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Phase assemblage study and cytocompatibility property of heat treated potassium magnesium phosphate–silicate ceramics

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Abstract This article reports the study on a new generation bioactive ceramic, based on $MgKPO_4$ (Magnesium Potassium Phosphate, abbreviated as MKP) for biomedical applications. A series of heat treatment experiments on the slip cast silica (SiO_2) containing MKP ceramics were carried out at 900, 1,000 and 1,100°C for 4 h in air. The density of the slip cast ceramic increases to 2.5 gm/cm^3 upon heat treatment at 900°C. However, no significant change in density is measured upon heat treatment to higher temperature of 1,000 and 1,100°C. On the basis of XRD results, the presence of $K_2MgSi_5O_{12}$ and dehydrated $MgKPO_4$ were confirmed and complementary information has also been obtained using FT-IR and Raman spectroscopy. In order to confirm the in vitro cytocompatibility property, the cell culture tests were carried out on selected samples and the results reveal good cell adhesion and spreading of L929 mouse fibroblast cells. MTT assay analysis with L929 cells confirmed non-cytotoxic behavior of MKP containing ceramics and the results are comparable with sintered HAp ceramics. It is expected that the newly developed MKP based materials could be a good substitute for hydroxyapatite (HAp or HA) based bioceramics.

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1 Introduction

In last decade, room-temperature setting chemically bonded ceramics is being developed for encapsulation and containment of hazardous and radioactive species. Some of the systems that have been developed are based on phosphates of Mg, Zr, Na, Ca and Fe. One of the systems, magnesium potassium phosphate ($MgKPO_4 \cdot 6H_2O$) has unique properties such as high strength and excellent chemical durability that has made it an attractive material for numerous structural and environmental applications. For example, the $MgKPO_4 \cdot 6H_2O$ compound, in particular, has been researched for a completely different application, i.e. the treatment of high level waste such as technetium, mercury and heavy metals as lead, cadmium and nickel [1, 2]. A critical literature search did not yield any report on the biomedical application of this material.

Hydroxyapatite (HAp) or, in general calcium phosphate (CaP), because of its composition closer to inorganic phase of human cortical bone as well as excellent biocompatibility, is proved to be a widely accepted bioactive implant material [3–7]. Some of the calcium phosphate materials in order of solubility include, tetracalcium phosphate ($Ca_4P_2O_9$) > amorphous calcium phosphate > alpha-tricalcium phosphate ($Ca_3(PO_4)_2$) > beta-tricalcium phosphate ($Ca_3(PO_4)_2$) ≫ hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) [8].

In the above perspective, it would be of interest to assess whether chemically bonded phosphate ceramics (CBPCs), like those based on magnesium potassium phosphate system can be used for biomedical applications. The biomaterial, MKP, used in our study is produced at low temperature by the acid–base reaction between magnesium oxide and potassium phosphate in aqueous environments. In general, some amount of silica (SiO_2) was also added during the synthesis process to improve the structural

integrity. In an exploratory set of experiments, the slip cast ceramic was heat treated at 900, 1,000 and 1,100°C for 4 h in air. Subsequently, the phase evolution was studied using XRD, FT-IR and Raman spectroscopy. An important aspect of the present study is to report the results of the cytocompatibility and cytotoxicity (MTT) to support the in vitro biocompatibility property of the investigated ceramic. It can be mentioned here that an important part of research to develop new biocompatible materials [9] focuses on characterizing the in vitro properties of biomaterials under scientifically controlled conditions. To this end, the in vitro cytotoxicity assays are considered as the primary biocompatibility screening tests for a wide variety of implant materials. For example, the tissue culture test methods are used as acute screening test for biocompatibility testing of materials [10]. Initially, the result of the heat treatment experiments on the slip cast MKP will be presented. This will be followed by the results of the in vitro cell adhesion and cell proliferation tests on heat treated ceramics using L929 fibroblasts cell lines.

2 Experimental procedure

2.1 Fabrication of MKP ceramics

The MKP ceramic was synthesized by reacting desired amounts (as per reaction 1) of calcined magnesium oxide (MgO) powder with potassium phosphate (KH_2PO_4) in aqueous conditions [1]. The nominal particle sizes for commercial MgO (Martin Marietta, Magnesia Specialities, Baltimore, MD) and KH_2PO_4 (FMC Corp., Philadelphia, PA) powders were 30 and 50 μm, respectively. The slurry formed was mixed for about 30 min and was subsequently poured/cast in molds. In approximately 2 h, the slurry sets up into a hard ceramic. The reaction for the binding phase can be represented as:

$$MgO + KH_2PO_4 + 5H_2O = MgKPO_4 \cdot 6H_2O. \quad (1)$$

The resulting hydrated $MgKPO_4 \cdot 6H_2O$ (MKP) phase is extremely stable, with a solubility product of 2.4×10^{-11} under ambient conditions [11]. Gibbs free energy change for the reaction described by reaction 1 is −969.8 kJ/mol at 25°C, which indicates the feasibility of spontaneous formation of MKP. Typically, the formation of $MgKPO_4 \cdot 6H_2O$ involves two steps: dissociation and dissolution of the binding components in water and an acid–base reaction between the dissociated ions. In addition to the base powders, various filler powders such as silica or silicates can be incorporated with MgO and KH_2PO_4. In the present study, up to 50 wt% of silica (commercially available, size <50 μm) filter was added in the dry powder mix. After the step described by reaction 1, heat treatment of the as slip cast ceramic pellets were carried out at 900, 1,000 and 1,100°C for 4 h in conventional pressureless sintering furnace. As discussed below, it was observed that there was chemical reaction between silica filler and MKP ceramic constituents during elevated temperature treatments. Through out the text the as-cast, and samples heat treated at 900, 1,000 and 1,100°C for 4 h are designated as MKP-1, MKP-2, MKP-3 and MKP-4, respectively.

2.2 Characterization of sintered ceramics

XRD analysis was carried out using Cu-K_α radiation ($\lambda = 1.54184$ Å) to identify different phases present in the starting material as well as in heat treated materials. The formation of any reaction product was studied by critical analysis of XRD data. The Fourier Transformed Infrared spectra (FT-IR, Vortex 70, BRUKER) were obtained in the range of 400–4,000 cm^{-1} using KBr pellets at room temperature to confirm the presence of phosphate group (PO_4^{3-}). Raman Spectra were recorded with the argon ion laser, attached with Acton AM505F spectrometer in the wave number region of 1,100–200 cm^{-1} at a laser power of 8 mW using an incident light with wavelength of 514.5 nm. The scattered radiation was collected at 180° (backscattering geometry) to the incoming beam and detected using a CCD cooled to −120°C. In addition to MKP-1, MKP-2, MKP-3 and MKP-4, the Raman spectra were also acquired from pure HAp (sintered at 1,200°C, 2 h). Compressive strengths of the as-set ceramic was evaluated on universal testing machine (Instron Corp, Norwood, MA) and the average compressive strength was measured to be 52 MPa after 14 days of post slip-cast setting.

2.3 Cell culture experiments

In this study, Mouse fibroblast (L929) cell lines were used for cell-culture experiments. L929 cell lines were obtained from CCMB Hyderabad, India and were cryo-preserved in a liquid N_2 container. Prior to seeding the cells on biomaterials surfaces, the cells were revived. The cryo-vial was rapidly thawed in a water bath at 37°C. Following this, L929 fibroblast cells were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS: Sigma Aldrich) and 1% penicillin/streptomycin solution (Sigma Aldrich). Culture plate was incubated in a standard incubator (5% CO_2 and 90% humidity) at 37°C temperature and the culture medium was replaced every 2 days interval. The sub-confluent monolayer of cells (approximately 5×10^5/ml, in 35 mm culture plate) was harvested from the culture plate using 0.5% trypsin and 0.2% EDTA solution (Sigma Aldrich).

2.4 Cellular adhesion tests

As stated in the Sect. 2.3, L929 cells were maintained in DMEM culture medium supplemented with 10% FBS and 1% penicillin/streptomycin solution. MKP-4 (1,100°C, 4 h) and control glass disc (gelatin coated) were used in the cell adhesion experiments. Also, pure HAp (sintered at 1,200°C, 2 h) was used to compare the results. All the samples were sterilized in an autoclave (121°C, 15 lb pressure) for 15 min and placed under ultraviolet light in a hood for 30 min. Following this, samples were soaked in 70% ethanol for 30 min for sterilization. The surfaces were then washed twice with warm phosphate buffer saline (PBS) and subsequently, the cells were seeded on the samples at density of 3×10^5/ml in four well culture plates. The seeded samples were then incubated in a CO_2 incubator with the previously described environment. After the stipulated time period (2–3 days), the samples were washed twice with PBS and then fixed with 1.5% glutaraldehyde in PBS. The cells, adhered on the materials surface, were dehydrated using a series of ethanol solutions (30, 50, 70, 95 and 100%) for 10 min twice and then further dried using critical point drier (CPD: Quramtech UK). The dried samples were sputter coated with gold and characterization of the cell-cultured samples was carried out using scanning electron microscope (SEM, Philips, Quanta) to understand the cell adhesion behavior.

2.5 MTT assay

First described by Mosmann [12] to detect mammalian cell survival and proliferations, MTT is a rapid colorimetric method. Mitochondrial enzymes of metabolically active cells react with yellow Tetrazolium salt (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT) and form purple formazan crystals. The fibroblast cells (L929) were seeded on sterilized samples at an approximate cell density of 3×10^5/ml (DMEM of cell suspension supplemented with serum and antibiotic). Subsequently, the culture plate was incubated for 48 h in CO_2 incubator. Thereafter, the medium was aspirated and samples were washed twice with PBS and fresh 200 µl DMEM was added (without phenol red and serum). In the next step, 10 µl reconstituted MTT (5 mg/ml in DMEM) was added in each well and plate was incubated for 10 h. In the meantime, the culture plate was viewed in the phase contrast microscope (Nikon-Eclipse80i, Japan) to check for the formation of purple formazan crystal. After the incubation, medium and MTT was removed and MTT–formazan crystal was dissolved by adding 200 µl of DMSO (stop solution). The culture plate was rocked for 15 min. The samples were removed from wells and optical density of the solutions were measured at 490 nm using ELISA automated microplate reader (Bio-Tek, model ELx800). In the present study, the MTT assay was carried out on MKP-(1–4), pure HAp (sintered at 1,200°C, 2 h) and glass slip (negative control). The quantification of the cell viability in terms of metabolically active cells, was calculated using the following formula,

$$\%\,\text{Viability} = (\text{Mean Absorbance of Sample}/\text{Mean Absorbance of Control}) \times 100.$$

3 Results and discussion

In the present study, the slip cast ceramics were heat treated at three different temperatures, i.e. 900, 1,000 and 1,100°C for time period of 4 h. The density of the heat treated ceramics are measured and compared with the untreated sample (Fig. 1). From the density data, it is clear that planned set of heat treatment conditions increases the density to some extent, when heat treated at 900°C for 4 h. However, on increasing temperature to 1,000 or 1,100°C, no significant change in density could be measured. In order to obtain structural information of the heat treated ceramics, various characterization tools, including X-Ray Diffraction, FT-IR and Raman Spectroscopy were utilized.

3.1 X-Ray diffraction pattern

XRD results, obtained with various MKP ceramics, heat treated at 900, 1,000 and 1,100°C for 4 h are shown in Fig. 2. The XRD peaks of MKP ceramics are very sharp, indicative of high degree of crystallinity of the samples. XRD spectra of MKP-1 (without heat treatment) shows the

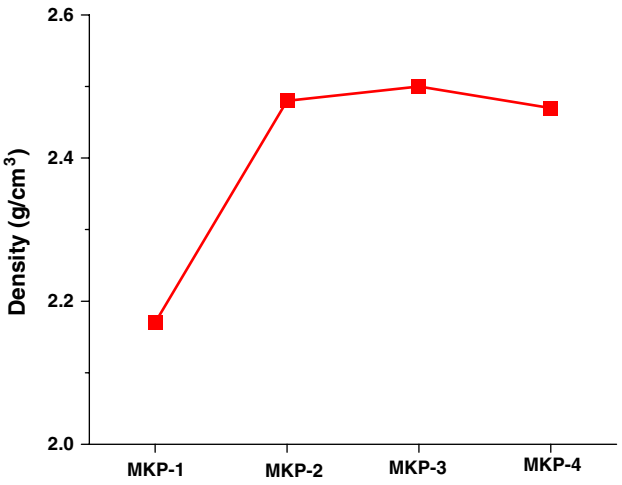

Fig. 1 A comparison of density of MKP as-cast (MKP-1) with MKP heat treated at 900°C (MKP-2), 1,000°C (MKP-3) and 1,100°C (MKP-4) for 4 h

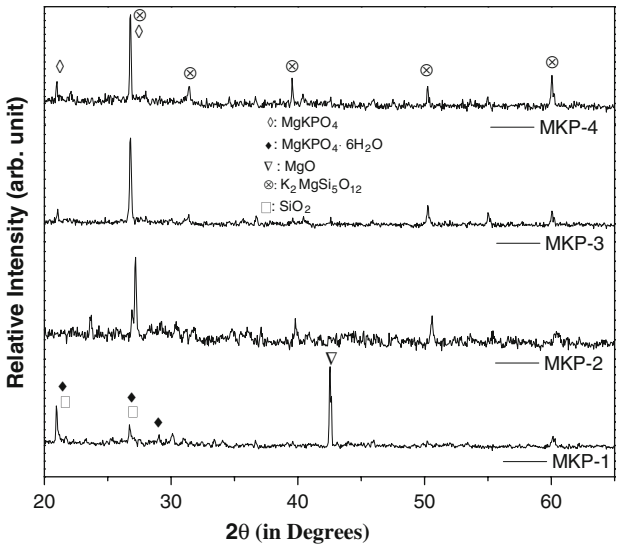

Fig. 2 XRD spectra obtained for MKP as-cast (MKP-1) and MKP sintered at 900°C (MKP-2), 1,000°C (MKP-3) and 1,100°C (MKP-4) for 4 h

presence of MgO (JCPDF # 87-0653) and $MgKPO_4 \cdot 6H_2O$ (JCPDF # 75-1076) phases. The critical search using JCPDF files reveals that the characteristic X-ray peaks of $MgKPO_4$ and $MgKPO_4 \cdot 6H_2O$ phases are close. Also, it should be understandable that MKP phase would loose water molecules upon heating to 900°C or above. In view of such considerations, it has been suggested that the heat treated ceramics should contain dehydrated $MgKPO_4$. On comparing the XRD plots from Fig. 2, it is clear that MgO phase completely disappears on sintering above temperature 900°C. The presence of potassium magnesium silicate ($K_2MgSi_5O_{12}$) phase (JCPDF # 10-0006) is detected in all the sintered samples. The major peaks, detected for the sintered samples within the XRD detection limit were found to be SiO_2, $MgKPO_4$ and $K_2MgSi_5O_{12}$. The strongest peak in all the sintered samples has major contribution from $K_2MgSi_5O_{12}$ phase and some contribution from $MgKPO_4$. It is to be noted here that the main peak from SiO_2 phase appears below 10^o (2θ), which was not covered during XRD measurements. No significant difference in terms of phase evolution in various heat treated ceramics could be noticed in Fig. 2. A closer look at Fig. 2 reveal that better stabilization of MKP phase as well as higher intensity phosphate peak can only be detected after heat treatment. From the above observations, it should be clear that MgO must have reacted with other constituting phases of baseline ceramic (MKP-1) to form $K_2MgSi_5O_{12}$. We propose two possible reactions:

$$MgKPO_4 \cdot 6H_2O = MgKPO_4 + 6H_2O. \quad (2)$$

$$2MgKPO_4 + 5SiO_2 = K_2MgSi_5O_{12} + P_2O_5 + MgO. \quad (3)$$

With the above reactions a glass with composition containing SiO_2, MgO and P_2O_5 should be formed. It should be noted that such glasses are well known as biomaterials and possibly the biological properties would be improved mainly due to this glassy phase formed. The free energy change of the above two reactions (2 and 3) in the temperature range of heat treatment could not be made, because of the non-availability of thermodynamic data of the phase $K_2MgSi_5O_{12}$. However, reaction 2 is more likely to occur as in the sintered product the presence of $MgKPO_4$ and absence of MgO was recorded.

3.2 FT-IR spectra

In order to obtain complementary information on the structural phases i.e. presence of phosphate (PO_4^{3-}) or other phases, FT-IR analysis was conducted. Figure 3 plots the FT-IR results for baseline MKP ceramic and samples heat treated at 900, 1,000 and 1,100°C for 4 h. The spectra show the characteristic band due to PO_4^{3-} groups at 938, 420, 1,125 and 567 cm^{-1} for v_1, v_2, v_3, v_4 vibrations, respectively [13]. Therefore, the peaks corresponding to PO_4^{3-} indicate the presence of magnesium potassium phosphate phase and this corroborates well with the XRD results (see Fig. 2). In the recorded FT-IR spectra, the absorption bands of silicate groups were also evident. The intense band at 980 cm^{-1} was assigned to the Si–O–Si asymmetric stretch [14]. A broad peak at around 3,450 cm^{-1}, corresponding to the absorbed moisture during sample preparation, is evident from the FTIR plots in Fig. 3. In addition to the above mentioned peaks, few other bands are also detected at around 2,000, 2,350 and

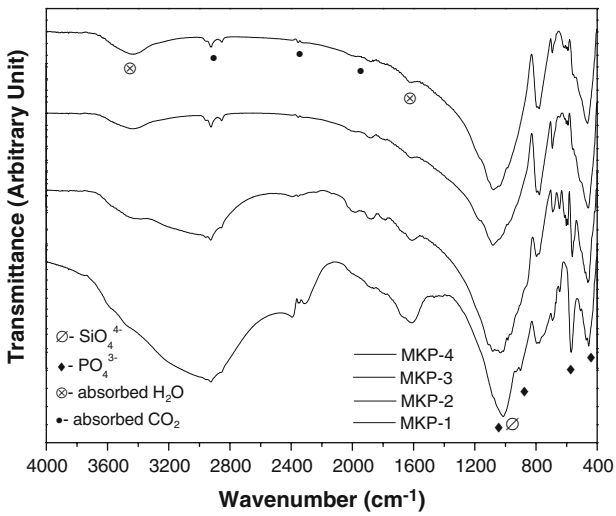

Fig. 3 FTIR spectra obtained for MKP as-cast (MKP-1) and MKP heat treated at 900°C (MKP-2), 1,000°C (MKP-3) and 1,100°C (MKP-4) for 4 h, revealing the bands characteristics of phosphate and silicate

2,930 cm^{-1}. These bands are not the characteristics of the samples, as FT-IR analysis of KBr pellet (free run) reveals that those peaks arises due to CO_2 and moisture absorption from the atmosphere during the KBr pellet preparation. It is possible that such FT-IR bands appear due to the presence of silicate glass phase.

3.3 Raman spectroscopy results

The Raman spectra of pure HAp, baseline ceramic and MKP ceramics heat treated for 4 h are shown in Fig. 4. The Raman bands of pure HAp at 1,076 and 1,049 cm^{-1} arise from v_3 PO_4 [15]. Also, the strong Raman peak located at 962 cm^{-1} band arise from v_1 PO_4 and one at 590 cm^{-1} band arise from v_4 PO_4. In addition, the Raman band at 431 cm^{-1} arises from v_2 PO_4. It can be noted that the strong band at 962 cm^{-1}, which arises from v_1 PO_4 is present in all the investigated samples (see Fig. 4). The strong Raman band at 465 cm^{-1} in the investigated ceramics (except pure HAp) arises due to symmetric stretching of oxygen of six-membered SiO_4 tetrahedra, v_s (Si–O–Si) [16]. In addition, the medium intensity lattice modes of quartz at 206 cm^{-1}, along with several weak lines at 263 cm^{-1}, 355 cm^{-1}, 395 cm^{-1} and 1,013 cm^{-1} are visible in the spectrum of heat treated MKP ceramics [17]. From the Raman results, the presence of phosphate and dominant presence of silicates can be confirmed in the heat treated ceramics. These results also corroborate well with our XRD and FT-IR results.

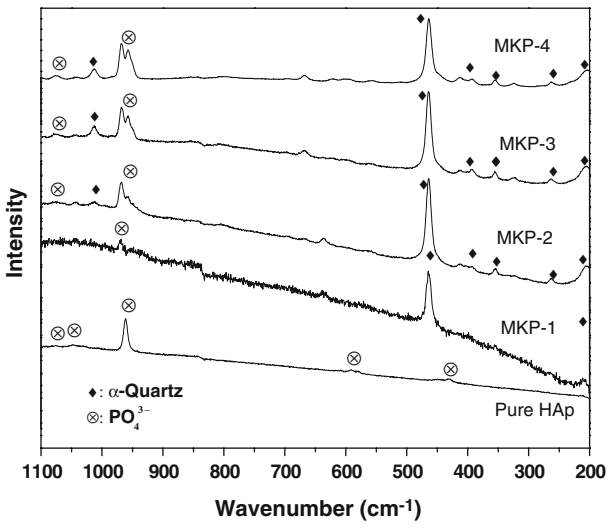

Fig. 4 Raman spectra obtained from pure HAp, MKP as-cast (MKP-1) and MKP heat treated at 900°C (MKP-2), 1,000°C (MKP-3) and 1,100°C (MKP-4) for 4 h

3.4 Cell adhesion assay

Figure 5 presents the scanning electron microscopy evidences of the adhesion of fibroblast L929 cells on MKP-4 ceramic, pure HAp and control glass disc, after 3 days of culture (see Fig. 5a–c). It is clear from Fig. 5a that mouse fibroblast L929 cells can adhere and proliferate on the

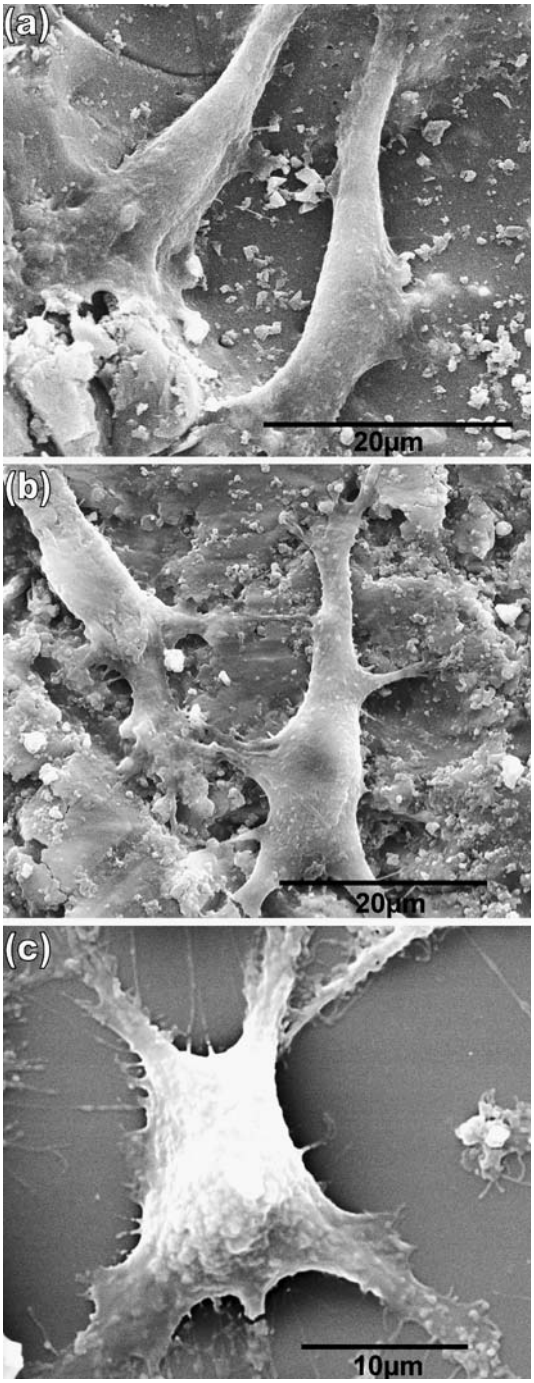

Fig. 5 SEM images, revealing the adhesion of L929 fibroblast cells on **a** MKP-4 (1,100°C, 4 h), **b** pure HAp and **c** control disc

MKP ceramics (MKP-4). Following this, some interesting observations are discussed for cell-adhesion test on MKP-4 samples. Cell proliferation, extra cellular matrix (ECM) formation and cell–cell contacts were the major observations in cell adhesion experiments. Also, the cell adhesions for MKP-4 are comparable with that of gelatin coated control discs. The SEM image (Fig. 5a) clearly shows cytoplasmic extension/cellular bridge formation on the surface of investigated ceramic. All such observations support good cytocompatibility property.

As far as the cell–material interaction is concerned, two components of interaction with the substrate may be recognized.

a) Adhesion, to allow the attachment and spreading that are necessary for cell proliferation.
b) Specific interactions, with adjacent tissue cells, and required for the expression of some specialized functions like cell motility etc.

Both the above factors, to a large extent, depend on various factors, like composition, surface roughness etc. As far as the presently investigated material is concerned, we have to understand the influence of both $K_2MgSi_5O_{12}$ and $MgKPO_4$ on the cell adhesion property. Although, the latter phase, being phosphate in nature, is likely to be biocompatible in nature. However, no such information is available on the $K_2MgSi_5O_{12}$ phase. Nevertheless, there have been a few reports on the biocompatibility property evaluation of silicate materials. For example, Gou et al. [14] reported good bioactivity, biocompatibility and mechanical properties of Ca_2SiO_4 material. Such silicate materials favor mesenchymal stem cell attachment as well as cell spreading [18]. In a different study, Wu et al. [19] reported the evaluation of new bredigite ($Ca_7MgSi_4O_{16}$) ceramic for biomedical applications and they showed the evidences of good osteoblast proliferation of such complex silicate ceramics. It was reported that calcium phosphate biomaterials induce bone formation at extra-skeletal sites without the need for additional osteogenic cells or BMP. In view of the above observations, it should be easy to realize the good cell adhesion behavior on the surface of the investigated ceramic, which contain $K_2MgSi_5O_{12}$ and $MgKPO_4$. It can be mentioned that silicate rich MKP ceramic biologically behave more like many of the bioglass/biocompatible glass ceramic substrate to support cell viability and proliferation.

Another observation, that requires some explanation, is the pronounced formation of extra-cellular matrix (ECM) after culturing for 3 days in the present case. On the basis of such observations, it is likely that the fibroblast cells, once adhering on the bioceramic surface, locally secrete proteins and polysaccharides, which in combination form ECM. Also, the ECM formation is indicative of the fact that the tissue formation on such ceramic surfaces will occur promptly at the implanted site. In particular, ECM contains two types of macromolecules, (a) polysaccharide chains of glycosaminoglycans (GAGs) and (b) fibrous proteins, including collagen, elastin, fibronectin and laminin. In addition to structural functions, all such proteins also have adhesive functions. In the present case, we believe, the occurrence of ECM formation also promotes cell attachment and spreading on ceramic surfaces.

3.5 MTT assay

It is well-known that MTT reagent directly reacts with the mitochondria (mitochondrial dehydrogenase) of metabolically active cells. Therefore, the reaction of MTT reduction is directly proportional to the number of growing cells. The measured optical density, which could be recorded using ELISA microplate reader, is directly proportional to the number of viable cells in the culture medium. Therefore, MTT is regarded as a quantitative assay to determine the cytotoxicity of the materials, detecting the viability/proliferation of the cells in solution, which contain the test samples. Figure 6 plots the MTT assay results obtained using mouse fibroblast cells (L929). In the present case, the result obtained with HAp is considered as baseline observation. In Fig. 6, the results are plotted with reference to pure HAp. Figure 6 shows that the numbers of metabolically active cells are either equal or more than pure HAp. All the test samples i.e. pure HAp, and MKP-1, MKP-2, MKP-3 and MKP-4 possess comparable cell proliferation.

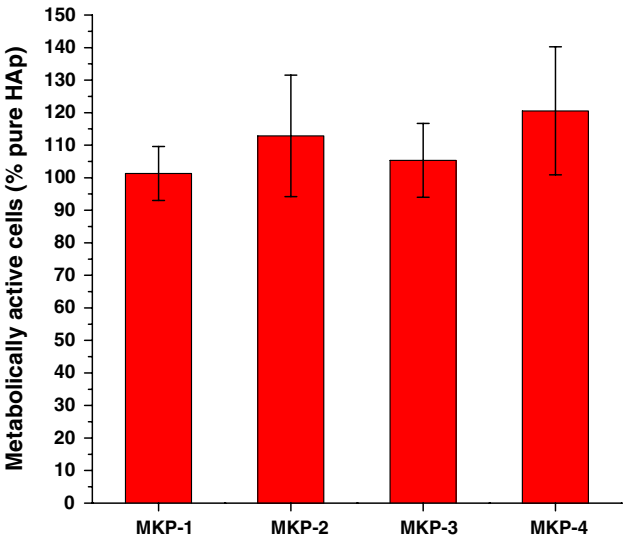

Fig. 6 MTT assay results obtained for MKP as-cast (MKP-1) and MKP heat treated at 900°C (MKP-2), 1,000°C (MKP-3) and 1,100°C (MKP-4) for 4 h. The MTT data shows the viability of fibroblast cells compared with pure HAp. The error bars indicate the standard deviation

Summarily, it can be said that the investigated ceramics are as non-cytotoxic as pure HAp.

In order to obtain an optimal balance of physical and biological properties, attempts will be made to develop MKP biocomposites by adding ceramic reinforcements, like mullite, alumina, and zirconia particulate/whiskers. Finally, the short term/long term implantation experiments in rabbits or other mammals will be carried out in vivo to assess the histocompatibility and osseointegration property. Such study is essential to realize their biomedical potential for hard tissue replacement applications.

4 Conclusions

In the present study, a planned set of heat treatment experiments on as slip-cast novel phosphate–silicate ceramics were carried out at 900, 1,000 and 1,100°C for 4 h in air. Also, the cytocompatibility property including cell viability of L929 mouse fibroblast cells were assessed. The following conclusions can be drawn from the work presented in this article:

a) The heat treatment of as slip-cast ceramics at 900°C for 4 h, increases the density to 2.55 g/cm^3 and no significant change in density is measured when heat treated at 1,000 or 1,100°C.
b) XRD analysis confirms the presence of crystalline $K_2MgSi_5O_{12}$ and $MgKPO_4$ phases in the heat treated MKP ceramics and MKP phase is found to be more stabilized when heat treated at 1,100°C. Complimentary information of the phosphate and silicate phases was obtained using FT-IR and Raman spectroscopy.
c) The cell viability study using MTT analysis of the MKP samples, heat treated for 4 h, clearly indicates that the newly investigated phosphate ceramics are as non-cytotoxic as sintered HAp.
d) The results of the cell culture experiments establish good cytocompatibility properties of MKP ceramics. The spreading/adhesion/proliferation characteristics of L929 cells on MKP containing ceramics are clearly comparable with that of pure HAp and control specimens.
e) Based on the present results, it can be concluded that the slip casting and heat treatment combination can lead to the development of low density biocompatible non-cytotoxic phosphate–silicate ceramics.

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