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In vitro and *in vivo* characterization of graphene oxide coated porcine bone granules

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ABSTRACT

Graphene oxide (GO) demonstrated to improve the wound healing properties of materials intended for bone replacement. The main objective of this study was the setting up of a simple and effective procedure for the production of GO-coated porcine bone (PB) granules and the characterization of the obtained material in order to improve its properties by exploiting chemical, physical, biological and mechanical features that the GO coating could confer to pre-formed PB granules. The obtained coating was homogeneously distributed on PB granule surface and demonstrated to confer PB an increased resistance to fracture load. Biological analyses evidenced no toxic effects of GO-coated PB samples on primary human gingival fibroblasts, and no inflammatory response around the grafted particles when implanted *in vivo* on a sheep model although GO-coated PB samples did not appear to improve new bone formation efficacy compared with the control within the investigated time. A small loss of GO was however detected, indicating the opportunity to investigate less GO concentrated samples. In conclusion, this study presents a novel and low cost approach to the development of functionalized biomimetic hybrid materials which can be applied to other bone substitute materials in order to improve their performances.

1. Introduction

Currently, an extraordinary interest is devoted to explore the potential of graphene for possible applications in biomedical and regenerative engineering. Graphene is a two dimensional allotrope of carbon with only one atom thickness, i.e. a planar sheet of condensed benzene rings. This structure confers graphene an extraordinary high mechanical stiffness [1], an exceptional high thermal [2] and electrical conductivity [3,4], and makes graphene ideal to be used as coating of materials that usually lack these properties. The strongest limitation of graphene practical applications is its poor solubility in both organic solvents and water, and therefore its difficult handling. Indeed, once exfoliated, graphene tends to reaggregate due to weak but extensive non-covalent interactions among its sheets [5]. Researchers have overtaken this drawback by exploiting chemical covalent functionalization [6–8]. In particular, oxidation appears to be the easiest and lowest cost

Abbreviations: GO, graphene oxide; PB, porcine bone; TEM, transmission electron microscopy; SEM, scanning electron microscopy; HGF, gingival fibroblasts; HA, hydroxyapatite; CNT, carbon nanotubes; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, buffered saline solution; v/v, volume/volume; DMSO, dimethylsulfoxide; SD, standard deviation; FTIR, fourier transform infrared spectroscopy; TGA, thermogravimetric analysis; UV, ultraviolet; vis, visible.

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functionalization available, enabling the insertion into graphene carbon backbone of a series of differently oxidized functionalities such as hydroxyl, ether and epoxyl groups on their basal plane and carboxyl and carbonyl mojeties located mainly at the sheet edges [9] that confer to graphene a hydrophilicity proportional to the degree of oxidation. Graphene oxide (GO) has thus been extensively investigated as an additive for biomedical materials thanking to its capacity to promote the adhesion, proliferation, and differentiation of various cells [10,11].

Nowadays, heterologous materials are included among the most commonly used biomaterials for bone replacement and bone regeneration, and they are considered excellent bioactive and osteoconductive materials [12]. However, these tissue derived materials, as well as the synthetic hydroxyapatite (HA) counterpart, are brittle and characterized by low fracture toughness and merely osteoconductive properties. These drawbacks could be overcome by using different materials such as alumina, titania and carbon nanotubes (CNT) [13]. We thought to improve pre-formed porcine bone (PB) granules features by using GO. As a matter of fact, despite functionalization alters graphene features [9], GO keeps its extraordinary mechanical stiffness and strength, and hopefully may act as an osteoinductive factor [14].

Actually, a few studies have been published on the use of GO for possible applications in biomedical and regenerative engineering, such as induction of differentiation of mesenchymal stem cells towards osteoblastic lineage [14–16] and formation of *in situ* hydroxyapatite HA via precipitation of calcium phosphate [17–19].

To our best knowledge no studies are available reporting on GO *in vivo* application for bone regeneration, or on the development of a GO coating for commercially available bone substitute materials. Thus, the main aim of the present study was to set up an easy and inexpensive protocol for the preparation of GO-coated PB granules and to characterize the obtained material through the investigation of the chemical, physical, biological and mechanical features that the GO coating could confer to pre-formed PB granules.

2. Materials and methods

2.1. Preparation of graphene oxide

Graphene oxide (GO) was prepared from graphite by using a modified Hummers method [20,21]. A flask containing a mixture of 0.2 g of graphite and 0.1 g of sodium nitrate in 4.6 mL of concentrated sulfuric acid was placed in an ice bath. Then 0.6 g of potassium permanganate was added slowly under continuous stirring. After 2 h, the reaction mixture was transferred in a water bath at 35 °C and stirred for 30 min. 9.2 mL of deionized water were slowly added into the solution (the monitored solution temperature was about 98 °C) and this temperature was ensured by heating for further 45 min. 27.8 mL of deionized water and 2.14 mL of 30% hydrogen peroxide were poured in the mixture to stop the reaction. The obtained light brown mixture was filtered through a sintered-glass filter (pore size 15–40 μm) and rinsed three times with 5% HCl and then with water. The solid was dried at 60 °C for 12 h. The obtained graphite oxide was redispersed in water, ultrasonicated for 45 min and centrifuged for 15 min at 9000 rpm. Rotary evaporation at 40 °C of the corresponding supernatant allowed to obtain exfoliated GO.

2.2. Preparation of GO-coated PB granules

25 mL of a homogeneous dispersion of GO in water at various concentrations (50, 75, 100 μg/mL), preventively ultrasonicated for 30 min and centrifuged at 5500 rpm for 15 min, were added to 50 mg of PB granules. After 15 min, solvent was evaporated at reduced pressure and controlled temperature (40 °C) on a rotary evaporator. At the end of the procedure, uniformly coated PB granules were obtained.

2.3. Materials

Synthetic Graphite ~200 mesh, 99.9995% powder was purchased from Alfa Aesar. PB granules (APATOS CORTICAL, OSTEOBIOL®) were a gift of Tecnoss dental s.r.l. Pianezza (TO), Italy. All other reagents of analytical grade were used as received. The Dulbecco's modified Eagle's medium DMEM, fetal bovine serum (FBS), antibiotics (penicillin and streptomycin) and fungizone were purchased from Euroclone, Pero, MI, Italy. MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) and DMSO (dimethylsulfoxide) were purchased from Sigma Aldrich, Saint Louis, MO, USA. Ivermectin (Ivomec ovini) was purchased from Merial Italia, Milano, Italy. Xylazine (Rompum®) was purchased from Bayer. Diazepam (Diazepam® 0,5), ketamine (Ketavet® 100), embutramide (TanaxH) and thiopental (Pentothal Sodium) were purchased from Intervet, Italia. Atropine sulfate was purchased from Fort Dodge. Halothane (Halotane®) was purchased from Merial. The glycolmethacrylate resin was purchased from Technovit 7200 VLC, Kulzer, Wehrheim, Germany.

2.4. Apparatus for chemico-physical characterization of the material

The morphology of GO nanosheets and GO-coated PB granules was evaluated by Scanning Electron Microscopy (JEOL 6360LV SEM microscope - JEOL, Tokyo, Japan) and Transmission Electron Microscopy (TEM, Zeiss Electron microscope 109). Samples for SEM were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h before being processed either with hexamethyldisilazane or at the critical point followed by gold-palladium coating. All SEM micrographs were obtained at 30 kV. GO samples for TEM imaging were prepared by dispersion of materials in water, followed by ultrasonication. A drop of suspension (5 μl) was placed onto carbon coated copper grids, dried in air and loaded into the electron microscope chamber. GO-coated PB granules were directly placed onto carbon coated grids. Raman spectra were recorded on an Invia Renishaw microspectrometer (50 or 100×) by using a laser source at 532 nm (power 5%, 3 or 5 accumulations/measurement). GO dispersion was drop-casted onto silicon wafers while PB or GO-enriched PB powder were gently compressed using a glass slide. The Fourier transformed infrared spectrum (FTIR) was measured with a Varian FTS 1000 spectrometer. The UV–Vis absorption spectrum was carried out by using a Varian Cary 100 UV–Vis spectrophotometer. Thermo-gravimetric analyses (TGA) on ca. 5 mg sample were performed under nitrogen by setting a temperature increment of 5 °C/min from 0 to 600 °C; the hybrid samples were grinded in a ceramic mortar. Measurements of average size and ζ-potential values were performed by using a 90Plus/BI-MAS ZetaPlus multiangle particle size analyzer (Brookhaven Instruments Corp.). For optical microscopy measurements the 50 μg/mL GO-coated PB granules were embedded in a glycolmethacrylate resin (Technovit 7200 VLC, Kulzer, Wehrheim, Germany), the specimens were sectioned with a high precision diamond disk at about 150 μm. The samples were stained with acid fuchsin and toluidine blue. Histomorphometry of newly formed bone, marrow spaces and residual biomaterial was carried out using a light microscope (Laborlux S, Leitz, Wetzlar, Germany) connected to a high resolution video camera (3CCD, JVC KY-F55B) and interfaced to a monitor and PC (Intel Pentium III 1200 MMX). This optical system was associated with a digitizing pad (Matrix Vision GmbH) and a histometry software package with image capturing