Original Article

Responses of rat bone marrow mesenchymal stem cells to graphene oxide films with different alkali treatment

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\begin{abstract}
Graphene oxide (GO) has been widely investigated in biomedical fields due to its excellent biocompatibility. In the present study, GO films were fabricated on titanium surface by cathode electrophoretic deposition with the help of Mg\textsuperscript{2+} and Al\textsuperscript{3+}. Responses of rat bone marrow mesenchymal stem cells to GO films with different alkali treatment, including urea hydrothermal treatment and sodium hydroxide hydrothermal treatment, were investigated. The results indicated that GO films with urea hydrothermal treatment could promote cell proliferation and improve alkaline phosphatase activity. On the contrary, GO films with sodium hydroxide hydrothermal treatment inhibited cell proliferation.
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\end{abstract}

1. Introduction

Titanium has been widely used as dental and orthopedic implants due to their superior biocompatibility and mechanical properties [1]. Nevertheless, implant failure still occurs because of poor osseointegration and lack of antibacterial activity [2]. Graphene oxide (GO) has been widely used in biomedical fields due to its unique physicochemical and biological properties, such as excellent biocompatibility [3], large specific surface areas [4], and antibacterial properties [5]. GO could promote cell adhesion and proliferation, induce osteogenic differentiation of rat bone marrow mesenchymal stem cells (rBMSCs) and exhibit superior antibacterial activity [6]. Based on this, GO was used for surface modification of titanium and its alloys to improve their biological properties.

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In this work, GO films were constructed on the titanium surface by electrophoretic deposition (EPD) with the help of Mg$^{2+}$ and Al$^{3+}$. Subsequently, two kinds of alkali treatment including urea hydrothermal treatment and sodium hydroxide hydrothermal treatment were performed. Responses of rBMSCs to GO films with different alkali treatment were investigated.

2. Materials and methods

Titanium ($10 \times 10 \times 1 \text{ mm}^2$) were cleaned by mixed acid solution (HF: HNO$_3$:H$_2$O = 1: 5: 4) and denoted as Ti. The electrolyte solution was prepared by mixing 0.5 mL of GO aqueous solution (6 mg/mL, purchased from Hangzhou Gaoxin Technology Co., Ltd.), 37.5 mg of Al(NO$_3$)$_3$·9H$_2$O (Sinopharm Chemical Reagent Co., Ltd.). $76.9 \text{ mg of Mg(NO}_3)_2$·6H$_2$O (Sinopharm Chemical Reagent Co., Ltd.) and 250 mL of ethanol. GO absorbed with metal ions could be deposited on Ti with EPD under a constant voltage of 40 V for 1 min, and the corresponding samples were denoted as GO-Ti. U-GO-Ti was obtained after hydrothermal treatment of GO-Ti with urea aqueous solution (6.5 mg/mL) for 10 h at 120°C in a sealed Teflon vessel. Alkaline thermal treatment of GO-Ti with 1 M NaOH and 6 mmol Na$_2$CO$_3$ was also performed under the same condition, and the corresponding samples were denoted as S-GO-Ti.

All the samples were examined by field emission scanning electron microscopy (FE-SEM, S-4800, HITACHI, JAPAN) with an accelerating voltage of 2.0 kV, X-ray photoelectron spectroscopy (XPS, PHI 5802, Physical Electronics Inc., USA) with an Mg K$_\alpha$ (1253.6 eV) source, X-ray diffraction (XRD, D8 Advance, Bruker, Germany), and Raman microscope system (LabRAM, Horiba Jobin Yvon, France).

The rBMSCs (Stem Cell Bank, Chinese Academy of Sciences, Shanghai, China) were cultured on various sample surfaces with the cell density of $5 \times 10^4$ cells/mL. After culturing for 4 days, cells were stained with live/dead cell staining kit (Thermo Fisher Scientific Inc., USA) and observed by the fluorescence microscope (Olympus, Japan). Cell proliferation was assessed by the alamarblue™ assay kit (Thermo Fisher Scientific Inc., USA) after culturing for 1, 4 and 7 days, and cell morphologies were examined with SEM. rBMSCs with a cell density of $5 \times 10^5$ cells/mL were cultured on various samples for 14 days to analyze the alkaline phosphatase (ALP) activity. Then cells were stained by an alkaline phosphatase kit (Beyotime, China) and observed by fluorescence microscope.

3. Results and discussion

Surface morphologies of various samples are presented in Fig. 1a. Ti had a rough surface with a ravined topography after acid etching. GO with wrinkles were observed on GO-Ti. Compared to GO-Ti, fluctuation of wrinkles became bigger and granular surface was observed on U-GO-Ti. Surface morphology of S-GO-Ti was quite different which flake-like structures appeared. XPS results are showed in Fig. 1b. Ti, O, and C elements could be detected from Ti sample. Mg and Al elements were observed while the Ti element disappeared on GO-Ti, which suggested that GO absorbed with metal ions was successfully deposited on titanium surface. N element was detected from U-GO-Ti. After sodium hydroxide treatment, the characteristic peaks of Ti element appeared on S-GO-Ti. Fig. 1c showed that the nitrogen fitting peaks had three peak positions at 398.2 eV, 399.5 eV, and 401.5 eV, corresponding to pyridinic-N, pyrrolic-N and quaternary-N, respectively [7]. The C 1s XPS spectra on U-GO-Ti could be fitted into four peaks including 284.8 eV, 286.4 eV, 287.5 eV, and 289.1 eV (Fig. 1d), which corresponded to carbon skeletons, epoxy/hydroxyl, carbon-nitrogen bonds, and carbonyl, respectively [8]. The results indicated that nitrogen element was indeed doped into GO on U-GO-Ti sample [9].

Contact angle values of Ti, GO-Ti, U-GO-Ti and S-GO-Ti were 74.67°, 55.38°, 70.01° and 0°, respectively, as shown in Fig. 2a. GO-Ti was more hydrophilic than Ti, due to the oxygen-containing functional groups on GO. The contact angle of U-GO-Ti was between Ti and GO-Ti, which may be attributed to the oxygen-containing functional groups were partly replaced by nitrogen atoms. S-GO-Ti has excellent hydrophilic properties due to the layer structure of LDHs.

Raman spectra of various samples are presented in Fig. 2b. Peaks of GO-Ti, U-GO-Ti and S-GO-Ti located at ~1350 cm$^{-1}$ and ~1580 cm$^{-1}$ are corresponding to the characteristic peaks of D and G bands of GO, respectively. It indicated that GO was successfully deposited on Ti. After thermal treatment with two different alkalies, GO still existed on U-GO-Ti and S-GO-Ti. XRD patterns of various samples are presented in Fig. 2c. Ti, GO-Ti and U-GO-Ti and S-GO-Ti presented the typical features of α-Ti. However, feature peak of Mg-Al layered double hydroxide at approximately 11.7° [10] was detected on S-GO-Ti, which suggested that Mg and Al ions existed in the form of Mg-Al LDH. Results of Tafel curves are presented in Fig. 2d. Corrosion potentials of Ti, GO-Ti, U-GO-Ti and S-GO-Ti were $-0.6157$ V, $-0.8835$ V, $-0.2147$ V, and $-0.4843$ V, respectively. U-GO-Ti exhibited the most positive corrosion potential, followed by S-GO-Ti, Ti and GO-Ti. It indicated that U-GO-Ti and S-GO-Ti could improve the corrosion resistance of the titanium surface.

Cell proliferation of various samples was investigated with SEM and alamarblue assay, and the results are presented in Fig. 3(a and b). After culturing for 1 day, cells on Ti, GO-Ti and U-GO-Ti spread well, while cells on S-GO-Ti were in bad state. As time went on, cell number on Ti, GO-Ti and U-GO-Ti increased while cells on S-GO-Ti were almost invisible. Cell proliferation rate acquired from alamarblue assay further confirmed the results that Ti, GO-Ti and U-GO-Ti exhibited good cytocompatibility. Among them, U-GO-Ti showed the highest cell proliferation rate, followed by GO-Ti and Ti. However, cell proliferation rate on S-GO-Ti was very low.

The live/dead cell staining kit results are shown in Fig. 3c. Live cells were stained with calcine-AM and generated green fluorescence, while dead cells were stained with PI and emitted red fluorescence. Lots of green fluorescence could be seen from Ti, GO-Ti, U-GO-Ti while the amount of red fluorescence on them was negligible. On the contrary, a small quantity of green fluorescence could be seen from S-GO-Ti. It indicated that Ti, GO-Ti, U-GO-Ti had no cell toxicity and rBMSCs could grow well on them, while S-GO-Ti was not suitable for cell growth. Fig. 3d displayed the ALP-positive areas of rBMSCs. A lot of ALP-positive areas could be found on GO-Ti and U-GO-Ti, especially for U-GO-Ti, while ALP-positive area on S-GO-Ti are rarely unseen.
As mentioned above, N-doping was achieved on U-GO-Ti after urea hydrothermal treatment, which endow the surface with more active sites [11]. Therefore, U-GO-Ti presented higher cell proliferation rate and more ALP-positive areas. For S-GO-Ti, flake-like structure appeared after sodium hydroxide hydrothermal treatment, which was not suitable for cell growth, not to mention improvement of ALP activity.
4. Conclusions

In summary, GO films were constructed on titanium surface by electrophoretic deposition. Cell responses of rBMSCs to GO films with different alkali treatment including urea alkali thermal treatment and sodium hydroxide alkali thermal treatment were investigated. Nitrogen-doped GO films were obtained after urea alkali thermal treatment while Mg-Al LDH/GO films were acquired after the sodium hydroxide alkali thermal treatment. GO films with urea alkali thermal treatment exhibited good biocompatibility, promoted cell proliferation and improved ALP activity. However, GO films with sodium hydroxide alkali thermal treatment showed cell toxicity and was not suitable for cell growth.

Conflicts of interest

The authors declare no conflict of interest.

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