Bioactivity of thermal plasma synthesized bovine hydroxyapatite/glass ceramic composites

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Abstract. Bone injuries and failures often require the inception of implant biomaterials. Research in this area is receiving increasing attention worldwide. A variety of artificial bone materials, such as metals, polymeric materials, composites and ceramics, are being explored to replace diseased bones. Calcium phosphate ceramics are currently used as biomaterials for many applications in both dentistry and orthopedics. Bioactive silicate-based glasses show a higher bioactive behaviour than calcium phosphate materials. It is very interesting to study the mixtures of HA and silicate-based glasses. In the present study; natural bovine hydroxyapatite / SiO₂–CaO–MgO glass composites were produced using the Transferred arc plasma (TAP) melting method. TAP melting route is a brisk process of preparation of glass-ceramics in which the raw materials are melted in the plasma and crystallization of the melt occurs while cooling down at a much faster rate in relatively short processing times compared to the conventional methods of manufacture of glass ceramics/composites. It is well known that; one essential step to the understanding of the biological events occurring at the bone tissue/material interface is the biological investigation by in vitro tests. Cell lines are commonly used for biocompatibility tests, and are very efficient because of their reproducibility and culture facility. In this study, we report the results of a study on the response of primary cultures of human fibroblast cells to TAP melted bioactive glass ceramics.

1. Introduction
Plasma technology encompasses a wide range of operations including plasma cutting, welding, melting and machining, spraying, chemical synthesis and mineral dissociation. Plasma technology is an enabling technology, which integrates processes associated with plasma material interaction with manufacturing, adds value to conventional materials and makes new types of materials and material processing techniques possible. Plasma technology has in recent years emerged as a novel technique for the manufacture of newer and better materials [1-3].

Bioactive glasses and glass–ceramics have been investigated for tissue engineering applications in bone repair and are successfully used in some clinical applications. Distinctive properties of bioactive glasses are the ability to convert to hydroxyapatite in body fluids and in aqueous solutions containing calcium and phosphate ions, and the ability to bond directly to bone. Since its bone bonding properties
were reported in 1971 by Hench et al. Calcium phosphate based glasses and glass–ceramics have been of interest for medical uses due to their unique properties. Certain ceramic materials, such as hydroxyapatite (HA), tricalcium phosphate (TCP) and selected compositions of silicate and phosphate glasses and glass ceramics, for example the commercially available Bioglass, react with physiological fluids and form tenacious bonds to hard and soft tissues through cellular activity. These materials are therefore known as “bioactive”. In last two decades, remarkable advances in the field of biomaterials have led to the development of bioglasses and bioceramics of various compositions for bone repair and prostheses applications. Hydroxyapatite (HA), β-tricalcium phosphate (β-TCP) and their composites are widely used due to their good biocompatibility and osteointegrative properties [4-6].

Bioactive silicate-based glasses show a higher bioactive behaviour than calcium phosphate materials [7]. Silicon possesses importance in bone formation and mineralization, and the possibility of producing soluble calcium phosphate phases; it is intriguing to study the mixtures of HA and silicate-based glasses. In the present study; natural bovine hydroxyapatite / SiO₂–CaO–MgO glass ceramics were produced using the Transferred arc plasma (TAP) melting method. It is well recognized that; one vital step to the understanding of the bioactivity is the biological study by in vitro tests. In this study, the results of the response of primary cultures of human fibroblast cells to TAP melted bioactive glass ceramics are reported.

2. Experimental procedure

2.1 Plasma synthesis of HA/ SiO₂–CaO–MgO glass ceramics.
Initially, The HA material used in this study was derived from the natural bovine bones (from the shaft portion of the bovine femurs). The 51.6% SiO₂ - 35.6% CaO -12.8% MgO (by wt %) glass ceramic was obtained by Transferred arc plasma (TAP) melting method. Detailed experimental technique involved in the SiO₂–CaO–MgO glass-ceramic synthesis was described elsewhere [9]. Homogeneous mixtures of HA/ 25 wt % SiO₂–CaO–MgO and HA/ 50 wt% SiO₂–CaO–MgO batches were obtained by dry mixing the respective compositions for 4 hours in a ball mill (In smart systems, India). After which the homogeneous mixtures were taken into the anode bed of the dc transferred arc plasma torch [8, 9]. For this experiment, a dc transferred arc plasma torch (Ion Arc Technologies Pvt. Ltd., India) was used for the purpose. The torch was operated at power level of 5kW. Argon at a flow rate of 10 lpm was used as the plasma forming gas, plasma was generated and discretely the mixtures were heated for 3 minutes respectively. During which they got melted in the plasma to form glass-ceramic melt. HA/SiO₂–CaO–MgO glass ceramics were produced by quenching the melts by applying forced air on it.

2.2 Characterization

(i) Phase and Microstructure. The synthesized glass-ceramic samples were studied for phase composition and microstructure using SEM and X-EDX analysis. The observation was carried out with an ESEM Quanta200 – FEI, worked in high vacuum mode. The phase composition was also studied by X-ray diffraction (X'Pert PRO PANalytical, working with the Cu Kα radiation in the angular range 2θ = 5–80°).

(ii) In-vitro bioactivity. Normal human skin fibroblasts were grown in 75 cm² cell culture flasks (Falcon, Becton-Dickinson, Cockeysville, MD, USA) in Dulbecco’s modified Eagle’s medium (with 10% fetal calf serum, 100 IU/ml penicillin G, 25 µg/ml streptomycin and 0.5 µg/ml fungizone. The cultured cells were then incubated at 37°C in 98% humidity, and 5% CO₂. Cell culture medium was changed every 2-3 days. When the cultures reached 90% confluence, the cells were detached from the flasks using a 0.05% trypsin-0.1% EDTA solution, washed twice, and used to evaluate their adhesion and growth onto the samples. Fibroblasts were seeded into 24 well plates. Each well contains small
pieces of the sample. Materials were sterilized in incubation in 70% ethanol. Following three washes with sterile DMEM, each well was seeded with \(10^3\) fibroblasts and cultured for 48 h. At the end of each incubating period fibroblasts that adhered to the materials were visualized using Hoechst staining. To do so, cells on the materials were fixed with 75% methanol and 25% glacial acetic acid solution for 5 min. Following 2 washes with PBS, each well was then supplemented with 0.5 ml Hoechst dye (1 μg/ml) and the specimens were incubated for 15 min at room temperature before being extensively washed with distilled water, observed under an epifluorescence light microscope (Axiophot, Zeiss, Oberkochen, Germany), and photographed using a digital camera.

3. Results and discussion

The XRD spectra were similar for all HA/50 wt% SiO\(_2\)–CaO–MgO samples (high nominal content of glass) and for HA/25 wt% SiO\(_2\)–CaO–MgO samples (low nominal content of glass). Regardless of the nominal composition, the peaks observed (Fig1) were all due to complex calcium phosphate silicate. The formation of complex calcium phosphate silicate is mainly attributed due to the combination of constituents of HA produced from bovine bone [6] and the raw material constituent’s melting due to high plasma temperature. Minor peaks could be ascribed to calcium silicon, CaSi. Only in HA/50 wt% SiO\(_2\)–CaO–MgO samples some traces of HA were detected, but the identification is uncertain due to the poor intensity of peaks. According to X-EDX analysis (Fig2, 3), light-grey areas are richer in Mg, Al and Si than dark-grey areas which, in turn, are richer in P. This could suggest that light-grey areas were originated by glass, while dark-grey areas derived from HA. However, even if the relative amounts are different, all the elements (O, Mg, Al, Si, and P, Ca + traces of C) can be detected in both areas. The microstructure of samples were basically uniform (with slight differences); It is possible to distinguish two phases (light-grey rounded-shape areas surrounded by a dark-grey matrix), with slightly different compositions. However both phases seem to contain O, Mg, Al, Si, P and Ca (in various proportions). Other elements, such as C, Fe and K, are occasionally present as well.

In HA/50 wt% (SiO\(_2\)–CaO–MgO) samples some traces of HA were detected, but the identification was uncertain due to the poor intensity of peaks. The X-EDX spectra of both glass ceramics revealed the presence of major Ca, P, Si, Mg and O elements with traces of Na, Al and S.
Figure 2. ESEM with X-EDX spectra of HA/50 wt% SiO₂–CaO–MgO

Figure 3. ESEM with X-EDX spectra of HA/25 wt% SiO₂–CaO–MgO
The human fibroblast cell culture results showed that the plasma synthesized samples were non-toxic for in vitro fibroblast cultures (Fig4).

**Figure 4.** Human fibroblast adhesion on (a) HA/50 wt% SiO$_2$–CaO–MgO, (b) HA/25 wt% SiO$_2$–CaO–MgO for 48 hours.

The cells spread throughout the material. Indeed, Hoechst staining revealed that fibroblasts adhere and exhibited a normal elongated and spread-out morphology as those cultured on cell culture surfaces. Of great interest, both the HA/SiO$_2$–CaO–MgO glass ceramics seem to highly promote fibroblast adhesion and growth. But in both cases, the fibroblasts density as visualized by cell staining showed that these cells grow well after seeding onto the materials. In general, these results showed that the glass ceramic samples showed good response to human fibroblasts.

4. Conclusion
HA/SiO$_2$–CaO–MgO glass-ceramics were produced using the Transferred arc plasma melting method. The synthesized glass-ceramic samples were studied for phase composition and microstructure using XRD, X-EDX and ESEM. Human fibroblasts were cultured on the synthesized glass ceramics. Overall, these results showed that the samples were non-toxic to human fibroblasts and promote cell growth.

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