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Short communication

Biological performance of titania containing phosphate-based glasses for bone tissue engineering applications ☆

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ABSTRACT

The interplay between glass chemistry, structure, degradation kinetics, and biological activity provides flexibility for the development of scaffolds with highly specific cellular response. The aim of this study was therefore to investigate the role of titania inclusion into the phosphate-based glass on its ability to stimulate osteoblast-like human osteosarcoma (HOS) cells to adhere, proliferate and differentiate. In depth morphological and biochemical characterisation was performed on HOS cells cultured on the surface of glass discs. Cell proliferation was also studied in the presence of the glass extract. Cell differentiation, through osteoblast phenotype genes, alkaline phosphatase (ALP) activity and osteocalcin production, was carried out using normal or osteogenic media. Both Thermanox® and titania free glass were used as controls. The data demonstrated that titania inclusion provides desired cytocompatible surface that supported initial cell attachment, sustained viability, and increased cell proliferation similar or significantly higher than Thermanox®. The modified glasses regulated osteoblastic cell differentiation as detected by osteoblast phenotype gene transcription and upregulated ALP and osteocalcin expression. Using osteogenic media had no significant effect on ALP activity and osteocalcin expression. Therefore, titania modified phosphate glasses may have future use as bone tissue engineering scaffolds.

1. Introduction

Bone regeneration is a natural process that occurs during bone remodelling. Where the defects are too large, however, the bone cannot heal itself. Bone tissue engineering has emerged as a promising route to stimulate the regenerative capacity of host bone cells to form new bone [1]. Bone tissue engineering utilises three-dimensional natural or synthetic scaffolds to provide the suitable environment for host cells to grow, and hence, proceed in tissue regeneration. These scaffolds are required to provide structural support for the host bone cells and should favourably affect bone formation by stimulating rapid cell adhesion, proliferation and finally regulate osteoblastic differentiation [2].

The fact that the major components of most phosphate-based glasses (e.g., phosphorus, sodium and calcium oxides) are also found in the inorganic phase of bone is a major contributing factor for the bioactivity of these glasses. Ions released from degradable glasses can modulate the host bone cells response [3–9], and they could be involved in various cellular processes at different levels [10]. For example, calcium "Ca" supports osteoblast proliferation, differentiation and extracellular matrix mineralisation; it also stimulates the Ca-sensing receptors in bone cells and subsequently increases growth factor expression e.g., IGF-I and IGF-II [11]. Inorganic phosphate "P" stimulates the expression of Gla protein (MGP) which is a key regulator in bone formation [12]. Accordingly, the control of the glass dissolution rate and hence the metallic ion release could be an attractive approach to drive the biological response in the desired route [10].

The interplay between glass chemistry, degradation kinetics, and biological response provides a wealth of information for the development of scaffolds with highly specific cellular response [13,14]. Titanium is a transition metal; it is highly reactive as an element and spontaneously forms a stable 'native' oxide layer (~3–7 nm thick) on its surface [15]. This oxide layer acts as an inorganic substrate that attracts molecules, proteins and then cells to attach and finally form bone [16]. Hence this oxide layer has been credited to the ability of titanium implants to facilitate bone apposition. Thus titanium-containing materials/implants have been successfully used in various craniofacial and orthopaedic applications [17,18]. Also other phosphate glasses of more complex composition have been investigated and found suitable for tissue engineering applications [9,19,20].

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Inclusion of titanium oxide, namely titania, into slowly degradable phosphate-based glasses [50 mol% P_2O_5, 40 mol% CaO, Na_2O (10 − x), and TiO_2 (x) where x varied from 1 to 5 mol%] has been reported to encourage attachment and viability of human osteosarcoma (HOS) cells. Increasing titania content (from 1 to 5 mol%) had a profound effect on cytoskeleton organisation, spreading and maturation of primary osteoblasts [5,21]. Currently, glass microspheres of approximately 10–200 μm in diameter based on the same system but with higher titania content (3–7 mol%) show considerable potential for use in a whole host of biomedical applications ranging from cancer radiotherapy and thermotherapy, to drug and protein delivery or bone tissue engineering applications [8]. The inclusion of titania into another relatively more degradable glass system [50 mol% P_2O_5, 30 mol% CaO, Na_2O (20 − x), and TiO_2 (x) where x varied from 1 to 5 mol%] that released higher level of Ti ions (e.g., 5 mol% titania containing glass of this system release at 0.0085 ppm·h^{-1} instead of 0.0051 ppm·h^{-1} for the same titania content in the previous system) also improved MG63 cell attachment, proliferation and osteoblast phenotype gene expression as well as the in vivo bioactivity of these glasses [5]. Inclusion of up to 15 mol% titania, the maximum possible content, has been attempted with this glass system to produce further control on glass structure and properties. The network structure and properties of this system containing up to 15 mol% titania have been elucidated using diverse analytical techniques [3], but its cytocompatibility has not yet been assessed. From a materials science perspective, slight changes in glass composition produce major changes in glass structure, degradation, ion release and consequently biological response [6,22]. The aim of this study was therefore to, for the first time, assess the biocompatibility of phosphate-based glasses doped with the maximum possible titania content [50 mol% P_2O_5, 30 mol% CaO, Na_2O (20 − x), and TiO_2 (x) where x varied from 5 to 15 mol%]. The influence of several glass characteristics and its degradation products on viability, proliferation and differentiation of osteoblast-like human osteosarcoma (HOS) cells has been investigated.

2. Materials and methods

2.1. Production of glasses

Using the precursor materials NaH_2PO_4, $CaCO_3$, P_2O_5, and TiO_2 (BDH, Poole, UK, all chemicals were >98% purity), four glass compositions with different titania (TiO_2) contents were produced by melt-quenching as described previously [3,4]. The P_2O_5 and CaO contents were fixed at 50 and 30 mol% respectively, while the Na_2O and TiO_2 contents varied. TiO_2 contents were 5, 10 or 15 mol% and accordingly the Na_2O contents were 15, 10, or 5 mol% respectively. The melting temperature of the as-prepared glasses was 1300–1350 °C. The melted glass was poured into a preheated graphite mould and annealed at 420 °C for 1 h to remove any stresses due to preparation and then slowly cooled to room temperature. These glasses were coded as PCN, PCNT5, PCNT10 and PCNT15 respectively. Discs of 15 mm in diameter and approximately 1 mm in thickness were used. The discs were polished using waterproof silicon carbide papers P# 120 for 30 s at 300 RPM, then P# 500, 1000 and 2400 for 1 min each and finally P# 4000 for 2 min to get a mirror-like surface on a Struers Rotopol-11 (Struers, UK).

2.2. Human osteosarcoma cell culture

Human osteosarcoma (HOS) cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air, in growth medium [Dulbecco's modified Eagle's Medium (DMEM, Gibco), supplemented with 10% foetal calf serum, and 1% penicillin and streptomycin solution (Gibco)]. The medium was changed at 2 day intervals.

All samples for biocompatibility tests were sterilised by dry heating at 180 °C for 3 h, and pre-incubated in 2 ml of growth medium for 24 h at a 37 °C humidified atmosphere of 5% CO_2 in air. Both titania free glass (PCN) and Thermanox® were used as controls. Cells were plated at a density of 3×10^4 cells/well in 24-well culture plates that provided a snug fit of the discs, in a 50 μl aliquot of medium (for initial attachment) prior to the addition of 1.5 ml of growth medium.

2.3. Cell viability and live dead staining

Determination of cell viability was carried out by incubating the specimens for 1 h in a standard growth medium containing 1 μl/ml calcein AM (acetomethoxy) to stain the live cells, and propidium iodide to stain the dead cells. The assessment of cell viability in three dimensions was performed using confocal laser scanning microscopy (CLSM, Bio-Rad, USA).

2.4. Cell proliferation assay

This study was carried out by growing HOS cells either directly on glass discs or in the presence of glass extracts. The extracts were produced by incubating glass discs in growth medium for 24 h. For the extract study, normal growth medium was used as a control. The proliferation assay was conducted up to 21 days for the direct study but only 7 days for the indirect one. At the required time point, the cultured cells were incubated in 10% alamarBlue® (Invitrogen, UK) for 4 h according to the manufacturer's instructions. The absorbance of the samples (n = 3 and duplicate reading for each one) was measured at 530 nm (A_{530}) and 590 nm (A_{590}) as excitation and emission wavelength using a Fluoroskan Ascent plate reader (Labsystems, Helsinki, Finland). The cell growth was presented as the average intensity of six replicate wells that was base line corrected and compared to the positive control cells.

2.5. Cellular differentiation

2.5.1. Gene expression

For this study, only titania containing glasses, coded as PCNT5, PCNT10 and PCNT15, were used and compared with Thermanox®. At 1 and 14 days, the total RNA was isolated from the lysed [with RLT-buffer (Qiagen, Germany) and β mercaptoethanol (Sigma)] and homogenised cells using RNeasy minikit (Qiagen) and eluted with 30 μl RNase-free water. RNA concentration was calculated by using Quanti IT™ RNA assay kit with the Qubit™ flourometer (Molecular Probes™, Invitrogen). Subsequently, the extracted total RNA was reverse-transcribed into cDNA using a high capacity cDNA archive kit (Applied Biosystems, Cheshire, UK) and an Eppendorf thermal cycler (Mastercycler, Eppendorf UK Ltd., Cambridge, UK). Samples were stored at −70 °C for further analysis.

cDNA for each sample was then amplified by real-time PCR-ABI PRISM® 7300 sequence detection system (Applied Biosystems, Cheshire, UK) using human TaqMan® gene expression assay (Applied Biosystems, Cheshire, UK). Conjugated to FAM (6-carboxyfluroescein) reporter dye, TaqMan® probes (Applied Biosystems, Cheshire, UK) were used to target corresponding nucleotide sequences for osteoblast phenotype genes [alkaline phosphatase (ALP), collagen type I alpha subunit I (COLIaI), core binding protein factor alpha 1 (Cbfa1) and osteonectin (Sparc)] in the cDNA single strands. Relative quantification Q-PCR, using the 7300 SDS software, was conducted against the expression of rRNA encoding housekeeping gene, 18s.

2.5.2. Alkaline phosphatase activity

For cell culture used in this study, both aforementioned normal growth medium (non-osteogenic), and osteogenic medium (prepared by adding 50 μg/ml ascorbic acid and 5 mM Na-β glycerophosphate to the normal growth medium) were used.

After 1, 7 and 14 days, the culture supernatants were removed and used for osteocalcin assay; samples were washed with phosphate buffer solution (PBS) (Gibco, UK) and then solubilised with 1% Triton-X by