SYNTHESIS, CHARACTERIZATION AND BIO-EVALUATION OF BIOACTIVE COMPOSITES SCAFFOLDS BASED ON COLLAGEN AND GLASS CERAMIC

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In this work, we report on the preparation and characterization of collagen-glass ceramic composites. The materials used for obtaining collagen-glass ceramic composites were collagen type I (Coll) as bioresorbable polymer and glass ceramic (GC) from CaO-SiO_2-P_2O_5 system as bioactive material. After incorporation of GC into Coll in different ratios (Coll/GC ratio was 25/75 wt% and 75/25 wt%) and freeze-drying of them, the obtained composite materials (Coll/GC) can be used, according to our results, as potential composite scaffold for bone tissue engineering. Coll/GC composite biocompatibility, assessed by an *in-vitro* test (SBF soaking for a period of 14 days, at a temperature of 37°C) and cytotoxicity of these on endothelial cells, was very good. The collagen-glass ceramic composites were characterized from the point of view of their mineralogical composition (by XRD) and morphology (by SEM). At the surface of the specimens soaked in simulated body fluid (SBF) for a period of 14 days, at a temperature of 37°C was observed the formation of mainly hydroxiapatite with a spherical morphology. Furthermore, the biocompatibility assays show that these collagen-glass ceramic composites are suitable for tissue engineering. Fluorescent microscopy of endothelial cells after 5 days in culture with this scaffolds revealed a great viability, also confirmed by the measurements of metabolic activity of endothelial cells.

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Keywords: Collagen, Glass ceramic, Composite scaffold, In-vitro test, Cytotoxicity.

1. Introduction

The biocomposite scaffold for tissue engineering combines bioresorbability of matrix and bioactivity of reinforcement additives [1-3].

The first bioglass was developed by Hench and then the glass ceramic materials were developed [3-16]. These materials with different chemical compositions can be produced in a wide range in order to serve various functions in the body. Also, they have a good bioactivity and can be both osteoconductive and osteoproductive.

Coll is an adequate support for cell attachment, offering the advantage of a natural biomaterial with haemostatic and wound healing properties. The positive effects of Coll on tissue regeneration and its interaction with the cells are the main causes of increased interest in the local treatment of affected tissue or in the formation of new tissues such as bone, skin or nerves [17-23]. Coll hydrogels are three-dimensional networks of fibrils formed randomly, which include large amounts of fluids [24, 25]. Water retention is due to the hydrophilic groups: amine, carboxyl and

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hydroxyl, which also allow hydrogen bond formation and ionic and hydrophobic interactions, that ensures their consistency.

In this study, it is assumed the incorporation of glass ceramic into the collagen matrix may improve the bioactivity, which was evaluated by an *in-vitro* test (SBF soaking for a period of 14 days, at a temperature of 37°C) and cytotoxicity of these on endothelial cells.

2. Materials and methods

2.1. Preparation of the composite materials

The collagen used in this study was type I, fibrilar bovine collagen obtained through chemical extraction (MW=300 000Da, 2.54% and pH=7), from National Institute of Textiles Leather Research & Development, Branch Leather and Footwear Research Institute, located in Bucharest.

The glass ceramics, with composition from $CaO\text{-}SiO_2\text{-}P_2O_5$ oxide system (63 wt% CaO, 27 wt% SiO_2 and 10 wt% P_2O_5), were obtained by the sol-gel method, according to our prevision work [26]; tetraethyl orthosilicate ($C_6H_{16}O_3Si$ – TEOS; Fluka, >98%), calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$), triethyl phosphate ($C_6H_{15}O_4P$ – TEP; Sigma-Aldrich, >99.8%) were used as raw materials. In order to obtain the final glass-ceramic masses, the dried gel was thermally treated at 700°C for 2h. This procedure leads to the polymer network dissociation, organic material burning and all decomposition processes completion. The resulting powder was then calcined in different conditions, 1100°C and 1200°C for 2h, and then slowly cooled in the furnace.

Coll/GC composite materials were obtained starting from Coll gel and glass ceramic powders, as it can be seen in fig. 1. Table 1 presents the materials compositions. A very dynamic homogenization was effected by mechanical stirring for 30 min.

Collagen cross-linking was performed with 1% glutaraldehyde solution (glutaraldehyde was 0.5% compared to Coll dry) in order to achieve Coll molecules stabilization, maintain this stability during use and increase the transfer time. The stability is also necessary in the freeze-drying process. Chemical cross-linking is essential for ensuring the stability of the matrix in contact with the physiological environment and it is responsible for the changes of the physico-chemical and morphological properties, for the drug transfer and their biological properties [17-19, 21, 24].

Obtaining composite materials as porous /cell by keeping the gel structure was achieved by drying the gels using the freeze-drying technique. The freeze-drying parameters were freezing at -55°C (2h), followed by drying at 0.1 mbar (12h at -55°C, followed by 8h at 0°C and 16h at 35°C).

Table 1 Composite materials composition

Composite materials symbol	MC1	MC2	MC3	MC4
Coll, wt%	25	75	25	75
CG (1100°C/2h), wt%	75	25	-	-
CG (1200°C/2h), wt%	-	-	75	25

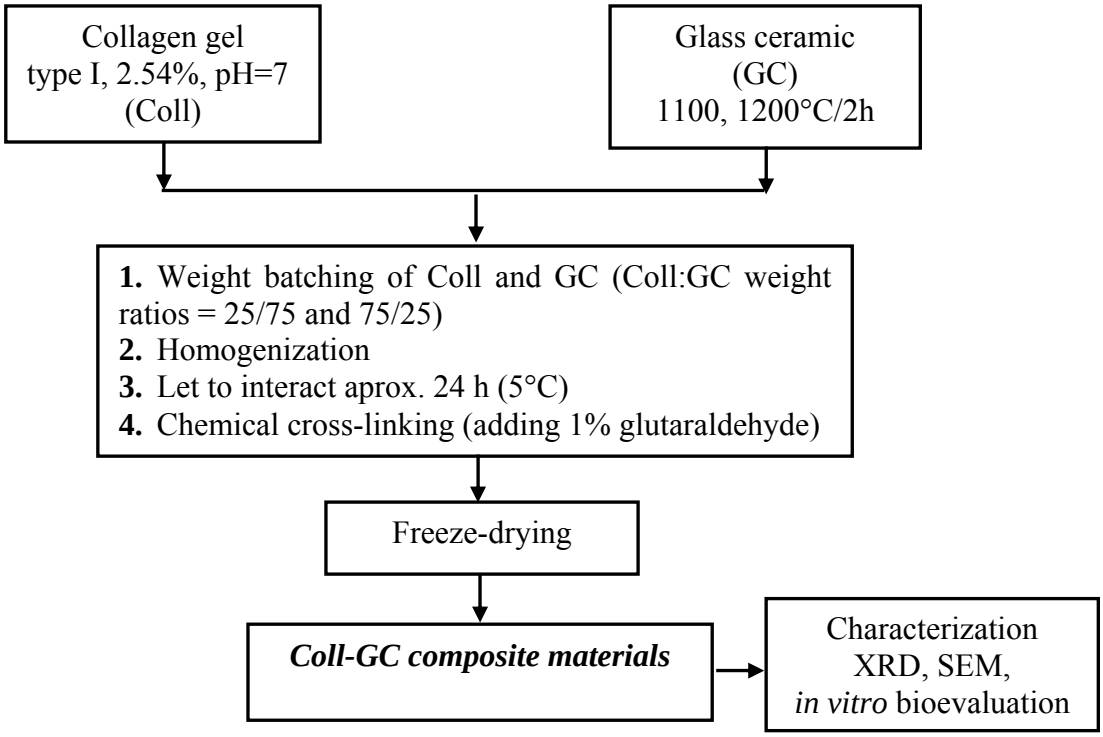

Fig. 1 Scheme of the synthesis of Coll/GC composite materials.

2.2. Characterization

For the identification of the crystalline phases of the powders (dried gel which was thermally treated at 700°C for 2h; glass ceramics from 1100°C/2h and 1200°C/2h) and composite materials, X-ray diffraction analysis was carried out on a Shimadzu diffractometer XRD 6000 - Ni-filtered CuKα (λ = 1.5406 Å) radiation, scanning speed of 2º/min in 2θ range of 10-60 deg.

The study of the synthesized materials microstructure was performed using two scanning electron microscopes: HITACHI S2600N and Quanta INSPECT F (equipped with electron field emission gun - EFG with a resolution of 1.2 nm). The samples were covered with a thin gold layer deposited by dc-sputtering.

The biocompatibility of the glass ceramic powders and composite materials was assessed by an *in-vitro* test and cytotoxicity of these on endothelial cells. The simulated body fluid (SBF) had a similar composition with human blood plasma (Table 2), according to Kokubo solution [27].

Table 2 Composition of SBF.

	Ionic concentration (mM)									
Solution	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}	Buffer	pH
SBF	142	5	1.5	2.5	147.8	4.2	1	0.5		7.25

The glass ceramic powders and composite materials were soaked in SBF solution (solid/liquid ratio of 0.5 mg/ml) and kept for 14 days, at 37°C (in a water bath), without SBF refreshing. After 14 days, the samples were soft washed with distilled water and dried at normal temperature. After drying, the powders were analyzed by XRD and SEM.

Cell viability and fluorescent microscopy

Human endothelial cells (EAhy926 cell line- ATCC -American Type Culture Collection, USA) were grown in DMEM (Dulbecco's Modified Eagle Medium) culture medium containing 10% FBS (Fetal Bovine Serum) and 1% penicillin and neomycin (Sigma Aldrich, St. Louis, MO, USA). For cell proliferation and viability assay (CellTiter96 Non-Radioactive Cell Proliferation

Assay kit, Promega, Madison, USA), EAhy926 cells were seeded in 96-well plate, at a density of 5 x 10^3 cells/well, in DMEM medium, supplemented with 10% FBS. The cells were incubated with collagen and glass ceramic scaffolds for 24 -72 h; controls were represented by endothelial cells grown in the same culture conditions, but without scaffolds. Cell proliferation assay was performed in triplicates, according to manufacturer's guidelines, at different time intervals. Briefly, 15 µl of Solution I was added in each well and incubated for 4 hours. Furthermore, 100 µl of Solution II was added in the 96-well plate, incubated for another hour and spectrophotometry measurements were performed at 570 nm using Mithras LB 940 (Berthold Technology, Germany).

Fluorescent microscopy was assessed using a RED CMTPX fluorophore (Life Technologies, Invitrogen, USA), a cell tracker for long-term tracing of living cells. The RED CMTPX dye was added in the culture medium at a final concentration of 5 µM, incubated 30 minutes for the dye to penetrate the cells. Furthermore, the cells were washed with PBS (Phosphate Buffer Saline) and visualized by fluorescent microscopy. The nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole) with 1mg/ml concentration. The photomicrographs were taken with a digital camera driven by software Axio-Vision 4.6 (Carl Zeiss, Germany).

3. Results and discussion

Fig. 2 presents the XRD patterns of the dried gel thermal treatment at 700°C and glass ceramic powders (1100°C/2h and 1200°C/2h). The XRD analysis of the dried gel thermal treatment at 700°C shows the presence of mainly two mineralogical phases, i.e. dicalcium silicate (C_2S, JCPDS [33-0303]) and calcium oxide (CaO, JCPDS [77-2010]). The characteristic peaks intensities of these mineralogical phases decrease by thermal treatment at 1100°C/2h and 1200°C/2h in the case of glass ceramics, suggesting their transformation into a new mineralogical phase. The XRD analyses of the glass ceramics indicate an important mineralogical phase, i.e. calcium phosphate silicate ($Ca_5(PO_4)_2(SiO_4)_6$, JCPDS [83-1494]).

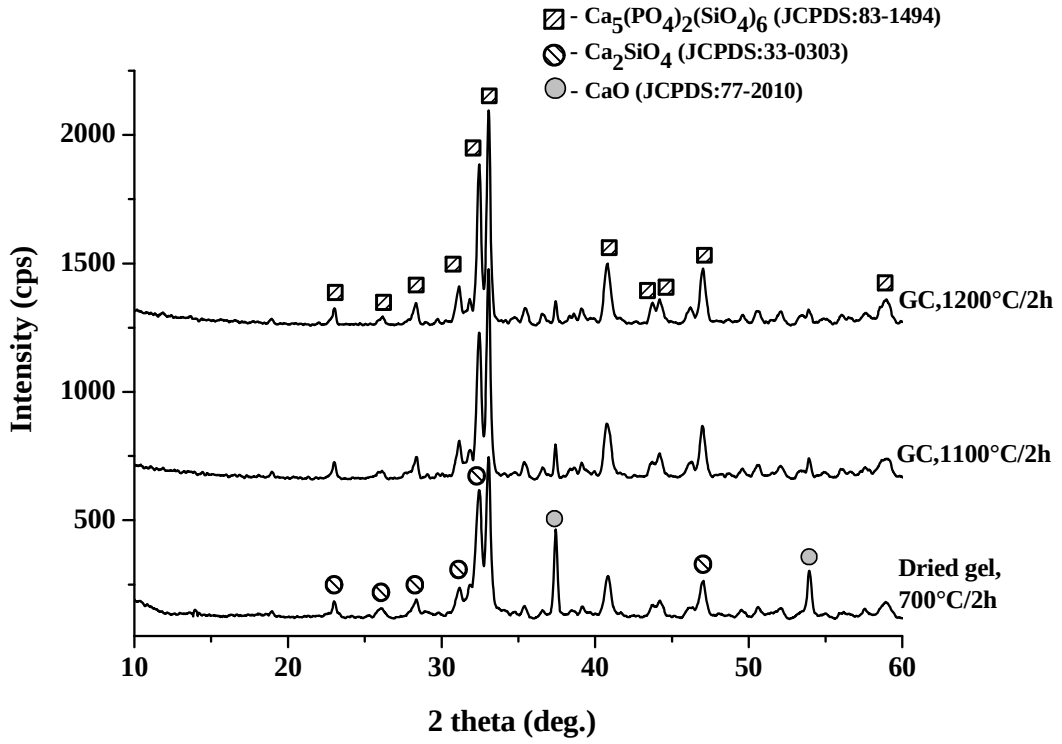

Fig. 2 The XRD patterns of the dried gel thermal treated at 700°C for 2h and glass ceramic powders (1100°C/2h and 1200°C/2h).

The morphological characteristics of the glass ceramic powders are show in figure 3. The increase of the calcining temperature from 1100°C to 1200°C leads to particle size increasing.

The SEM images for the composite materials (figure 4) display the loading of Coll fibres with GC powders, that is a good incorporation and compositional homogeneity.

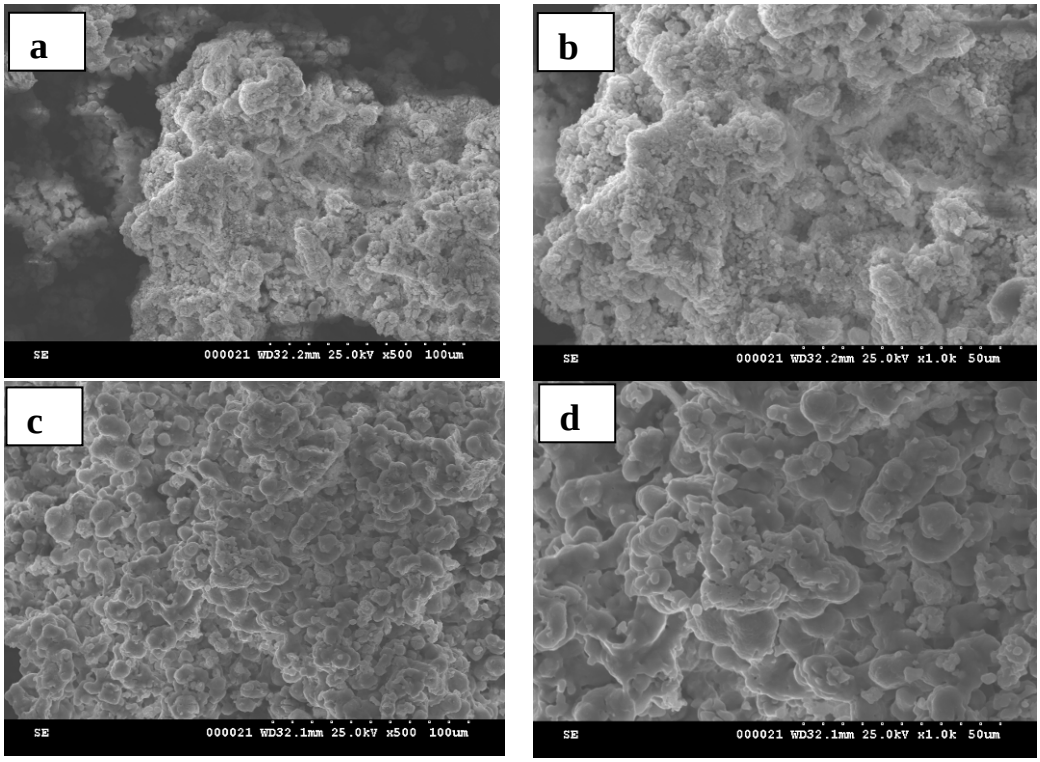

Fig. 3 SEM images of glass ceramic powders obtained at: (a,b) 1100°C/2h and (c,d) 1200°C/2h.

Coll/GC composites biocompatibility, assessed by an *in-vitro* test (SBF soaking for a period of 14 days, at a temperature of 37°C), was evaluated by XRD analyses (figure 5), which show the formation of hydroxyapatite. The biocompatibility was also evaluated by SEM investigation; it can be noticed that this hydroxyapatite is in the form of microcrystals, with spherical morphology (figure 6), with the same characteristics like the hydroxyapatite formed by SBF soaking of the glass ceramics for a period of 14 days, at a temperature of 37°C (figure 7).

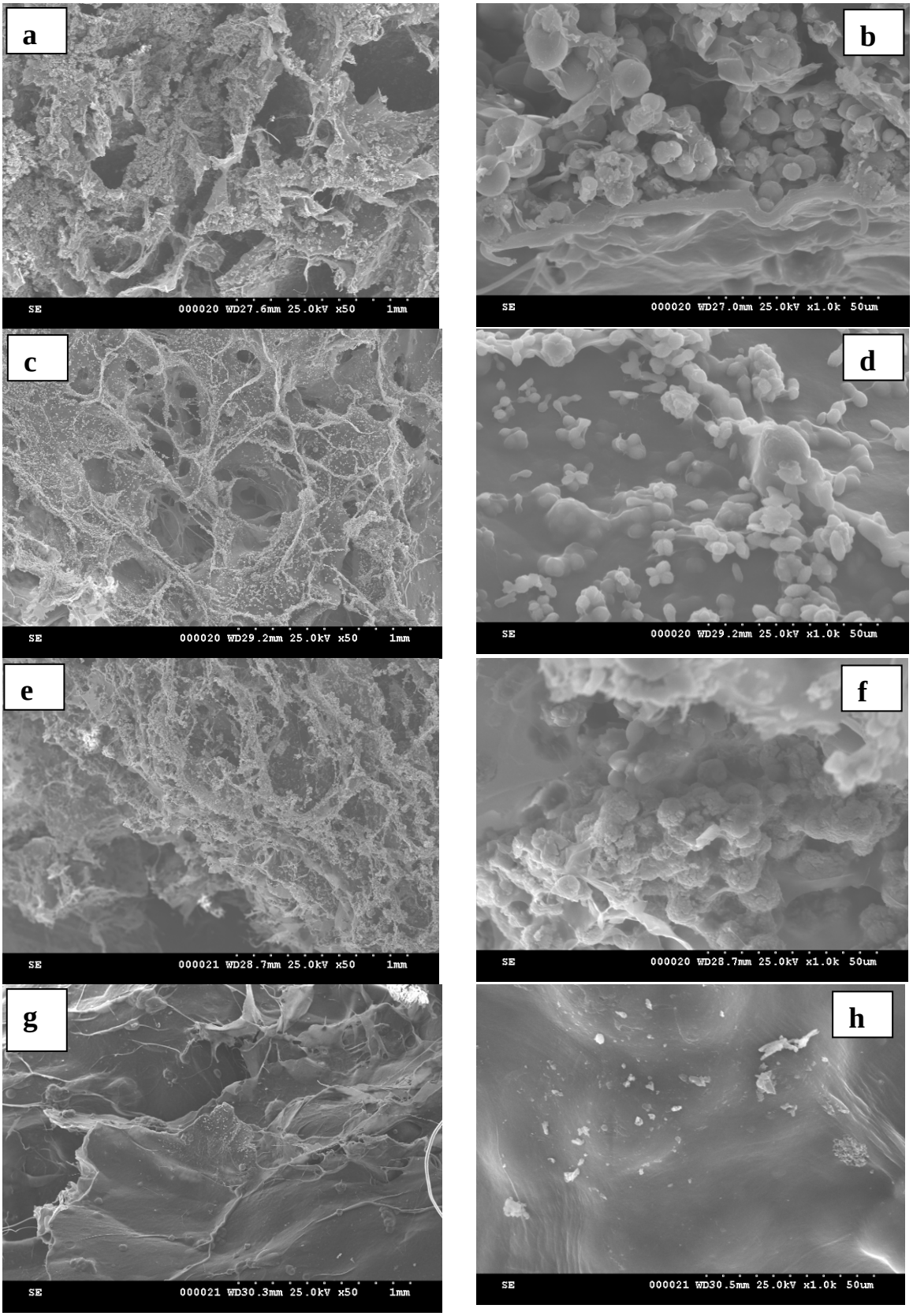

Fig. 4 SEM images of the composite materials: (a, b) MC1, (c, d) MC2, (e, f)MC3 and (g, h) MC4.

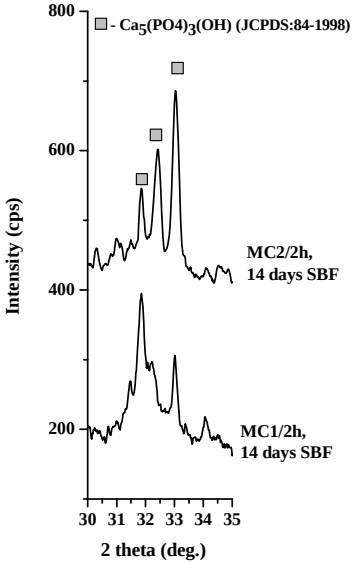

Fig. 5 The XRD patterns of MC1 and MC2 soaked in SBF for 14 days.

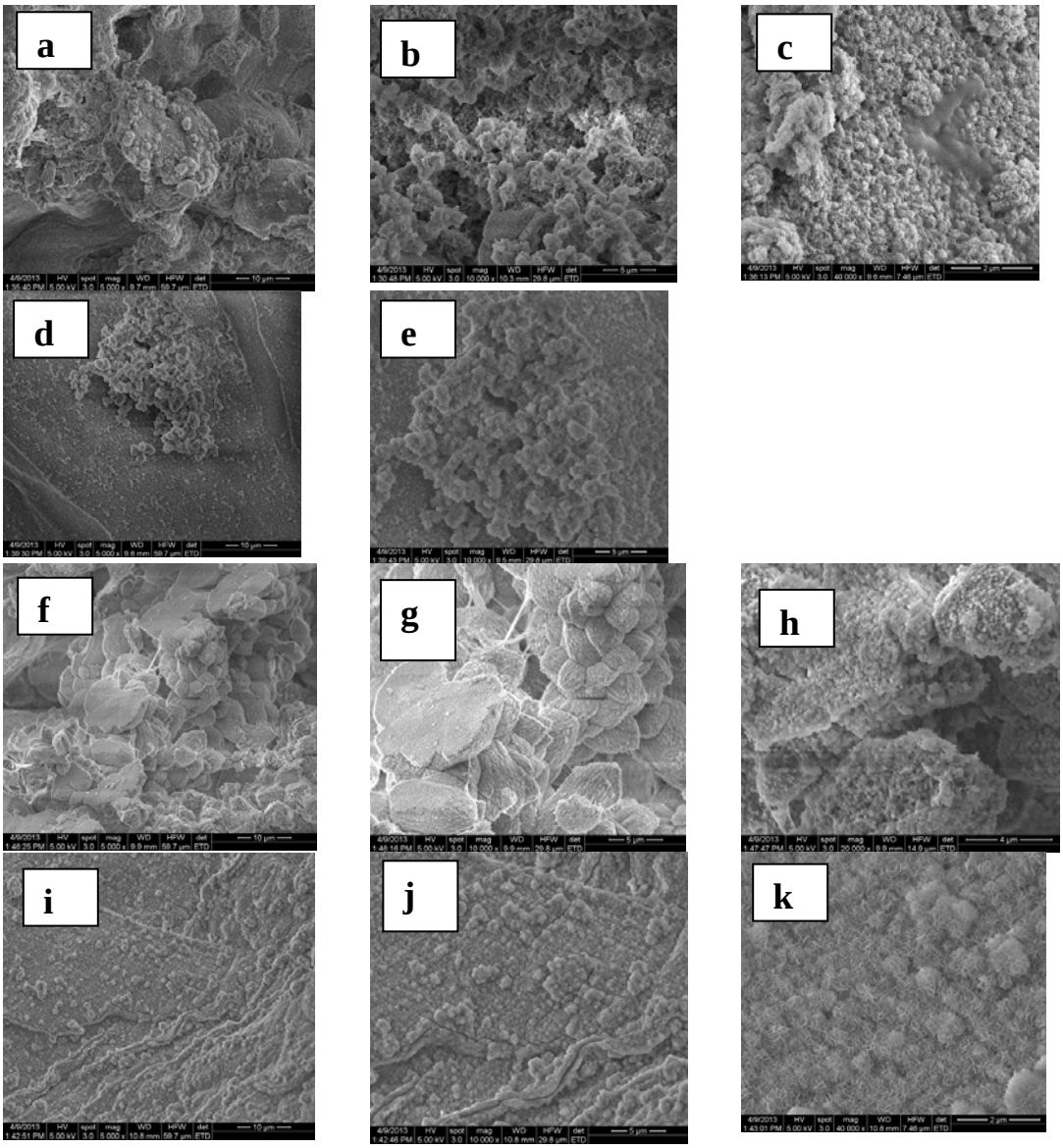

Fig. 6 SEM images of the composite materials soaked in SBF for 14 days:
(a, b, c) MC1, (d, e) MC2, (f, g, h) MC3, (I, j, k) MC4.

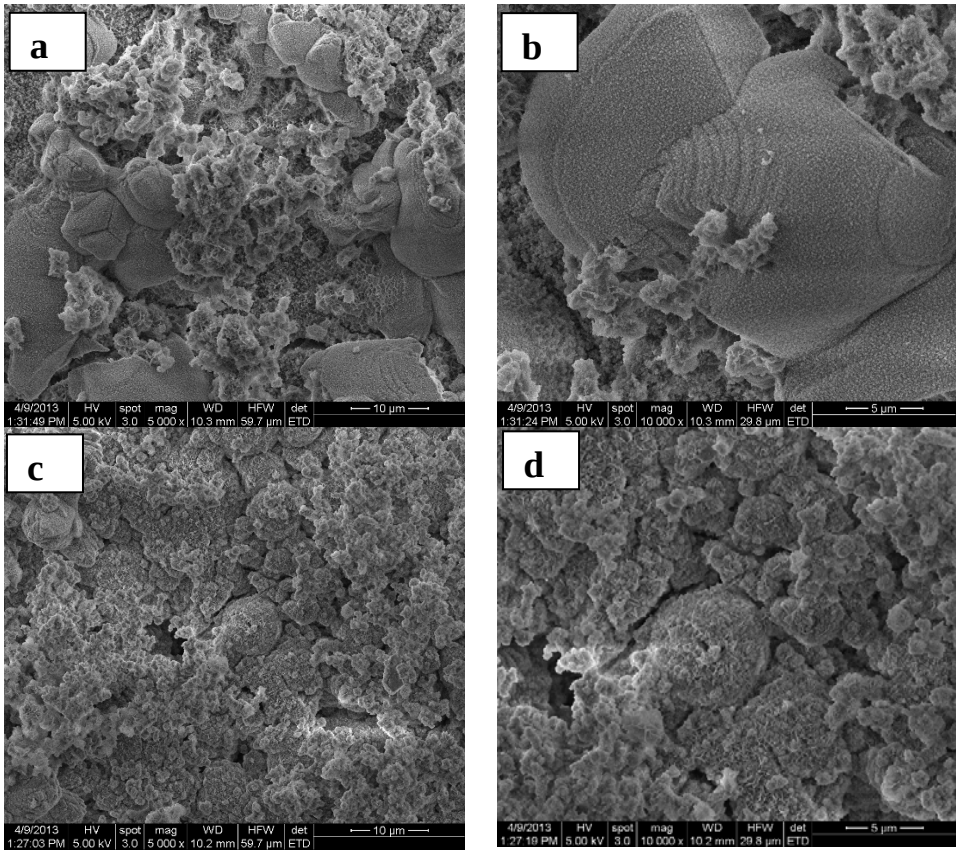

Fig. 7 SEM images of the glass ceramics soaked in SBF for 14 days: (a, b) 1100°C/2h and (c, d) 1200°C/2h.

Cell viability assay shows that, in the presence of collagen and glass ceramic scaffolds, endothelial cells are viable, cell proliferation being maintained comparable to control, suggesting that this type of scaffolds are biocompatible (figure 8). Furthermore, the fluorescent microscopy of endothelial cells labeled with RED CMTPX cell tracker has confirmed the viability of this cell after 5 day in the presence of composite scaffolds. In the viable cells, the RED CMTPX dye reacts with tiol group, in a glutathione S-transferase–mediated reaction, being transformed into a cell-impairment fluorescent dye (577-602 nm); the presence of fluorescent cells is equated to viable endothelial cells (figure 9).

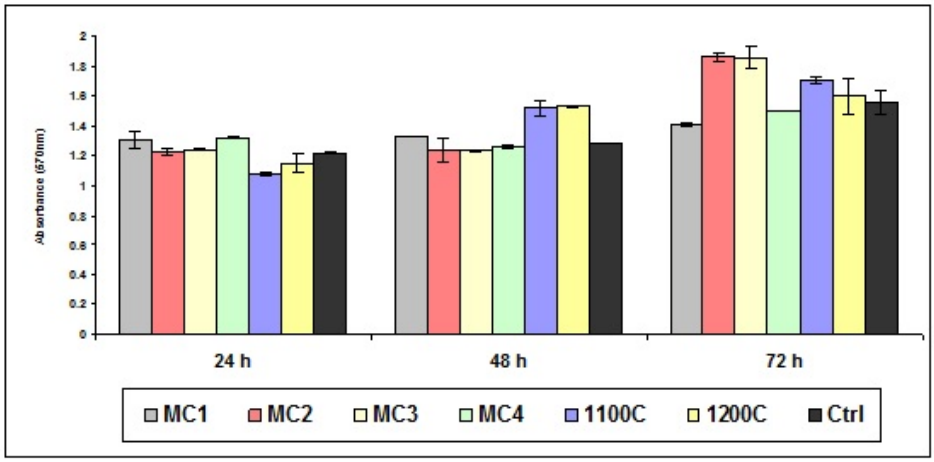

Fig. 8 MTT test for the evaluation of endothelial viability in the presence of Coll/GC composite materials and glass ceramics (1100°C/2h and 1200°C/2h) at different time intervals (n=3, p< 0.05).

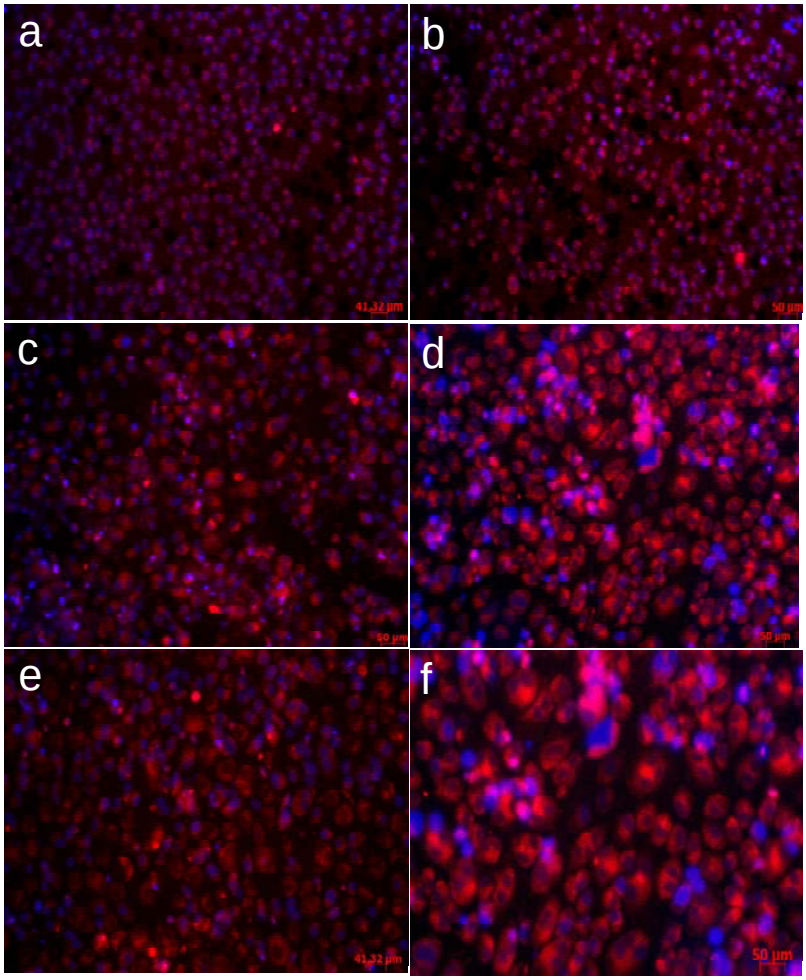

Fig. 9 Photomicrographs of endothelial cells labeled with vital RED CMTPX cell tracker in the presence of the composite scaffolds based on collagen and glass ceramic: (a) GC-1100°C/2h, (b) GC-1200°C/2h (c) MC1, (d)MC2, (e) MC3, (f) MC4, nuclei stained with DAPI (1mg/ml).

4. Conclusions

In this study, glass ceramics with the composition 63 wt% CaO, 27 wt% SiO_2 and 10 wt% P_2O_5 were prepared by the sol-gel method. The XRD analysis indicates the occurance of an important mineralogical phase such as calcium phosphate silicate, obtained after thermal treatments at 700°C and 1200°C. Moreover, Coll/GC composite materials with good homogeneity and porous structure were obtained starting from Coll gel and glass ceramic powders. Coll/GC composites biocompatibility tests reveal the formation of hydroxyapatite after soaking in SBF solution; the hydroxyapatite is in the form of microcrystals, with spherical and lamellar morphology.

Regarding cell viability, our results indicate that in the presence of collagen and glass ceramic scaffolds, endothelial cells are viable, cell proliferation being maintained comparable to control. This assay together with the SEM investigation suggests that this type of scaffolds is biocompatible and promise the bioactivity improving.

References

[1] X. Li, J. Chang, J. Mater. Sci.: Mater. Med., **16**, 361 (2005).
[2] J. Yong, Y.H. Ma, M. Jiang, Wear, **225**, 734 (2003).
[3] P.N De Aza, Z.B Luklinska, M.R Anseau, M Hector, F Guitián, S De Aza, Biomaterials, **21**, 1735 (2000).
[4] L.L. Hench, J. Mater. Sci.: Mater. Med., **17**, 967 (2006).
[5] J.R. Jones, Acta Biomaterialia, **9**, 4457 (2013).
[6] L.-C. Gerhardt and A.R. Boccaccini, Materials, **3**, 3867 (2010).
[7] L. Radev, V. Hristov, I. Michailova, B. Samuneva, Cent. Eur. J. Chem., **7**, 317 (2009).
[8] L. Radev, V. Hristov, I. Michailova, B. Samuneva, Cent. Eur. J. Chem., **7**, 322 (2009).
[9] C. Vitale-Brovarone, E. Verne, L. Robiglio, P. Appendino, F. Bassi, G. Martinasso, G. Muzio, R. Canuto, Acta Biomaterialia, **3**, 199 (2007).
[10] A. Saboori, M. Rabiee, F. Moztarzadeh, M. Sheikhi, M. Tahriri, M.. Karimi, Mater. Sci. Eng. C, **29**, 335 (2009).
[11] O. Bretcanu, S. Spriano, C.B. Vitale, E. Verne, J. Mater. Sci., **41**, 1029 (2006).
[12] R. Ravarian, F. Moztarzadeh, M. Solati Hashjin, S.M. Rabiee, P. Khoshakhlagh, M. Tahriri, Ceram. Int., **36**, 291 (2010).
[13] G. E. Stan, A. C. Popa, D. Bojin, Dig. J. Nanomater. Bios., **5**, 557 (2010).
[14] D.G. Li, D.L. Zhou, Y. Lin, T.H. Pan, G.S. Chen, Q.D. Yin, Mater. Sci. Eng. C, **30**, 148(2010).
[15] M.N. Rahaman, D.E. Day, B.S. Bal, Q. Fu, S.B. Jung, L.F. Bonewald, A.P. Tomsia, Acta Biomaterialia, **7**, 2355 (2011).
[16] A. Balamurugan, G. Balossier, S. Kannan, J. Michel, A.H.S. Rebelo, J.M.F. Ferreira, Acta Biomaterialia, **3**, 255 (2007).
[17] X. Ding, A.W.G. Alani, J.R. Robinson, Extended release and targeted drug delivery system. In: D.B. Troy, Remington, The Science and Practice of Pharmacy, 21st edition (Lipincott Williams and Wilkins, Philadelphia; 2006).
[18] W. Friess, Biopolymers for parenteral drug delivery in cancer treatment. In: D.M. Brown editors, Drug delivery systems in cancer therapy (Humana Press, 2004).
[19] N. Ahmed, C.E. Mora-Huertas, C. Jaafar-Maalej, H. Fessi, A. Elaissari, Polymeric drug delivery systems for encapsulating hydrophobic drugs. In: D. Douroumis, A. Fahr, editors, Drug delivery strategies for poorly water-soluble drugs (John Wiley & Sons Ltd, Oxford, UK, 2013).
[20] A. Kumari, S.K. Yadav, S.C. Yadav, Colloid. Surf. B, **75**, 1 (2010).
[21] W.Friess, Eur. J. Pharm. Biopharm., **45**, 113 (1998).
[22] K. Panduranga Rao, J. Biomat. Sci-Polym. E., **7**, 623 (1995).
[23] D.G. Wallace, J..Rosenblatt, Adv. Drug Deliver. Rev., **55**, 1631 (2003).
[24] Z. Ruszczak, W. Friess, Adv. Drug De liver. Rev., **55**, 1679 (2003).
[25] K. Fujioka, M. Maeda, T. Hojo, A..Sano, Adv. Drug Deliver. Rev., **31**, 247 (1988).
[26] S.I. Jinga, A. Badanoiu, G. Voicu, I. Vasile, Romanian J. Mater., **43**, 396 (2013).
[27] T. Kokubo, H. Takadama, Biomaterials, **27**, 2907 (2006).