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Double Minerals Ions Co-substituted Nano-Hydroxyapatite Based Biocomposite Implant for Biomedical Applications

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The biocomposite of cerium(III)-copper(II) minerals co-substituted hydroxyapatite (Ce-Cu-HA)/poly(methyl methacrylate) (PMMA) was developed using a process of solvent evaporation. The analysis of X-ray diffraction (XRD), Fourier transform infrared (FT-IR), scanning electron microscope-energy dispersive X-Ray spectroscopy (SEM-EDX) confirmed the formation of Ce-Cu-HA in the PMMA matrix. In addition, the fabricated biocomposite demonstrated high antibacterial activity on *Escherichia coli*, which might be attributed to the action of Ce^{3+} and Cu^{2+} ions release on the substrate that blocks the cell transport pathways contributing to apoptosis. Biocomposites have been tested in osteoblast-like cells for their biological properties (biocompatibilty, alkaline phosphatase (ALP) activity and calcium deposition). Based on the present findings, it was proposed that Ce-Cu-HA/PMMA biocomposite could be a possible therapeutic candidate for bone tissue regeneration.

Keywords: Hydroxyapatite, Biocomposite, Biocompatibility, Antibacterial activity, Osteogenic, Poly(methyl methacrylate).

INTRODUCTION

Owing to the environmental and sustainability concerns, this decade has seen a vast progress in the development of green materials through the production of biocomposites. Biocomposites dependent on poly methacrylic acid have improved physico-chemical properties relative to standard biocomposites [1-3]. The development of moderate hard tissue biocomposites consisting of an organic matrix and an inorganic strengthening component has attracted a great attention for both scientific and application purposes [4]. It can be demonstrated by integrating the beneficial properties of inorganic fillers with that of polymeric materials [5]. However the development of polymer biocomposites with attractive characteristics is a problem for examiners due to the variations in surface energy between polymers and inorganic nanoparticles [6]. Hence, surface alteration of nanoparticles is often needed, in particular for biochemical activity, to resolve the imbalance problem and to develop interactions between polymers and inorganic nanoparticles, leading to fine presence of nanoparticles and improved properties for polymer biocomposites [7].

Residues of impurities monomer units contribute to adverse reactions and weak characteristics of the finished goods that are not suitable in the healthcare profession. These days, orthopaedic composites have become recognized as the ideal implant materials; relative to bone amalgam, composites have increased durability, less protection issues and positive medical outcomes. Orthopaedic biocomposites exhibit of resin matrix and assorted inorganic nanoparticles. Hydroxyapatites (HAs) are primarily used as bone replacements in medical applications biocompatible and biodegradable, easy processing, chemical inertness and compositional similarity to the natural bone [8]. Therefore, the low strength of hydroxyapatite limits its therapeutic usage under load-bearing circumstances.

From a material engineering point of view, the crystalline nature of minerals substituted hydroxyapatite is vital, given the low concentrations of minerals ion replacement occurring in both human tooth and bone minerals phase [9]. The current research is an effort to establish a combined minerals ion replacement hydroxyapatite combining two vital trace minerals, *i.e.* cerium and copper [10,11]. While several publications on the

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physico-chemical characteristics of hydroxyapatite and minerals substituted hydroxyapatite have already been reported. No article has been reported on the substitution of Ce and Cu in hydroxyapatite. Nikitina *et al*. [12] reported the synthesis of Ce and Cu co-substituted hydroxyapatite.

For the use of PMAA in orthopedic implants, the reinforcement of the polymer with bioceramic nanoparticles is essential. In addition, the introduction of hydroxyapatite into PMMA can affect biocompatibility on the biocomposite [13]. For such purposes, biologically active phase-strengthening polymer matrix material such as hydroxyapatite incorporate the usual biologically active behaviour of the improved mechanical properties in bioceramics to make the biocomposites equivalent to mammalian bone tissues. In orthopedic, PMMA based biocomposites have a wide variety of uses [14]. Due to its versatility, the use of such a type of substance will maintain to rise in frequency and use. The fast-moving mechanism of these substances indicates continual improvements in this field. Concerning the significance of Ce and Cu as essential functions, the aim of this study is to examine the physico-chemical and biological properties of Ce-Cu-HA/PMMA biocomposites.

EXPERIMENTAL

Analytical grade poly(methyl methacrylate) (PMMA), $Ca(NO_3)_2{\cdot}4H_2O$, $Cu(NO_3)_2{\cdot}6H_2O$, $Ce(NO_3)_2{\cdot}6H_2O$ and K_2HPO_4 were used as the sources for Ca, Cu, Ce and P, respectively. All the chemicals were purchared from Sigma-Aldrich India. and used without further purification. The double distilled water was employed throughout the experiments.

The fabricated biocomposite (Ce-Cu-HA/PMMA) was characterized with FT-IR (Perkin-Elmer, USA), SEM-EDX (Curl Jdiss Supra 40-2007, Germany) and XRD (PANalytical) techniques.

Fabrication of Ce-Cu-HA nanoparticles: Calcium precursor (0.9 M) was mixed with Ce (0.05 M) and Cu (0.05 M), respectively. The Ca, Ce and Cu precursor mixture was added dropwise to the phosphate precursor solution (0.6 M) in equal ratio with constant stirring for 1 h and raised the pH of the solution to 10 using ammonia solution. The suspension was digested on the microwave irradiation for 0.5 h with 750 W. The resultant precipitate was rinsed twice with double distilled water and dried at 100 ºC in a hot air oven.

Fabrication of Ce-Cu-HA/PMMA biocomposite: The Ce-Cu-HA/PMMA biocomposite were prepared *via* solvent evaporation technique. The prepared 5 wt.% Ce-Cu-HA nanoparticles (2M) was mixed gradually in the PMMA precursor solution (20 wt.% PMMA precursor was prepared in acetone) in the volume ratio 20:5 (Ce-Cu-HA/PMMA). The solution was casted in a glass petri-dish and finally stored at 20 ºC. The solvent was allowed to evaporate slowly and the Ce- Cu-HA/PMMA biocomposite was obtained.

Bacterial activity: The bacterial properties of fabricated biocomposite have been studied against two Gram-negative microorganisms (*Klebsiella* and *E. coli*) *via* the inhibition zone method [15]. The inoculums of the two microorganisms were set up from the crisp overnight medium (Tripton soy medium with 0.6% yeast remove) which were brooded at 37 ºC. The subsequent medium cultures were utilized for the dispersion tests. The agar dilution test was performed at Muller-Hinton agar and it was done by pouring agar into petri plates to shape 4 mm thick layers, which includes 2 mL thick inoculums of the studied bacteria. Petri plates were left to desiccate in air and then, the fabricated biocomposite was tested with various concentrations impregnated into the well against the bacteria at room temperature. The concentrations of biocomposite utilized as a part of the test were 25-100 µL, individually. The concentrations of fabricated biocomposite tests were conducted by dissolving 0.2 g of biocomposite in 2 mL of DMSO. The inhibition zone was checked by measuring the width of the zone of restraint (mm) around the well.

Biocompatibility study: The biocompatibility of Ce-Cu-HA/PMMA was done by utilizing the human osteoblast cells also evaluated *via* MTT assay. The osteoblast cells were seeded at 3×10^3 cells per well in 96-culture plates for 24 and 48 h, furthermore Dulbecco′s altered Eagle′s solution was also included to support the cell development. After this progression, the cells were rinsed over and again by serum free solution and treated with different concentrations of Ce-Cu-HA/PMMA biocomposite (200 and 1000 µg/mL) and subsequently incubated for 24 and 48 h at 37 ºC and then measured at 570 nm by using micro plate reader. Also, a control was set up without Ce-Cu-HA/PMMA biocomposite and to these osteoblast cells with composite wells, 0.45 mg/mL of MTT solution was added and preserved in the CO_2 compartment for 4 h at 37 ºC and measured. To acquire the dependable result, the experiments were conducted in the triplicate.

$$\text{Cell viability (\%)} = \frac{\text{Composite OD}}{\text{Control OD}} \times 100$$

ALP, calcium deposition and collagen formation studies: The ALP activity of osteoblast cells co-colonization on the fabricated biocomposite was examined using ALP colorimetric test. The fabricated biocomposite seeded with osteoblast cells for 1, 3 and 7 days were rinsed twice with PBS followed by the addition of lysis buffer solution and then *p*-nitrophenyl phosphate solution. The solution was incubated for 60 min at 37 ºC. The response was blocked by NaOH and furthermore, the absorbance was noted at 405 nm [16].

The calcium deposition on the fabricated biocomposite was estimated by alizarin red stain [17]. To a subsequent 1, 3 and 7 days of osteoblast cells seeding, the composite were mixed with absolute alcohol for 1 h at 4 ºC, rinsed three times throughly with double distilled water marked by alizarin red marker for 0.5 h. Following a series of double distilled water rinsed, the marker was removed by cetylpyridinium chloride and the absorbance of the removed marker was noted at 562 nm.

Collagen formation through osseous cells on the fabricated biocomposite was established *via* sirius red colorimetric plate assay. Subsequent to 1, 3 and 7 days of cell colonization, the cells were seeded on the fabricated biocomposite with Bouin′s solution. The solution was eliminated after 1 h and the specimen was rinsed with double distilled water, dehydrated with afterwards marked by dye for 1 h. Subsequently, the specimen was

rinsed with HCl and the bound dye was suspended in NaOH. The absorbance of the dye solution was noted at 550 nm [18].

RESULTS AND DISCUSSION

FTIR studies: The FTIR spectra of the PMMA, Ce-Cu-HA and Ce-Cu-HA/PMMA composite are shown in Fig. 1. Fig. 1 exhibits the main characteristic peaks of the phosphate group $(PO_4)^{3-}$ and the hydroxyl group $(OH)^-$ in the Ce-Cu-HA structure which are the characteristics of hydroxyapatite. The peaks ~ 1100 cm^{-1} are associated with phosphate group while the peak at 3460 cm^{-1} is due to the vibrations of the hydroxyl group in Ce-Cu-HA [19,20]. Absorption peaks at 2992 and 2951 cm^{-1} are assigned to the methyl group ($-CH_3$) asymmetric stretching modes of PMMA [6,21]. Moreover, the band at 1728 cm^{-1} identified to the ester carbonyl group (C=O) stretching of PMMA (Fig. 1) [6].

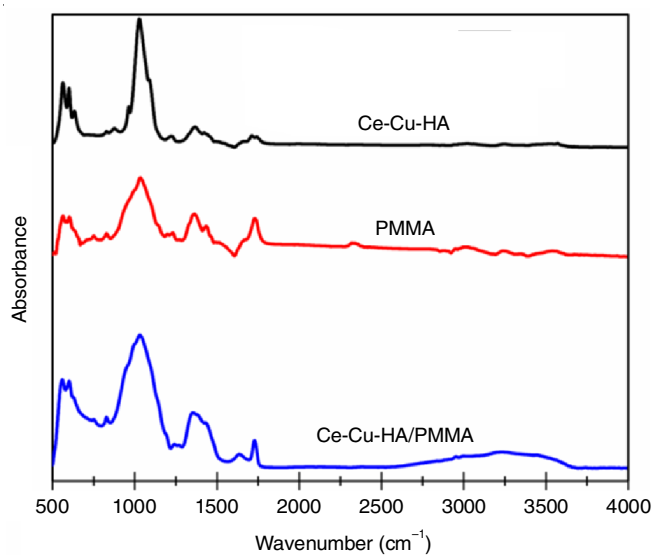

Fig. 1. FT-IR spectra of Ce-Cu-HA (a), PMMA (b) and Ce-Cu-HA/PMMA biocomposite (c)

Fig. 1c illustrates the intense vibration peak compared to C=O at 1710 cm^{-1} and the band at 1284 cm^{-1} incurring to the C-O both are assigned to the distinguishing functional groups of the PMMA matrix. Further, peaks at 1053 and 1022 cm^{-1}, which are specific aspects of the phosphate group of the Ce-Cu -HA. In addition, the band at 1236 cm^{-1} could be associated to the hydrogen bonding of PMMA with the Ce-Cu-HA group. This result also implied some molecular interactions between the Ce-Cu-HA and the PMMA in the biocomposite.

XRD studies: The XRD patterns of Ce-Cu-HA, PMMA, and Ce-Cu-HA/PMMA biocomposite are shown in Fig. 2. XRD patterns of Ce-Cu-HA (Fig.2a) demonstrates an extensive reflection plane in the array of 25° to 53° of 2θ values, which matches to the attribute planes of hydroxyapatite crystal phase (JCPDS card # 9-432) [22]. The XRD pattern for PMMA (Fig. 2b) demonstrate planes at 2θ (14°, 22°, 29° and 41°) and comparative intensities acquired for the polymer contest with the JCPDS card no. 13-0835 file, recognizing it as PMMA [23]. In the XRD pattern of Ce-Cu-HA/PMMA biocomposite (Fig. 2c), the corresponding planes at 2θ (14°, 22°, 29° and 41°)

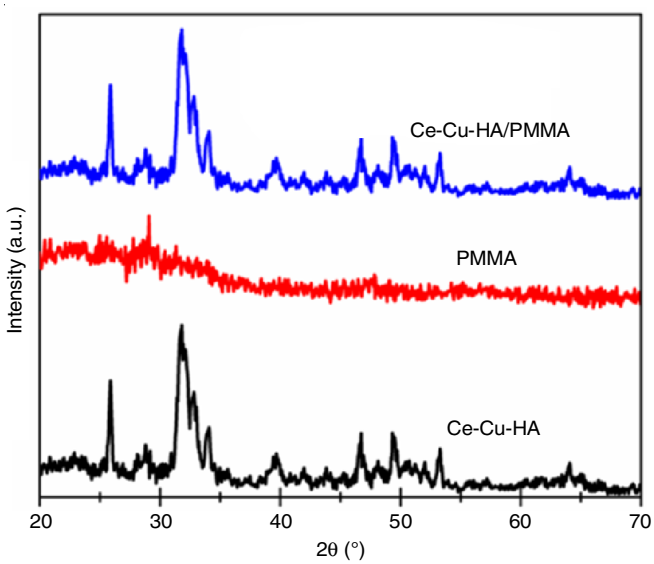

Fig. 2. XRD spectra of Ce-Cu-HA (a), PMMA (b) and Ce-Cu-HA/PMMA biocomposite (c)

correspond to PMMA, while the 2θ (25°, 28°, 31°, 34°, 39°, 46°, 48° and 53°) correspond to hydroxyapatite [24,25]. The XRD study demonstrates the vicinity of Ce-Cu-HA within the PMMA polymer matrix.

Morphological and elemental analysis: The elemental composition surface structure of Ce-Cu-HA and Ce-Cu-HA/PMMA biocomposite are shown in Fig. 3.The SEM images (Fig. 3a) of Ce-Cu-HA nanoparticles revealed that the elements are in the form of spherical with agglomeration and dense nature. Fig. 3c shows the SEM micrographs of Ce-Cu-HA/PMMA biocomposite which display the structure of particles as beads shaped. Thus, it is observed that addition Ce-Cu-HA into PMMA matrix leads to the formation of the fine beads. In addition, the EDX spectrum validates the existence of Ca, P, Ce, Cu, C and O atoms and confirms the Ca/P ratio as 1.6725 and 1.6602 in Ce-Cu-HA and Ce-Cu-HA/PMMA nanocomposites, respectively.

Bacteriostatic effect: The bactericidal action of fabricated Ce-Cu-HA/PMMA biocomposite at four different concentrations (25, 50, 75 and 100 µL) were tested against the two Gram-negative microorganisms *viz. Klebsiella* and *E. coli*. It is found that inhibition zone increased significantly with the increase of Ce-Cu-HA/PMMA biocomposite concentration. This may be owing to the existence of metals, for instance, Ce and Cu in the fabricated composite [26]. Thus, it is confirmed that as the concentration increases, the restraint area for both microbes' was too enhanced which shows the positive antibacterial action against both the studied microbes. Since metals are the components of cartilage, its existence even in small level may show a significant character in strengthening the antibacterial action of apatite.

Biocompatibility study: The biocompatibility of Ce-Cu-HA/PMMA biocomposites was calculated by an MTT assay. The MTT assay showed that osteoblast cells display usual metabolism and development in the existence of Ce-Cu-HA/PMMA. After one day of culturing, osteoblast cell propagation was two times faster on changed surfaces contrasted to control.

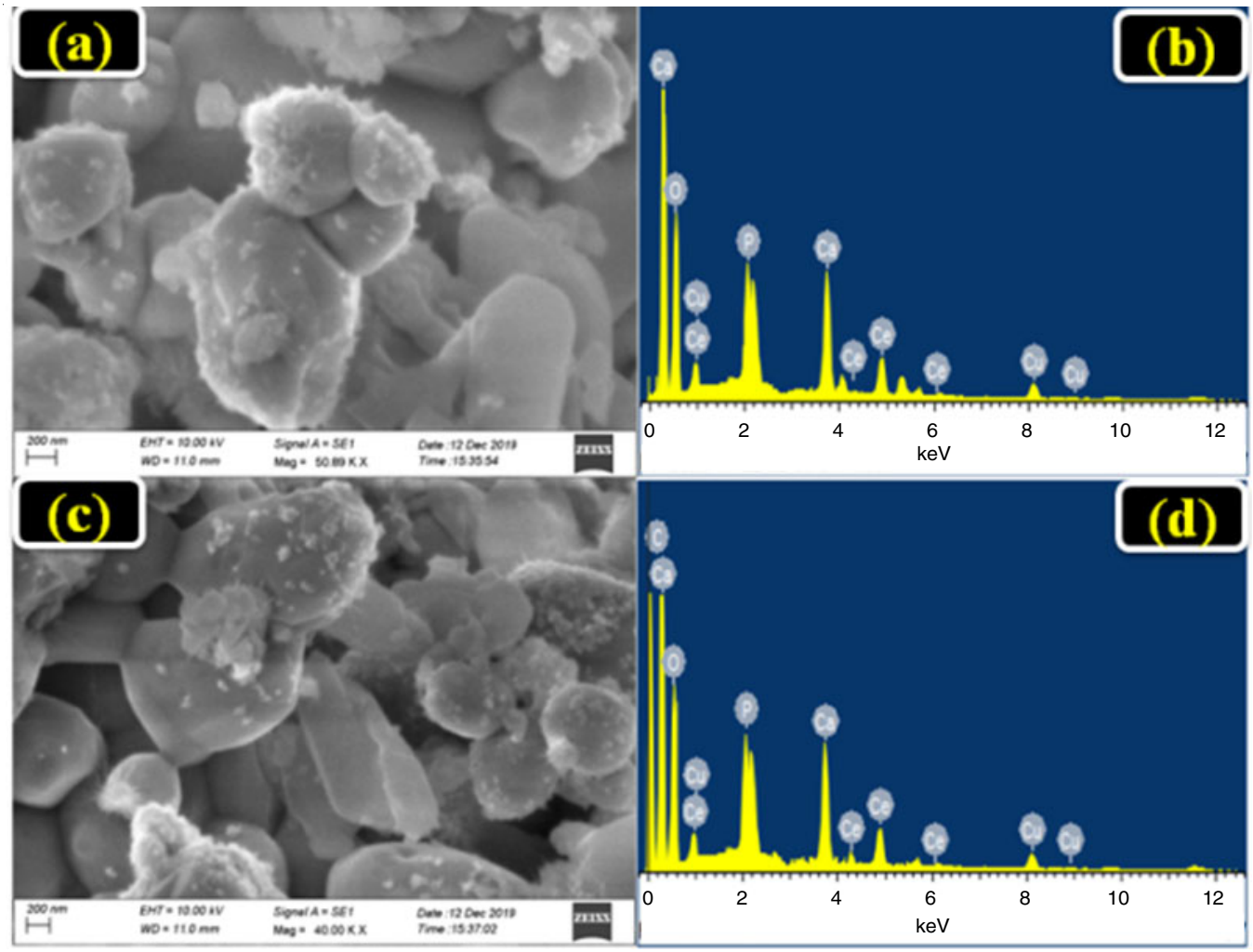

Fig. 3. SEM-EDX images of Ce-Cu-HA (a-b) and Ce-Cu-HA/PMMA biocomposite (c-d)

The similar result was also detected after 3rd day of incubation (Fig. 4).These findings showed the bioavailability ability of biocomposites and validate their use as new surfaces for the production of *in vitro* cultured cells.

Fluorescent imaging pictures also support the feasibility of the physiological procedure, which indicates that osteoblast cell survival was preserved after 3rd days in the presence of Ce-Cu-HA/PMMA. The morphology of attached osteoblast cells had a fairly regular composition on all substances (Fig. 5). These findings are in accordance with other investigators who have shown that Ce-Cu-HA/PMMA biocomposite materials pose strong cytocompatibility, osteogenic characteristics and enhance substrate stability among hydroxyapatite and cellular substrate [27].

The ALP, calcium deposition and collagen formation of the osteoblast cells co-cultured on the Ce-Cu-HA/PMMA biocomposite are shown in Fig. 6. The ALP activity was determined in a co-cultured osteoblast cells on Ce-Cu-HA/PMMA biocomposite (Fig. 6a). While anticipated, the control illustrated the slightest ALP action. The minerals (Ce & Cu) substituted HA in the PMMA polymer matrix produced in major up directive of ALP action in biocomposite as compared to the control. These outcomes recommended that the Ce-Cu-HA/PMMA biocomposite advances the ALP action of the osteoblasts cell differentiation.

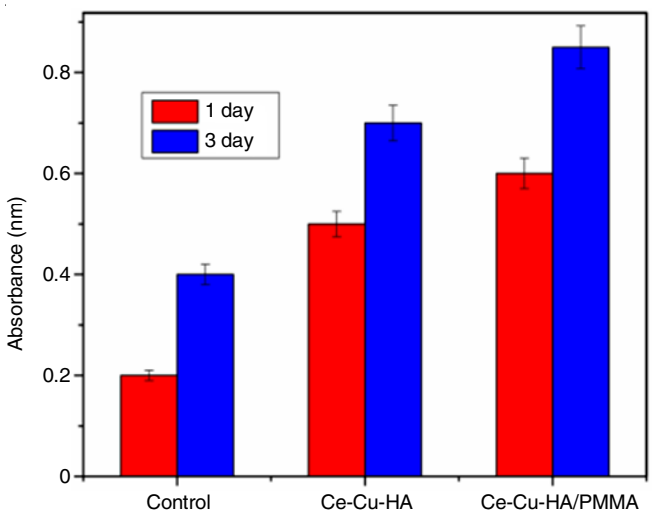

Fig. 4. Osteoblast cells proliferation profiles after growing on control and biocomposites materials for up to 3rd day

The development of calcium deposition on the composite co-colonization with bone cells is the marker for the osseous

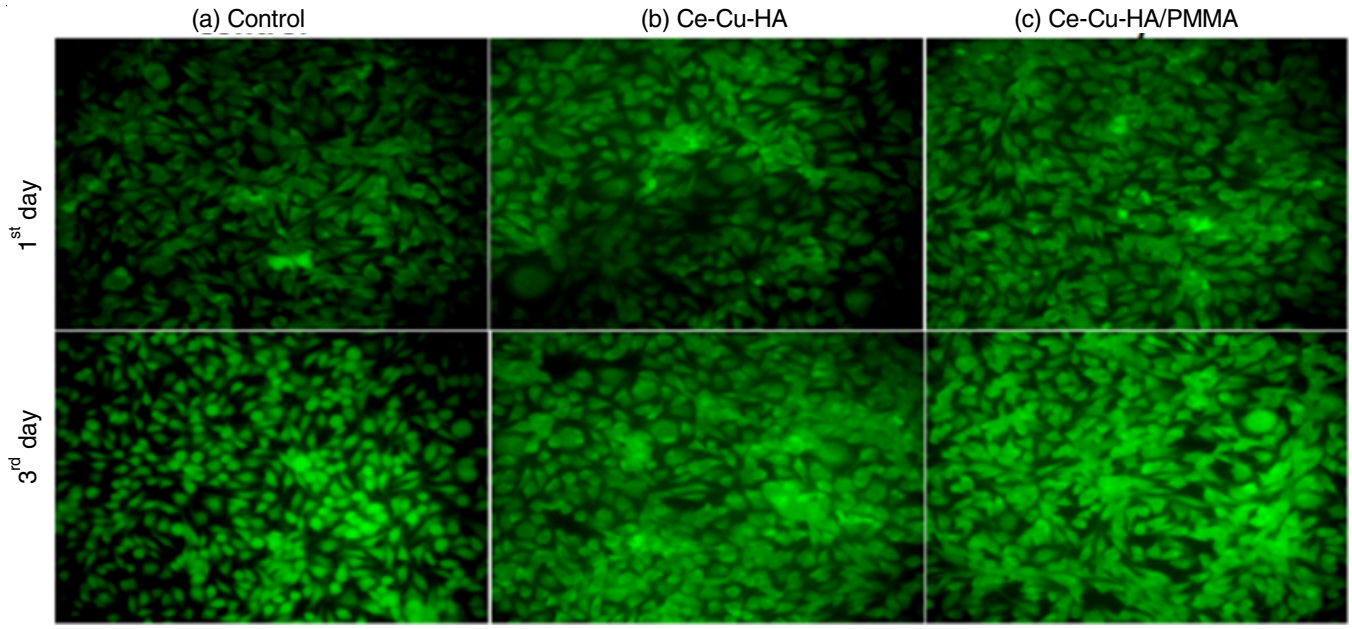

Fig. 5. Fluorescence microscopic images of the osteoblast cell treated for 1st and 3rd days biocomposite materials: (a) control, (b) Ce-Cu-HA and (c) Ce-Cu-HA/PMMA

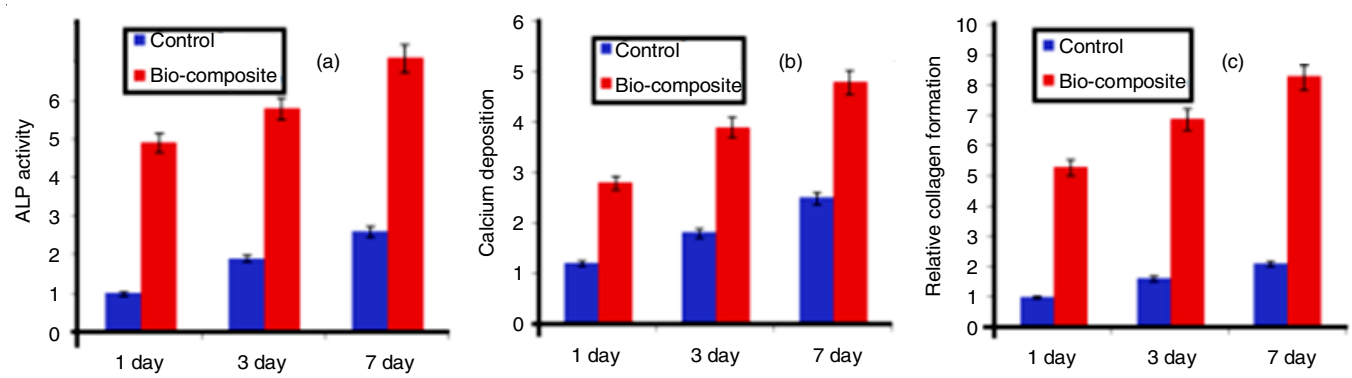

Fig. 6. ALP activity (a), calcium deposition (b) and collagen formation (c) on fabricated biocomposite

cell integrations. Fig. 6b demonstrates the comparative mineralization to normalize the control sample. The calcium deposition consequences were reliable through ALP outcomes.

Collagen is a significant part of bone cell propagation and hard tissue development. The collagen making in the composite by osteoblast cells designates the construction of extracellular matrix inside the biocomposite. The collagen comfortable produced *via* osseous cells seeded on Ce-Cu-HA/PMMA biocomposite as shown in Fig. 6c. A fewer quantity of collagen was detected in control sample, while the sum collagen inside biocomposite was particularly superior. Consequently, the outcomes implied that the encouraging properties of Ce-Cu-HA/PMMA biocomposite towards the osteogenesis.

Conclusion

The Ce-Cu-HA/PMMA biocomposite were fabricated by the solvent evaporation technique. The FTIR and XRD patterns of the prepared biocomposite confirmed the presence the functional groups and the crystalline structure of Ce-Cu-HA/PMMA. The bactericidal results revealed that the fabricated biocomposite demonstrated a strong bactericidal property against *Klebsiella* and *E. coli* (Gram-negative bacteria). Further, *in vitro* cytocompatibility, ALP, calcium deposition and collagen formation results revealed that Ce-Cu-HA/PMMA composite has excellent biocompatibility and cell feasiblility at lower levels of concentrations. Based on the bactericidal property and cytocompatibility results, the fabricated Ce-Cu-HA/PMMA biocomposite can be a potential candidate for bone tissue applications.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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