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Copper-doped 45S5 bioglass nanoparticles for tissue engineering applications: A comparative study

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ABSTRACT

In this study, 45S5 bioglass and copper-doped 45S5 bioglass nanoparticles were prepared using sol gel technique, copper doped in the structure of 45S5 bioglass at three concentration to determine a biocompatible and effective concentration that is appropriate for Tissue engineering application. The prepared nanoparticles were analyzed using Fourier Transform Infrared Spectroscopy (FTIR), as well as X-ray diffraction (XRD) analysis and EDAX. The nanostructures of the prepared samples were analyzed using Scanning Electron Microscopy (SEM). The effects of the doped copper were studied on the cytotoxicity of the 45S5 bioglass nanoparticles structure indirectly using MTT analysis by L929 mouse fibroblast cells. It was shown that increasing the concentration of copper can decrease the glass transition temperatures, and affecting the structure of 45S5 bioglass, although increasing the copper content were cytotoxic. MTT assay revealed 1% copper is suitable for cell viability and is no toxic.

Keywords: *45S5 Bioglass nanoparticles, copper, cell viability.*

1. INTRODUCTION

Biomaterials are widely used in tissue engineering to restore the function of diseased tissues and organ in the form of implants and biodegradable scaffolds [1, 2]. During tissue engineering process, cell behavior affected by bioactive materials. Bio glasses and glass ceramics, have currently proved their importance as hopeful biomaterials for tissue engineering due to their capability to maintenance bone cell growth [3], attachment to both soft and hard tissues, ability to restoration defect sites and manageable degradation degree in vivo [4–6]. Bioactive glass and glass ceramic can be successfully used as substitutes for bone regeneration in orthopedic, dental and craniofacial surgery and in the field of bone tissue engineering, as granules, implants and nano particles [7], the ability of bioactive glass to bond to bone and osteoblasts stimulation made these materials good candidates for using in tissue engineering [8]. Bioactive derived scaffolds are suitable for bone tissue engineering uses, since their ability to serve as a mineralization agent through hydroxy-carbonated apatite (HCA) layer formation on their surface [7,8]. Microstructure of the scaffolds as well as the Glass compositions are essential factors that control the degradation degree and transformation to an HA-like biomaterial, response to cells. 45S5 bioglass (composed of: 45% SiO_2, 6% P_2O_5, 24.5% CaO, 24.5% Na_2O) is an extremely biocompatible material with an antibacterial effect [5, 9]. It was earlier reported that bioactive glasses have good osteoconductivity and bioactivity and manageable bio-degradability. These benefits make bioactive glasses favorable scaffold materials for bone tissue engineering [10]. 45S5 Bioglass® may possibly improve osteogenesis over a straight control over genes that regulate cell proliferation [11]. For production the glasses with improved bioactivity, the sol-gel route preferred, compared to melt-derived route [12]. Via this method particulated and porous scaffolds can be manufactured, through ease of control of textural properties, and once contact with simulated body fluids rapidly formed an apatite layer on the surface of bioactive glasses [11–13]. Various elements can doped in the structure of sol-gel derived bioglasses, such as Cu, Zn and Sr. Reactions on the surface of bioglass encourage the release of soluble ions such as Si, Ca, P and doped ions, [10, 14, 15]. With these ionic products, bioglass has been stated to be able to intensely affect cell behaviors, in particular, neovascularization of endothelial cells (ECs), which finally stimulates angiogenesis and generally, stimulates the vital tissue growth [16]. Recent studies have revealed that bioglass can stimulate neo-angiogenesis, critical to tissue engineered constructs [17], also for wound healing. And this ability of bioglasses has providing an alternative method to the use of costly growth factors for stimulating neovascularization [18] of tissue engineered scaffolds. bioglass has been proved to be able to stimulate angiogenesis of ECs and increase the secretion of angiogenic growth factors from fibroblasts, including VEGF and bFGF. It has been well known that these angiogenic growth factors are critically important for wound healing [19]. In addition, the bioactive glass activated fibroblast skin tissue engineering grafts could largely increase the blood vessel formation, improve the production of collagen I, and stimulate the differentiation of fibroblasts into myofibroblasts in the wound site, which finally accelerate wound healing [19, 20].

We investigated the effects of copper ion on 45S5 bioglass structure with the goal to find a proper ratio of doped copper ion to modify the 45S5 bioglass nanoparticles for application in tissue engineering. This study demonstrates that the copper ion doped 45S5 bioglass, at proper ion ratio is no toxic, and cell viable on the surface of bioglass, that is beneficial for both hard and soft tissue

engineering because of angiogenic properties of copper ion and bioglass.

2. EXPERIMENTAL SECTION

2.1. Sol-gel method.

Bioglass 45S5 (45 wt% SiO_2, 6 wt% P_2O_5, 24.5 wt% CaO, 24.5 wt% Na_2O) and copper containing 45S5, respectively containing 1% and 3% copper ion instead of Ca ion in the structure of bioglass were synthesized by sol-gel process. For this reason, tetraethyl-orthosilicate (TEOS, $Si(OC_4H_9)_4$, 99.99 %, Sigma Aldrich), triethyl phosphite ($P(OEt)_3$, $P(C_2H_5O)_3$, 99.5 %, Sigma Aldrich), calcium nitrate tetrahydrate ($Ca(NO_3)_2$ x $4H_2O$, 99.60 %, Sigma Aldrich), copper nitrate and sodium nitrate ($NaNO_3$, 100.40 % Sigma Aldrich) were used in the hydrolysis and polycondensation reaction. Nitric acid (0.1 M) was used as catalyzer of TEOS and $(P(OEt)_3$. The mentioned reactant were one after the other added to the mixture at intervals of 45 minutes under constant magnetic stirring at room temperature like our previous report for fabrication of submicron bioglass fibers [21], with some modifications. The resulting sol was kept at room temperature for about 3 days, aged at 40 ℃ for 72 h and lastly desiccated in oven at 120 ℃ for 36 h, allowing the removal of water. In the subsequent, the achieved product was thermally preserved in air at different temperatures (i.e. 600, 650, 700, 800℃) in the subsequent conditions: heating rate 10 ℃/min, cooling rate 20 ℃/min, with 20 minutes soaking time.

2.2. Powder characterization.

2.2.1. Differential scanning calorimetry and thermo gravimetric analysis (DSC-TGA).

The thermal behavior of the sol-gel derived 45S5 bioglass nanoparticles was investigated by simultaneous thermogravimetry and differential thermal analysis (DSC–TGA; sample weight of about 50 mg, heating rate of 10 ℃/min, peak temperature of 1,000 ℃).

2.2.2. Fourier Transform Infrared Spectroscopy (FTIR).

The functional groups of sol-gel derived samples were studied by FTIR with Bomem MB 100 spectrometer. For FTIR analysis, the first 1 mg of the fine particles were carefully mixed with 300 mg of KBr (infra-red grade) and palletized under vacuum. Then the pellets were evaluated in the region 400-4,000 cm^{-1} at the scan speed of 23 scan/min through a spectral resolution of 4 cm^{-1}.

2.2.3. Scanning electron microscopy (SEM) and energy dispersive X-ray analyzer system (EDAX).

Scanning electron microscopy (SEM; Philips XL30 microscope equipped with an, Oxford Instrument, INCA, England). Was used for investigation the morphology of the sol-gel derived bio glass nanoparticles and presence of desired ions in the structure of synthesized nanoparticles. The micrographs were obtained using accelerating voltage between 15-25 kV.

2.2.4. X-ray diffraction (XRD) analyses.

X-Ray diffraction patterns of sol-gel derived bio glass were recorded on a Philips X-ray diffractometer with Co-K_α radiation (λ = 1.78901 Å). The scans of the selected diffraction peaks were performed in step mode (step size 0.02°, measurement time 1s, measurement temperature 25 ℃, and standard: Si powder). Crystallographic identification of the phases of synthesized sol-gel derived bio glass nanoparticles was accomplished by comparing experimental XRD patterns to standards compiled by the International Center for Diffraction Data (ICDD).

2.3. Cell viability assay (MTT assay).

Cell viability evaluated by 3- [4,5-dimethylthiazol-2-YL] -2,5 diphenyltetrazolium bromide (MTT) assay on days 1, 3 and 5. For this experiment 5 mg/ml MTT solution was prepared by dissolving the MTT powder (Sigma, Germany) in warm PBS (37 ◦C). About 1×10^4 cells / well in 96 microtiterplates (NUNC, Denmark) were incubated for 24 h, 20 µL of MTT solution was added to each well for 4 h were incubated in a CO2 5% in incubator at 37◦C. The solution was then removed and the scaffolds were blotted with filter paper. dimethylsulfoxide- 99.5% (DMSO, Sigma, Germany) was added to each well and the plate was shaken for 5 min with stirring. Then absorbance of 100 µL of this solution was read at 570 nm with an ELISA plate ELISA reader.

2.4. Statistical analysis.

All experiments were performed in fifth replicate. The results were given as means ? standard error (SE). Statistical analysis was performed by using One-way ANOVA and Tukey test with significance reported when $P < 0.05$. For investigation of group normalizing, Kolmogorov–Smirnov test was used.

3. RESULTS SECTION

3.1. Differential scanning calorimetry and thermogravimetric analysis (DSC-TGA).

To attain the right heat behavior, DSC and TGA analysis of sol-gel derived bioglass nanoparticles were performed. As shown in figure DSC-TGA curves gained from powder among room temperatures toward 1000 ℃. Endothermic and exothermic peaks detectable in the DSC curves. Endothermic peaks resemble to the elimination of sodium nitrite and other nitrogen compounds. And, the exothermic peak shows the formation of a crystalline phase and phase transformation.

At 75◦C, an endothermic peak observed, relates to the release of materially adsorbed water, which was not detached through drying. TGA trace shows that all water and polycondensation reaction products, between 75 and 160◦C were removed. The other endothermic peaks, starting at 300 ℃, are associated with pyrolysis reaction of free organic species, peaks observed among 600-700℃ related to removal of nitrates which are commonly removed in the thermal stabilization process. Hence all nitrates were omitted in this range of temperature. Above 700 ℃ no substantial weight loss was detected (Figure 1), and this

temperature the structure of 45S5 bioglass nanoparticles will be entirely stabilized. Cu was integrated in the structure of 45S5 bioglass nanoparticles as Cu^{2+}. Copper decreases the glass transition and descents melting onset temperature [21]. The optimum stabilization temperature, which all the nitrogen components were removed, is around 700 ℃ conferring in a previous studies [21, 22].

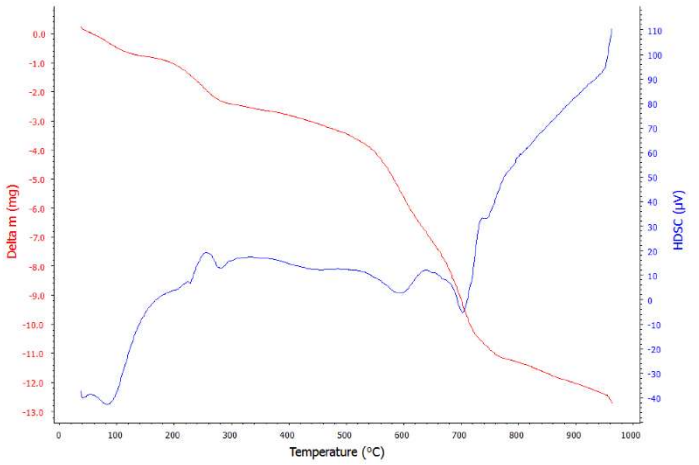

Figure 1. Differential scanning calorimetry and thermogravimetric (DSC-TGA) analysis of 45S5 nanoparticles.

3.2. SEM and energy dispersive X-ray analyzer system (EDAX).

The morphology of bioglass powder were observed with SEM. Figure 2 illustrated SEM micrographs taken from the heat preserved nanoparticles and sintered bio glasses at different temperatures. As shown in these micrographs the diameter of nanoparticles were beneath 100 nm. Addition of copper in the structure of bioglass affecting the crystal formation and sintering the nanoparticles when temperature increased. The presence of copper ion (Figure 3) in copper doped bioglasses investigated with an energy dispersive X-ray analyzer system (EDAX).

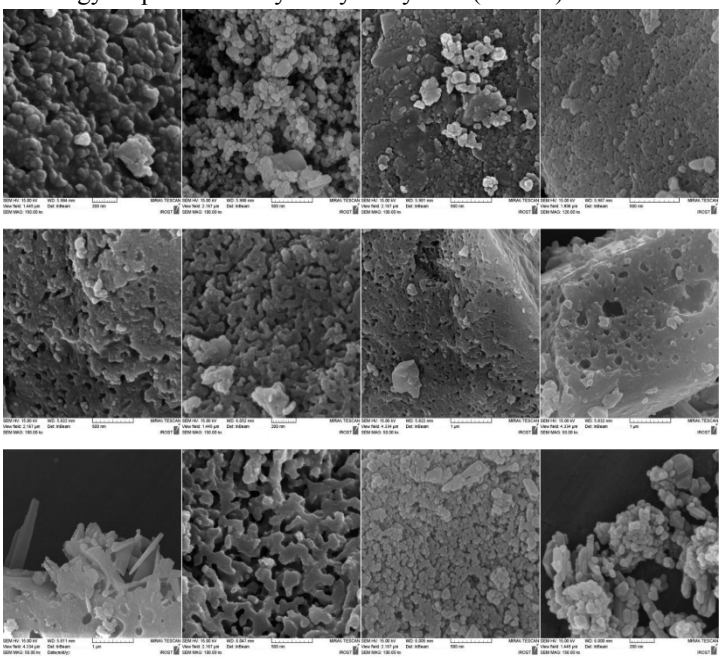

Figure 2. Scanning electron microscopy (SEM) micrograph of sol-derived copper doped 45S5 bioglass nanoparticles.

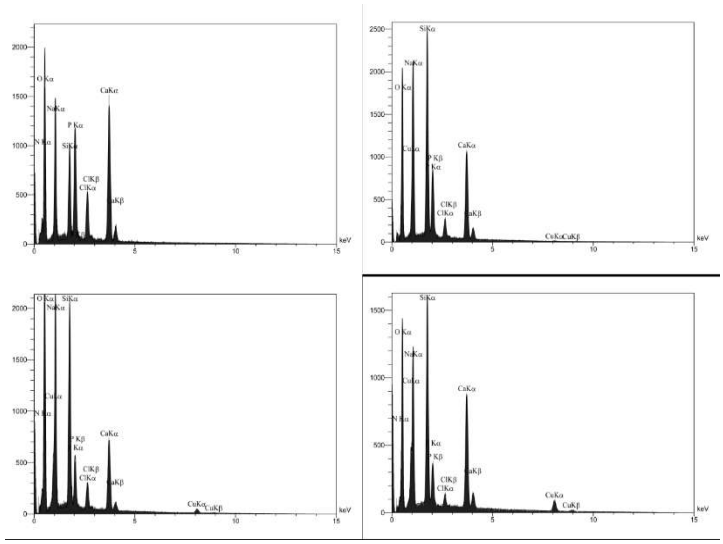

Figure 3. EDAX of bioglass nanoparticles, 45S5, 1%, 3% and 5% copper doped bioglass nanoparticles.

3.3. Crystallographic properties of nanoparticles (XRD analysis).

Bioglass nanoparticles were analyzed using XRD. Figure 4 demonstrations diffractograms gained from XRD for the bio glass nanoparticles samples at different temperatures. The diffractogram has weak peaks demonstrating a type of amorphous or semi-crystalline nature. At 600 -650 ℃ these peaks attributed to sodium nitrate and halite, At 700 ℃ and above this temperature these peaks can be attributed to combeite, according to the ICCD database. While adding copper to bio glass network does not affect the emergence of sodium calcium silicate phases.

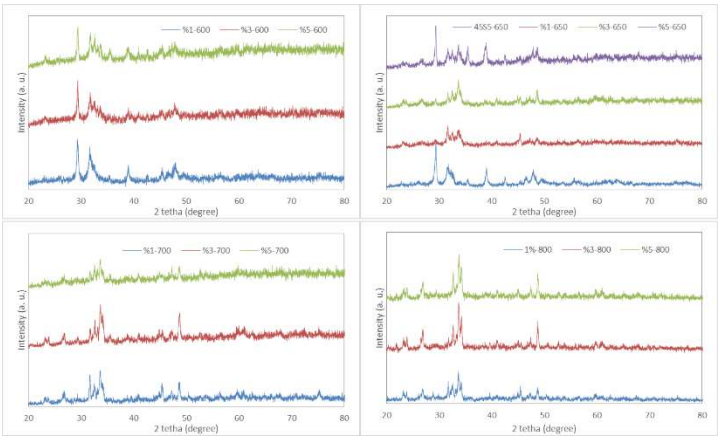

Figure 4. XRD patterns of sol-derived copper doped 45S5 bioglass nanoparticles after heating at 600 ℃, 650 ℃, 700 ℃ and 800℃.

3.4. FTIR analysis.

FTIR analysis was performed in the spectral range of 400-4000 cm^{-1}, for Heat treated copper doped 45S5 bioglass nanoparticles (Figure 5). FTIR spectra of copper doped 45S5 bioglass nanoparticles demonstrated a number of characteristic spectral bands found at: 464, 940 and 1084 cm^{-1}, which are related to the silicate network and attributed to the Si-O-Si bending vibration, Si-O stretching vibration and asymmetric stretching vibration of Si-O-Si, respectively [22]. At 600 ℃ around 1380 cm^{-1} a sharp peak observed related to nitrate residues, increasing the temperature above 650 eliminate this peak, moreover in the samples above 700 ℃ this peak completely omitted [7, 8].

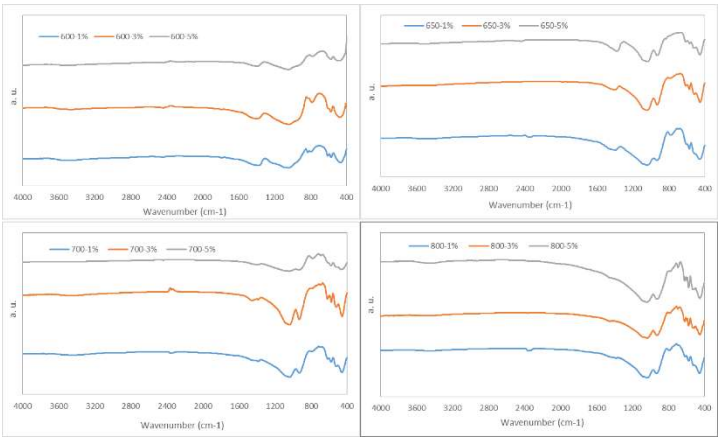

Figure 5. FTIR spectrum of sol-derived copper doped 45S5 bioglass nanoparticles after heating at 600 ℃, 650 ℃, 700 ℃ and 800℃.

3.6. MTT assay.

MTT assay results shown, 45S5 bioglass nanoparticles did not have any negative effects on proliferation rate of fibroblast compared with plastic cell culture surfaces. Increasing the copper content in 1%, increasing the viability, but other ratio of doped copper decreased the viability of cells. MTT assay was performed at days 1, 3, and 5 on bioglass 45S5 and bioglass 45S5 containing different ratio of copper ion. Significant differences were saw between 5 % copper-doped 45S5 bioglass and control group while insignificant differences were observed in all the times mentioned among 1 % copper doped bioglass and 45S5 nanoparticles vs control group, which indicates that 1 % copper doped bioglass and 45S5 nanoparticles had no cytotoxicity ($p < 0.05$).

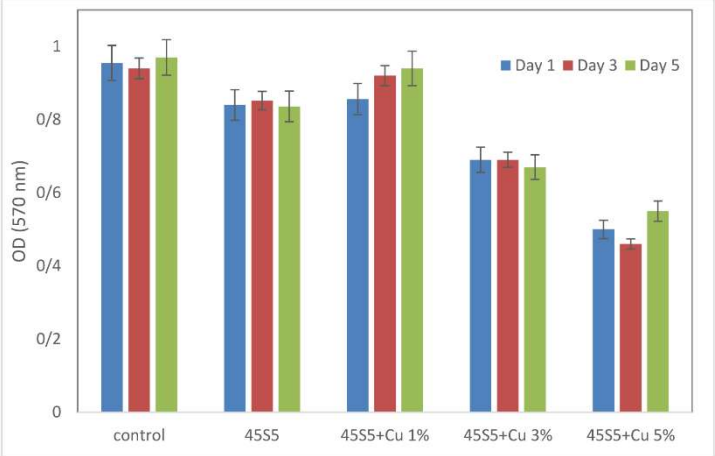

Figure 6. MTT assay analysis for cell culture on scaffold after Day 1, Day 3 and Day 5.

4. CONCLUSIONS

In this study the effect of three ratio of copper doped in the structure of 45S5 bioglass nanoparticles were studied. Sol gel method was used to preparation of 45S5 bioglass nanoparticles. Structural analysis were performed to investigation the desired phase of bioglass by XRD and FTIR. SEM micrographs showed that bioglass nanoparticles were uniform and the diameter of the particles were approximately below 90 nm. EDAX mapped the copper in the structure of copper doped 45S5 bioglass. The effect of copper in the structure of 45S5 bioglass was investigated, increasing the copper content in the structure of bioglass, decrease the crystal formation. Although increasing the ratio of copper in the structure of 45S5 bioglass can improve the elimination of nitrate and organic product, without formation of crystalline phase, but also increase the cytotoxicity of 45S5 bioglass nanoparticles. So among the 1, 3 and 5 % of copper doped in the structure of 45S5 bioglass, 1 % is the proper copper ratio doped in the structure of bioglass, because of desired effect on the structure of 45S5 bioglass nanoparticles and no toxic effect in the fibroblast cells. Cellular biocompatibility assays showed that nanopartcles containing 1 % copper ion in the bioglass structure had more viability in comparison with other ratio of copper, copper ion improved growth of the fibroblast on cell culture flask compared to 45S5 without copper ion and the other ratio of copper. Hence, the results of this study are suggestive for the potential of the appropriate copper doped 45S5 bioglass, as bioactive nanomaterial for tissue engineering applications.

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6. ACKNOWLEDGEMENTS

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