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Bone Scaffold Biomimetics Based On Gelatin Hydrogel Mineralization

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Key Words: Biomimetics, Tissue Engineering, Bone Scaffold Mineralization, Hydroxyapatite, Magnesium Phosphates, Calcium Phosphates.

Abstract: Apatite phase Calcium and Magnesium Phosphate doped nanocomposite scaffold has been synthesized in physiological environment by gelatin hydrogel double diffusion technique. Several analytical methods, such as X-ray diffraction (XRD), infrared spectroscopy (FTIR), energy dispersive spectroscopy (EDS) and scanning electron microscopy (SEM) were applied to characterize physicochemical properties of the studied samples.

The results showed that nanocomposite scaffolds were porous with three-dimensionally interconnected microstructure, pore size ranging from 200 to 300 μm nanocrystalline precipitated minerals were dispersed evenly among gelatin fibers. A mineral containing amorphous calcium phosphate and brushite precipitate was formed within the gelatin matrix at 4°C. After incubation in SBF solution at 37°C for 7 days, the mineral phase was changed to nanocrystalline hydroxyapatite. It should be well-known that precursor phases inside a scaffold implanted into the bone are equal to biomimetic adaptation of precursors to hydroxyapatite that is very similar to the bone and has an attentive level of biocompatibility. Therefore, the result confirms the significance of biomimetic calcium and magnesium phosphate bone tissue scaffolds in developing new biomaterials for bone regeneration.

1. Introduction.

Biomimetic synthesis of inorganic compounds such as calcium carbonate, silicon dioxide, and apatite has received increasing attention over the last few years [1,2]. Many researchers have recently detected and theorized the basic principles involved in biomineralization process [3] and innovated bone resembling materials utilizing biomimetic strategies [4]. Mostly, as a model system, gelatin hydrogel diffusion has been adopted by several authors to study mineralization of bone, as well as the calcium phosphates formation [5]. Bone mineral consists of an appetite structure, Hydroxyapatite mineral (HA), $Ca_{10}(PO_4)_6(OH)_2$, which is widely used to produce highly biocompatible ceramic materials for orthopedic and dental applications [6-9, 10]. The combination of apatite and collagen fibrils provides hardiness and flexibility against bone fracture. Because of biocompatibility of bone mineral, HA can broadly be used as a main substance for hard tissue implantation [11]. Therefore, biological apatites are completely different from pure HA in stoichiometry, crystallinity and mechanical properties [12]. Biomimetic synthesis of bone materials is a central aim in biomaterial studies. Much attention has recently been dedicated to the development of new biomimetic and non-stoichiometric apatite, which likely results in the prevalence of biodegradability and bioactivity against stoichiometric HA [13].

Due to its minimal inflammatory responses and tissue injury, gelatin hydrogel is commonly used in biotechnology and medicine [14,15]. As a result of its' hydrophilicity, Gelatin has great affinity with nHA and shaping through the gel which has perfect combination with the matrix, perfectly homogenizes with HA in the precipitation phase of aqueous solutions conducted by double diffusion technique. In this system, calcium and phosphate ions concentration decrease during a diffusion gradient [16]. Therefore, polymers synthesized under these conditions are very similar to bone tissue.

Particles strongly attach to the matrix in this nanocomposite scaffold and reveal better mechanical properties [17]. In the mineralization process, amorphous calcium phosphate (ACP) functions as a precursor phase, temporary source of calcium (Ca) and phosphorous (P) for bone mineralization. The amorphous phase facilitates the improvement of the mechanical properties [18].

Bone mineral apatites structural and physical instablity, together with their fierce reactions are essential factors in the formation and dissolution of crystals in tissues.

In this research study, double diffusion method was adopted to provide a biomimetic GEL-ACP nanocomposite scaffold in a physiologically relevant environment and to investigate ACP's phase conversion to HA during incubation in a simulated body environment.

2. Materials and Methods

2.1. Material of Synthesis. All reagent grade chemicals were purchased from Merck Company and used without further purification. B Gelatin, Calcium nitrate-tetrahydrate [Ca $(NO_3)_2.4H_2O$], Magnesium nitrate-hexaydrate [Mg $(NO_3)_2.6H_2O$], Disodium hydrogen phosphate [$Na_2HPO_4.2H_2O$], Glutardialdehyde solution 25%, HCL, SBF solution [NaCl, $NaHCO_3$, KCl, $Na_2HPO_4.2H_2O$, $MgCl_2.6H_2O$, $CaCl_2$ $.2H_2O$, Na_2SO_4 and $(CH_2\ OH)_3\ CNH_2$]

2.2. Synthesis Method. HA samples with Mg substituting for Ca^{+2} with x values of 0, 8 samples were synthesized using 100 ml aqueous solutions with various Mg/ (Mg + Ca) molar ratios. The total concentrations of Ca^{+2} and Mg^{+2} were initially 0.2 M and concentration of PO_4^{-3} was 0.12 M. The pH value of solutions was adjusted to 7.4 by adding Tris and HCl. The principle of synthesizing bivalent Mg cation (Mg^{+2}) substituted apatite by the precipitation method is expressed in the following equation:

$$(10\text{-}x)Ca^{+2} + xMg^{+2} + 6PO_4^{-3} + 2OH^- \longrightarrow Ca_{(10-x)}Mg_x(PO_4)_6(OH)_2 \quad 0 \le X \le 10 \qquad (1)$$

The synthesisation started with preparing gelatin (10% weight per volume, w/v), placement into a 13.5 × 9 × 4.5 cm mould, refrigeration at 4°C for 24 hr, then the provisional walls were removed and the prepared calcium nitrate solution was poured to one side of gel, and transferred to the refrigerator and held at 4°C for 2 days. Consequently, disodium hydrogen phosphate solution was added to the other side of gel, moved to the refrigerator and held at 4°C for 5 days. Once removed from the refrigerator, the precipitation was cut into the desired size, moved to -80°C and aged for one hour. It was then put in a freeze dryer overnight. Scaffolds were then soaked in 25% glutardialdehyde (GA) solution overnight for cross-linking. To remove probable GA residue from scaffolds structure, scaffolds were washed with distilled water, ultrasounded three times and finally freeze-dried to maintain their porous structure. The scaffolds were soaked at 37°C in Kokubo solution (SBF) which contains inorganic ion concentrations similar to human blood plasma. The surface structures of the prepared scaffolds were examined by X-ray Diffraction (XRD) after soaking in SBF.

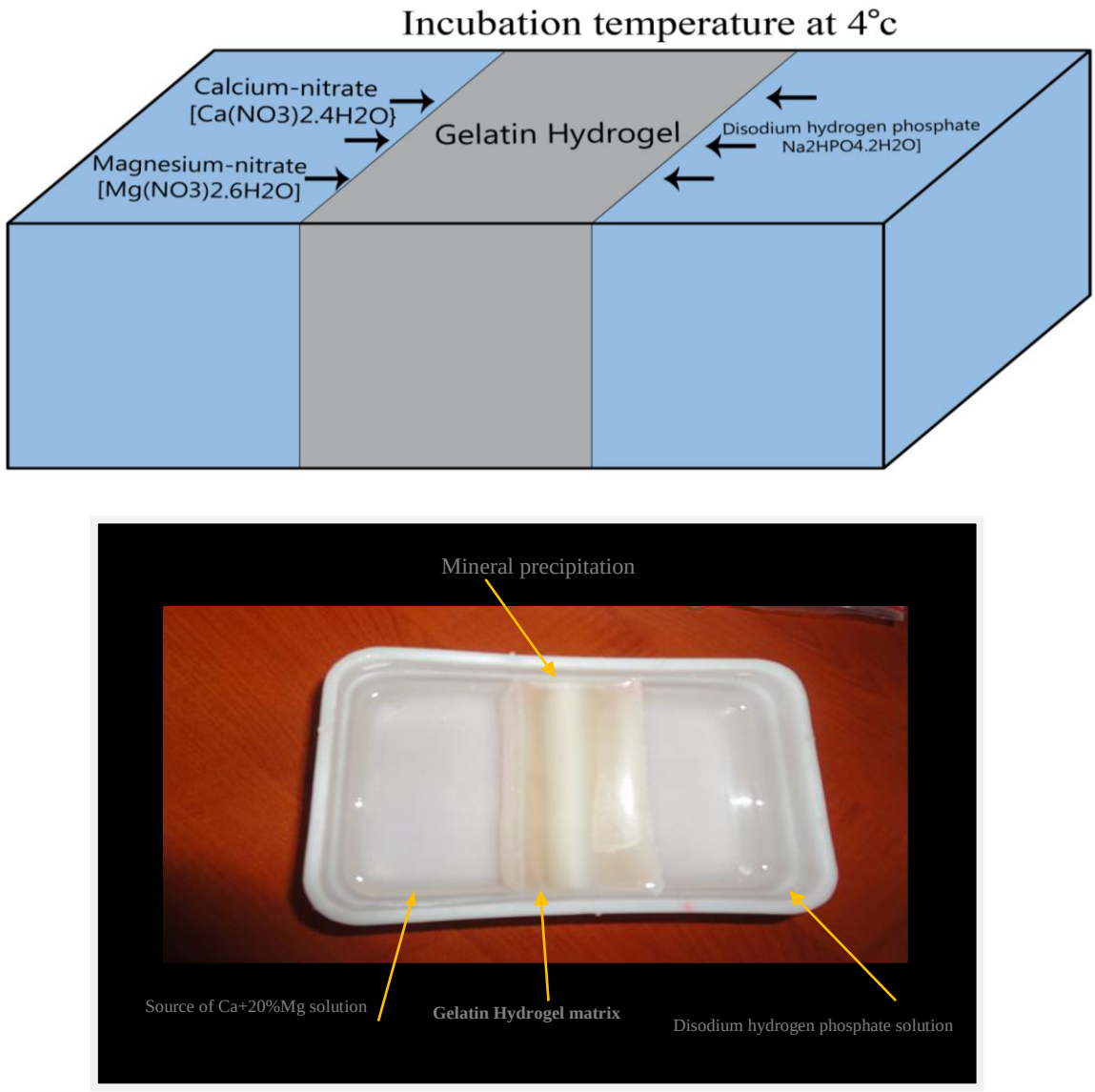

Figure 1. Schematic view of reactor used for bio mineralization of apatite within GEL hydrogel via double diffusion.

2.3. Powder Characterization

2.3.1. X-Ray Diffraction. The resulting samples were analysed by X-ray diffraction (XRD) using an EQUINOX3000 diffractometer. This instrument was set to apply voltage and current settings of 40 kV and 30 mA respectively and to use Cu-Kα radiation (1.5405 Å). A scan speed of approximately 6°/min was conducted. For qualitative analysis, XRD diagrams were recorded in the interval $10° \leq 2\theta \leq 80°$ with 0.1° resolution.

2.3.2. Energy Dispersive X-ray Spectroscopy. The chemical compositions of the samples were studied by the X-ray microanalysis, using energy dispersive spectroscopy (EDS), Thermo Noran (USA).

2.3.3. Fourier Transform Infra-Red Spectroscopy. FTIR spectroscopy was used to identify the functional groups of resulting powders. The synthesized powder was examined by Fourier transform infra-red spectroscopy (FTIR) with JASCO FT/IR410 spectrometer. To undertake this analysis, 1 mg of the powder sample was carefully mixed with 300 mg of potassium bromide (KBr) (infrared grade) and pelletized under vacuum. Then, the pellet was analysed in the range of 400 – 4000 cm^{-1} at the scan speed of 2 mm/sec with 4 cm^{-1} resolution.

2.4. Scaffold Characterization

2.4.1 Porosity Measurement of Scaffolds. Porosity of HA and Mg-HA scaffolds was measured by the following formulas that d = scaffold thickness, A = area scaffold, w_s = molecular weight of scaffold, ρ_s = scaffold density.

$$porosity\ percentage: \frac{d \times A - \left(\frac{W_s}{\rho_s}\right)}{d \times A} \times 100 \quad (2)$$

2.4.2. Scanning Electron Microscopy Analysis. The scanning electron microscopy techniques were utilized to study the structural morphology of scaffolds. (SEM, Seron AIS2100 with 3.5 nm resolution, 15~300,000 Accelerating Voltage and Diffusion Pump vacume system). The scaffolds were coated with a thin layer of gold. Three electron microscope images of each of the scaffolds were randomly selected to measure pore size and the large diameter of all pores were measured by Image J software and the average pore size was reported.

2.4.3. Cytotoxity Testing. Cytotoxicity evaluation is the early phase of biocompatibility test. It serves as a reliable, convenient, reproducible screening method to identify cell death or serious injury of cells [39]. Chinese hamster ovary (CHO) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich). It took 24 hrs to incubate the scaffolds culture medium. Once incubation was finished, the CHO cells were seeded onto the scaffolds at a density of 105 cells/scaffold allowing them to incubate for another 24 hours.

2.4.4. MTT Assay. Cell viability was measured using MTT assay. The assay is based on the reduction of tetrazole by living cells. Cleavage of the tetrazolium rings turns the pale yellow MTT into dark blue formazan crystals, the concentration of which is directly proportional to the number of metabolically active cells. Therefore, the production of formazan can reflect the level of cell viability on the material. This reduction takes place only when mitochondrial reductase enzymes are active [40]. Scaffolds were incubated in the DMEM medium (Dullbecco's Modified Eagle Medium).

The Wistar rat cells were seeded in 96-well cell culture plates (1-104 cells/cm^2), and reached 80% confluence in 24 hrs. The 24 hr extracts were placed in contact with the monolayer for 24 hrs. The viability test using MTT assay was performed and four samples were measured. The culture medium was replaced by a solution of MTT (Sigma, USA) and the cells were incubated for 4 hrs. The viable cells, those with functional mitochondrial dehydrogenase, were able to reduce the yellow MTT to a purple formazan product [19,20]. The medium was discarded and the precipitated formazan was dissolved in 0.5 mL DMSO containing 6.25% (v/v) 0.1M NaOH. The final product was quantified by microplate spectrophotometer (BMG LABTECH, GmbH, Germany) at a wavelength of 540 nm and expressed as optical density (OD) units after blank subtraction.

3. Results

3.1. Porosity Measurement. Diffusion of Ca^{+2} and Mg^{+2} phosphate from adjacent chambers into the middle results in formation of white precipitates. Porosity percentage of the HA and Mg-HA scaffolds were estimated to be about 88.78% and 86.82 respectively. It is important to realize that bone mineral (biological apatite) is non-stoichiometric, of which different types exists and which consists of foreign ion substitutions containing sodium, magnesium, potassium, carbonate and fluoride.

3.2. FTIR Spectral Analysis. The FTIR spectrum of the nanocomposite exhibited a number of characteristic spectral bands related to gelatin and HA (Figure 2). Chief among them were protein spectrums such as: N–H bending vibration at 1260cm-1 for the amide III, N–H bending vibration at 1560cm-1 for the amide II, C=O stretching vibration at1670cm-1 for the amide I, C–H bending vibration at 2952cm-1 for the amide B and band at 3570cm-1 indicate the presence of O–H groups [21]. The second bond at 2363cm-1 appeared after cross-linking of Gel with GA.

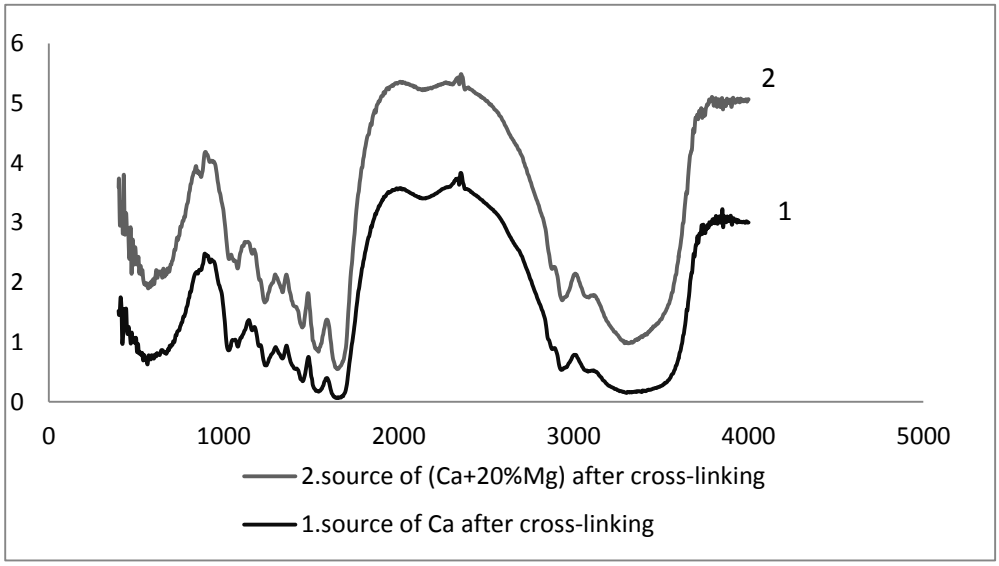

Figure 2. FTIR spectra obtained form (a) Nano composite samples containing GEL and precipitated minerals formed at 4°C and (b) after incubation in SBF solution at 37°C.

3.3. X-Ray Diffraction. The XRD results for HA, Mg-HA is presented in Figures 3, 4, 5, 6. All the samples showed the characteristic apatitic structure. XRD reflections show that two different minerals are present in the scaffold composite. Some reflections are from HA, a biologic nonapatitic calcium phosphates containing amorphous Calcium Phosphate such as dicalcium phosphate dehydrate (DCPD), $CaHPO_4.2H_2O$ (Brushite), other in sample source of (Ca+20%Mg) may be from $Mg_3(PO_4)_2$.

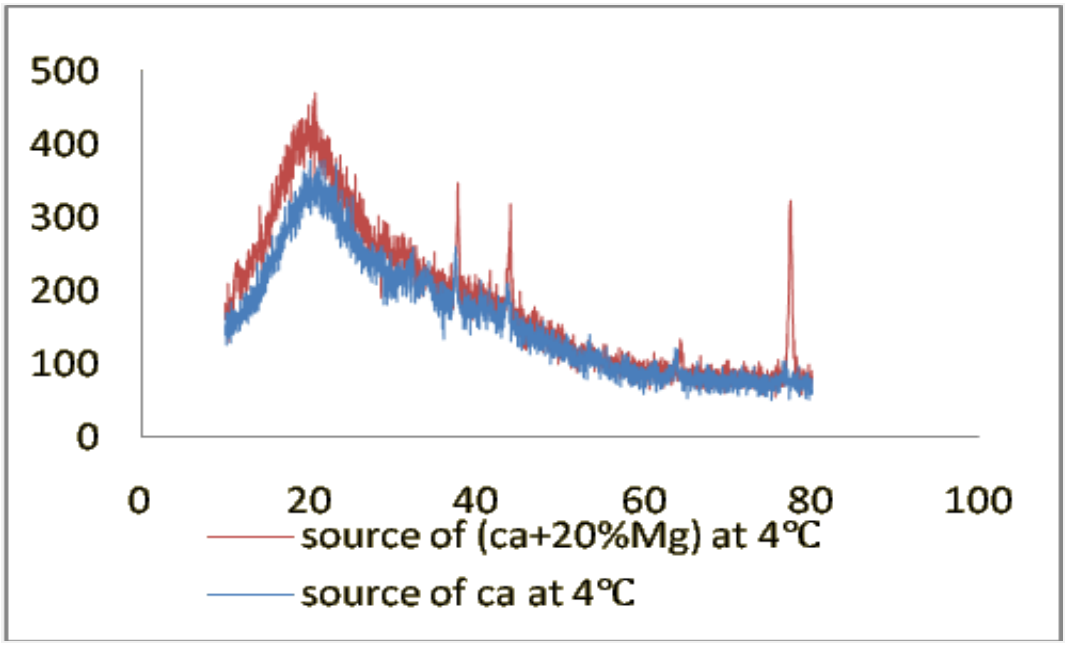

Figure 3. XRD spectra for source of Ca and (Ca+20%Mg)

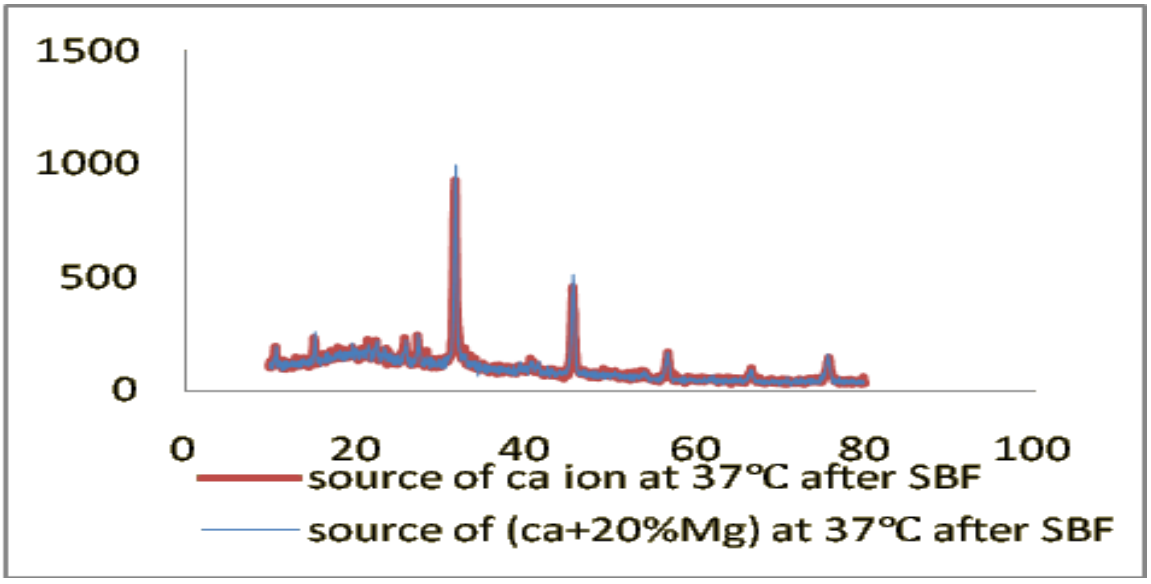

Figure 4. XRD spectra for source of Ca at 4°C and (Ca+20%Mg) at 37°C.

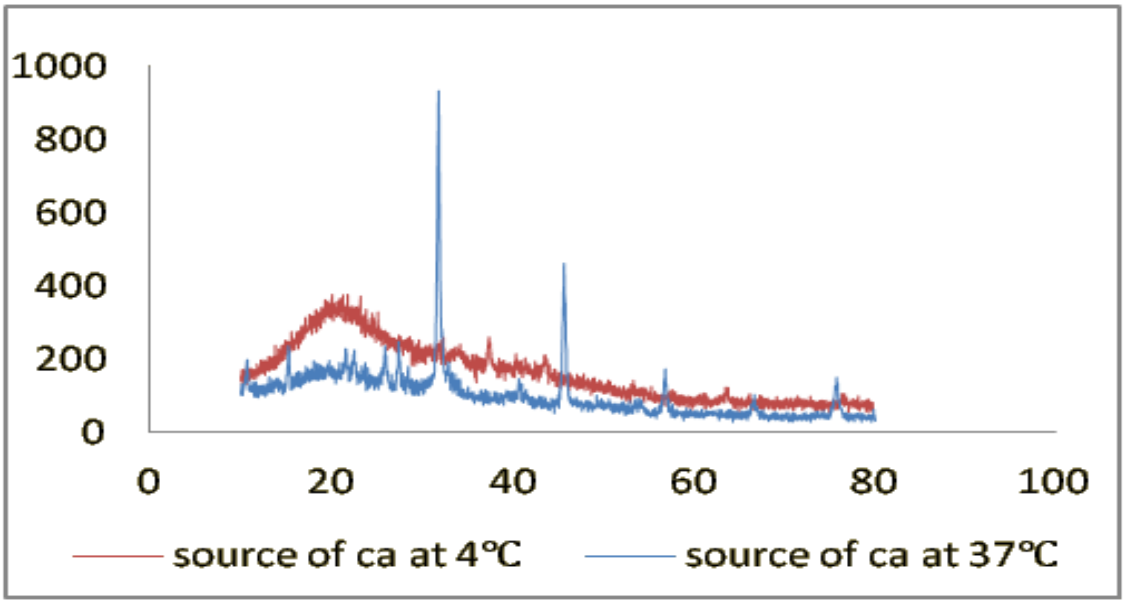

Figure 5. XRD spectra for source of Ca at 4°C, 37°C.

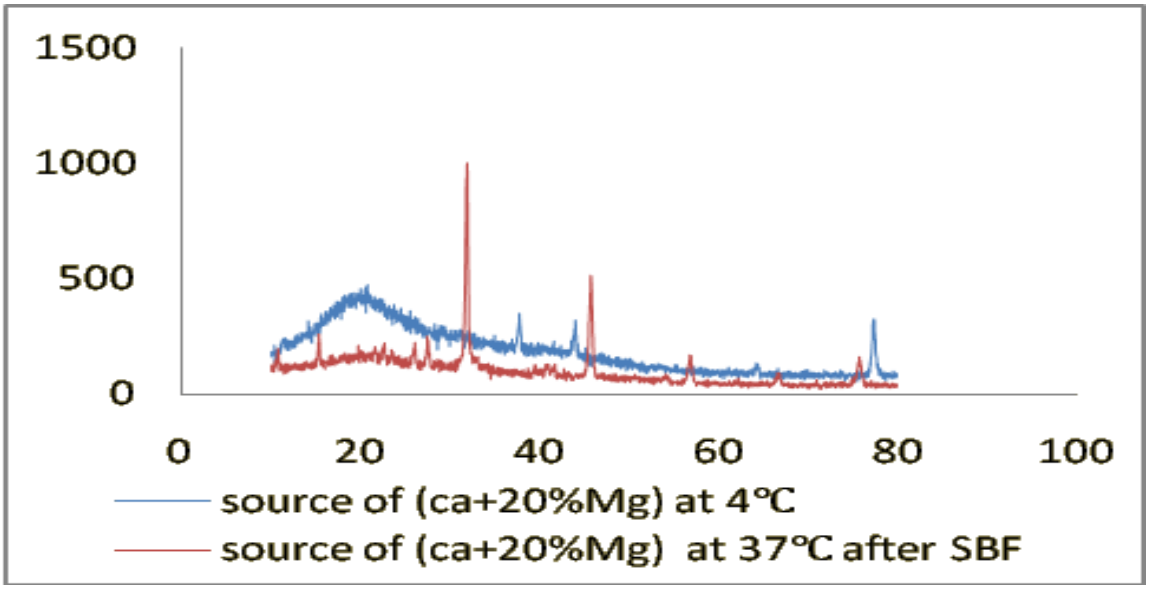

Figure 6. XRD spectra for source of (Ca+20%Mg) at 4°C and 37°C.

3.4. Scanning electron microscopy. Figure 7 shows SEM of HA and Mg-HA after soaking in the SBF for 7 days. It is seen from these figures that the composites are porous, with pore size of about 200-300 μm.

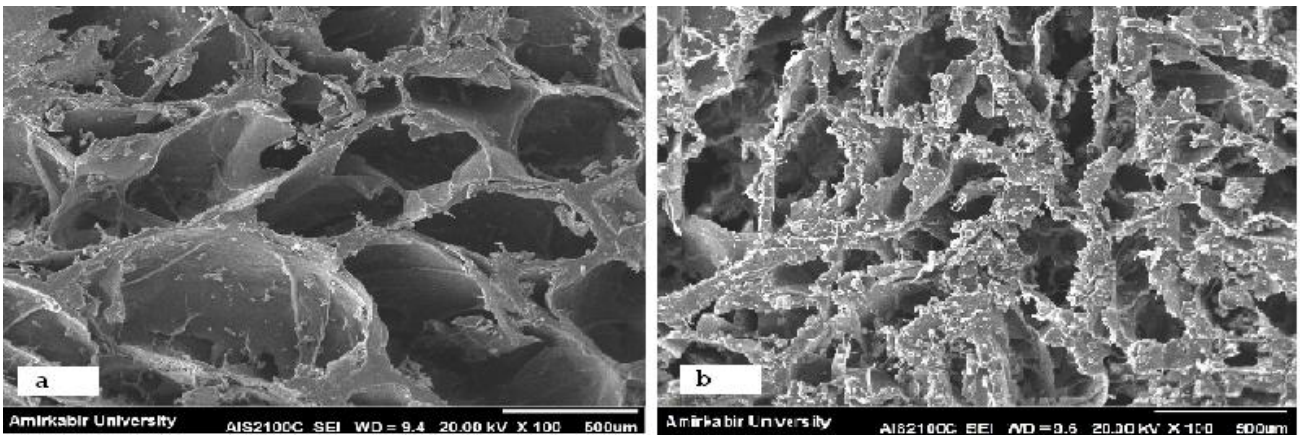

Figure 7. SEM micrographs obtained from surface of the synthesized biomimetic nanocomposite scaffold showing porous structure and nucleation and growth of the precipitate-phase nanocrystals on the surface of pore walls after being soaked in SBF solution (a) for source of Ca (b) source of (Ca+20%Mg)

3.5. Energy Dispersive Spectroscopy (EDS). The EDS spectrum (Figure 8) HA confirmed the presence of calcium, phosphate and magnesium peak of HA. The Ca/P ratio was found to be 1.42 and 1.46 respectively that is related to samples of source of Ca and (Ca+20%Mg).

Table 1. EDS analysis and chemical involved in prepared biomimetic GEL/Apatite Nanocomposites Scaffold for source of Ca and source of Ca+20%Mg.

	Source of Ca+20%Mg		**Source of Ca**	
Elt	**W%**	**A%**	**W%**	**A%**
C	57.79	70.07	57.98	70.49
O	23.55	21.44	25.04	22.85
Na	6.46	4.09	2.45	1.55
Mg	0.52	0.31	-	-
P	1.81	0.85	2.25	1.06
Cl	5.18	2.13	6.52	2.69
Ca	2.65	0.96	3.19	1.16
Au	2.04	0.15	2.58	0.19
	100.00	100.00	100.00	100.00

3.6. Cytotoxicity. For the scaffolds to be used in the bone implants, they should be biocompatible with CHO cells. The scaffolds should not be toxic to the bone cells for bone applications. Thus the scaffold materials were subjected to cytotoxic studies with CHO cells (Figure 9).

3.7. MTT Assay. MTT results show that composite scaffolds showed a little decreased OD value after 24 hr, (Figure 10). This could be due to the low crystallinity of HA leading to dissolution of calcium and phosphate into the media which in turn leads to the increase of intracellular Ca and phosphate concentration which may induce cell death [23]. The results show that the composite scaffolds are cytocompatible and no morphological change was observed in RMSCs (Rat Mesenchymal Stem Cells) placed in direct contact with composite scaffold. Both of the scaffolds supported 65-80% cell viability compared to the control.

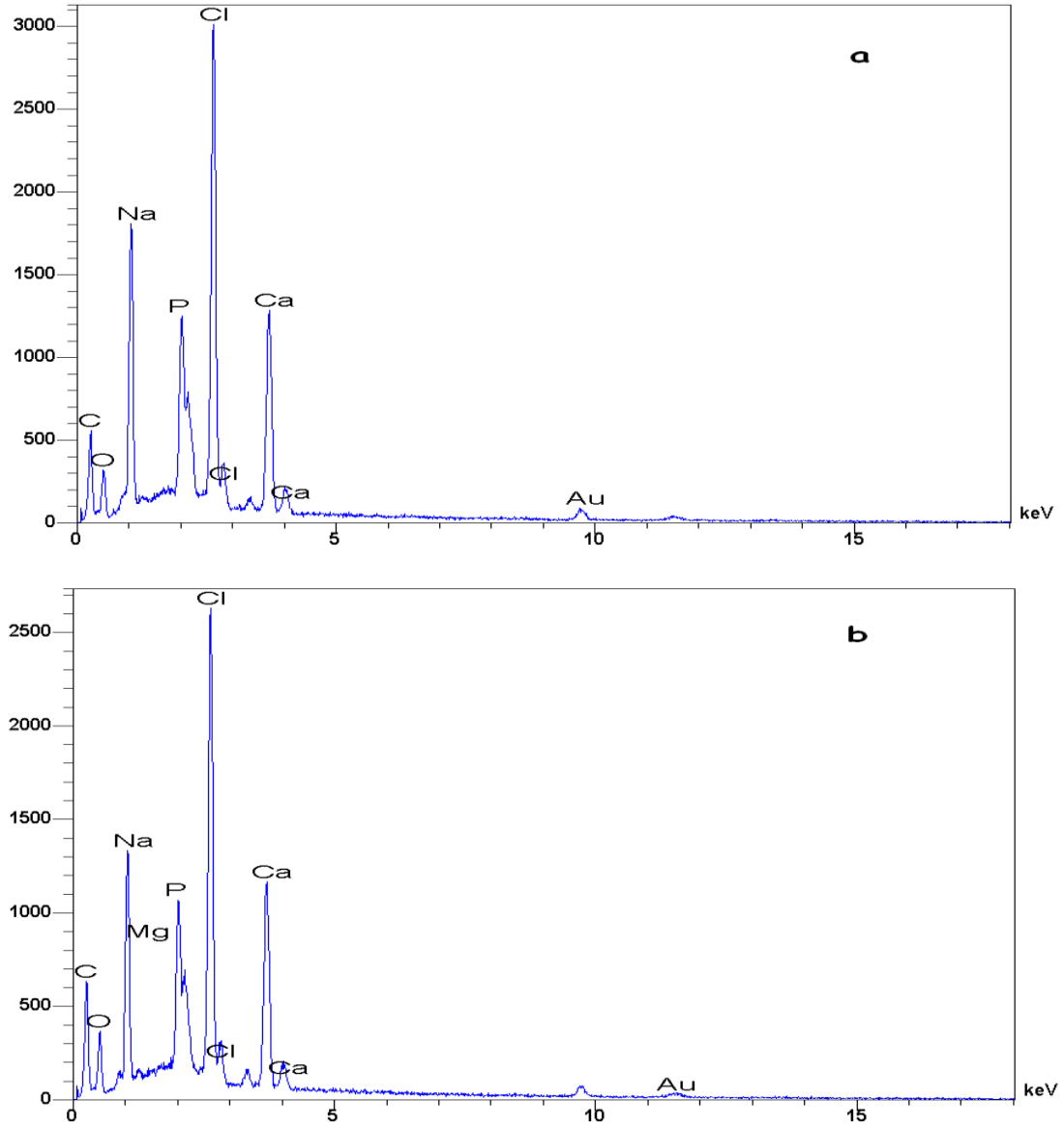

Figure 8. The EDS spectrum obtained from nanocomposite samples containing GEL and precipitated minerals formed related to samples of source of(a) Ca and (b) (Ca+20%Mg).

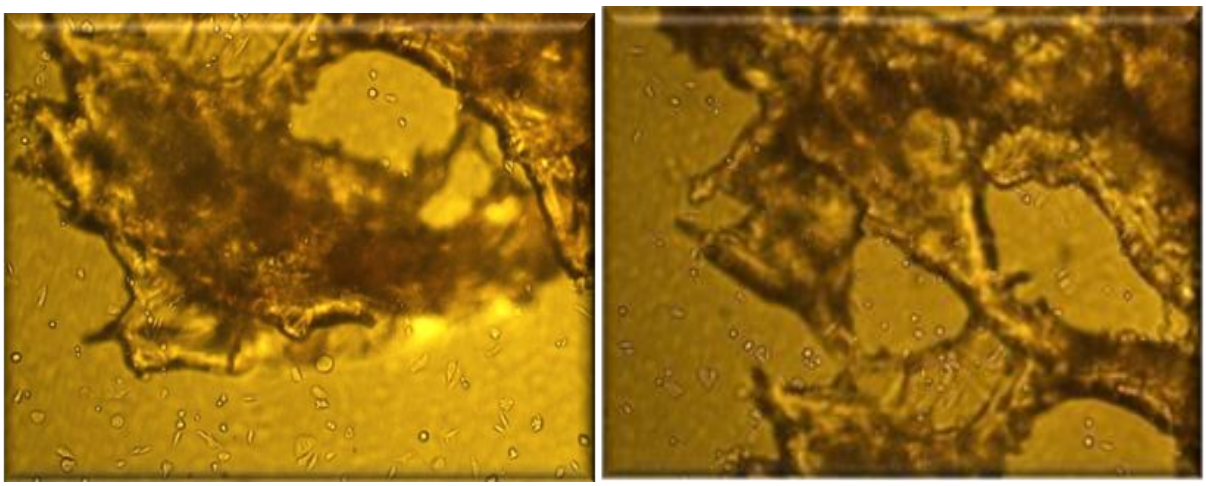

Figure 9. The scaffolds were subjected to cytotoxic studies with CHO cells. They were found to be biocompatible and non-toxic.

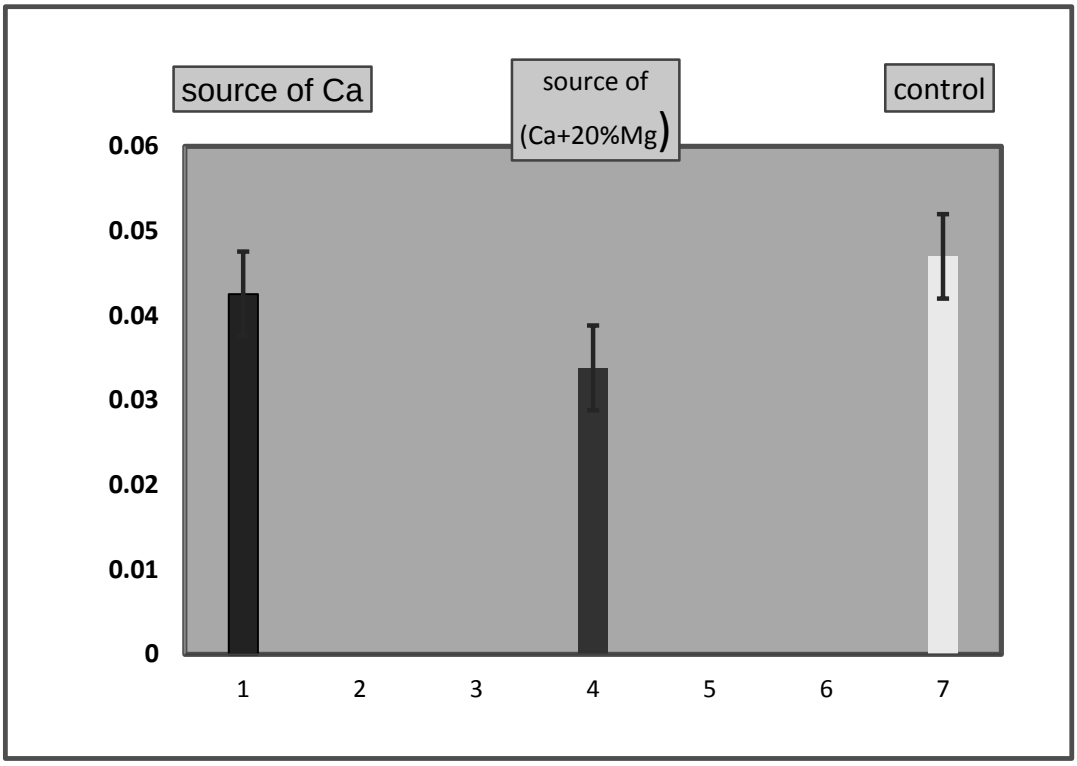

Figure 10. The toxicity of the extraction medium was tested on Wistar rat cells. Results are expressed as the OD of viable cells.

4. Discussion

In short, HA and Mg-HA nano composite scaffolds were synthesized by double diffusion technique. In this study we investigated the simultaneous diffusion effect of both Mg^{+2} and Ca^{+2} ions on the one hand and the phosphate ion diffusion into gelatin hydrogel matrix on the other.

Both Magnesium and Calcium have similar behaviour chemistry and both are group II metals [24]. Magnesium ions are used as substitutes in calcium phosphate-based biomaterials [25].

Porosity percentage of the HA and Mg-HA scaffolds were measured to be about 88.78% and 86.82% respectively. The resemblance of our achievement to those of other researchers' [26] indicates the credibility of this experiment. The FTIR spectrum of the nano composite showed several spectral bands related to gelatin and HA. The peak of the N-H bending vibration of amide III, II, C-H bending vibration and the peak of the hydroxyl groups confirm this result, and also have been shown by other authors [21].

The XRD diagrams demonstrate that the two differently formed mineral phases are related to the DCPD (brushite) and Mg_3PO_4. This investigation reveals that ACP and DCPD change to HA after being incubated in SBF solution at 37°C for seven days. The same result has been reached by other observers [27-31]. Thus, if the GEL/ACP scaffold is implanted in the body, it might eventually be converted to HA crystals as determined by a process similar to natural bone formation in the body. Precipitation of calcium phosphate mineral using the method defined can provide a more homogenous combination and desirable linkage of ceramic particles with GEL matrix.

The SEM results show that porous and interconnected scaffolds have been produced. Cytotoxity test result confirms the scaffolds are not toxic to the cells for bone applications.

MTT results indicate that composite scaffolds are cytocompatible and no morphological change was observed.

5. Conclusion

In the present study, a double diffusion system is employed to prepare HA, Mg-HA in gelatin hydrogel system. Double- diffusion gel precipitation systems are the most realistic and applied of the cell-free *in vitro* methods of studying biomineralization processes. These methods demonstrate how matrix molecules develop to nucleate, inhibit, or modify mineralization. Mg substitution decreases the crystallinity of apatite and destabilizes the apatite structure.

Acknowledgements.

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