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Direct cytotoxicity evaluation of 63S bioactive glass and bone-derived hydroxyapatite particles using yeast model and human chondrocyte cells by microcalorimetry

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Abstract In this study, the cytotoxicity evaluation of prepared 63S bioactive glass and bone-derived hydroxyapatite particles with yeast and human chondrocyte cells was carried out using isothermal micro-nano calorimetry (IMNC), which is a new method for studying cell/biomaterial interactions. Bioactive glass particles were made via sol–gel method and hydroxyapatite was obtained from bovine bone. Elemental analysis was carried out by XRF and EDXRF. Amorphous structure of the glass and completely crystalline structure of HA were detected by XRD analysis. Finally, the cytotoxicity of bioactive glass and bone-derived HA particles with yeast and cultured human chondrocyte cells was evaluated using IMNC. The results confirmed the viability, growth and proliferation of human chondrocyte cells in contact with 63S bioactive glass, and bone-derived HA particles. Also the results indicated that yeast model which is much easier to handle, can be considered as a good proxy and can provide a rapid primary estimate of the ranges to be used in assays involving human cells. All of these results confirmed that IMNC is a convenient method which caters to measuring the cell-biomaterial interactions alongside the current methods.

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1 Introduction

The evaluation of biocompatibility is one of the most important assessments to be performed prior to clinical use of biomaterials. At the time being, there are a number of different methods in use for evaluating biocompatibility, including scanning electron- and light microscopy [1, 2], measurement of cell proliferation [3], enzyme reactions [4] or RNA expression [4, 5], as well as cytotoxicity assays [4]. These methods are time consuming and the results depend extremely on the human skill [2].

There is a need for a convenient method for screening of biocompatibility and cytotoxicity (as a criterion of biocompatibility), a method that can directly evaluate cell growth as well as cell adhesion to biomaterials surfaces. Furthermore, it should be possible to sample the medium for analysis of compounds released by the cells [6].

All living organisms produce heat as a result of their metabolism. Metabolic heat generated by single cells ranges from about 1 to 80 pW. Normal human connective tissue cells (e.g., fibroblasts, adipocytes) have reported metabolic rates of ~25–80 pW per cell, and chemical reaction-based theoretical calculations of single-cell metabolism are in the same range. In contrast, most microbes produce significantly smaller amounts of heat (~1–3 pW per cell). Despite the low heat output of microorganisms, their exponential replication in culture allows their detection by microcalorimetry within hours even when the starting numbers are low (e.g., 1–10 colony-forming units (CFUs)/specimen) [7, 8].

Isothermal micro-nano calorimetry (IMNC) is capable of measuring the heat production or consumption rate in the μW range with a calorimeter operating at nearly a constant temperature. Therefore, this technique allows direct and continuous monitoring of the metabolic activity of living cells [9]. Microcalorimetry is a nonspecific analytical tool for measuring the heat produced or consumed over time by chemical reactions, including heat generated by complex biologic processes in cultured cells. Basic microcalorimetry data are recordings of heat flow rates, frequently referred to as heat power–time curves [8–11].

As a result, IMNC can be extensively used for monitoring the growth and activity of microorganisms [12], human cells or cell lines [13, 14] and evaluating the cell-biomaterial interactions. Since it measures only heat production in a passive way (i.e., through a thermopile), IMNC allows study of specific biological processes and recovery after exposure to effectors without disturbing the cell culture [6].

The biocompatibility of bioactive glass and bone-derived HA particles with osteoblast cells has previously been investigated by different researchers [15–17]. In this study, direct cytotoxicity of these well-known bioceramics with yeast and cultured human chondrocyte cells was evaluated using IMNC. We used the yeast *Saccharomyces cerevisiae* as an experimental model, which can be easily manipulated to evaluate different biological effects (inhibition of cellular growth and cytotoxicity) induced by the materials considered [18, 19]. Although a single cell, it is true eukaryote, and share fundamental cell processes with metazoan systems. This offers a unique tool to the cell biologist, providing complementary approaches and insights into functions of larger eukaryotes [18]. The usefulness of eukaryotic organisms such as yeast in materials cytotoxicity screening has been demonstrated [19–21].

In the study reported here, there is first a description of how the 63S bioactive glass and bone-derived HA particles were prepared. Then, the primary characterization was carried out to illustrate the present phases and elements in their compositions. Finally, the direct cytotoxicity evaluation of the bioceramics particles with yeast and human chondrocyte cells was performed using isothermal microcalorimetry.

2 Materials and methods

2.1 Synthesis and preparation

2.1.1 Bioactive glass

Colloidal solutions (sols) of 63S composition (63 mol% SiO2, 28 mol% CaO, 9 mol% P2O5) were prepared by mixing distilled water, 2 N hydrochloric acid (Merck), tetraethyl orthosilicate (TEOS, Merck), triethyl phosphate (TEP, Sigma-Aldrich) and calcium nitrate (Merck) [22]. The initial procedure involved mixing TEOS (28 ml) and ethanol (40 ml, Merck) as an alcoholic media. Distilled water was added to solution and allowed to mix until the solution became clear. The H2O:(TEOS) molar ratio was 4:1. After 30 min, TEP (2.3 ml) was added to the stirring solution. After another 20 min, calcium nitrate (12 gr) was added. The solution was then stirred for an additional hour. The gel was heated (60°C, 10 h), dried (130°C, 15 h) and thermally stabilized (600°C, 2 h) according to established procedures [22, 23]. The produced gel was ground with a mortar and pestle to disagglomerate the particles. Finally the particles were sieved to make a distribution of particles of size less than 50 μm (L3–M50 50 μm stainless steel sieve and Sonic Sifter Separator, Advantech Manufacturing Co., New Berlin WI, USA). Bioactive glass particles were sterilized at 180 °C for 1 h.

2.1.2 Bone derived HA

A femur of an adult bovine was obtained from a slaughterhouse and boiled in water for 12 h to render it aseptic and loosen any attached soft tissues. Then it was washed and cleaned carefully to remove visible tissues, fats and any other readily visible foreign materials on the bone surface. To remove the internal organic content (e.g., collagen) and water, the bone was then heated in an electric furnace under ambient conditions, at 700°C, with a 2 h holding time. The resulting white solid specimens were first ground and crushed with a mortar and pestle to produce a powder. The powder was then sieved to produce a distribution of particles of size less than 50 μm (L3–M50 50 μm stainless steel sieve and Sonic Sifter Separator, Advantech Manufacturing Co., New Berlin WI, USA). Bone powders were sterilized at 150°C for 1 h, rinsed in distilled water and incubated in 1% phosphoric acid. They were rinsed again in sterile distilled water, and sterilized at 200°C.

2.2 Characterization

In order to detect the phases and the elements present in the chemical compositions, the basic characterization experiments were carried out.

2.2.1 Elemental composition analysis

The elemental composition of bioactive glass particles was confirmed by X-ray fluorescence spectroscopy (XRF), (PW2404, PHILIPS) and energy dispersive X-ray analysis (EDX) technique (SUPRA 40 VP FE-SEM). Elemental

analysis of the natural HA particles was carried out using an energy dispersive x-ray fluorescence spectrometry (EDXRF) instrument (SPECTRO XEPOS, SPECTRO Analytical Instruments GmbH, Germany). A spectral resolution of less than 160 eV for Mn K-α was achieved and the maximum count rate was 120,000 cps. The high spectral resolution at high count rates results in reduced measuring times and improved measurement accuracy.

2.2.2 X-ray diffraction

X-ray diffraction (XRD) technique (Philips X'Pert-MPD system with a Cu Kα wavelength of 1.5418 A°) was used to analyze the structure of the prepared bioactive glass. The diffractometer was operated at 40 kV and 30 mA at a 2θ range of 20–80° employing a step size of 0.02°/s.

2.3 Direct cytotoxicity evaluation with IMNC

Monitoring the cell growth in solid substrates is often difficult. However, in this context microcalorimetry offers a way to measure the growth and activity of the cells over time in the presence of solid compounds such as bioactive glass and hydroxyapatite particles.

2.3.1 Yeast experiment

Yeast (*Saccharomyces cerevisiae*) were grown and maintained on yeast peptone dextrose (YPD) medium. Overnight cultures were performed before the experiment. The culture was diluted ca 5000 times in order to have an optical density at 600 nm below 0.05. 3 ml of the diluted culture was added to increasing amounts of bioactive glass and hydroxyapatite (0–50 mg/vial) in 4 ml calorimetric ampoules. The ampoules were sealed and placed in equilibration position in the microcalorimeter (TAM48, Waters/TA) for at least 15 min. Following equilibration, ampoules were lowered in measuring position equilibrated again for 45 min. After this second equilibration time, growth related heat-flow was recorded for ca. 300 h.

2.3.2 Chondrocyte experiment

Trypsinized human chondrocyte culture was performed in Dubelco's modified eagle's medium containing 10% fetal bovine serum, D-glucose (4.5 mg/ml), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, penicillin (100 units/ml), streptomycin (100 μg/ml), and L-glutamine (0.29 mg/ml). In addition, the medium was added with growth factors (transforming growth factor (TGF-1, 1 ng/ml), fibroblast growth factor-2 (FGF-2, 5 ng/ml), and platelet-derived growth factor type bb (PDGF-bb, 10 ng/ml),) previously shown to increase the proliferation rate [24]. The cultures were aseptically transferred in microcalorimetric ampoules containing 3 mg of bioactive glass or hydroxyapatite particles. This quantity was determined using the results of the yeast experiment assuming that human cell would be more sensitive to calcium concentration than yeast. There was also a control sample containing only chondrocyte cells and the culture medium. For making sure that the measured heat is only of the cell growth, the second control sample dedicated to bioceramic particles in the culture medium (without cells).

3 Results

3.1 Elemental composition analysis

3.1.1 EDX and XRF results for bioactive glass

The result of EDX microanalysis of the glass particles showed the elements present in the composition. The peaks of O, Si, P and Ca indicate the consisting elements of prepared bioactive glass particles (Fig. 1).

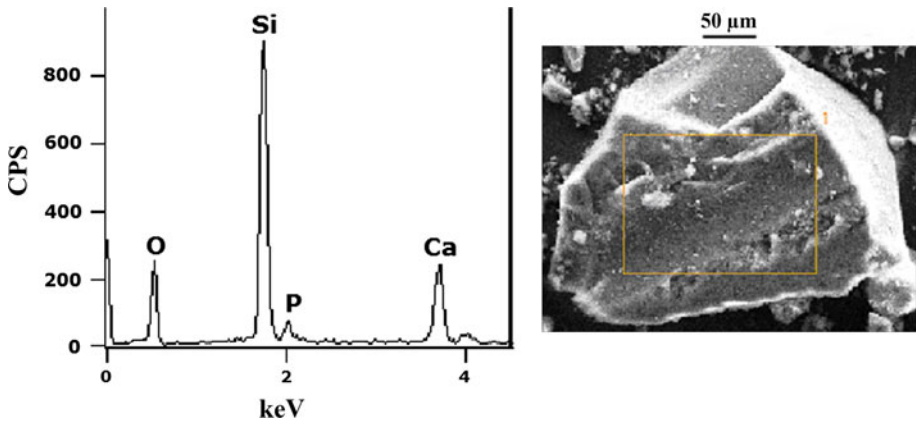

Fig. 1 *Left panel* Energy dispersive X-ray analysis (EDX) of the bioactive glass particles. The peaks of O, Si, P and Ca indicate the consisting elements of prepared bioactive glass. *Right panel* SEM micrograph of one representative particle analyzed (before sieving)

The existent elements in prepared bioactive glass particles and estimated composition measured by X-ray XRF, is shown in Table 1. The molar percentage of oxides was expressed by computer according to the elemental analysis and considering the assumption that all the elements were in oxidized form.

3.1.2 EDXRF results for HA

The elementary composition results for the HA particles are listed in Table 2. As expected, the main elements detected calcium and phosphorus with an average Ca/P molar ratio of 1.93. As for minor components, silicon, magnesium and sodium were detected. Furthermore, traces of potassium, strontium, zinc, barium, vanadium, aluminum, manganese, lead, cupper and iron were found. The results shown are the mean values of several examinations of particles by EDXRF.

Table 1 Bioactive glass estimated oxidic composition measured by X-ray fluorescence (XRF)

Oxide	Molar %
SiO_2	62.17
CaO	28.47
P_2O_5	9.25
LOI (loss on ignition)	0.11

Table 2 Elemental composition (mean value (standard deviation)) of the bone-derived HA particles measured by EDXRF

Element	Concentration (% w/w)
Ca	41.77 (1.14)
P	16.71 (0.52)
Si	0.74 (0.18)
Mg	0.63 (0.20)
Na	0.40 (0.18)
K	0.24 (0.11)
Cl	0.12 (0.19)
Sr	0.11(0.16)
S	0.083 (0. 10)
Zn	0.016 (0.02)
Ba	0.011(0.01)
V	0.0045 (0.009)
Al	0.0020 (0.00052)
Mn	0.001 (0.00041)
Pb	0.0009 (0.001)
Cu	0.0005 (0.00036)
Fe	0.0001 (0.00024)

3.2 XRD analysis

The XRD pattern of the prepared glass after heating at 600°C for 2 h did not contain diffraction maxima, indicative of the internal disorder and the glassy nature of this material. The XRD patterns indicated that the obtained bioactive glass particles were amorphous (Fig. 2), proving the sol–gel method could prepare pure glasses.

The XRD spectrum showed that the HA particles prepared from bovine bone were highly crystalline (Fig. 3). The HA spectra ($2\theta = 31.83°$) exactly conformed to natural hydroxyapatite XRD pattern that is reported in the literature [25]. Also only a minimal amount of MgO ($2\theta = 42.9°$) was detected in XRD pattern. There was no CaO detectable by XRD.

3.3 Cytotoxicity evaluation

HA and bioactive glass are bioceramics which contain calcium in their composition. Bioactive glass is rich in silicon as well (in form of SiO_2). Both of these elements (Ca and Si) can be toxic and may cause cell death [26, 27]. It has been demonstrated that SiO_2 component in biomaterials has toxic potential when dissolved in the body [26]. The main reason for toxicity of Ca is precipitation of inorganic phosphates and compromising energy of metabolism due to Ca high concentration. It is noticeable that release kinetics of calcium depends on the nature of the particles and their crystalinity. Therefore that it is of great importance to perform this cytotoxicity assay.

3.3.1 Yeast experiment

Saccharomyces growth in YPD which was added with bioactive glass exhibited normal growth (i.e., comparable to the control (0 mg bioglass)) for bioactive glass concentration up to 3.3 mg/ml (10 mg/vial). For higher concentration, decrease in maximum activity and delays in growth were

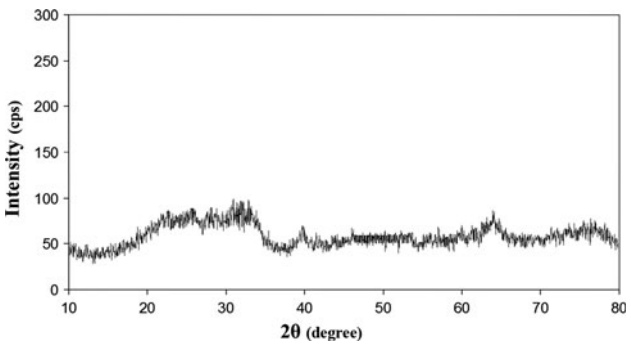

Fig. 2 XRD pattern of the prepared bioactive glass particles. Intensity of diffraction versus angle of radiation (2θ,). No peak of diffraction could be observed

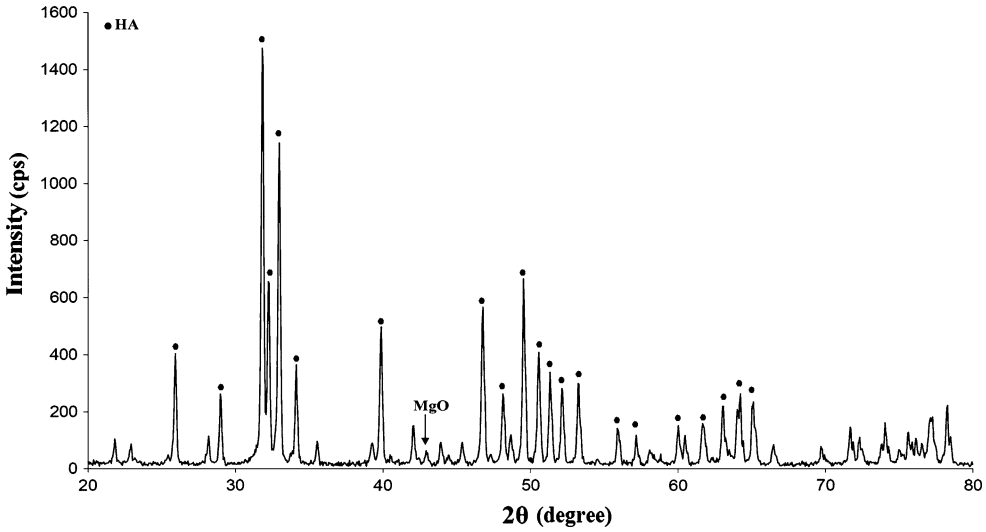

Fig. 3 X-ray diffraction spectrum of the HA particles

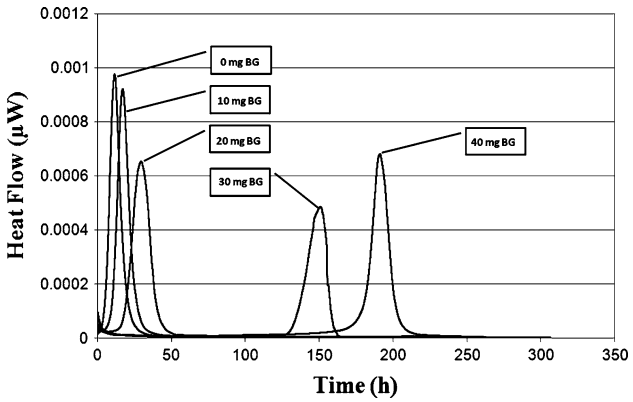

Fig. 4 Microcalorimetric curves showing the effect of bioactive glass particles (BG) on yeast growth

observed (Fig. 4). For concentration of 40 mg bioactive glass, in comparison with 30 mg bioactive glass, more activity of yeast was observed. However, the delay in yeast growth was evident for 40 mg bioactive glass. No yeast growth was detected for concentration of 50 mg bioactive glass.

For *Saccharomyces cerevisiae* cultures added with bone-derived hydroxyapatite, no deleterious effect on the growth is observed for concentrations up to 6.6 mg/ml (20 mg/vial). In addition, only a minimal effect on growth has been spotted for the concentration of 10 mg/ml (30 mg/vial). For higher concentrations up to 15.5 mg/ml (40 mg/vial), mostly minimal delays in growth were recognized. Finally, for concentration above 15.5 mg/ml (50 mg/vial) strong decrease in the maximum activity and delays in growth were distinguished (Fig. 5).

3.3.2 *Chondrocyte experiment*

As can be perceived from Fig. 6, chondrocyte growth was not significantly affected by bioactive glass and HA concentrations of 3 mg. Only a minimal effect was observed

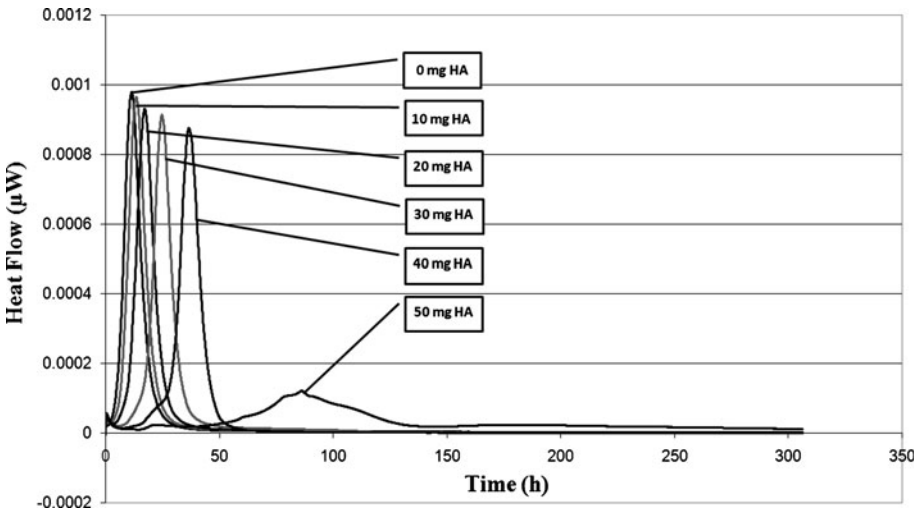

Fig. 5 Microcalorimetric curves illustrating the effect of HA particles on yeast growth

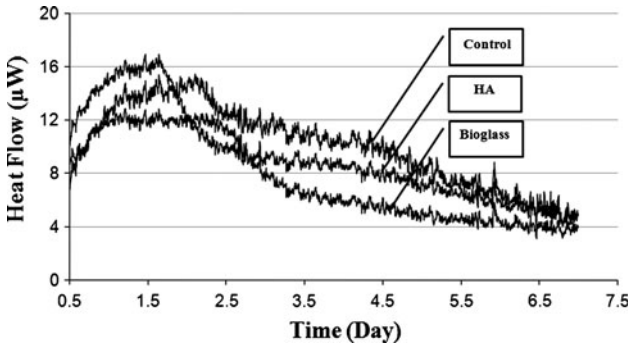

Fig. 6 Microcalorimetric curves showing the effect of adding bioactive glass and HA particles to cultured human chondrocyte cells on chondrocyte growth

(slightly lower than maximal activity). All the cultures followed a similar growth pattern and decayed at a similar rate. The reason for this decease is the closed system of IMNC and consequently, inability of renewing the cultures. It can be concluded that for such concentrations of 63S bioactive glass and bone-derived HA, growth of human chondrocytes is not essentially affected (Fig. 6).

The detected heat from the ampoules which contain bioactive glass and HA, is comparable to the control sample and proves the metabolic activity of the cells. There was no detected heat for the second control sample. This shows that the detected heat was absolutely assigned to the cell activity. The results gained from the direct cytotoxicity evaluation of HA and bioactive glass with chondrocytes and performed through IMNC demonstrated the fact that these bioceramics had no deleterious effect on chondrocyte growth and are completely non-cytotoxic.

4 Discussion

The characterization results of bioactive glass particles showed that the composition of prepared bioactive glass was in good compatibility with documented composition for sol–gel 63S bioactive glass [28].

The obtained Ca/P ratio (1.93) for hydroxyapatite is an approximate value as overlapping of peaks of different elements is possible, but this possibility was not investigated. EDXRF showed small amounts of trace elements including silicon, magnesium, sodium, potassium, strontium, zinc and barium (Table 2). The existence of these elements in HA could be advantageous if this bioceramic is implanted in the human body adjacent to bone containing viable bone cells. For example, Tian et al. have shown the positive influence of strontium on bone tissue in an in vivo experiment [25]. The role of trace elements in bone metabolism has been discussed in the literature [29].

The obtained result from XRD showed that there was no crystalline phase in bioactive glass particles and this is completely in agreement with previous reports [22, 23]. The XRD result for HA was in agreement with previous reports [30, 31]. It was confirmed by XRD analysis that prepared natural HA particles were mainly a highly crystalline hydroxyapatite ceramic.

The IMNC results showed that chondrocytes can live on, grow and proliferate in contact with both bone-derived hydroxyapatite and 63S bioactive glass particles.

This may not be a novel result, but it would be valuable when firstly gained through IMNC (that is very simple and easy to handle) and secondly when the human chondrocyte cells were in direct contact with the bioceramic particles. Conventional methods for biocompatibility and cytotoxicity assay (e.g., MTT) do not suggest a way for direct evaluation of the particles' cytotoxicity. These methods usually evaluate cytotoxicity indirectly (using the extraction of materials) [32, 33] or are designed for bulk structures (not for particles) [34, 35].

The results also suggest that yeast model can be used as a preliminary step to determine the range of material amounts (bioceramic particles) needed for the experiments with human cells. In other words, the weight amount of needed bioceramic particles for the human cell experiment can be initially determined using yeast model assay.

These concentrations of particles could be obtained perfectly by coating implants. In these conditions, the biocompatibility of coated implants with chondrocyte cells can be assured. These types of coatings are already in use. Through recent years, the coating of metallic [22, 36], polymeric [37] and ceramic implants [38] has been focused by researchers. The obtained results from this study confirmed the growth, proliferation and viability of chondrocyte cells in contact with bone derived HA and 63S bioactive glass.

Finally, this study suggests that isothermal microcalorimetry is a promising novel method for evaluating the cytotoxicity of materials to microorganisms such as human cells. This culture method detects heat, resulting from cellular metabolism and growth. IMNC can be used as a primary stage of biocompatibility assessments and yields complementary results when compared to the conventional in vitro methods.

5 Conclusion

The cytotoxicity of particulate materials such as bioactive glass and hydroxyapatite particles can be evaluated using microcalorimetry method. This is a modern method for in vitro study of biomaterials biocompatibility and cytotoxicity which can be used alongside the old conventional

assays. The obtained results showed that there was no negative, pernicious and toxic effect on chondrocyte growth and they could live on, grow and proliferate in contact with bone-derived hydroxyapatite and 63S bioactive glass particles. The results also suggested that yeast model (much easier to handle) could be used as a preliminary step to determine the ranges for human cell experiments. Bioactive glass and natural HA prepared and used in this study are well-known bioceramics which have considerable biomedical applications. Repairing the defects in hard and soft tissues, stimulating cells to proliferate as well as their recent usage in drugs and gene delivery as a carrier, are the most common applications of these bioceramics.

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