Materials Science & Engineering C 90 (2018) 365–378

Contents lists available at ScienceDirect

Materials Science & Engineering C

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

# Evaluation of long-term biocompatibility and osteogenic differentiation of graphene nanosheet doped calcium phosphate-chitosan AZ91D composites

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ARTICLE INFO

*Keywords:*
Magnesium alloy
Graphene
Long-term
Biocompatibility
Osteogenic differentiation

ABSTRACT

To improve the biodegradable performance and osteoinductivity of magnesium (Mg) alloy, a novel graphene nanosheet (GNS) doped micro-arc oxidized-AZ91D (MAO-AZ91D) based calcium phosphate (CaP)-chitosan (CS) (GNS-CaP-CS/AZ91D) composite was fabricated. Their long-term in vitro biocompatibility, osteoinductivity and the related signaling pathway were evaluated. The electrochemical test, Mg concentration and SEM results showed that the corrosion rate of GNS-CaP-CS/AZ91D became much slower, whose corrosion degree with immersion time being 90 d was similar to that of AZ91D being 16 d. Good biocompatibility and non-cytotoxicity were observed during the whole immersion process of GNS-CaP-CS/AZ91D. Extracts from GNS-CaP-CS/AZ91D especially in the early stage (16 days and before) could significantly enhance alkaline phosphatase (ALP) activity, calcium mineral deposition and osteoblast-related genes expression of human bone marrow mesenchymal stem cells (hBMSCs). GNS released from GNS-CaP-CS/AZ91D composites might serve a critical role to induce osteogenic differentiation through ERK/MAPK pathway in the long-term immersion process, while, PI3K/Akt signaling only significantly in the early stage. Moreover, Mg element could promote osteogenic differentiation through ERK/MAPK pathway and the maximum concentration was about the range between 200 and 500 ppm.

## 1. Introduction

Biomaterial implants can provide mechanical support during bone healing and establish a suitable environment for specific types of cells to generate new bone tissue [1]. An ideal bone implants should possess good biocompatible, controllable degradation and resorption rate to match cell/tissue growth in vitro or in vivo. Meanwhile, the implant must be suitable for cell attachment, proliferation, and differentiation and its degradation products must be no toxic and preferably with osteoconductivity and osteoinductivity features. In addition, the mechanical properties must be sufficient to match the tissues at the site of implantation [2]. As a biodegradable medical material, magnesium (Mg) alloy has gained significant attention. Mg is an essential element to the human metabolism as a cofactor for many enzymes and a stabilizer for the structures of DNA and RNA [3]. The daily intake of Mg for a normal adult is about 300–400 mg and excess Mg ions can be gradually dissolved, absorbed, consumed or excreted in the human body. Moreover, owing to its degradability in human body fluids, repeated surgery is unnecessary to remove an implant made of Mg. In addition, the specific density (1.7–2.0 g/cm$^3$), young's modulus (41–45G Pa) and yield strength (100–200 MPa) of magnesium alloys are closer to those of human bones (density 1.7–2.0 g/cm$^3$, young's modulus 10–30 GPa, yield strength 130–180 MPa), which will avoid stress shielding [4–9]. Nevertheless, there are still many shortcomings limited its application, such as poor activity, low tensile strength, high corrosion rate and so on [5]. Moreover, cell survival on corroding Mg alloy surfaces is primarily limited by hydrogen liberation and pH increase in the degradation process. To overcome these drawbacks, surface modification methods such as coatings on Mg-based materials are proved to improve its corrosion resistance [10–12].

Graphene, a novel and potentially useful nanomaterial among different carbon allotropes [13,14], possess a series of advantages, such as no cytotoxic effect on human osteoblasts and mesenchymal stromal cells [15–18], better cell attachment and proliferation ability [19–21], excellent apatite mineralization property [22,23] and accelerating osteogenic differentiation osteogenic-related genes expressions [16,17,24–26]. Meanwhile, its excellent mechanical properties are employed as reinforcing material to improve the mechanical property

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https://doi.org/10.1016/j.msec.2018.04.082
Received 27 August 2017; Received in revised form 15 April 2018; Accepted 26 April 2018
Available online 27 April 2018
0928-4931/ 

of composites [27,28]. Human mesenchymal stem cells (hMSCs) have capability of self-renewing ability, strong proliferative ability and multi-differentiating potential. It not only repair mesoderm tissue such as bone, cartilage, tendon, muscle, blood vessel and hematopoietic system [29,30], but also be used as a seed to repair the nerve tissue derived from ectoderm and lung, liver and others derived from the endoderm [31–33]. These cellular behaviors, such as attachment, proliferation, and differentiation into certain lineages could be influenced by a lot of physical, chemical, and environmental factors, especially substrate topography, extracellular matrices, stem cell-growth factor/chemical inducer interactions, and stem cell substrate interactions [34]. Therefore, the applications of hMSCs in the field of regenerative medicine are currently crucial. Osteoinductivity is a key process to direct induce mesenchymal cells differentiate into osteoprogenitor cells, osteoblast cells, and then form bone tissue. Although it was reported that grapheme-based materials could promote cell growth and differentiation [19,20,22,23], the effects of graphene-containing bone implants on stem cell osteoblastic differentiation and underlying molecular mechanisms, however, are poorly understood. Furthermore, it has been reported that the mitogen-activated protein kinase (MAPK) signal pathway plays important roles in inducing stem cells differentiation [35,36]. In the MAPK family, extracellular regulated protein kinases (ERK) 1/2 and p38 MAPK (p38) involve in biomaterials induced differentiation of stem cells have been widely documented [37,38]. The phosphatidylinositol-3 kinase (PI3K) pathway could profoundly modulate cell proliferation, osteoblastic differentiation and bone growth [39], and previous study has shown that graphene could induce the upregulation of PI3K/Akt pathway [40]. In short, it is essential to explore the role of different extracts in long-term immersion process on ERK/MAPK, p38/MAPK and PI3K/Akt signal pathway.

In order to enhance its apatite mineralization property and osteogenic differentiation of stem cells and stimulate bone reparation and regeneration, a novel GNS doped MAO-AZ91D based CaP-CS composite (GNS-CaP-CS/AZ91D) was fabricated with electrophoresis deposition (EPD) combined with conversion in a phosphate buffer solution (PBS) [41,42]. To date, studies on various biodegradable materials mainly focus on the characteristics and biocompatibility of the material itself and the short-term effects of degradation products in vitro and in vivo [35,43–47]. However, the long-term influence of products was rarely investigated which are crucial for cell survival, proliferation, and differentiation. In this study, we primary investigate the effects of different GNS-CaP-CS/AZ91D extracts on the survival, proliferation and osteogenic differentiation of human osteoblasts (SaOS-2) and human bone marrow mesenchymal stem cells (hBMSCs) during the long-term immersion process. Furthermore, the expression of bone-specific genes and the roles of ERK/MAPK, p38/MAPK and PI3K/Akt pathways involved in the regulation of osteogenic differentiation of hBMSCs were also investigated.

## 2. Experimental

### 2.1. Fabrication of GNS-CaP-CS/AZ91D composites

AZ91D (Dongqi Magnesium Alloy Products Co., Ltd., China) was first carried out with MAO procedure as the previous work [41]. The chemical composition of AZ91D were 8.6 wt% Al, 0.74 wt% Zn, 0.24 wt % Mn and 90.42 wt% Mg. GNS (Nantong Hongxing carbon industry co., LTD., China) doped MAO-AZ91D based CaP-CS coating was performed as described below. 0.25 g CS (Mw = 200,000) with a deacetylation degree of about 85% (Sigma-Aldrich) and 1.0 g *n*HA particles prepared by wet chemical precipitation technology were sequentially added into 200 ml of an aqueous solution containing 9 ml of acetic acid under agitation. Another solution was obtained by adding 1.5 g *n*HA particles into 300 ml of absolute ethanol. The 0.02 g/l GNS was added to the mixture of these two solutions sonicated for 1–2 h and aged for 24–30 h. The EPD cell was composed of AZ91D as the cathode and the titanium alloy coated iridium tantalum as the anode. The space between two electrodes was about 10–15 mm. The EPD process was performed at a voltage of 40 V for 20 min at room temperature. After the EPD process, the obtained samples were soaked in a conical flask with PBS (10 g/l $NaH_2PO_2$ + $Na_2HPO_4$) at 37 °C for 5 d. The PBS was replaced every day. The obtained samples were rinsed with distilled water and dried at room temperature.

### 2.2. Electrochemical test

An electrochemical test was conducted to evaluate the corrosion resistance of the GNS-CaP-CS/AZ91D composites and untreated AZ91D alloys according to previous study [48]. The experimental setup was a standard three-electrode system with the sample as the working electrode, a saturated calomel electrode as the reference electrode, and a platinum mesh as the counter electrode. To calibrate the reference electrode, five reference electrodes were chosen to measure the electric potential difference between each two electrodes, respectively. And then, the addition of the potential difference absolute value was calculated compared each electrode with the other, in which the smallest one was as the corrected reference electrode. The results of the measurements were analyzed by using the EG&G EQUIVCRT software. Prior to the polarization, the samples were immersed in 500 mL of modified simulated body fluid (m-SBF) maintained at 37 °C for 15 min to establish a relatively stable free corrosion potential [49,50]. The polarization curves were obtained at a scan rate of 5 mV/s, and the changes of the corrosion potential ($E_{corr}$) and the corrosion current density ($i_{corr}$) values were monitored as a function of time. The corrosion current density ($i_{corr}$) was converted into the corrosion rate (CR) according to the Faraday's Law.

### 2.3. Immersion into the m-SBF

After immersion in the PBS, four of each of the GNS-CaP-CS/AZ91D and untreated AZ91D samples were put into conical flasks with 250 ml of the m-SBF for 12 different time points (6 h, 12 h, 1 d, 2 d, 8 d, 16 d, 24 d, 34 d, 44 d, 60 d, 90 d and 120 d) at 37 °C respectively. The ratio of the specimen surface area ($cm^2$) to the m-SBF volume (ml) was 1:250. At each time point, the samples were removed from the m-SBF, rinsed with de-ionized water and dried at room temperature and the extracts of each time point were retained for experiments after sterilization with the disposable filter (Milipore) which specification was 0.22 μm. The concentrations of Mg, Ca and P element in different extracts were measured using an inductively coupled plasma optical emission spectrometer (ICP-OES, 5300DV, Perkin Elmer, USA). In addition, the pH values of the extracts were also measured.

In order to observe the corrosion condition and products, part of the obtained GNS-CaP-CS/AZ91D samples (1 d, 2 d, 8 d, 16 d, 24 d, 34 d, 44 d, 60 d and 90 d) were examined with a scanning electron microscopy (SEM, HITACHI S-4800, Japan). The other part of GNS-CaP-CS/AZ91D and AZ91D samples were immersed in a solution comprising 200 g/l CrO3 and 19 g/l AgNO3 for 10–15 min to remove the corrosion products [48]. After that, they were quickly cleaned with distilled water, dried in warm flowing air and then examined with a SEM to examine their surface morphology and corrosion property under the protection of the GNS-CaP-CS film compared with untreated AZ91D.

### 2.4. Cell culture

The human osteosarcoma cell line SaOS-2 (ScienCell, USA) and hBMSCs (ScienCell, 7500, USA) were used in this study. SaOS-2 were cultured in McCoy's 5A Medium (Sigma) with 15% heat inactivated fetal bovine serum (FBS, ScienCell, USA) and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% $CO_2$. hBMSCs were cultured in Mesenchymal Stem Cell Medium (MSCM, ScienCell, 7501, USA) at 37 °C in a humidified atmosphere of 5% $CO_2$.