Carbohydrate Polymers 92 (2013) 167–175

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

ZrO_2 surface chemically coated with hyaluronic acid hydrogel loading GDF-5 for osteogenesis in dentistry

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ARTICLE INFO

Article history:
Received 20 June 2012
Received in revised form 28 August 2012
Accepted 24 September 2012
Available online 4 October 2012

Keywords:
Zirconium dioxide
Photo-cured hyaluronic acid hydrogel
Growth and differentiation factor-5
Bone morphogenetic protein-2
Release kinetics
Bone formation

ABSTRACT

The objective of this study was to modify zirconium dioxide (ZrO_2) with photo-cured hyaluronic acid hydrogel (pcHAgel), and to subsequently evaluate the bone regeneration potential of the modified ZrO_2. In the present study, HA grafted onto a ZrO_2 substrate was investigated for its biocompatibility and other properties. We describe the positive influences of ZrO_2 surface-modified with pcHAgel (Zr-3) containing two different loads of growth and differentiation factor-5 (GDF-5) to aid new bone formation as compared to the same amount of BMP-2 (Zr-4–7). We characterized the Zr-3 for their surface morphology and chemical properties. Atomic force microscopy (AFM), scanning electron microscope (SEM), and X-ray photoelectron spectroscopy (XPS) showed that the pcHAgel was successfully grafted onto the ZrO_2 surface. The sustained release of GDF-5 and BMP-2 were observed to occur in the Zr-4–7. *In vitro* cell tests showed a higher level of MG63 cell proliferation and differentiation on Zr-4–7 than on Zr-3. The Zr-3 is a good biomaterial to deliver osteogenic differentiation factors such as BMP-2 and GDF-5, and GDF-5 can be useful as an effective alternative to aid new bone formation as compared to BMP-2.

1. Introduction

Osseointegration is one of the most important criteria for the success or failure of bone anchored metallic implants in dentistry (Brånemark, Brånemark, Rydevik, & Myers, 2001). One of the most critical challenges to osseointegration is successfully anchoring metallic implants through stable attachment to alveolar bone. Successfully anchored metallic implants undergo a process which homogeneously combines with alveolar bone by directly depositing new bone onto their surfaces through favorable interactions with osteoblasts (Webster, Ergun, Doremus, Siegel, & Bizios, 2000).

Over the last several decades, titanium (Ti) and its alloys have been introduced as suitable materials for tooth reconstruction due to their chemical stability, mechanical strength, and excellent biocompatibility (Pourbaix, 1984). However, despite these functional merits, there has been a steady growing demand in the market for enhanced aesthetic features as well.

Zirconium dioxide (ZrO_2) is replacing Ti as a material for dental implants due to its aesthetic quality as it appears very similar to original teeth as well as having good chemical resistance, mechanical strength, and excellent biocompatibility (Chevalier, 2006). It has a flexural strength of ~900 MPa, a fracture toughness of up to 10 MPa/$m^{0.5}$, and an elastic modulus of ~210 GPa, which are superior mechanical properties than that of Ti (Garvie, Hannink, & Pascoe, 1975; Piconi et al., 1998). To improve the performance of Zr, its surface has been modified by using various methodologies, including ultraviolet (UV) light treatment, for enhanced bone integration (Att et al., 2009).

Recently, incorporation of a thin hydrogel layer on metallic solid surfaces has been reported to provide several advantageous functions for biomedical applications including micro-patterning and controlled drug release (Choi, Konno, Matsuno, Takai, & Ishihara, 2008; Sidorenko, Krupenkin, Taylor, Fratzl, & Aizenberg, 2007; Tokarev & Minko, 2010). For example, Ishihara's group reported that a surface-treatment method using 2-methacryloyloxyethyl phosphorylcholine (MPC) and a photo-labile linker could selectively regulate the attachment of MC-3T3 E1 cells on a glass surface (Choi et al., 2008). They also reported that a multilayered phospholipid polymer, synthesized with MPC, *n*-butyl methacrylate (BMA), and 4-vinylphenylboronic acid units (VPBC), coated on a titanium alloy surface could control the release of a hydrophobic antineoplastic agent, paclitaxel (PTX). This technique can be applied to provide localized drug delivery from metal-based biomedical

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http://dx.doi.org/10.1016/j.carbpol.2012.09.044

devices (Choi et al., 2008). Hydrogel formation on a metallic solid surface has been fabricated by several methodologies, including layer-by-layer (LBL) self-assembly, photopolymerization, and radical polymerization (Choi et al., 2008; Yakushiji et al., 1999). Photopolymerization is one of the most adaptable methods for hydrogel coating on metallic solid surfaces by providing control over temporal and spatial reaction kinetics (Clapper, Sievens-Figueroa, & Guymon, 2008; Hiemstra, Zhou, Zhong, Wouters, & Feijen, 2007; Hutchison, Stark, Hawker, & Anseth, 2005). Moreover, photopolymerization can be achieved in a single, rapid-step process (Clapper et al., 2008; Hiemstra et al., 2007; Hutchison et al., 2005).

Hyaluronic acid (HA), a linear D-glucuronic acid and N-acetyl-D-glucosamine copolymer, has been known as a good macromolecule for many biomedical applications due to its hydrodynamic characteristics, viscous properties, and excellent water uptake (Gerecht et al., 2007; Kogan, Soltés, Stern, & Gemeiner, 2007). It has good hydrophilicity due to negatively-charged functional groups (Kogan et al., 2007).

Recent attention has been drawn to growth and differentiation factor-5 (GDF-5), another member of the BMP family (Hötten et al., 1996). The GDF-5 has been recognized as an important factor in limb development (Buxton, Edwards, Archer, & Francis-West, 2001; Hötten et al., 1996). The GDF-5 has also been expressed in bovine and rat tooth germs in cells associated with periodontal ligament (PDL) formation and cells located along the alveolar bone and cementum surfaces during the course of root formation, suggesting that GDF-5 may play regulatory roles in the development of the periodontal attachment (Morotome, Goseki-Sone, Ishikawa, & Oida, 1998; Sena et al., 2003). Recent *in vivo* studies with the addition of rhGDF-5 have reported significantly enhanced alveolar bone and cementum formation in a canine intra-bony defect model (Lee et al., 2010), as well as bone formation in both pre-clinical and clinical sinus augmentation studies (Gruber et al., 2009; Koch, Becker, Terheyden, Capsius, & Wagner, 2010). GDF-5 also interacts with limb-building BMP (Brunet, McMahon, McMahon, & Harland, 1998) and is more cost effective than osteogenic bone morphogenetic protein-2 (BMP-2) (Kim et al., 2011).

In this study, we designed and prepared surface functionalized ZrO_2 modified with photo-cured HA hydrogels containing two different amounts of BMP-2 and GDF-5 (10 and 50 ng) (**ZrO_2-4–7**) (Fig. 1), and evaluated their influence on new bone formation. To our knowledge, little is known about the surface modification technique of ZrO_2 with hydrogel containing osteogenic differentiation factors.

2. Materials and methods

2.1. Materials

Hyaluronic acid (HA) (Mw: 1700 kDa) was purchased from Lifecore Biomedical Co. (Chaska, MN, USA). Zirconium dioxide (ZrO_2) disks (diameter: 1 cm) were kindly obtained from DIO Co. (Busan, Republic of Korea). 2-Morpholinoethanesulfonic acid (MES), 2-aminoethylmethacrylate (AEMA), dexamethasone, alkaline phosphatase (ALP) assay kit, ascorbic acid, cetylpyridinium chloride and β-glycerophosphate were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). A cytocompatible photoinitiator, 4-(2-hydroxy ethoxy)phenyl-(2-hydroxy-2-propyl)ketone (Irgacure D-2959), was purchased from Ciba Geigy Ltd. (Basle, Switzerland). 1-Ethyl-3-(3-dimethyaminopropyl)-carbodiimide (EDC), and N-hydroxysuccinimide (NHS) were purchased from Tokyo Chemical Industry CO., Ltd. (TCI, Japan). Growth and differentiation factor-5 (GDF-5) was purchased from Preprotect Inc. (Rocky Hill, NJ, USA). Bone morphogenetic protein-2 (BMP-2) was purchased from R&D Systems (Minneapolis, MN, USA). Fetal bovine serum (FBS), penicillin/streptomycin, high-glucose Dulbecco's Modified Eagle's Medium (DMEM) and trypsin were purchased from GIBCO BRL (Carlsbad, CA, USA). Human osteosarcoma cell line (MG-63) cells and RAW 264.7 (mouse macrophage) cells were purchased from the Korean Cell Bank (Seoul, Republic of Korea).

2.2. ZrO_2 surface-modified with photo-cured HA hydrogels (*pcHAgel*) containing two amounts (10 and 50 ng) of BMP-2 and GDF-5 (**ZrO_2-4–7**)

The functionalized **ZrO_2-4–7** were engineered according to three procedures (Fig. 1). In the first procedure, a concentrated aqueous NaOH solution (2.5 M) was added to pristine **ZrO_2-1** and heated to 60 °C for 24 h in order to form active OH groups on the surface (**ZrO_2-1′**) (Uchida, Kim, Miyaji, Kokubo, & Nakamura, 2002). After washing with distilled water several times, the activated **ZrO_2-1′** was mixed in anhydrous toluene. To this mixture 2-aminopropyltriethoxysilane (APTES) (5 mL, 5% (v/v) solution) was added, and then reacted at 120 °C for 24 h. This reaction formed APTES-conjugated ZrO_2 (**ZrO_2-2**) (Fig. 1A). For the second procedure, 2-aminoethyl methacrylate (AEMA)-conjugated HA (**HA-AEMA**) was synthesized according to our previous report (Bae et al., 2011). Briefly, HA (1 g) was dissolved in MES buffer solution (50 mM, pH 6.5), and then NHS (1.06 g, 0.09 mol) and EDC (3.50 g, 0.22 mol) were added to the reaction solution. After stirring for 1 h, AEMA (400 mg) was added, and then continuously stirred at room temperature for 24 h. After dialysis (MWCO: 3500) against distilled water for 3 days, the aqueous solution of functionalized **HA-AEMA** was filtered, evaporated and lyophilized with blocking penetration of light (Fig. 1B). The reaction was confirmed by ^{1}H NMR (Varian Unity Plus 300, Varian Inc., Palo Alto, CA, USA) spectrometer (300 MHz) and ATR-FTIR (TENSOR 37, Bruker, USA) as reported previously (Bae et al., 2011). After dissolving **HA-AEMA** (1 g) in MES buffer solution (10 mL, pH 6.5), the solution was chemical conjugated to **ZrO_2-2** using NHS (0.11 g, 0.09 mol) and EDC (0.35 g, 0.22 mol). The reaction was carried out at room temperature for 24 h in order to conjugate **HA-AEMA** onto **ZrO_2-2** through amide bonds (**ZrO_2-3**). After washing with distilled water several times, a mixture of 0.05% (w/v) cytocompatible photoinitiator Irgacure D-2959 in distilled water (10 μL), two amounts of functional factor BMP-2 and GDF-5 (10 and 50 ng) were added, respectively. Following this each of the mixture put onto **ZrO_2-3** was exposed to UV light (CL-1000 UV-crosslinker, 365 nm, UVP) (Jeon, Bouhadir, Mansour, & Alsberg, 2009) at room temperature for 5 min. This formed the photo-cured HA hydrogel film on **ZrO_2-3** containing either BMP-2 or GDF-5 (**ZrO_2-4–7**) as depicted in Fig. 1C.

2.3. Surface characterization of ***ZrO_2-1***, ***2***, and ***3′***

Scanning electron microscopy (SEM, S-2300, Hitachi, Japan) observations were carried out on dried **ZrO_2-1**, **2** and **3′**gold-coated by using a sputter-coater (Eiko IB, Japan) under an accelerating voltage of 15 kV. X-ray photoelectron spectroscopy (XPS) measurements were carried out with a Thermo Electron (U.K.) at a grazing angle of 90° under high vacuum ($<3.1 \times 10^{-9}$ Torr). A monochromatic aluminum Kα X-ray radiation (photoelectron energy = 1486.6 eV) was used and the wide-scanned XPS spectra was obtained at a pass energy of 187.8 eV. Static contact angle measurements using a sessile drop method was carried out on three Zr specimens at 20 °C with 3 μL of distilled water droplet under a relative humidity of 60%, in advance ajusted to 71.8 mN/m of surface tension, as measured by a telescopic goniometer (Pheonix 300, SEO, Republic of Korea). Tapping mode atomic force microscopy (TM-AFM) observations were carried out in wet condition by using a NANOS® TM-AFM system (NanoInk, Inc., USA). The