$ are reported in Table 1. Previous studies have demonstrated that high hydrophilicity is desirable for cell adhesion to promote osteoconductivity [19, 20]. As it can be seen Ti_{CS} and $Ti_{CS/GO}$ showed the most hydrophilic surface properties. Particularly, the contact angles of the samples were significantly decreased on the Ti_{CS} samples. In Ti_{CS} sample the highest hydrophilicity was observed among the other samples and Ti being 109.4 ± 1.27 in control and 60.5 ± 0.33 in Ti_{CS} (Table 1). This result may refer to the presence of positive charge amino functional groups of Cs. The combination of these groups with GO nano-layers may provide a convincing reason to high level of hydrophilicity in composite sample rather than Ti_{GO} sample. The surface topography of different samples was evaluated via AFM and the images are shown in Figure 2. The values of Ra are also reported in Table 1. Universally, there is an agreement that surface roughening improves bone integration and stability of implants [20]. The roughness increment was found to be 33.58 ± 2.09,

14.1 ± 2.08, 4.82 ± 2.55 nm and 27.45 ± 1.56 nm for $Ti_{CS/GO}$, Ti_{CS}, Ti_{GO} and Ti, respectively (Table 1). It can be seen that the highest roughness was observed on the surface of $Ti_{CS/GO}$ sample. This result may relate to the folding and bending characteristics of GO multi-layer sheets on Cs polymeric chain which may cause surface crumbling. This finding is in agreement with our AFM and SEM results [21].

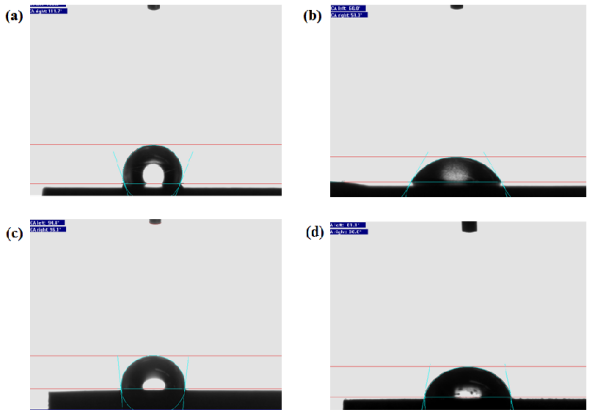

Figure 1. Water contact angle measurements of samples (a) Ti $_{GO}$, (b) Ti $_{Cs}$, (c), control (d) Ti $_{Cs/GO}$.

Table 1. Roughness and wettability values of different samples and control * P<0.05 compared to control, Ti $_{Cs/GO}$, Ti $_{Cs}$, and control, ** P<0.05 compared Ti $_{Cs}$, Ti $_{GO}$, and control.

Substrate	Contact angle (deg)	R_a (µm)
Ti_{Cs}	60.5 ± 0.33	14.1 ± 2.08
Ti_{GO}	109.4 ± 1.27*	4.82 ± 2.55
$Ti_{Cs/GO}$	82.65 ± 0.46	33.58 ± 2.09**
Control	95 ± 0.81	27.45 ± 1.56

Surface morphology of the coatings

The surface characteristics of each samples was examined using scanning electron microscopy (SEM). As shown in the Figure 3, Ti_{GO} consist of a multilayer sheets structure and the individual GO layer sheet stacked one above the other and exhibited a wrinkled structure in some parts. It is possible to recognize the edge of each layer from the image. Comparing with Ti_{CS}, the $Ti_{CS/GO}$ demonstrated a more coarse morphology. This may contribute to the presence of GO in the composite sample. The Figure 3 clearly shows the folding and bending behavior of GO sheets on $Ti_{CS/GO}$. The Ti_{CS} sample on the other hand showed a continuous structure with high uniformity. The presence of a large amount of oxygen-functional groups in GO structure enabled it to make physical and electrostatic interactions with Cs, resulting in a well dispersed GO sheets in the Cs matrix [11, 22].

Cell attachment and proliferation

MTT assay involves a reduction reaction which reduces MTT reagent to a blue formazan product when incubated with viable cells and the absorbance of formazan indirectly reflects the level of cell viability in culture. Figure 5 shows the number of viable MG63 cells that adhere to the sample surface. After 2 hours of culture, no significant difference is seen in between the cell adhesion rates all samples. However after 4 hours, the cell adhesion to the Ti_{CS} and $Ti_{CS/GO}$ samples were increased in comparison with the other samples. After 6 hours, the cell adhesion to the Ti_{CS} and $Ti_{CS/GO}$ surfaces was significantly higher than that of Ti_{GO} and Ti samples. At all time points, the cell adhesion in Cs was higher than all the other samples (P<0.05) (Fig. 4).

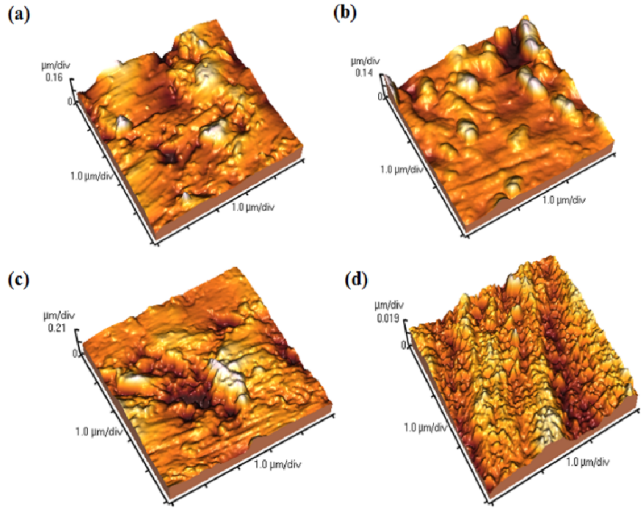

Figure 2. AFM images and root mean square roughness values (rms) of (a) Ti $_{GO}$, (b) Ti $_{Cs}$, (c) Ti $_{Cs/GO}$, (d) control.

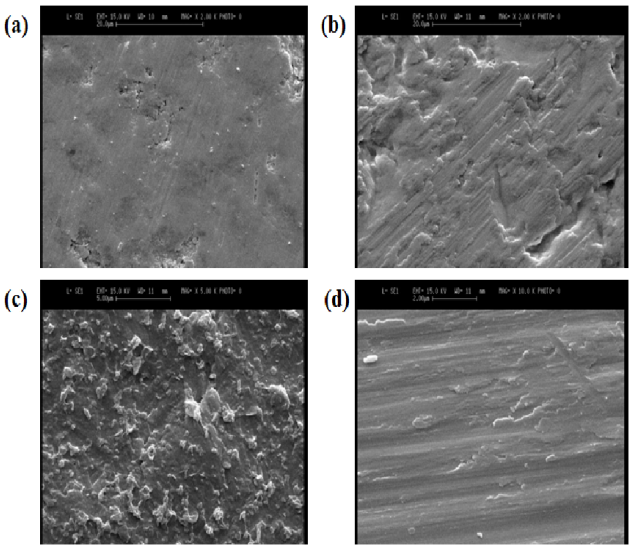

Figure. 3 Scanning electron microscope (SEM) images of each coatings and control. (a) Ti $_{Cs,}$ (b) Ti $_{GO}$, (c) Ti $_{Cs/GO,}$ and (d) control.

This is believed to be related to the attraction between the net positive charge of CS and the net negative charge of MG63 cells [23]. Moreover, previous studies have suggested that cell attachment could be further improved on the rougher surfaces.

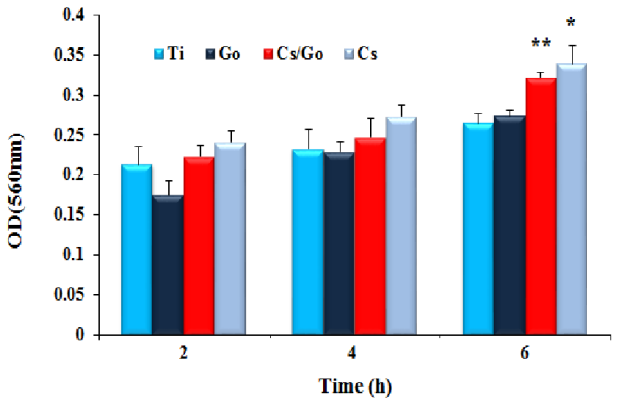

Figure. 4 MG63 osteoblast cells adhesion on titanium surfaces after 1, 2 and 4 h. Data is presented as mean ± SD; n = 3. * P<0.05 compared to Ti $_{Cs/GO}$, Ti $_{GO}$ and control, ** P<0.05 compared to Ti $_{Cs}$, Ti $_{GO}$ and control.

The reported increased adhesion to rougher surfaces has been explained with an increase of the surface area available for adhesion. As we mentioned above, the composite sample showed the highest surface roughness characteristics among the other samples. So, these findings may explain why the cells prefer to attach more on Ti_{CS} and $Ti_{CS/GO}$ samples. Cell proliferation of the samples was also evaluated via MTT assay, after direct cultivation of the cells on the samples for 1, 3 and 7 days. Cells grown on uncoated samples were used as control. The results are shown in Figure 5. After 1 day of culture, there was no significant difference between MTT values detected in the cells seeded on control and the coated samples. After 3 days, Ti_{CS} and $Ti_{CS/GO}$ samples had MTT values slightly higher than that of the Ti. After 7 days of culture, the Ti_{CS} and $Ti_{CS/GO}$ surfaces displayed significantly higher MTT values than the control (P<0.05).

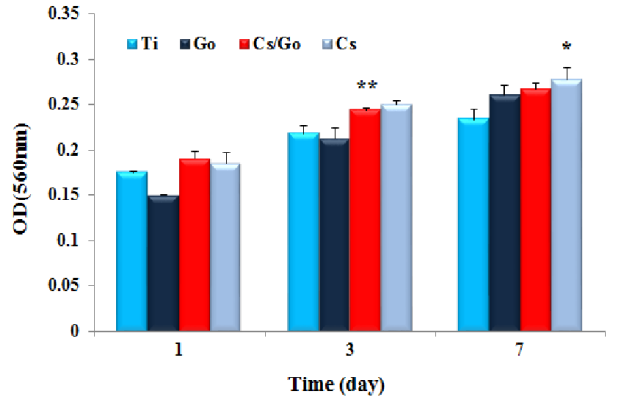

Figure 5. Proliferation of MG63 cells after 1, 3 and 7 days on titanium surfaces was measured with MTT assay. Each bar represents the mean of cell proliferation ± SD (n=3). * P<0.05 compared to Ti $_{Cs/GO}$, Ti $_{GO}$ and control, ** P<0.05 compared to Ti $_{Cs}$, Ti $_{GO}$, and control.

SEM observations (Fig. 6) confirmed the proliferation results. It is well known that cell attachment is highly depending on expression and adsorption of extracellular matrix proteins such as fibronectin, vinculin, and actin. Previous reports have corroborated that increased surface hydrophilicity can enhance protein adsorption. Our contact angle results confirmed that the Ti_{CS} and $Ti_{CS/GO}$ samples demonstrated the higher hydrophilic properties than the other sample. Moreover, due to similar structure of CS to glycosaminoglycan, an exracellular matrix molecule, CS has the potential to promote extracellular matrix proteins expression in human osteoblast cells [12, 22].

Cell morphology

Scanning electron microscopic (SEM) was used to study the morphology of MG63 cells after culturing for three days on different Ti surfaces (Fig. 6). The results are shown in Figure3. The observations demonstrate interaction between cells in $Ti_{CS/GO}$ and $Ti_{CS/GO}$. In comparison with control and Ti_{GO} samples, a large population of prominent filopodia and lamellipodia extensions were observed in Ti_{CS} and $Ti_{CS/GO}$ samples.

Our SEM images clearly showed that the MG63 cells completely covered the Ti_{CS} and $Ti_{CS/GO}$ samples. More cell contacts of the filopodias were also observed in these samples than that of Ti_{GO} and control samples. The better morphology on these samples can be ascribed to their surface roughness properties. Surface roughness, in fact, can modulate the local regulatory factors produced by osteoblast-like MG-63 cells at the molecular level. These findings is in agreement with previous studies which supported that surface roughness, and wettability can influence cell behavior and enhance cell adhesion, proliferation, and differentiation on titanium surfaces.

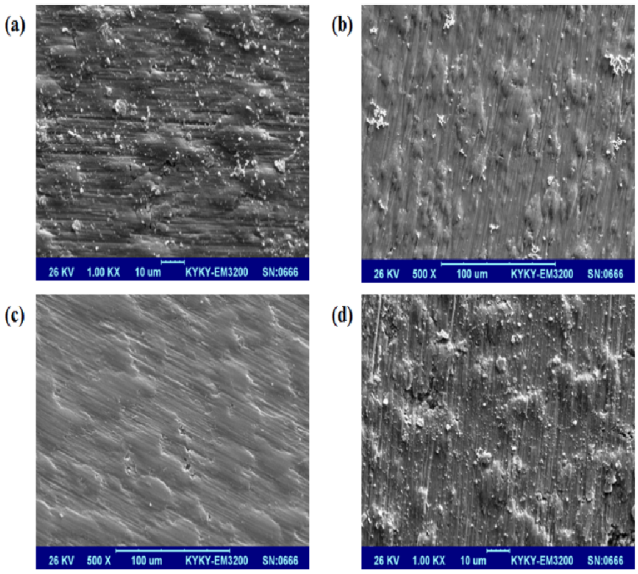

Figure. 6 Scanning electron microscope (SEM) images of MG63 cells cultured on titanium samples after 2 days of incubation, (a) Ti $_{GO}$, (b) Ti $_{Cs}$, (c) Ti $_{Cs/GO}$, (d) control.

Corrosion property of the samples

To evaluate the corrosion vulnerability of the samples under aggressive electrochemical environment, the polarization experiment was performed. Figure 7 illustrates the potentiodynamic polarization curves of each sample. The corrosion potential value (E_{corr}) of the samples was extracted from the polarization curves and the results are

listed in Table 2. As it can be seen the E_{corr} value of Ti_{GO} (-0.1 mv) and $Ti_{CS/GO}$ (-0.15 mv) samples were significantly higher than Ti_{CS} (-0.2 mv) and pure Ti (-0.23 mv) samples. No significant difference was also observed between E_{corr} value of Ti_{CS} and pure Ti samples. This notable corrosion resistance in Ti_{GO} and $Ti_{CS/GO}$ samples can be attributed to the closer stacking of GO multilayer sheets during spin coating and drying. This ability offers great potential for GO sheets to act as a barrier in prohibiting the electrolyte from reaching the metal surface [11].

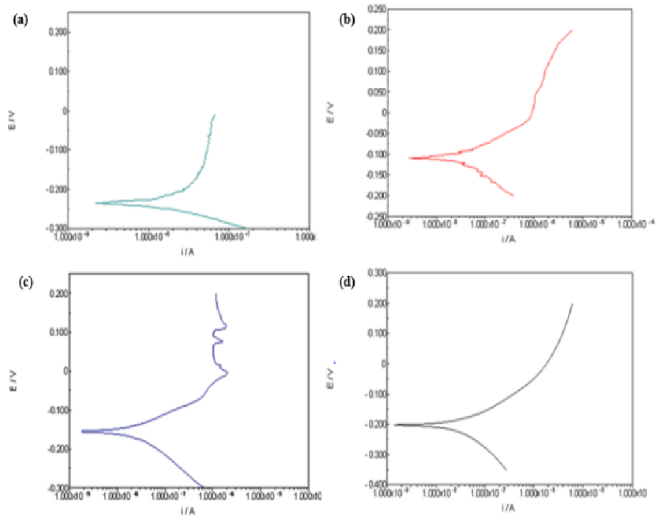

Figure 7. Potentiodynamic polarization plots for control sample and coated samples.

Table 2. Corrosion potential value of different samples and control * P<0.05 compared to Ti_{Cs} and control, Ti, ** P<0.05 compared Ti_{Cs} and control.

Substrate	Corrosion potential value (Ecorr)
Ti_{Cs}	-0.2 mv
Ti_{GO}	-0.1 mv*
$Ti_{Cs/GO}$	-0.15 mv**
Control	-0.23 mv

Conclusion

In this study, we successfully fabricated Cs, GO and Cs-GO nanocomposite coatings on Ti substrates through spin coating method to evaluate the osteogenic properties of each coatings and uncoated Ti substrates. Our results indicated that the addition of GO into Cs had a positive effect on the attachment and morphology of MG63 cells grown on Ti substrates. The resulting Cs/GO coatings also showed higher surface wettability compared to pure Ti substrates. More importantly, the nancomposite coating could reduce the bacterial growth to the surface and provide effective corrosion protection of the Ti substrates. Therefore, our study suggests that combination of Cs and GO may be a promising coating material to increase the osteogenic properties of Ti based implants.

Acknowledgments

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