**Bretcanu O, Miola M, Bianchi CL, Marangi I, Carbone R, Corazzari I,
Cannas M, Verné E.**

**[In vitro biocompatibility of a ferrimagnetic glass-ceramic for
hyperthermia application.](#)**

Materials Science & Engineering: C 2017, 73, 778-787.

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DOI link to article:

<http://dx.doi.org/10.1016/j.msec.2016.12.105>

Date deposited:

13/01/2017

Embargo release date:

23 December 2017

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Accepted Manuscript

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PII: S0928-4931(16)31357-1
DOI: doi: [10.1016/j.msec.2016.12.105](https://doi.org/10.1016/j.msec.2016.12.105)
Reference: MSC 7298

To appear in: *Materials Science & Engineering C*

Received date: 16 September 2016
Revised date: 3 November 2016
Accepted date: 20 December 2016

Please cite this article as: Oana Bretcanu, Marta Miola, Claudia L. Bianchi, Ida Marangi, Roberta Carbone, Ingrid Corazzari, Mario Cannas, Enrica Verné, In vitro biocompatibility of a ferrimagnetic glass-ceramic for hyperthermia application. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Msc(2016), doi: [10.1016/j.msec.2016.12.105](https://doi.org/10.1016/j.msec.2016.12.105)

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***In vitro* biocompatibility of a ferrimagnetic glass-ceramic for hyperthermia application**

**Oana Bretcanu^{a#}, Marta Miola^a, Claudia L. Bianchi^b, Ida Marangi^{c°},
Roberta Carbone^{c^}, Ingrid Corazzari^d, Mario Cannas^e, Enrica Verné^{a*}**

^a Applied Science and Technology Department, Institute of Materials Physics and Engineering
Politecnico di Torino, C.so Duca degli Abruzzi 24, 10129 Torino, Italy.

[#]present affiliation: Newcastle University, School of Mechanical and Systems Engineering,
Newcastle upon Tyne, United Kingdom.

^b Department of Chemistry, University of Milan, Via Golgi 19, 20133 Milan, Italy.

^c Department of Experimental Oncology, European Institute of Oncology, Milan, Italy .

[^]present affiliation: Tethis S.p.A Genextra Group, Via Russoli, 3, 20143 Milano, Italy.

[°] present affiliation: ATS INSUBRIA, Sede Territoriale di Varese, Via O. Rossi, 9, 21100 Varese,
Italy.

^d Department of Chemistry, University of Torino, Via Pietro Giuria 7, 10125 Torino, Italy, “G.
Scansetti” Interdepartmental Centre for Studies on Asbestos and other Toxic Particulates, Via Pietro
Giuria 7, 10125 Torino, Italy

^e Department of Medical Science, Human Anatomy, University of Eastern Piedmont, Novara, Italy

***corresponding author: e-mail: enrica.verne@polito.it**

tel: +39 011 0904717, fax: +39 011 0904624

Abstract:

Ferrimagnetic glass-ceramics containing magnetite crystals were developed for hyperthermia applications of solid neoplastic tissue. The present work is focused on *in vitro* evaluation of the biocompatibility of these materials, before and after soaking in a simulated body fluid (SBF). X-ray diffraction, scanning electron microscopy, atomic absorption spectrophotometry, X-ray photoelectron spectrometry and pH measurements were employed in glass-ceramic characterisation. The free-radical mediated reactivity of the glass-ceramic was evaluated by Electron Paramagnetic Resonance (EPR) spin trapping. Cell adhesion and proliferation tests were carried out by using 3T3 murine fibroblasts. Cytotoxicity was performed by qualitative evaluation of human bone osteosarcoma cells U2OS cell line. The results show that almost two times more 3T3 cells proliferated on the samples pre-treated in SBF, compared with the untreated specimens. Moreover a decrease of confluence was observed at 48 and 72 hours for U2OS cells exposed to the untreated glass-ceramic, while the powder suspensions of glass-ceramic pre-treated in SBF did not influence the cell morphology up to 72h of exposition. The untreated glass-ceramic exhibited Fenton-like reactivity, as well as reactivity towards formate molecule. After pre-treatment with SBF the reactivity towards formate was completely suppressed. The concentration of iron released into the SBF solution was below 0.1 ppm at 37°C, during one month of soaking. The different *in vitro* behavior of the samples before and after SBF treatment has been correlated to the bioactive glass-ceramic surface modifications as detected by morphological, structural and compositional analyses.

Key words: ferrimagnetic, glass-ceramic, magnetite, cell culture, hyperthermia, cancer.

1. Introduction

Magnetic materials are widely used for different biomedical applications such as magnetic resonance imaging contrast agents, immunoassay, detoxification of biological fluids, hyperthermia, drug delivery, cell separation, etc [1, 2]. More recently, magnetic materials (nanoparticles) have been included in the category of bio-targeted systems and therapeutic tools [3] which can be guided to target sites by means of an external magnetic field and subsequently heated for hyperthermia cancer therapy. Magnetic nanoparticles are assessed as both carriers for drugs and as contrast agents for magnetic resonance detection. All these applications require appropriate biological behaviour in terms of biocompatibility and non-toxicity. In vitro biological tests have general applicability and widespread use for evaluation of a large range of devices and materials, being a critical factor for biomedical applications.

In the last decades, magnetic glass-ceramics have been developed for the magnetic induction of hyperthermia, which is one of the therapies for cancer treatment [4, 5, 6, 7, 8]. This method exploits the potential of magnetic materials to generate heat under an alternating magnetic field. The heat generation depends on the materials properties, magnetic field parameters and tissue characteristics [9]. Magnetic particles are implanted in the tumour site and an external oscillating magnetic field is applied. The magnetic materials will develop heat, raising the temperature of the surrounding tissue and thus, the cancer cells are damaged or destroyed [10].

In our previous studies [11, 12, 13, 14] ferrimagnetic glass-ceramics with the composition in the system $\text{SiO}_2\text{--Na}_2\text{O--CaO--P}_2\text{O}_5\text{--FeO--Fe}_2\text{O}_3$ were obtained by two different synthesis methods: coprecipitation derived and traditional-melting derived. The microstructure, thermal and magnetic properties, bioactivity and specific power loss were analysed. Focusing the attention on the bioactivity mechanism of glasses and glass-ceramics [15], it was shown that it starts with a rapid ions exchange between the alkaline ions from the glass surface and the hydrogen ions from the solution. This step is followed by the formation of silanols on the surface of the glass, which then undergo polycondensation to form a silica gel layer. Silica gel promotes the adsorption of calcium and phosphate ions from the solution, which subsequently react, forming a hydroxyapatite layer.

The first stages of bioactivity, up to the formation of silanols on the glass surface, give rise to a slight enhancement of pH values due to the depletion of the hydrogen ions in the solution. This feature can be also observed in solutions such as TRIS buffer or Kokubo's simulated body fluid (SBF) [16] and should be carefully controlled when bioactive glass samples are placed in contact with different cell lines, during biological evaluations [17].

If iron oxides are present into the glass or glass-ceramic, some effort should be done to assess that ion leaching can occur without production of toxic ions *in vivo*, taking into account that, however, the body has a mechanism for the management of Fe^{3+} , so its low-level release should not produce any toxic effects [18]. Moreover, when particles come in contact with cells and tissues, the interactions between particles surface and the living matter can have detrimental outcomes. As observed with toxic particulates including lunar dust simulants and volcanic ashes [19, 20], the presence of redox active centres (more likely iron) is responsible for the oxidative potential of such particulates. In particular, they can drive the release of reactive oxygen species (ROS), such as hydroxyl, superoxide and hydroperoxide radicals. A mechanism through which iron (or other redox-active metals) centres are implied in the generation of $\text{HO}\cdot$ radical is the Fenton-like reaction which occurs in the presence of H_2O_2 , as in the phagolysosomal vesicles after phagocytosis. A complementary reaction driven by redox active centres is the homolytic cleavage of the C-H bond of biomolecules (including lipids, proteins and nucleic acids). In this paper we focus on the surface reactivity and ion leaching of ferrimagnetic glass-ceramics in the system $\text{SiO}_2\text{--Na}_2\text{O--CaO--P}_2\text{O}_5\text{--FeO--Fe}_2\text{O}_3$, and on their biological properties by analysing the in-vitro biocompatibility on 3T3 murine fibroblasts and U2OS human bone osteosarcoma osteoblasts. Moreover, the capability of SC45 to generate $\text{HO}\cdot$ radicals via a Fenton-like reaction and to cleave the C-H bond of formate ion was investigated by electron paramagnetic resonance (EPR) spin trapping.

2. Materials and methods

2.1. Sample preparation

A ferrimagnetic glass-ceramic with the nominal composition $24.7\text{SiO}_2\text{--}13.5\text{Na}_2\text{O--}13.5\text{CaO--}3.3\text{P}_2\text{O}_5\text{--}14\text{FeO--}31\text{Fe}_2\text{O}_3$ (wt.%) was obtained by a traditional melting method, quenching the melt from 1550°C melting temperature onto a rectangular brass mould as described in [12]. The resulted glass-ceramic, named SC45, contains magnetite crystals, embedded in an amorphous matrix. Magnetite crystals are formed during quenching. The obtained bars were annealed at 600°C for 12 hours and then were polished with SiC abrasive papers, up to 2400 grit. A part of the bars was cut in small slices (around $5 \times 5 \times 2\text{mm}^3$). Another part was ground in a planetary ball mill and the obtained powder was sieved under $20\mu\text{m}$.

2.2. Characterisation of the ferrimagnetic glass-ceramic

2.2.1. X-ray diffraction (XRD)

X-ray diffraction (XRD) patterns were registered by a Philips X'Pert diffractometer with Cu K α radiation, between 10° and 75° (2 θ), at 40kV and 30 mA. The phase identification was performed by X'Pert HighScore program, with PCPDFWIN database.

2.2.2. Scanning electron microscopy (SEM)

The glass-ceramic morphology was analysed by scanning electron microscopy (SEM), using a FEI, QUANTA INSPECT 200 model. In order to analyse the crystals morphology, the polished glass-ceramic bulk samples were first etched for 4min with a solution of 5% volume of HF:HNO₃ in a molar ratio 1:1, and then coated by a thin layer of silver.

2.2.3. pH analysis

The variation of the pH during soaking the samples in 15ml SBF, prepared after Kokubo's protocol [21] over one month period, was measured by a pocket sized pH-meter (Hanna Instruments). Both bulk and powder samples were used. The bulk samples had dimensions around 5 x 5 x 2 mm³, while the powders (0.2 g/sample) had the particles size less than 20 μ m. The samples were kept at 37 °C in an incubator. The SBF solutions were refreshed twice a week for both powders and bulk samples. For the bulk samples, the pH was measured without refreshing the SBF solution. Four samples were used for each set of experiments and the average pH value was calculated.

2.2.4. Atomic absorption spectrophotometry (AAS)

Samples containing 0.2 g of SC45 powder were immersed in 15ml SBF solution and respectively distilled water, and kept at 37°C in an incubator for a maximum of four weeks. The variation of the concentration of Ca, Si, Na and Fe released during soaking the SC45 powder samples in both distilled water and SBF was measured by a Perkin-Elmer 1100B Atomic Absorption Spectrometer equipped with an air-acetylene flame burner. Each chemical element was measured individually. The experiments were performed in triplicate.

2.2.5. X-ray photoelectron spectroscopy (XPS)

X-Ray photoelectron spectroscopy (XPS) spectra were registered on a Surface Science Instrument (M-Probe) with a monochromatic Al K α X-ray source. The detailed spectra of oxygen, carbon and silicon regions (O1s, C1s and respectively Si2p) were analysed separately. The take-off angle to the substrates was 90°, the pressure was approximative 10⁻⁸ Torr and the pass energy used to determine

the elemental composition was 30 eV. Two sets of bulk samples were analysed: SC45 samples before and after soaking in SBF for one week (samples denominated SC45+SBF).

2.2.6. *Electron paramagnetic resonance (EPR)/ spin trapping*

The generation of free radicals was monitored by electron paramagnetic resonance (EPR) spectroscopy using a Miniscope MS 100 (Magnettech, Berlin, Germany) spectrometer. 5,5-Dimethyl-1-Pyrroline-N-Oxide (DMPO) was employed as the spin trapping agent. The instrument settings were the following: microwave power 10 mW, modulation 1000 mG, scan range 120 G and the centre of field approximately 3345 G.

2.2.6.1 . *Surface-driven Fenton reactivity (target molecule H_2O_2)*

SC45 was suspended in a H_2O_2 (0.04 M) buffered solution (potassium phosphate buffer, KPB 125 mM, pH 7.4) at the dose of 45 mg/ml in the presence of DMPO (0.075 M) as spin-trapping agent. During the experiment, the suspension was continuously stirred. 50 μ l of the suspension were withdrawn after 10, 30 and 60 minutes of incubation and the EPR spectra were recorded. The experiments were performed in triplicate. In order to assess the persistence of the reactivity of SC45 after prolonged contact with biofluids, the same experiments were carried out on SC45 previously incubated with SBF for one week (SC45+SBF samples). Blanks experiments were performed in parallel in the absence of SC45 powders.

2.2.6.2. *Generation of carbon centred radicals (target molecule formate ion)*

SC45 was suspended in a sodium formate (1 M) buffered solution (KPB, 250 mM, pH 7.4) at the concentration of 45 mg/ml in the presence of DMPO (0.075 M) as spin-trapping agent. During the experiment, the suspension was continuously stirred. 50 μ l of the suspension were withdrawn after 10, 30 and 60 minutes of incubation and the EPR spectra were recorded. Same experiments were performed on SC45+SBF samples. The signals were double integrated and the values obtained were expressed as arbitrary units. Blanks experiments were performed in parallel in the absence of SC45 powders. All the experiments were carried out in triplicate.

2.2.7. *Cell adhesion and proliferation tests*

Adhesion and proliferation studies were performed using 3T3 murine fibroblasts. The cells were cultured at 37 °C in 95% air, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Invitrogen Corporation), 1% penicillin streptomycin solution (Cambrex) and 1% L-Glutamine (Cambrex);

SC45 and SC45+SBF (after one week of soaking in SBF) bulk samples were first sterilized in an oven at 180 °C for 3 h before the cellular tests. The cell density was 2×10^4 cells/cm² for adhesion tests and respectively 5×10^3 cells/cm² for proliferation tests. Both adhesion and proliferation tests were performed in triplicate. The negative control (CTR) was carried out on polystyrene without adding any glass-ceramic sample. After 3 h for adhesion and respectively 72 h for proliferation, the samples were rinsed twice in a phosphate buffered saline solution (PBS) and then stained with a 0.025% Acridine Orange solution. Cell number, morphology and vitality on each material were analysed using a fluorescence microscope (Leitz Aristoplan) at 250x magnification. The resulted cell number is expressed as a mean of 10 random fields of 0.2 mm² for each sample. The experiments were carried out in triplicate and thus 30 random fields (n=30) were analysed for each set of samples (SC45, SC45+SBF and CTR), for both adhesion and proliferation tests. The data were expressed as mean $\pm$ standard deviation. Considering CTR=100%, the relative percent of 3T3 cells attached to SC45 and SC45+SBF samples can be calculated by dividing their corresponding mean cell numbers by the mean cell number of the control samples.

Cell morphology was also studied by SEM analysis. In this case, a cell density of 1×10^4 cells/cm² was used. After 36 h of culture, the cells were washed with 0.1M sodium cacodylate buffer and then fixed for 30 min at 4°C in a solution containing 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4. The samples were then dehydrated in a series of ethanol solutions (50%, 70%, 85%, 95% and 100%) and fixed in hexamethyldisilazane. The obtained samples were gold coated using an ion sputter and then examined using a SEM Philips 525 microscope at an accelerating voltage of 10 kV.

2.2.8. *Citotoxicity*

In-vitro cytotoxicity assessment of SC45 glass-ceramics was performed by qualitative evaluation of osteoblast cells morphology based on confluence, vacuolization and cell death analysis. U2OS human bone osteosarcoma cells were used for this purpose.

Ferrimagnetic glass-ceramic powders before (SC45) and after one week of soaking in SBF (SC45+SBF) were sterilized at 180 °C for 3 h in an oven. U2OS cells were seeded into 6-wells plates at a density of 5×10^4 cells/well in DMEM medium supplemented with 10% fetal bovine serum 1% penicillin streptomycin solution and 1% L-Glutamine at 37°C under a humidified atmosphere with 5% CO₂. Different concentrations of glass-ceramic particles were suspended in serum free medium using an ultrasonic bath for 10min to prevent agglomeration. After 24h, the medium with 10% FBS was removed and replaced by 3 ml/well particle suspensions at the following dilutions: 1:10, 1:100, 1:1000, 1:10000 and 1:100000. Controls were carried out with

U2OS cells treated with an equivalent volume of serum-free medium (3ml/well) without any particles. Cells were incubated for further 72 h. After time intervals of 24 h, 48 h and 72 h of incubation with particle suspensions at different concentrations, cells were examined microscopically by using an inverted microscope (Olympus). The bright field images were recorded at 10X magnification.

2.2.9. Statistical analysis

The data are expressed as mean $\pm$ standard deviation. Statistical analysis of data was carried out using Student's t-test, considering a statistical significance level of 0.05 (95% confidence interval). A calculated p-value higher than 0.05 ($p > 0.05$) indicates no significant difference.

3. Results

3.1. X-ray diffraction

Figure 1 displays a typical XRD pattern of SC45 glass-ceramic powder. This material contains only one crystalline phase, magnetite, which forms during cooling from the melting temperature. The quantity of magnetite crystals was evaluated in a previous work [12] and was estimated around 34 wt%. The theoretical quantity of magnetite is 45 wt% and therefore, the rest of 11 wt% remain in the amorphous matrix.

Figure 1 XRD spectrum of a SC45 powder sample (●=magnetite)

3.2. Scanning electron microscopy

SEM micrographs of the chemically etched surface of the SC45 glass-ceramic samples are shown in Figure 2. The acid solution removed the amorphous phase, leaving visible magnetite crystals. As can be seen, the magnetite is organised in small columns, formed by crystals with dimensions between 5-8 μm (Figure 2.a). A higher magnification SEM image of the central part of the Figure 2.a is presented in Figure 2.b. The foreground crystal has an octahedral shape, typical of cubic crystalline phases. Its mean size is 7.5 μm .

Figure 2 SEM images of a SC45 bulk sample after chemical etching at different magnifications

3.3. pH analysis

The variation of the SC45 samples pH during soaking in SBF for one month is presented in Figure 3. It can be noticed that the pH values of the powders sample are higher than the ones of the bulk samples. Considering the pH data with refresh, the slope of the powders variation (blue line in Figure 3) in the first week is assessed between 7.9 and 8, while for the bulk samples (green line in Figure 3) the slope is assessed between 7.5 and 7.6. The powders have higher specific surface area and are much more reactive. Their pH has a rapid increase in the first two weeks of soaking, even if the SBF solution was refreshed twice/week. This can be explained by the fast ionic exchange between the powders surface and SBF solution, as discussed in the next sections. The highest pH value (8) is reached after one week in SBF. After two weeks, the pH starts to decrease, maintaining a slow variation around the 7.7 value, due to the lower ionic dissolution and SBF refresh.

The pH of the bulk samples progressively increases during one month of soaking, reaching values under 7.7. There is no significant difference between the pH values of the bulk samples with or without the refresh (green and red lines in Figure 3).

Figure 3 pH variation of SC45 samples during SBF soaking (R=with refresh; NR=without refresh)

3.4. Atomic absorption spectrophotometry (AAS)

The variation of the concentration of Ca, Na, Si and Fe ions released in a SBF solution during one month of SC45 powders soaking at 37 °C is shown in Figure 4 (the vertical axis is a $\log_{10}$ scale). Na values are close to 10000 ppm (3-5 orders of magnitude higher than the other ions). Na is released from bioactive glass-ceramics due to the ionic exchange with the solution. SBF contains a high quantity of Na and the variation of this ion during one month is not significant due to the instrument limitation. On the contrary, the values of iron released in SBF at 37 °C are under 0.1 ppm (mg/l).

Figure 4 Variation of concentration of different ions released in SBF solution during one month of SC45 powder immersion at 37°C (vertical axis is a $\log_{10}$ scale)

This result is quite important for the biocompatibility assessment of the SC45 glass-ceramics. Both Si and Ca values slightly increase during one month in SBF, due to the ionic exchange between the powders samples and solution.

In order to analyse the concentration of these ions released in a basic (simplified) solution, distilled water was considered. The variation of the ions released at 37°C during two weeks of immersion of SC45 powders in distilled water can be seen in Figure 5.

Figure 5 Variation of concentration of different ions released in distilled water solution during two weeks of SC45 powder immersion at 37°C (vertical axis is a $\log_{10}$ scale)

In this case, Na concentration continuously increases after 3 days and the slope is of three orders of magnitude (vertical axis is a $\log_{10}$ scale). Na concentration starts from under 1 ppm values and reaches values around 80 ppm after two weeks in distilled water, indicating a fast ions exchange. Silicon concentration rapidly increases during the first 3 days and after then displays a slow raise. Iron is completely released in the first day of soaking and after then its concentration remains almost constant. Fe values are also in this case under 0.1 ppm. Ca concentration increases in the first 24 h and after then slightly decreases up to values around 1 ppm (after 2 weeks). Therefore, considering the distilled water, Na and Ca exhibit a more evident variation of their concentration, compared with Si and Fe ions.

3.5. X-ray photoelectron spectroscopy (XPS)

XPS spectra of the silicon, carbon and oxygen regions before (SC45 sample) and after 7 days of pre-treatment in SBF (SC45+SBF sample) are shown in Figure 6.

Figure 6 XPS graphics of the Si2p (a, b), C1s (c, d) and O1s (e, f) peaks for the SC45 samples before (a, c, e) and after one week of soaking in SBF (b, d, f).

Before the SBF treatment, the SC45 sample presents one silicon peak at around 102 eV, characteristic to silica glass (Figure 6.a). After SBF pre-treatment, XPS detects a new peak at higher energy (103.9 eV), typical of silica gel (Figure 6.b). Similarly, the carbon regions before (Figure 6.c) and after SBF (Figure 6.d) are different. The carbon peak at around 286 eV was attributed to carbonates species. It can be noticed that this peak increases after immersing the samples in SBF. Before SBF soaking, the oxygen peak (Figure 6.e) was deconvoluted into three spectral bands at

about 530 eV, 532 eV and 533 eV. The low energy peak at 530 eV is characteristic to oxygen in different metal oxides. The peak at 532 eV is attributed to oxygen in silica. The higher energy peak at approximately 533 eV is due to oxygen in silanols, hydroxyls or silica gel. After SBF pre-treatment, the silica gel peak of the oxygen region (533 eV) considerably increases (Figure 6.f), in accordance with the results obtained for the silicon region. The silicon peak is monoenergetic before SBF and bienergetic after SBF, due to the formation of a silica gel layer on the sample surface. The increase of the carbonates peak after SBF pre-treatment indicates a possible formation of a thin layer of hydroxycarbonate apatite (HCA), according to the bioactivity mechanism of bioactive glasses [22].

3.6. Radical reactivity of SC45 towards molecules of biological relevance

Two free radical driven reactions were employed to investigate the oxidative potential of SC45, namely: i) the Fenton-like reactivity towards H_2O_2 , and ii) the rupture of a C-H bond in the formate molecule.

In the first experiment, referred to as Fenton-like reaction, H_2O_2 is allowed to interact with redox-reactive transition metal ions (generally iron) at the particles surface and the generation of $\text{HO}\cdot$ radicals is observed. The release of $\text{HO}\cdot$ may take place when a particle is exposed to the oxidative environment reach in H_2O_2 of lysosomal fluid following phagocytosis by macrophages and polymorphonucleated cells (PMN) [23]. Moreover it was observed that also osteoblasts can engulf particles through lysosomal-like elements [24].

The second free radical reaction investigated is based on the cleavage of C-H bond of formate ion which is employed as a simple model of several biomolecules (including peptides, proteins and lipids). In the presence of reactive dusts, such reaction possibly occurs via a direct surface-assisted homolytic cleavage driven by redox-reactive transition metal (including iron) at the particles surface. The carbon centred radical produced, i.e. $\cdot\text{CO}^-$, reacts with DMPO forming a stable paramagnetic adduct which is detectable by EPR spectroscopy. The results obtained for both experiments are reported in Figure 7. Employing DMPO as spin-trapping agent, the ability of SC45 to induce $\text{HO}\cdot$ radical upon contact with H_2O_2 was investigated. In Figure 7A the EPR spectrum recorded after 60 minutes of incubation shows the typical signal of DMPO- $\text{HO}\cdot$ adduct indicating that SC45 is able to react with H_2O_2 via a Fenton-like mechanism.

In order to assess the persistence of such reactivity after contact with biofluids, the same experiment was carried out on SC45 previously incubated in SBF at 37 °C for one week (SC45 + SBF). Also in this case the EPR signal of DMPO- $\text{HO}\cdot$ adduct was recorded. The signal intensities are virtually the

same with both the samples indicating that one week of incubation with SBF did not decrease the surface Fenton-like reactivity of SC45.

Figure 7 EPR spectra recorded after 60 minutes of incubation of a suspension of SC45 (45mg/ml) and SC45+SBF (45 mg/ml) in a potassium phosphate buffered solution (KPB, pH 7.4) of **A)** H_2O_2 and **B)** sodium formate, in the presence of DMPO as spin trapping agent. Blank experiments were carried out in the same conditions without SC45 powders.

In Figure 7.B the EPR spectra recorded after 60 minutes of incubation of SC45 with a buffered solution of sodium formate and DMPO are reported. A rather intense signal was detected. The effect of a prolonged contact with biofluids on the reactivity of SC45 was investigated employing SC45+SBF. In this case, no signal was detected indicating that the incubation in SBF caused the complete inactivation of the reactive centres responsible for such reactivity.

3.7. Cell adhesion and proliferation tests

The average percentage of 3T3 fibroblast cells attached to the SC45 and SC45+SBF samples after 3h of incubation is presented in Figure 8. These results are relative to the polystyrene control which was considered 100%. Therefore, around 60% of the cells adhered to the glass-ceramic surface. Considering the 95% confidence interval, there is no significant difference between the SC45 and SC45+SBF samples.

Figure 8 Average percentage of 3T3 cells relative to the polystyrene control, attached to the materials after 3 hours (adhesion). CTR was considered 100%.

Figure 9 shows the relative percent of 3T3 cells attached to the samples surface after 3 days of incubation. Around 25% cells proliferate on the SC45 samples, whereas for the SC45+SBF samples, around 45% of cells are attached. Therefore, almost two times more cells proliferated on the pre-treated samples.

Figure 9 Average percentage of 3T3 cells relative to the polystyrene control, attached to the materials after 3 days (proliferation). CTR was considered 100%.

Student's t-test shows a significant difference between the two sets of samples. The difference probably arises from different morphological surface features. The surface chemical reactions which occur during the SBF soaking modify the surface characteristics of the SC45+SBF glass-ceramic samples, leading to a favourable environment for the biological species.

SEM images of the 3T3 cells attached to the SC45+SBF and polystyrene control, after 36h of incubation are displayed in Figure 10.

Figure 10 SEM images of the 3T3 cells attached to the SC45+SBF (a and b) and polystyrene control (c and d) after 36h

It can be immediately noticed the different morphologies of the two surfaces: the rough and the irregular surface of the SC45+SBF sample (Figure 10.a and 10.b) in relation to the flat surface of the CTR (Figure 10.c and 10.d) sample. The cells grow normally on both substrates, spreading their

filopodia over the materials surface. Higher magnification images of fibroblast cells layer extended over the SC45+SBF and CTR samples can be seen in Figure 10.b and Figure 10.d, respectively. The cells size is larger for the CTR samples. Regarding the SC45+SBF substrate, the 3T3 cells maintain their morphological characteristics, with distinct flat shape and extended filopodia, but have smaller size compared to the CTR reference.

3.8. Citotoxicity

Optical microscopy panel images of the U2OS cells at different times of incubation, using SC45-powders suspensions at different dilutions are presented in Figure 11. The results show that cells do not display a clear sign of apoptosis or major alterations in the cell growth. However, at lower dilution rates (1:10, 1:100) the number of cells grown into the well plates is much lower than the one for higher dilution rates. The morphology of the cells for higher dilution rates of the SC45-powders suspensions (1:1000, 1:10000 and 1:100000) looks similar to the one of the control. The cells reach about 80% confluency after 72h of cells culture. For the 1:10 dilution rate, U2OS cells have around 30% confluency, whereas for 1:100 dilution rate they are about 40% confluency after 72 h of incubation. It seems that the presence of SC45 particles agglomerations does not affect the cells attachment (see Figure 11.c, panel 48h, 1:10 dilution). Similar results were obtained for SC45+SBF-powders suspensions (Figure 12). Even for lower dilution rates, the cells morphology and density are similar to the ones of the control. A layer of normally spread cells can be observed for all samples (including 1:10 dilution one). This qualitative evaluation demonstrated no change of cellular morphology for cells exposed to SC45+SBF-powders suspensions. After 72h incubation, a confluent layer of cells was formed in all these wells.

a) 0h

b) 24h

c) 48h

d) 72h

Figure 11 Optical microscopy panel images of the U2OS cells before (a) and after 24h (b), 48h (c) and 72h (d) in SC45-powders suspensions at the following dilutions (DIL): 1:10; 1:100; 1:1000; 1:10000; 1:100000. CTR- is the control sample without SC45-powders suspensions. All images are recorded at 10X magnification.

a) 0h

b) 24h

c) 48h

d) 72h

Figure 12 Optical microscopy panel images of the U2OS cells before (a) and after 24h (b), 48h (c) and 72h (d) in SC45+SBF-powders suspensions at the following dilutions (DIL): 1:10; 1:100; 1:1000; 1:10000; 1:100000. CTR- is the control sample without SC45+SBF-powders suspensions. All images are recorded at 10X magnification.

4. Discussion

The biological applications of magnetic materials require a previous evaluation of toxicity. These features depend on physical state, chemical composition and surface morphology. Thus, *in vitro* biological evaluation is a key factor for biocompatibility assessment of the ferrimagnetic glass-ceramics.

These ferrimagnetic glass-ceramics containing magnetite crystals have been studied in the past for hyperthermia applications. An accurate characterisation of these materials has been previously done. The microstructure, crystals morphology, thermal and magnetic properties, power loss and bioactivity behaviour of these glass-ceramic materials have been analysed in our previous studies [11, 12, 13, 14]. The process parameters have been optimised in order to obtain magnetite as the unique crystalline phase embedded into the amorphous structure. As shown in Figure 1, XRD analysis evidenced that magnetite was the only crystalline phase formed during cooling from the melting temperature. Magnetite crystals exhibited octahedral shape and dimensions less than 10µm (Figure 2). As shown in [12], not all the iron oxides crystallized as magnetite; around 11wt% remain inside the glassy phase. The effect of iron ions that might be released into surrounding tissues was studied by AAS. Due to higher specific area of powders samples compared to the bulk ones, the amount of ions released by powders is expected to be higher than the corresponding amount of bulk specimens. Therefore, the release of iron ions was analysed on powders samples in both SBF and distilled water fluids. The results showed that the iron concentration into both fluids at 37°C does not exceed 0.1mg/l, (see Figure 4 and Figure 5). Therefore, the presumable cytotoxic effect of these glass-ceramic due to the iron release is minimal. The concentration of silicon and calcium released into SBF solution slightly increases during one month of immersion at 37°C. These results are in agreement with the previous studies on the reaction kinetics of bioactive glasses [22]. On the first stages of immersion of a bioactive glass in an aqueous solution, an ion exchange process between the network-modifying ions and H_3O^+ ions occurs, followed by the breaking of the glass network. Breaking of -Si-O-Si-bonds involves the silica release into solution and the silanols formation. By polycondensation of the silanols, a silica-rich layer is formed on the glass surface. Due to the continuous ionic exchange process between the materials surface and solution, the concentration of calcium and phosphate ions released into SBF increases. When SBF solution becomes saturated with calcium and phosphate, these ions precipitate, forming a calcium phosphate rich layer, on the top of the silica gel layer. Later on, during the last stage of *in vitro* bioactivity reactions in acellular SBF, the calcium phosphate rich layer crystallizes to a hydroxycarbonate apatite (HCA) structure, incorporating carbonate anions from solution.

Therefore, the increase of Na, Ca and Si concentrations, determined by AAS studies (Figure 4 and Figure 5) is confirmed by Hench's studies on the bioactivity mechanism. The ion exchange process leads to an increase of the pH value. In fact, Figure 3 shows a rapid increase of the pH during the SBF soaking for both bulk and powder samples. High values of pH, over the physiological limits, might damage the cellular membrane leading to cell death. To reduce the initial pH increase, the samples can be pre-treated into aqueous solutions before biological evaluation.

XPS studies on the ferrimagnetic glass-ceramic samples (Figure 6) show the formation of a silica gel layer after SBF soaking, in accordance with AAS and pH measurements. The increase of the carbonates peak after SBF pre-treatment (Figure 6.d) indicates a possible formation of a thin layer of hydroxycarbonate apatite (HCA), according to the bioactivity mechanism of bioglasses.

In order to assess the oxidative potential of SC-45, the free radical-mediated reactivity was measured. SC-45 exhibited the capability to generate HO• radical in the presence of H₂O₂ (Fenton-like reactivity) (Figure 7.a), as well as to cleave the C-H bond of formate molecule (Figure 7.b). After pre-treatment with SBF the HO• radical yield remained unvaried, whereas the reactivity towards formate was completely suppressed. This difference can reside in the different nature of the reactive sites involved. It is expected that the prolonged contact with SBF promoted the oxidation of Fe²⁺_{surf} to Fe³⁺_{surf} at the surface of SC-45. Fenton reaction is driven by Fe²⁺ following eq. (1):

Moreover, H₂O₂ is able to reduce Fe³⁺_{surf} to Fe²⁺_{surf} (eq.2); Fenton reaction is also observed in the presence of Fe³⁺ [25]:

This reaction restores the former supply of Fe²⁺_{surf} oxidized to Fe³⁺_{surf} in SBF. This can account for the sustained HO• release observed with SC45+SBF. At the contrary, the homolytic cleavage of C-H bond of formate has been observed only with Fe²⁺ [25]. In our experimental conditions, the depletion of Fe²⁺_{surf} following its oxidation in SBF can account for the complete suppression of such reactivity.

The SBF pre-treatment may stimulate different biological species, enhancing the materials integration [26]. Therefore, a comparison study between untreated and pre-treated samples was accomplished. Consequently, the *in vitro* biocompatibility of these ferrimagnetic glass-ceramics was assessed by using two set of samples: SC45 (untreated) and SC45+SBF (samples pre-treated in

SBF for one week). The results showed a better biological behaviour of the glass-ceramic sample pre-treated in SBF solution, compared with the untreated one. In fact, on the first stage of the biological assessment (adhesion test), no significant difference between the SC45 and SC45+SBF samples is obtained. Almost 60% of 3T3 cells adhered to the both substrate surfaces (Figure 8). Regarding the proliferation test, the percentage of the cells which grew on the SC45+SBF samples is higher than the one of the SC45 (Figure 9); about 80% more cells proliferated on the pre-treated samples. In this case, the t-test probability is less than 0.05, so the difference between the SC45 and SC45+SBF samples is significant. SC45+SBF samples favour the 3T3 proliferations, probably due to the silica gel layer formed on the samples surface after one week of SBF soaking. Figure 10 shows the typical observed morphologies of 3T3 cells after 36h of incubation. SEM images evidence the presence of healthy cells with a good level of spreading. A clear micrograph of the cells filopodia which spread over the SC45+SBF sample surfaces can be seen in Figure 10.b.

To evaluate toxicity we performed bright field images with inverted microscope to analyze cell death (apoptosis) and cell density (cell growth) at 0h, 24h, 48h and 72h incubation. This qualitative evaluation demonstrated no change of cellular morphology for cells incubated with the ferrimagnetic glass-ceramic powders suspensions.

Morphological examination of cells which had been incubated with SC45 powder suspensions does not reveal any cytotoxic effect (Figure 11). However, we observed a decrease of confluence for U2OS cells exposed to the 1:10 dilution rate at 48 and 72 hours; the cells maintain their normal flat morphology with a distinct cell shape, but were not longer confluent after 3 days of incubation.

Incubation of U2OS cell lines with SC45+SBF powder suspensions at intervals of 24h, 48h and 72 hours did not influence the morphology of the cells (Figure 12). A layer of normally spread cells can be observed with only minor changes of confluence, vacuolization and morphological parameters.

5. Conclusions

In vitro biological characterisation of a ferrimagnetic glass-ceramic containing magnetite crystals have been carried out by using two sets of samples: samples pre-treated in a simulating body fluid (SBF) and samples without the pre-treatment. The SBF pre-treatment induces the formation of a silica-gel layer on the materials surface, which stimulates the cellular function of biological species. The concentration of iron released into the SBF solution does not exceed 0.1mg/ml at 37°C, during one month of soaking. Free radical mediated reactivity has a recognized role in particle toxicity. HO• release (Fenton reactivity) and cleavage of C-H bond of formate ion were assessed as indexes of the oxidative potential of the SC45 glass-ceramic samples. SC45 was active in both the tests. Pre-

treatment with SBF completely annealed the reactivity towards C-H bond of formate, very probably due to the oxidation of $\text{Fe}^{2+}_{\text{surf}}$ centres. Cell adhesion and proliferation tests were carried out on 3T3 murine fibroblasts cell line. The adhesion test shows that there are not significant differences between the untreated and SBF treated samples during the first hours of incubation. The difference appears after one day of cell culture. The proliferation test reveals that there is a significant difference between the two sets of samples after 3days of incubation. About 80% more cells were attached to the pre-treated samples after 3days of incubation, compared to the untreated samples. Cytotoxicity was performed by qualitative evaluation using U2OS osteoblast cells. Morphological examination of cells does not reveal any cytotoxic effect. The cells incubated with untreated samples display a decrease of confluence. It seems that the SBF pre-treatment of these ferrimagnetic glass-ceramic acts as a surface activation process, which leads to an increase of the cells activity.

In conclusion, the results reported in the present paper indicate that the SBF pre-treatment can contribute to an increase of the biocompatibility of SC45 glass-ceramics by modifying their surface reactivity. The SBF pre-treatment can be also considered as a surface activation process, which implies the stimulation of the cellular functions, facilitating the tissue integration.

Acknowledgments

The Piedmont Region (Italy) is kindly acknowledged for the research grant supplied. (Progetto Regionale sulla Ricerca Scientifica Applicata, settore Salute e Scienze Mediche 2005-2006, “Biomateriali per terapie non invasive nel trattamento in ipertermia dei tumori”).

References

ACCEPTED MANUSCRIPT

In vitro biocompatibility of a ferrimagnetic glass-ceramic for hyperthermia application

Highlights

- *In vitro* characterisation of a ferrimagnetic glass-ceramic containing magnetite crystals have been carried out, before and after treatment in Simulated Body Fluid (SBF).
- The SBF pre-treatment stimulates the cellular function and acts as a surface activation process, which increases the cells activity.
- Pre-treatment with SBF annealed the reactivity towards C-H bond of formate.

1 D. Bahadur, J. Giri, *Sadhana*, 2003, **28**, 639–656.

2 A. K. Gupta, M. Gupta, *Biomaterials*, 2005, **26**, 3995–4021.

3 S. Pietronave, M. Iafisco, D. Locarno, L. Rimondini, M. Prat, *Journal of Applied Biomaterials & Biomechanics*, 2009, **7**, 77-89.

4 N. F. Borrelli, A. A. Luderer, J. N. Panzarino, *US Patent 4323056*, year 1982

5 Y. K. Lee, S. Y. Choi, *Journal of the American Ceramic Society*, 1996, **79**, 992-996.

6 S. H. Oh, SY Choi, YK Lee, K. N. Kim, *Journal of Biomedical Materials Research*, 2001, **54**, 360-365.

7 Y. Ebisawa, F. Miyaji, T. Kokubo, K. Ohura, T. Nakamura, *Biomaterials*, 1997, **18**, 1277-1284

8 D. Arcos, R. P. del Real, M. Vallet-Regi, *Journal of Biomedical Materials Research*, 2003, **65A**, 71–78.

9 B. N. Gray, S.K. Jones, *United State Patent 6167313*, year 2000.

10 G. H. Nussbaum, *Physical aspects of hyperthermia*, American Institute of Physics, 1982

11 O. Bretcanu, S. Spriano, E. Verné, M. Cöisson, P. Tiberto, P. Allia, *Acta Biomaterialia*, 2005, **1**, 421-429

12 O. Bretcanu, E. Verné, M. Cöisson, P. Tiberto, P. Allia, *Journal of Magnetism and Magnetic Materials*, 2006, **305**, 529-533.

- 13 O. Bretcanu, S. Spriano, C. Vitale Brovarone, E. Verné, *Journal of Materials Science*, 2006, **41**, 1029-1037.
- 14 O. Bretcanu, E. Verné, M. Cöisson, P. Tiberto, P. Allia, *Journal of Magnetism and Magnetic Materials*, 2006, **300**, 412-417.
- 15 E. Verné, O. Bretcanu, C. Balagna, C. L. Bianchi, M. Cannas, S. Gatti, C. Vitale-Brovarone, *Journal of Materials Science: Materials in Medicine*, 2009, **20**, 75-87.
- 16 J. M. Oliveira, R. N. Correia, M. H. Fernandes, *Biomaterials*, 2002, **23**, 371-379.
- 17 M. Bosetti, M. Cannas, *Biomaterials*, 2005, **26**, 3873-3879.
- 18 K. Hurrell-Gillingham, I. M. Reaney, I. Brook, P. V. Hatton, *Journal of Dentistry*, 2006, **34**, 533-538
- 19 F. Turci, I. Corazzari, G. Alberto, G. Martra, B. Fubini, *Astrobiology*, 2015, **15**, 371-380.
- 20 C. J. Horwell, G. W. Stannett , D. Andronico, A. Bertagnini, I. Fenoglio , B. Fubini , J. S. Le Blond, B. J. Williamson, *Journal of Volcanology and Geothermal Research*, 2010, **191**, 222–232.
- 21 T. Kokubo, H. Kushitani, S. Sakka, *Journal of Biomedical Materials Research*, 1990, **24**, 721-734
- 22 L. L. Hench, J. *An introduction to bioceramics*, Imperial College Press, 2013, chapter 3, 49-69.
- 23 E. Gazzano, F. Turci, E. Foresti, M. G. Putzu, E. Aldieri, F. Silvagno, I. G. Lesci, M. Tomatis, C. Riganti, C. Romano, B. Fubini, N. Roveri, D. Ghigo, *Chemical Research in Toxicology*, 2007, **20**, 380-387.
- 24 C.H. Lohmann, Z. Schwartz, G. Kokster, U. Jahn, G. H. Buchhorn, M. J. MacDougall, D. Casasola, Y. Liu, V. L. Sylvia, D. D. Dean, B. D. Boyan, *Biomaterials*, 2000, **21**, 551-561.
- 25 F. Turci, M. Tomatis, I. G. Lesci, N. Roveri, B. Fubini, *Chem. Eur. J.*, 2011, **17**, 350 – 358
- 26 R. Barbucci, *Integrated Biomaterials Science*, Kluwer Academic/Plenum Publishers, New York, chapter 5, 2002.