Environmental Toxicology and Pharmacology 57 (2018) 41–45

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

Research paper

Fabrication of capsaicin functionalized reduced graphene oxide and its effect on proliferation and differentiation of osteoblasts

Liansuo Zhai*, Lin Li, Qi Zhang

Department of Orthopedics, Jiyang County People's Hospital, Jinan, Shandong, 251400, China

ARTICLE INFO

Keywords:
Capsaicin
Stabilization
Rgo
Osteoblasts

ABSTRACT

Herein, we report a new simple and biological approach for the preparation of capsaicin adsorbed reduced graphene oxide (RGO), where capsaicin acts as a stabilizing and deoxygenating agent. The capsaicin that is decorated on graphene surface plays an important role as a capping agent to avoid the aggregation of graphene sheets. The capsaicin functionalized RGO stimulated the differentiation and proliferation of osteoblasts to a larger extent, which is a significant feature for the use of biomaterials in biomedical application such as in bone tissue engineering, more speciafically in the case of diseases such as osteoporosis.

1. Introduction

Reduced graphene oxide (RGO) is a new 2D carbon nanomaterial that has fascinated scientific attention because of its exceptional electronic, physical and chemical features (Akhavan et al., 2012). Hence RGO and its hybrid materials have been used in various technical and scientific fields, such as in field-effect transistors, solar cells, sensors, polymer composites and in pharmaceutics (Geim and Novoselov, 2007; He et al., 2010; Wang et al., 2011; Stankovich et al., 2006; Yin et al., 2010a,b; Dreyer et al., 2010).

Several reports have been found in literature for the fabrication of RGO using micromechanical cleavage, chemical vapor deposition (Eizenberg and Blakely, 1979) and deoxygenation of GO by chemical (Bagri et al., 2010; Park and Ruoff, 2009; Stankovich et al., 2007a,b). However, reduction of GO by chemical methods is regarded to be potential because of its cost effectiveness for production in large scale. Moreover, RGO produced by using chemical reagents has the tendency of easy aggregation with hydrophobic nature because of the presence of vander Waals and π-π stacking interactions between the graphene sheets (Wang et al., 2008; Si et al., 2008).

Therefore, the prevention of RGO aggregation is of great prominence, in order to sustain the features of RGO in individual graphene sheets. Stabilization is an approach to overcome the problems of RGO agglomeration, via covalent or non-covalent approaches. From the past few years, a novel methods has been reported for fabrication of RGO sheets by a green and non-toxic approach, using biomolecules such as glucose, plant extracts and Vitamin C as deoxygenating and stabilizing agents (Paredes et al., 2011). Although, several green synthetic approaches have been developed for graphene synthesis, challenges remained for the production of functionalized reduced graphene oxide to tune its properties for use in various biomedical applications.

On the other hand, the design of nanomaterials that can control the differentiation and proliferation of cells is an important area of research in regenerative nanomedicine. For instance, functionalized gold nanoparticles are already reported as osteogenic agents by improving the bone tissue regeneration via encouraging the differentiation and proliferation of osteoblasts (Heo et al., 2014; Yi et al., 2010). Hence, the development of new functionalized nanomaterials for the differentiation and proliferation of osteoblasts is on demand.

In the present report, we showed an environmental friendly method for the fabrication of RGO using capsaicin as a deoxygenating and capping agent. The −OH functionalities that are available on capsaicin plays key role for the fabrication of functionalized RGO. Further to know the potential applicability of prepared functionalized RGO for use in the tissue engineering field, we have collected the osteoblast cells from 24 h old SD rats and studied the effect of fabricated RGO on their differentiation and proliferation.

2. Experimental section

2.1. Materials

Hydrogen peroxide (H_2O_2, 30%), Sodium nitrate ($NaNO_3$), $KMnO_4$, H_2SO_4 (98%), graphite powder (99.9995%, 100 mesh), and other reagents, solvents were obtained from Sigma-Aldrich, Shanghai.

* Corresponding author.
E-mail address: Liansuozhai11@gmail.com (L. Zhai).

https://doi.org/10.1016/j.etap.2017.11.012
Received 27 June 2017; Received in revised form 11 November 2017; Accepted 17 November 2017
Available online 21 November 2017
1382-6689/ © 2017 Published by Elsevier B.V.

2.2. Synthesis of capsaicin functionalized reduced graphene oxide (RGO)

Graphene oxide for the fabrication of RGO, was synthesized from graphite powder by using a reported modified Hummer's method (Perera et al., 2012). To prepare RGO, about 500 mg of capsaicin was added to 50 mL of GO (1 mg/mL) followed by stirring at room temperature for about 20 min. The subsequent solution was alkalined using NH_4OH to maintain a pH of 12. Then the reaction solution was heated at 90 °C in oil bath for about 6 h to observe the colour change of the solution from brown colored GO to black colored RGO signifying the reduction of GO.

2.3. Osteoblast proliferation and adhesion

2.3.1. Cell culture

Rat calvariae were obtained from 24 h old SD rats and dissected followed by washing several times using PBS buffer to discard the tissue debris. Later the obtained rat calvariae were made into 1 × 1 mm pieces followed by incubating in 0.25% trypsase at 37 °C for about 20 min. The supernatant was then removed and the calvariae were washed again with PBS buffer followed by digestion in DMEM medium having type I collagenase (0.075%) at 37 °C for about 60 min. The osteoblasts cells that are released throughout the digestion process were collected, and centrifuged at 1000 rpm for about 10 min. As obtained osteoblast cells were then seeded in DMEM medium containing streptomycin, 1% penicillin and 10% FBS followed by culturing in 5% CO_2 atmosphere at 37 °C to obtain confluence of 80%.

2.3.2. CCK-8 assay and image analysis by acridine orange staining

A CCK-8 assay is followed for the present study rather than other cell viability techniques such as because of its advantages including less handling time with high sensitivity, which is due to the higher solubility of CCK reagent in medium than MTT reagent which avoids the cell death during cell viability experiments. For CCK-8 assay, Osteoblasts at a cell concentration of 1 × 10^4/mL were seeded in 200 μL of DMEM/well into a 96-well plate. Cell Counting Kit-8 (CCK-8) assay was used to study the proliferation of osteoblasts cultured on the sterilized pellets in the 96-well plate after seeding at 24, 48, and 72 h of time. In brief, the cells obtained were washed thoroughly with PBS buffer after discording the cell culture medium. Later, about 200 μL of culture medium without serum, containing 10% CCK-8 reagent, was mixed with each specimen in the 96-well plates, followed by incubation at 37 °C for about 2 h. The optical absorption of the cell suspension present in each well was recorded using a microplate reader at 450 nm. Further, to better evaluate cell proliferation, we also conducted acridine orange staining for the cells cultured with the prepared materials for 1, 3 and 5 days. The osteoblasts were washed with PBS buffer and AO reagent (100 μg/mL) was added and left for 5 min. Later, the obtained samples were washed with PBS buffer for about two times and visualized under inverted microscope.

2.4. Characterization

A JASCO Ultraviolet-visible (UV-vis) spectrophotometer was used for absorption measurements of GO and RGO. Samples for UV–vis measurements were prepared by dispersing GO and RGO in water. Double distilled water was used for blank measurements. The functionalization of capsaicin on the surface of RGO was known by using JASCO FTIR 4100 spectroscopy instrument over the range of 4000–500 cm^{-1}. GO and RGO samples for measurement were prepared by pelletizing the products with KBr. A Bruker D8 Advance diffractometer was used for X-ray diffraction (XRD) measurements over the range of 3–80°, with λ = 1.54 Å, step size of 0.02° and a scan rate of 4°/min. Also, the measurements were performed at an operating voltage of 40 kV and 40 mA. On the other hand, the morphology of GO and RGO was studied by using Field-emission scanning electron microscopy

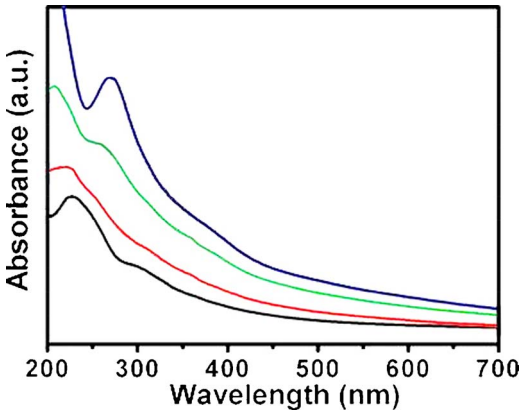

Fig. 1. UV–visible spectrum showing the reduction of GO.

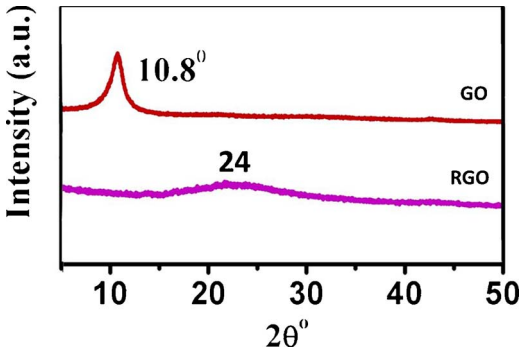

Fig. 2. XRD patterns of GO (black) and RGO (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(FESEM) and transmission electron microscopy (TEM) instruments. A JSM-7001F instrument was used to take FESEM images. Similarly, A JEM-2100 microscopic instrument was used to take TEM images. Sample for TEM analysis were prepared by dropping a diluted dispersion of GO or RGO onto the surface of copper grid and dried in an oven under vaccume. Raman spectrometric measurements were performed using A J-Y T64000 Raman spectrometer with 514.5 nm incident laser. Further the morphology and thickness of graphene sheets were also studied by using NT-MDT Solver P47-PRO studied using atomic force microscopy (AFM) instrument. SXM, ULVac-PHI Inc instrument was used for C1S X-ray photo electron spectroscopy (XPS) measurements for GO and RGO. Sample for XPS measurements were prepared by uniformly decorating the GO and RGO powder on the surface of a silicon wafer.

3. Results and discussion

During the course of GO reduction, the colour of the reaction suspension was changed from brown colour to black, signifying the restoration of electronic conjugation. The reduction of GO was monitored by taking the optical absorption spectra of RGO with the course of time. The RGO samples for UV–vis measurements were prepared by collecting a portion of reaction mixture at different time intervals and washed for 3 times and dispersed in water to obtain a dilute solution for measurements. From Fig. 1, GO exhibited an optical absorption peaks at 230 nm and 300 nm (shoulder peak). With the course of time, the characterized peak of GO at 230 nm gradually red-shifted to 271 nm, representing the restoration of electronic conjugation. It is found that the existence of intense peak at 271 nm corresponding to the formed graphene which was mainly because of the restored sp2 carbon network. On the other hand, In a comparative study for evaluating the efficiency of various reducing agents, Merino et al. have proposed that the maximum red-shift value that is obtained after reduction of GO can

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