Functionalized graphene oxide coating on Ti6Al4V alloy for improved biocompatibility and corrosion resistance

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A R T I C L E   I N F O

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Cell viability
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A B S T R A C T

The present study focused on the development of magnesium-functionalized graphene oxide (FGO) coating on titanium alloy (Ti6Al4V) by electrophoretic deposition. Graphene oxide (GO) was synthesized by modified Hummers’ method and functionalized with magnesium ions. X-ray diffraction, infrared spectroscopy (IR) and Raman spectroscopy were employed to confirm the synthesis of GO and GO-coatings on Ti6Al4V. Functionalization of GO with Mg ions was confirmed by energy dispersive X-ray spectroscopy. The surface morphology of coated samples was examined through scanning electron microscopy. Reduction of FGO coating (labelled as rFGO) by heating at 200 °C was confirmed by IR. The rFGO coated Ti6Al4V was found to be hydrophilic in nature as determined by contact angle measurement which showed reduction in the contact angle of Ti6Al4V from 95.4° to 42.1°. The percent cell viability over the coated sample was appreciably improved compared to as-received Ti6Al4V sample owing to hydrophilicity of the former. The positive shift in open circuit potential and increase in polarization resistance was observed after coating Ti6Al4V samples with FGO. The significant decrease in the corrosion current density and negative polarization loop in the reverse scan of samples also confirmed the improved corrosion resistance of rFGO-coated Ti6Al4V over uncoated Ti6Al4V in the PBS solution. Furthermore, the impedance spectroscopy revealed that the preferential adsorption of ionic species (indicated by large Rads) at the surface improved the barrier characteristics of rFGO coated samples and exhibited an order of magnitude higher Rs compared to as-received samples.

1. Introduction

Commercially pure titanium and their alloys are the favorite choices for application in hard tissue engineering, such as the orthopedic and dental implants. Titanium owes this prominence to its exceptional properties like biocompatibility, resistance to corrosion, and high mechanical strength [1–3]. An implant is considered successful if it offers effective and quick osseointegration when infused into the body. However, the incomplete osseointegration may result in osteolysis, inflammation and eventually the loosening of the implant [4,5]. The issue can be addressed by treating the surface of the implant. Several techniques and approaches have been reported to modify the implant surfaces such as roughening by machining or acid treatment [6], hydroxyapatite coatings [7], hydroxyapatite hybrid coatings [8,9], etc. The advantages of these bioactive surfaces are enhanced cell viability, osseointegration, and bone regeneration [2,10]. However, the detachment of hydroxyapatite from titanium surfaces [11] and decrease in the rate of osteoblast differentiation due to dissolution of phosphorous ions from hydroxyapatite [12] have been reported in the literature. These problems could lead to the failure of the implant in service.

Recently, graphene has been extensively studied for biomedical applications and has exhibited substantial potential as a bioactive material [13–17]. The aromatic scaffold nature of graphene and graphene oxide (GO) plays a vital role at the cell-biomaterial interface and helps in promoting the cell behavior, starting from attachment to proliferation and differentiation [18]. The non-covalent interaction of reduced graphene oxide (rGO) with proteins and growth factors can promote the
local concentration of extracellular matrix (ECM), providing the pre-concentration platform to cells [2,15]. Therefore, using graphene-based coatings to grow important cell lines could be a possible solution to eradicate the problems of other types of coatings, such as in the case of hydroxyapatite coatings. Several studies have been published so far but extensive research is still required to qualify graphene as an advanced biomaterial. Jung et al. [19] have studied the drug-loaded rGO, electrostatically coated, on caliber-rolled titanium alloys. The coated alloys demonstrated a significant cell growth compared to uncoated alloys. Furthermore, the hemocompatibility and biocompatibility of graphene coated titanium alloy have been reported by Podila et al. [20]. They reported that the limited charge transfer between graphene and fibrinogen could inhibit the platelet activation thus preventing the embolization phenomenon. The successful growth of neuronal cells and human osteoblasts on graphene [21] and negligible in-vitro toxicity exhibited by graphene have been reported as well [20]. A combinatorial approach of using functionalized nanomaterial coatings could improve both biocompatibility and corrosion resistance of the implant. The presence of functional nanomaterials could help in overcoming the issues of low cell-adhesion, and low cell proliferation on conventional biomaterial surfaces [22]. Marimuthu et al. [15] coated titanium plates with sodium (Na) functionalized GO via spin-coating and reported an improvement in the cell viability and corrosion rate of Ti plates. It is well established that the metal ions can electrostatically interact with the hydroxyl, carboxyl, and epoxy groups at the basal plane and edges of GO [23]. Just like Na, magnesium (Mg) is an essential nutrient of the body being an integral part of bones and teeth [24] and could be used to functionalize GO for biocompatible coatings.

The electrophoretic deposition (EPD) of graphene on metallic substrates from the colloidal suspensions of graphene oxide (GO) is considered as the suitable procedure. This is because the presence of the basal plane functional groups on GO [25], could help in the formation of uniform dispersion in polar solvents to make colloidal suspension and hence it can be deposited on conductive substrates [26,27]. For titanium, the cathodic-EPD route is preferred because titanium tends to anodize when used as anode [28]. In cathodic-EPD, Ti6Al4V is made cathode (negatively charged) and to deposit a negative species (GO), cations (Mg2+) can be introduced in the GO suspension. Cations can functionalize negatively charged GO and thus provide driving force that helped GO to migrate toward negatively charged Ti6Al4V. Moreover, such process facilitates the deposition of cation-functionalized graphene oxide (FGO) on the electrode of opposite polarity. Briefly, in this study, GO functionalized with magnesium (Mg) was deposited on Ti6Al4V alloy to explore the potential of coating for osseointegration. Modified Hummers’ method was adopted to prepare GO from natural graphite followed by functionalization with Mg2+ ions. FGO was electrophoretically deposited on the Ti6Al4V substrates at 50 V for 5 min followed by thermal reduction at 200 °C. Finally, the structural features, biocompatibility and electrochemical performance of the coated samples were examined and their wettability, cell adhesion, percent cell viability and corrosion resistant behavior were discussed.

2. Materials and methods

2.1. Synthesis and functionalization of GO

Graphite powder was oxidized according to modified Hummer’s method [25]. Briefly, 69 ml sulfuric acid (H2SO4) (96%), 3 g graphite powder and 1.5 g sodium nitrate (NaNO3) were added in a 3-neck flask. The temperature was maintained at 0 °C in an ice bath. Then 9 g of potassium permanganate (KMnO4) was added gradually to the mixture while keeping the temperature of the mixture below 20 °C. After KMnO4 addition, the mixture was heated to 35 °C and stirred for 7 h. Additional KMnO4 (9 g) was added, followed by further stirring for 12 h. The mixture was cooled to room temperature (−25 °C) by adding cold water and then 5 ml of 30% hydrogen peroxide (H2O2) was added. Finally, the suspension was filtered and washed successively with DI water, 30% hydrochloric acid (HCl) and ethanol (C2H5OH). The washed product (graphite oxide) was dried overnight at 80 °C in a vacuum oven and exfoliated in deionized (DI) water via ultrasonication to prepare GO suspension. GO powder was retrieved from the suspension after centrifugation at ~3500 rpm.

GO was functionalized with Mg2+ ions using the method as reported in [29,30]. For this purpose, GO and Mg(NO3)2·6H2O (1:1 by weight) were added to a mixture of DI water and isopropyl alcohol (1:9 by volume) and ultrasonicated for 2 h. The functionalized GO is designated as FGO in this manuscript and was used to deposit on the titanium substrates.

2.2. Preparation of Ti6Al4V samples and EPD of FGO

Ti6Al4V disk samples were cut from a 0.625 inch diameter rod. Samples were wet ground sequentially by using SiC abrasive papers of grit size from 180 to 1200. Finally, the samples were ultrasonically cleaned in acetone and DI water for ~30 s.

EPD of FGO on Ti6Al4V samples was carried out by using DC Power Supply (SGA1000X50C-0AAA). The working samples were connected to the negative terminal of the DC source. The platinum wire was used as a counter electrode. The electrodes were immersed in the suspension of FGO (2 mg/ml) prepared in isopropyl alcohol and DI water. The deposition time of 5 min was allowed to produce a uniform layer of GO coating at 50 V. These conditions for the coating process were optimized after a series of experiments. To achieve the uniformity and full surface coverage of rFGO on Ti6Al4V, the selected potential and time were considered in this study. The randomly selected SEM images obtained after coating at 50 V for 1 and 2.5 min, 30 V for 5 min and 40 V for 5 min are shown in the supplementary data, Fig. S1. However, the SEM image of coating obtained at 50 V and 5 min (Fig. 3b) was found to give full coverage of the substrate and hence these parameters were taken as optimized for deposition of FGO. After coatings, the samples were air dried for 2 h followed by heating at 200 °C for an hour. The heating resulted in the removal of some of the functional groups from the FGO, and hence it was called as rFGO [31]. The samples were sonicated in DI water for 1 min. In the following discussion, the uncoated and coated Ti6Al4V samples are termed as ‘As-received’ and ‘rFGO coated’, respectively.

2.3. Cell line for biocompatibility tests

MC3T3-E1 Subclone 4 (ATCC® CRL-2593™) pre-osteoblast cells used in this study were cultured in minimum essential medium alpha modification (MEM alpha) having 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin. The mixture will be referred to as the cell culture media in the following discussion. To get enough number of cells for the experiments, the cell culture was performed at 37 °C in a humidified atmosphere containing 5% CO2. Cells were allowed to grow in petri dishes for several days meanwhile the cell media was changed after every 48 h.

2.4. Physical and chemical characterization

X-ray diffraction (XRD) patterns (2θ = 5–80°) of graphite and GO powders were obtained by using X-ray diffractometer (Rigaku MiniFlexII Desktop). The Cu Kα radiation (λ = 0.154 nm) source at a scanning rate of 1°/min−1 was used to acquire diffraction patterns. Raman spectra (Kaiser Optical Systems Inc.) of as-synthesized GO and rFGO-coated samples were acquired by impinging laser beam (λ = 532 nm) excitations. Infra-Red (IR) spectra of GO powder and coated samples were obtained using FTIR-ATR spectrometer (Nicolet™ iS™ 50) in the Attenuated Total Reflection (ATR) mode to investigate the existence of functional groups. The as-synthesized GO was drop cast
on a glass slide before taking IR and Raman.

The morphology and chemical composition of as-received and coated samples were observed through a scanning electron microscope (SEM) coupled with energy dispersive spectroscopy (EDS) facility. To further investigate the nature of deposited FGO, the FTIR-ATR spectra of coated samples before and after heating were acquired. The contact angle was measured to estimate the surface wettability of the surfaces by using the sessile drop method on a contact angle goniometer (Attention Theta- DSC Q 2000) and D.I. water was used as a medium.

2.5. Electrochemical analyses

To evaluate the corrosion behavior of as-received and rFGO coated samples, open circuit potential (OCP), potentiodynamic cyclic polarization (PCP), linear polarization resistance (LPR) and electrochemical impedance spectroscopy (EIS) techniques were used. All electrochemical tests were performed in a three-electrode cell setup connected with the potentiostat (Reference 3000 Gamry Inc.). The saturated calomel (SCE) (+240 mV vs. SHE) was used as a reference electrode, graphite was used as a counter electrode, whereas, the samples under study were made the working electrodes in the cell. Phosphate buffer saline (PBS), having pH = 7.34, was used as an electrolyte and the constant temperature ~25 °C was maintained, following the previously published reports on different biomaterials [32–34]. The composition of PBS solution is given in Table 1. The samples were analyzed in triplicate to validate the reproducibility of the results. The OCP was initially stabilized for 2 h to achieve 0.01 mV/s stability before each electrochemical test. The cyclic polarization scans were initiated from −500 to 1500 mV vs. OCP at a scan rate of 1 mV/s. The LPR measurements were conducted within ± 20 mV potential range vs. OCP at 0.1 mV/s scan rate. The potentiostatic EIS measurements were carried out by applying 5 mV AC potential amplitude within 100 kHz–10 mHz frequency range at 0 V DC bias potential versus OCP.

2.6. Cytotoxicity assessment

The effect of ions release on the percent viability of MC3T3-E1 pre-osteoblast cells was assessed by using an MTS assay (G3580, Celltiter 96® Aqueous One Solution Reagent, Promega Corporation) [1,35,36]. As-received and rFGO coated samples were immersed in the cell culture media for 10 days duration. The first time the media was changed and collected after 24 h and after that every 48 h. The collected media was used to culture the cells and to determine the cell viability. Initially, 20,000 cells were cultured in MEM alpha mixed with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin medium at 37 °C in a humidified atmosphere containing 5% CO2. The cells were plated in a 96-well plate containing 200 μl of culture media per well. The cells were incubated for 24 h and the media was replaced with the media exposed to samples under investigation. Pure culture medium with cells was used as a control. The cells were further incubated for 24 h. After 24 h exposure, 100 μl of the media was removed from the wells. The remaining 100 μl media was treated with 20 μl of Celltiter 96® Aqueous one solution reagent. Finally, the 96-well plate was placed in the incubator for 4 h and after that the optical density was measured by using ELx800™ BioTek absorbance microplate reader with a 490-nm absorbance excitation filter.

<table>
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<tr>
<th>Table 1</th>
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<tr>
<td>Chemical composition of PBS solution (g/L).</td>
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<tr>
<td>NaCl</td>
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Fig. 1. (a) XRD pattern, (b) IR spectra, and (c) Raman spectra of graphite and graphene oxide.

2.7. Cell surface interaction

The effect of coating on pre-osteoblast cells interaction with Ti6Al4V alloy was investigated by exposing the cells to as-received and
rFGO coated substrates. MC3T3-E1 pre-osteoblast cells were grown in the cell culture media. 20,000 cells were allowed to grow on the as-received and rFGO coated samples. The samples were incubated for 24 and 48 h and stained using NucBlue® Live ReadyProbes™ Reagent and ActinGreen™ 488 ReadyProbes® Reagent. The adhesion and growth of the cells on the surface after 24 and 48 h was observed using EVOS® FL Cell Imaging System (AMF4300, Invitrogen Inc.).

3. Results and discussion

3.1. XRD

The diffraction patterns of precursor graphite and as-synthesized (GO) are presented in Fig. 1a. The characteristics peak at 26.5° corresponding to the (002) plane was observed for graphite. This peak was appreciably diminished in the case of GO and a peak at 10.8° emerged. This peak was associated with the (001) plane (d-value = 0.78 nm) suggested the successful oxidation of graphite to graphene oxide in the presence of oxidizing agents as cited in the literature [37,38].

3.2. IR spectroscopy

IR spectra of both graphite and GO are shown in Fig. 1b. The precursor graphite was insensitive to the IR waves and the transmittance peaks at 2325–1981 cm⁻¹ were characteristics of the diamond crystal used in the ATR mode [39,40]. On the other hand, the GO exhibited a broad peak at 3368 cm⁻¹ attributed to the O–H bond stretching vibrations which likely be corroborated to the C–OH and physically adsorbed moisture. The peaks at 1722, 1619 and 1051 cm⁻¹ appeared in the IR spectrum of GO powder were corresponded to the stretching of C=O, C–OH and C–O bonds, respectively [41,42].

IR spectra of as-received and rFGO coated samples, before and after heating at 200°C, are presented in Fig. 2a. It was observed that compared to the as-received sample, the coated sample presented the characteristic peaks of FGO, confirming the deposition of FGO. The broad peak at 1558 cm⁻¹ in the rFGO sample (solid line) could be attributed to C=C and C=O stretching. The presence of this peak and absence of a peak at 3368 cm⁻¹ suggested that FGO has been partially reduced into graphene, perhaps due to the post-heating of coated samples. Uthaisaw et al. [41] has reported a similar peak (~1558 cm⁻¹) in their work and was attributed to the migration of epoxide groups toward the edge regions and to the conversion of these into carbonyl groups.

3.3. Raman spectroscopy

Raman spectra of graphite, GO, and rFGO coated sample are presented in Figs. 1c and 2b, respectively. Graphite exhibited a G band peak at 1579 cm⁻¹ which were associated with the stretching vibrations of in-plane sp² bonded carbon atoms. In the case of GO, the G band vibrations originated at relatively higher wavenumber (1597 cm⁻¹) compared to graphite also confirmed the oxidation of graphite powder. Another band, designated as the D band, was observed at 1359 cm⁻¹ and this was related to the defects/irregularities in the hetero-atoms plane of attached carbon chains and/or to the formation of grain boundaries [42]. The origin of D band in the Raman spectrum of GO also suggested the oxidation of graphite.

The coated sample showed both G and D bands peaks in the Raman spectra (Fig. 2b). Compared to GO (1359 cm⁻¹), the D band peak position was shifted to slightly higher wavenumber number (1363 cm⁻¹) in the case of rFGO. The presence of D band in the coated sample suggested the non-crystalline nature of the graphite and confirmed that the presence of layered graphene sheets in the deposit over the surface of Ti6Al4V substrate. For rFGO coated sample, the slight shift in both D and G bands to higher wavenumber could either be associated with the destruction of sp² hybridized C=C network or to the presence of an oxygen-containing functional groups in its structure [27]. The excessive sonication and partial reduction of FGO to rFGO could also be the possible reason of variation in the peak position.

3.4. SEM-EDS

SEM images of as-received and rFGO coated Ti6Al4V alloy are shown in Fig. 3a and b, respectively. The SEM images depict significant topographical changes, after surface treatment with rFGO. The flattened morphology with grinding marks on the as-received surface was replaced by the flaky coating. The purity of the new coating and its chemical states were studied with the help of EDS. The presence of significant amount of ‘Mg’ in the rFGO deposit is exhibited in the Fig. 3d. The EDS also indicates the presence of aluminum (Al) and vanadium (V) contents, which is the composition of the alloy. These features of coatings, in SEM and EDS, suggest the deposition and effective functionalization of reduced GO. The cross-sectional SEM image of rFGO coated sample is presented in Fig. S2a. The coating thickness ranged between 6 and 8 µm over the surface. Moreover, the EDS line scan of the cross-section is presented in Fig. S2b, which shows the distribution of elements across the cross-section.

3.5. Wettability and cell adhesion

For any new surface, the wetting ability is measured to analyze its surface energetics and interaction of the liquid molecules with a solid surface. Wettability has a substantial importance in biomedical applications involving cell-biomaterials interactions as surface wettability significantly affects various biological events at sub level and sub-cellular level. The protein adsorption and adhesion phenomenon are the key factors in cell adhesion and proliferation, which could be influenced by wettability [43].
This is generally observed that hydrophilic surface enhances cell proliferation and adhesion. Similarly, in the case of mineralization of proteins on the surface of the implant, a hydrophilic surface is favorable [44]. The quantitative measurement of contact angle data is reported in Fig. 4. The contact angle of rFGO coated sample was almost one-half (42.1°) to that of the as-received Ti6Al4V sample (95.4°). These measurements suggested an increase in the hydrophilic character of the rFGO coated surface. This decrease in the contact angle can also be related to the increased surface energy of the coated sample owing to the presence of polar functional groups within the GO structure. Fig. 8 can give us a better explanation in this regard. The as-received surface of the alloy indicates hydrophobic behavior, therefore, less protein adsorption leads to lower mineralization (Fig. 8a & b). Hence the cell morphology is different in comparison with that of samples coated with rFGO. It is noticed that the cells make colonies throughout the surface and there is a lack of filopodia network which helps in communication and cell migration. However, the cells on the rFGO coated samples (Fig. 8c & d) have shown stellar morphology and have clear and spread cell structure.

3.6. Electrochemical analysis

The stability of biocompatible surface coatings and susceptibility to localized corrosion could decrease the overall service life of the implant or sometimes it may be life-threatening for the patients. Therefore, the ex-situ evaluation of the electrochemical response of the implant materials is very important. For this purpose, the electrochemical tests were conducted to evaluate the nature of rFGO coated sample in comparison with the as-received Ti6Al4V alloy in PBS solution. The OCP of both as-received and coated samples was recorded for 8 h in PBS to achieve 0.01 mV/s potential stability. The initial OCP for the as-received sample was −559.4 mV which increased to −391.2 mV in the initial 1 h (Fig. 5a). After 4 h, the OCP gradually shifted to the much positive value (−272.8 mV) and finally achieved stable value (−205.3 mV) in 8 h of exposure to the PBS solution. In the case of rFGO coated sample, the initial OCP was +17.21 mV, (Fig. 5a). Within 1 h of exposure, the OCP of the coated sample shifted to a positive potential (+29.66 mV). The OCP value for rFGO coated sample with a minor change finally attained a constant value +34.97 mV, after 8 h of exposure in the PBS solution. The OCP trends are shown in Fig. 5a indicated a positive shift in the surface potential which was most likely associated with the adsorption of ionic species within the graphitic layers of the coated sample. On the other hand, the positive shift in OCP of the as-received Ti6Al4V sample was related to the development of surface film upon extended exposure to the electrolyte.

To further confirm the electrochemical response of the coated sample in comparison with the as-received sample, the PCP scans were obtained. The PCP trends of the as-received and the coated samples are shown in Fig. 5b and the estimated electrochemical parameters are presented in Table 2. All the scans were obtained vs. OCP, stabilized for 2 h before each experiment. Qualitatively the E\textsubscript{corr} (mixed potential) of the coated sample shifted to relatively more positive potential (−205.3 mV) compared to the as-received sample (−349.5 mV vs. SCE) which was expected from the state of the surface and the nature of the interfacial redox reactions under applied conditions. For instance,
in PBS solution (pH = 7.34) reduction of water over as-received Ti6Al4V could occur at relatively negative potential. The formation of intermediate (Hads) species may specifically adsorb at the surface via following reactions (1) & (2) [45].

\[
\begin{align*}
2\text{H}_2\text{O} + 2e^- &\rightarrow \text{H}_{\text{ads}} + \text{H}_{\text{ads}} + 2\text{OH}^- \quad (1) \\
\text{Ti} + x\text{H}_{\text{ads}} &\rightarrow \text{TiH}_x \quad (2)
\end{align*}
\]

Compared to the OCP value, the negative \(E_{\text{corr}}\) value of coated sample was associated with the ingress of cationic species toward the surface during cathodic polarization followed by adsorption at the rFGO layers containing functional groups. It has been reported in the literature that the existence of C=O functional groups over graphitic materials could facilitate the adsorption of H\(^+\) ions during cyclic polarization [46,47].

The limited corrosion tendency of the as-received sample was estimated from the large \(\beta_a = 201 \text{ mV/dec}\) in the Tafel region. Independent to the potential, the large polarization in the anodic scan corresponded to the formation of a passive film at \(I_{\text{pass}}\) (27.89 \(\mu\text{A/cm}^2\)). On the other hand, the coated sample, within the Tafel region exhibited the similar behavior but the large polarization without representing the passivation limiting current tendency akin to the as-received sample at large overpotential. This behavior indicated the change in the surface nature of the coated sample due to the presence of rFGO over the Ti6Al4V. The presence of thick/dense nano-platelets having surface functional groups, i.e. C=O over the graphitic layered structure could preferentially limit the electrolyte approach at the interface. The lower corrosion rate (as given in Table 1) registered by the coated sample (0.0116 mpy) than the as-received sample (0.0516 mpy) suggested the barrier characteristics of rFGO coating over Ti6Al4V. Furthermore, the presence of Mg in the rFGO coated sample and the presence of anionic species (e.g. PO_4^{3-}) in the electrolyte under applied conditions could interact and could further enhance the polarization effects at the coating/electrolyte interface as evident in this case [48].

The negative anodic current density upon reverse scans (negative loop) was observed in both samples, which predicted the hindered localized attack of the ionic species [49]. The very limited corrosion tendency, observed in the case of rFGO coated sample, could be beneficial for the improvement of implant’s service life. But rigorous analyses are still required for the qualification of this coating to be applicable for bioimplants and is in progress (Table 2).

To further investigate the instantaneous electrochemical behavior of as-received and coated sample, the LPR method was used. The scans were obtained within ± 20 mV vs. OCP potential range to study the pure reversible electrochemical character of the as received and coated samples without influencing the intrinsic behavior of the surface i.e. the small potential range was selected to avoid any irreversible charge transfer processes. It was found that within cathodic polarization region (\(i < 0\)) initially, the current increased rapidly at constant potentials which proceeded by the potential dependent current response. The slope of these LPR scans (at \(i = 0\)) represented the polarization resistance (\(R_p\)) as shown in Fig. 6. The \(R_p\) of the as-received sample (0.914 MΩ cm\(^2\)) was significantly lower than the rFGO coated sample (4.6 MΩ cm\(^2\)). The potential independent increase in the current within the cathodic polarization regime and large polarization effect (larger \(R_p\)) presented by the coated sample was in support with the PCP results and can be attributed to their strong tendency for hydrogen adsorption and their barrier characteristics in the PBS solution.

The EIS analyses of both as-received and coated samples were also carried out to investigate the electrochemical integrity of the rFGO coating and to evaluate the nature of possible reactions at the sample/electrolyte interface. The small AC potential (5 mVrms) amplitude was applied to promote the progress of electrochemical reversible reactions at the interface and to avoid any permanent change. The Nyquist and
bode plots are shown in Fig. 7a and b, respectively. The spectra were simulated with the equivalent electrical circuit models (EEC) as presented in Fig. 7c. The quantitative information of each element (in EEC) was obtained by fitting the experimental spectra by using Gamry Echem Analyst software version 6.25. The quantitative information of these elements is given in Table 3. The single constant time Randle circuit corresponding to the parallel combination of double layer capacitance and charge transfer resistance is used to fit the impedance spectrum of as-received sample. The rough surface of the rFGO coated sample and presence of surface functional groups over the rFGO could lead to non-uniform charge distribution in the electrical double layer. This charge relaxation corresponded to the non-faradaic capacitive response of the double layer at the coating/electrolyte interface. This is represented as constant phase element (Φdl) in the EEC. In addition, the charge transfer processes (faradaic response) in the EEC would proceed either via specific adsorption/desorption of the ionic species on the rFGO and/or due to the dissolution of the substrate. In case of rFGO coated sample, the overall charge transfer resistance (Rct) was further dissociated into the faradaic capacitive (Φdl) and resistive (Rads) response, attributing to the adsorption and desorption processes, respectively. The electrolyte (PBS) resistance (Rads) was almost similar at the interface of both samples (29–36 Ω·cm²) as determined from the high frequency regime of the impedance spectra. The relaxation coefficient (nads) of both the samples was < 1.0 which represented the non–ideal double layer capacitance conforming to the non–uniform surficial charge distribution. An order of magnitude higher Rads of rFGO coated sample (1.93 × 10⁶ Ω·cm²) compared to as-received sample (6.17 × 10⁴ Ω·cm²) validated the barrier characteristics of the surface coating. However, the relatively smaller nads of rFGO coated sample compared to as-received sample indicated the large surface roughness affiliated with the surface morphology of the rFGO surface layer. The significantly high Rads of the rFGO coated sample (2.44 × 10⁶ Ω·cm²) also suggested the specific adsorption of ionic species at the surface functional groups present on the rFGO. In other words, the rFGO surface coating on the Ti6A4V would have preferential affinity toward ionic species which may specifically adsorb at the active sites (surface functionalities) available on the rFGO. This behavior also supported the PCP and LPR results, validating the improvement in the barrier characteristics of the rFGO coated sample possibly due to the preferential adsorption of ionic species. Almost similar value of Φdl to the Rads (1.34 × 10⁻⁵ S·s⁻¹·cm⁻²) for rFGO coated sample also suggested the preferential adsorption of ionic species at the surface which could appreciably limit the dissolution tendency of the substrate.

### Table 3

<table>
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<th>Electrochemical parameter</th>
<th>As-received</th>
<th>rFGO coated</th>
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<td>Rads (Ω·cm²)</td>
<td>29.74</td>
<td>6.17 × 10⁵</td>
</tr>
<tr>
<td>Φdl (S·s⁻¹·cm⁻²)</td>
<td>2.72 × 10⁻⁵</td>
<td>1.93 × 10⁵</td>
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<tr>
<td>nads</td>
<td>0.92</td>
<td>0.99</td>
</tr>
<tr>
<td>Rads (Ω·cm²)</td>
<td>33.47 × 10⁶</td>
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</tr>
<tr>
<td>Rads (Ω·cm²)</td>
<td>36.83</td>
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3.7. Cell-surface interaction and biocompatibility

The human physiological environment is a complex mixture of organic species, which has corrosive effects on the metallic implants. The corrosion of the implant results in the release of metal ions and causes metallosis or bioaccumulation within the adjacent tissues. Depending on the nature of ions, they may disrupt the cells or cause adverse reactions in the form of inflammations by introducing apoptosis and necrosis. These effects ultimately result in the implant loosening [1]. Therefore, the biocompatible and suitable coating is important for both long and short-term implantable materials. Fig. 9 exhibits the percent cell viability of MC3T3 pre-osteoblast cells in applied dosage of ionized media from the as-received and rFGO coated surfaces. The one-way ANOVA test was performed on this data with the significance levels of 0.05 and 0.01. The means, reported in this study, were not found to be significantly different. However, the bar plots indicate the reduction in cell viability after the first day and proceed to the second day in the as-received sample while the trend is different for the coated surface. This behavior may be attributed to the severe ions leaching from the surface due to loosely bonded atoms and oxide layer defects. This behavior also satisfies the OCP data as the starting potential of the as-received surface (−559.4 mV) is far lower than rFGO coated sample (−272.8 mV). For the rFGO-coated sample, the OCP shows stable behavior throughout the test, which is an indication of less reactivity, and no or less release of ions in the electrolyte. Similarly, it is obvious from the PCP results that the corrosion potential is improved, and lower corrosion current density is achieved after rFGO is coated. Thus, the surface treatment is an indication of the less surface dissolution and provided a safe podium for osteoblast to proliferate. Therefore, the cell viability seems to get better and stabilized for 4, 6, 8 and 10 days in case of rFGO-coated while the cell viability for the as-received surface is lower in comparison.

To study the cell and surface interaction and the influence of leached metal ions on the cell morphology, adhesion, and biocompatibility, the MC3T3-E1 cells were proliferated for 24 and 48 h. The stained cell images are shown in Fig. 8. It is generally observed that in order to maintain the structural and functional homeostasis and ionic reactivity, the cell signaling system can alter the equilibrium in the form of protein adsorption which results in a change in cell morphology [50]. Similarly, the wettability of the surface can influence the cell morphology and its adhesion. The hydrophilic surface helps in the mineralization of proteins on the surface of the implant [44]. These are some of the parameters which can affect the cell adhesion and osseointegration. From Fig. 8, the cell morphology on the as-received surface has clumps which might be attributed to the hydrophobicity of the surface. However, the proliferation seems less after 48 h of...
incubation, respectively. However, the average number of cells on rFGO coated sample were 7.2 and after 24 h and 48 h incubation, respectively. The proliferation of cells on these samples was directly related with the surface wettability and morphological features of the cells as discussed above.

4. Conclusion

Graphene oxide, synthesized by modified Hummer’s method, was successfully functionalized by doping Mg^{2+} ions and deposited on Ti6Al4V alloys via EPD method. Thermal reduction of FGO coated samples at ~200 °C was achieved as evaluated from IR spectra. The presence of rFGO coating provided a hydrophilic surface for better cell adhesion and proliferation. An improvement in the electrochemical properties and cell viability was observed for rFGO coated Ti6Al4V. The corrosion rate of the as-received Ti6Al4V (0.0516 mpy) significantly decreased after rFGO deposition (0.0116 mpy). Moreover, significantly higher values of R_p for rFGO coated sample (4.6 MΩ·cm²), calculated from the LPR scans were in support of the PCP results. From the impedance spectroscopy, the rFGO coated sample exhibited an order of magnitude higher R_p compared to as received samples. Furthermore, the preferential adsorption of ionic species at the surface functionalities (limited faradaic response indicated by the large R_eq) improved the barrier characteristics of the rFGO coated sample. Although the rFGO represented better cell adhesion and proliferation of MC3T3 E1 pre-osteoblast cells, further research work is still required.

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Appendix A. Supplementary data

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References


