IJBPAS, November, 2020, 9(11): 2873-2886

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

CERIUM AND LANTHANUM SUBSTITUTED CALCIUM PHOSPHATE COATING ON TITANIUM ALLOY FOR ELECTRODEPOSITION METHOD: IMPROVEMENT OF ANTIBACTERIAL AND BIOACTIVE PROPERTIES

C. SANTHA [1], S. SATHISHKUMAR[1], S. SIVAKUMAR[1], C. SRIDEVI[2] AND A. KAMARAJ[1*]

1: PG and Research Department of Chemistry, Sri Vijay Vidyalaya College of Arts and Science, Dharmapuri, Tamilnadu, India

2: Department of Chemistry, PGP College of Arts and Science, Namakkal- 637 207, Tamilnadu, India

***Corresponding Author: A. Kamaraj: E Mail: sathish.sskg@gmail.com**

Received 15th March 2020; Revised 18th April 2020; Accepted 11th May 2020; Available online 1st Nov. 2020

https://doi.org/10.31032/IJBPAS/2020/9.11.5227

ABSTRACT

Cerium and Lanthanum substituted hydroxyapatite composite was novel biomaterial was coating used on titanium alloy for electrodeposition method. These Ce, La-HAP composite coatings were examined by Fourier transform infrared spectroscopy (FT-IR), X-Ray diffraction (XRD), Scanning electron microscopy (SEM) equipped with energy dispersive X-ray analysis (EDAX). Hence, the antibacterial action of the composite coatings were explored by gram positive bacteria and gram negative bacteria. These results clearly presented that good antibacterial activity. Since, the *In vitro* cell viability and alkaline phosphate activity of the coatings was studied by MC3T3-E1 human osteoblastic cell lines. The *in vitro* cell proliferation analysis discovered that the Ce, La-HAP coating is initiate appropriate for the development of freshly cell growth which evidences the superior bioactive environment of the coating. Therefore, Ce, La-HAP composite coating on titanium alloy can play a significant role in dental and orthopedic applications.

Keywords: Ce, La-HAP Composite coating, Electrodeposition, Antibacterial activity, biocompatibility

1. INTRODUCTION

The primary necessity for any bio material to be retained the human body is that it must revealed better bioactivity and stability in physiological body fluid **[1-2]**. The metal that is to be applied as implants need of good corrosion resistance and enhanced biocompatibility. Till day titanium alloy is the utmost frequently used biometals in orthopedic devices owing to excellent bioactivity and superb corrosion resistance. In adding, hydroxyapatite [$Ca_{10}(PO_4)_6$ OH_2] has been significantly used as a biocompatible material on metals like titanium alloy used for enhancement of bone development **[3-6]**. Then the ordinary hydroxyapatite bio material main role in new bone growth and tissue regenerated functions. The synthetic hydroxyapatite bioceramics with small amount of mineral ions improved its biomedical applications. Recently going to hot research topics achieved the different ionic substitutions such as lanthanum (La^{3+}),Cerium (Ce^{3+}), Europium (Eu^{2+}), Terbium (Tb^{2+}) etc., in hydroxyapatite ceramics have been clearly indicated the good bone growth and better biological performances **[7-9]**. Moreover , Cerium (Ce^{3+}) and Lanthanum(La^{3+}) ions an essential and interesting for substituted in normal hydroxyapatite due to major role in better antibacterial agent, tissue regeneration and best medical applications. Cerium ions have been used an antibacterial agents in medical field on long term applications. Numerous research evidences have showed that cerium ion can inspire the used to antibacterial applications and better cell proliferation reactions **[10]**. Then the cerium ions (Ce) performance in equal to calcium ion in organs. Because, the cerium ion present in little amount can encourage the metabolism in organisms **[11-12]**.

The electronegativity value of cerium ion is 1.06 and the ionic radius is 0.107 nm while both values are equal to calcium ions as electronegativity value of 1.01 and ionic radius of 0.100 nm and similarly lanthanum ion alsoLa^{3+} (1.016 A °). Since, the cerium ions substituted in calcium ion in the lattice of hydroxyapatite structure. The correlation among Lanthanum and Hydroxyapatite also is of particularattention becoz, of its inhibitory effect on the demineralization of dental enamel. In general, La and other rare earths are known as Ca-substituting ions in apatite structure. Many researchers have been achieved the little amount of lanthanum substituted hydroxyapatite composites for esteemed information **[13]**. These investigations reported the good bond strength of hydroxyapatite to lanthanum bonding structure and better bioactivity against human osteoblast cell lines. *In vitro* and in vivo studies with La-HAP composites are super performance in biological evidences.

Several methodologies are in research for the development of calcium phosphate bio ceramic coatings onto the implantation surface areas such as chemical spraying process, plasma spray coating, pulsed laser deposition technique, electrophoretic deposition method, sol-gel system and chemical vapor deposition process etc. **[14-18]**. these approaches of coatings need the before prepare of the coating bio material is high power beam source and high vacuum chamber. Since, they are practically very high expensive and may not get the favored arrangement in the various coating techniques. But then the electrochemical deposition develops many advantages alike coating at a very simple technique, low temperature compare than other techniques, little expense and uniform deposition of fully surface coverage coatings.

Hence the present work planned to achieved a Ce, La-HAP composite coating on titanium alloy for electrode position method. The antibacterial performances of Ce, La-HAP composite coating on titanium were observed. Hence, the *in vitro* characterizations of Alkaline phosphate activity and cell proliferation analysis were evaluates in human cell lines in order the reliability of Ce, La-HAP coating.

2. EXPERIMENTAL WORK

Preparation of titanium alloy

`The titanium alloy specimens having ions composition (wt %) C-0.038, Al-5.7, O-0.106, N-0.035, V-3.87, Fe-0.18 and balanced titanium with dimensions cut into 10x10x3 mm used as a substrate material. Before the electrode position technique, all titanium specimens were initially abraded using various grade 400-1500 grit silicon carbide sheets. After grinding the titanium specimens were washed with deionized water and ultrasonically cleaned in ethanol, deionized water and acetone for 15 minutes.

Preparation of electrolyte solution

The electrolyte for electrode position prepared by dissolved the analytical grade solution of chemicals like 0.4 M (Ca $(NO)_3$. $6H_2O$),0.05M Ce $(NO)_3.6H_2O$, 0.05M La $(NO)_3.6H_2O$ and 0.3 M $(NH_4)_2HPO_4$ in DI water and the chemicals mixed in molar ratio (Ce,La +Ca)/P of 1.67. These prepared electrolyte solutions kept in a magnetic stirrer for 2h and adjusted the pH 4.5 by adding NH_4OH solution.

Electrochemical deposition of Ce, La-HAP composite on Titanium alloy

The electrochemical deposition of Ce, La-HAP composite coating on titanium alloy specimen was performed by regular three electrode system using electrochemical workstation (CHI 760 USA). Since, Platinum electrode was used by counter electrode, saturated calomel electrode (SCE) and Titanium alloy specimens as a reference electrode and working electrode. The Ce, La-HAP composite coating on titanium alloy carried out in galvanostatically technique at various weight percentages at 1 wt%, 2 wt% and 3 wt%. After the electrode position Ce, La-HAP composite coating specimens were washed with deionized water. Then the coating samples dried in air and analysis of further investigations.

Surface Characterization

The Ce, La-HAP coated samples were investigated for the functional groups by Fourier transform infrared spectroscopy (Perkin elmer RX1 FTIR spectrometer) and the frequency range from 4000 cm^{-1} to 400 cm^{-1} with a number of 32 scans and spectral resolution of 4 cm^{-1} by KBr pellet method. The Phase composition and crystalline nature of coating scraped samples were explored by X-ray diffraction PAN alytical X'Pert PRO diffractometer in the 2 theta angle between 20^0-60° with Cu Kα radiation (1.5406 Å). The surface morphology and actual composition of as coating samples were estimated by a high resolution scanning electron microscopy (HRSEM, JSM 840A, JEOL-Japan) equipped with energy dispersive X-Ray analysis (EDAX).

Antibacterial activity

The antibacterial activity of the Ce, La-HAP coating at various concentrations have been explored against two prokaryotic bacterial strains such as *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) by agar disc diffusion method. The Mullar−Hinton agar plates were equipped by pouring 15 mL of a molten medium into sterile Petri plates. The plates were allowed to solidify for ∼15 min and 0.1% of inoculum suspension was swabbed uniformly over the agar until the inoculums became invisible. Various concentrations 25 μL, 50 μL, 75 μL, 100 μL, and 125 μL of Ce, La-HAP coating were loaded onto 5 mm sterile individual discs, followed by incubation of plates at 37 °C for 24 h. The zone of inhibition was observed by measuring the width of the inhibited zone **[19]**.

Cell culture

The cell proliferation of MC3T3-E1 cells onCe, La-HAP was studied using MTT assay on day 1, 3, 5 and 7 days. To determine the cytotoxicity of the samples at different conditions, MC3T3-E1 cells were seeded in 12-well plates at 104 cells/mL in a humidified 5% CO_2 atmosphere. Each time, 400 μL of MTT reagent (1 mg/mL) was added to each well and incubated for 4 h under the same conditions. Finally, MTT reagent was removed and 400 μL of dimethyl sulfoxide (DMSO, Sigma−Aldrich) was added for dissolving the form as an crystals and the absorbance was measured at 560 nm in an ELISA microplate reader and then the cell viability (as a percentage) was calculated, with respect to the control, as follows

% Cell viability = [A] Test / [A] Control x 100 [20].

Alkaline phosphate activity

Alkaline phosphate activity of Ce, La-HAP composite coating at different concentrations at 10,20,50 and 100 μg/mL in human osteoblast cell lines (MC3T3-E1)were assessed by using a test kit according to the study protocol. Produced on hydrolysis of free phenol in P-NPP (P-nitrophenyl phosphate) with Alkaline phosphate as catalyst respond with 4-amino-antipyrine in the appearance of alkaline potassium ferricyanide to react a red colored complex with absorbance obtained at 520 nm is directly proportional to the alkaline phosphate activity in the Ce, La-HAP composite powders. After that 7 day of culture medium the supernatant was separated and 100 ml in 1% TritonX- 100 mixture (lysis solution) was additional to each well and treated for 2 hours. After, 30 ml of the resulting of human cell (MC3T3-E1) lysates were moved to each fit of fresh 96-well plate and sophisticated with 50 ml carbonated buffer solution (pH = 10) and 50 ml 4-amino-antipyrine substrate solution at Room temperature. Next, 150 ml potassium ferricyanide (chromogenic agent) was additionally added into miscellaneous solution and its absorbance was obtained at 520 nm by a spectrophotometer. Then standardization, the total protein content was obtained at using a BCA (Bicinchoninic Acid) protein analyze kit. Since, the alkaline phosphate activity was stated and standardized by the total protein contented (U/gprot) **[21]**.

Statistical Analysis

All the studies were performed for the Ce, La-HAP coating in triplicate and repeated three times (mean ± standard deviation) (SD).Statistical analysis was performed using analysis of variance (ANOVA) with Tukey's multiple comparison tests (Prism, version 5.0). The difference observed between samples was considered to be significant at $P < 0.05$.

3. RESULTS AND DISCUSSION

The FT-IR study of Ce, La-HAP coated on Ti alloy samples are indicated in **Figure 1**

The absorption bands founded at 3479 $cm^{-1}$and 1681$cm^{-1}$are ascribed to stretching and bending modes of adsorbed water molecule of Ce, La-HAP coating respectively. The vibrations of stretching and librational modes of hydroxyl (OH^-) ions present at 3548 $cm^{-1}$and 665cm^{-1}. Further, the characteristic peak for Phosphate $(PO_4)_3$ found at 475cm^{-1}, 575cm^{-1}, 601cm^{-1}, 965cm^{-1}, 1029$cm^{-1}$and 1085 cm^{-1}respectively. The vibration at 475cm^{-1} is founded to Phosphate bending vibration ($\square_2$). In addition, triply restored ($\square_4$) bending vibrations presented at phosphate group of 584cm^{-1} and 606cm^{-1}.The bands were present at 966cm^{-1}($\square_1$),1036 $cm^{-1}$and 1092 cm^{-1}($\square_3$) correspond to the symmetric stretching modes of phosphate groups. Therefore, the FT-IR investigation confirmed the formation of Ce, La-HAP coating on titanium alloy and no other peaks were detected.

XRD analysis

Figure 2 Presented in the XRD pattern of Ce, La-HAP composite coating on titanium alloy in The Pattern in these figure clearly indicated for Ce, La-HAP are in well arrangement with the standard data for Hydroxyapatite (ICDD card No.09-0432).In this regards the major diffraction peaks of Ce, La-HAP are obtained at 2□ values 25.8^0, 29.17^0, 30.67^0, 31.7^0, 32.52^0, 46.95^0, 50.43^0 and 52.96^0. The association of slightly left by substitutions of minerals ions. Since the development and contraction in the HAP lattices. These X-Ray diffraction peaks for Ce, La-HAP coating on Titanium alloy which demonstrated of the coating that the better purity and high crystalline nature.

SEM analysis:

The SEM micrographs of different weight percentages at 1 wt%, 2 wt%, and and 3 wt% Ce, La-HAP coating on titanium alloy displayedin **Figure 3a-c**. Ce, La-HAP coating preparation revealed porous with sphere-shaped designed nanoparticles.

Morphology at various weight percentages. **Figure 3a** demonstrations a non-uniform nanoparticles of Ce, La-HAP coating on titanium alloy for 1wt%. Then, the coatings 2 wt%, indicated the fully cover and uniform spherical like surface morphology. On more increases weight percentage (3 wt%) displayed agglomerated and non-uniform morphology compare then low weight percentages at 2 wt% and 1 wt%. Therefore, the weight percentages of 2 wt% display the uniform porous with sphere-shaped like surface morphology and fixed the best condition suitable for Ce, La-HAP composite coating on titanium alloy. The EDAX spectrum of Ce, La-HAP composite coating on titanium alloy represented in Figure 3d. The EDAX spectrum for Ce, La-HAP composite coating indicates the presence of Ca, P, O, La, and Ce ions. These consequences confirms the Ce, La-HAP composite on titanium alloy.

Antibacterial activity

Figure 4 demonstrated the antibacterial activity of Ce, La-HAP coating were assessed against the double prokaryotic bacterial strains through disc diffusion technique. The double bacterial strains such as *E. coli* (gram positive bacterium) and *S.aureus* (gram negative bacterium) used in bacterial protocol. Figure 4 revealed against *E. coli* and *S.aureus* of the zone of inhibition around the Ce, La-HAP composite samples used different concentrations at 25µL, 50µL, 75µL, 100µL and 125 µL. while compared the *E. coli* and *S.aureus* bacterial strains of Ce, La-HAP coating, *S.aureus* bacterial strain indicated developed antibacterial activity at higher concentration125µL which is obviously detected from the around zone of inhibition. Hence, increasing the concentration of Ce, La-HAP samples the antibacterial activity also increases. The reason may be present of lanthanum and cerium mineral ions play a key role in enhanced the antibacterial performance. Thus excellent and broad antibacterial activities of Ce, La-HAP composite coating against *E. coli* and *S.aureus* strains exhibited that the more suitable for orthopedic applications.

Cell viability

The cell viability of Ce, La-HAP coated samples estimated by MC3T3-E1 osteoblastic cell lines. In **Figure 5** displayed the cell viability of MC3T3-E1 cell lines calculated at different days such as 1, 3, 5 and 7 days. The human osteoblastic cell lines absorbance of 570 nm directly proportional to number of living cells in normal culture (MC3T3-E1) medium detected in spectrophotometer. The cell viability of Ce, La-HAP coating is exhibited to develop on increasing the days from one to seven days on MC3T3-E1 cell lines. These consequences evidently identified at significantly higher cell viability at 7 days compare then 1, 3 and 5 days. Therefore, the cell viability results are without toxic nature and good agreement of cell growth. Accordingly, the cell viability and cell growth results indicates that the Ce, La-HAP composite coating is developed the orthopedic applications.

Alkaline Phosphate Activity

ALP activity is preliminary marker for osteogenic differentiation and control the metabolism of phosphates. The alkaline phosphate activity of MC3T3-E1 cell lines on

Ce, La-HAP composite coating with various concentrations such as 10µg/mL, 20µg/mL,50µg/mL and 100 µg/mL at different culture periods on 1,3,5 and 7 days represented at **Figure 6**. Meanwhile, the alkaline phosphate activity of cells cultured on Ce, La-HAP composite coating substrates apparently improved complete the period. Then, the alkaline phosphate activity of MC3T3-E1 cells growth on composite coating at three day was improved phosphate activity compare than one day. Though the increasing the culture period of seven day suggestively greater than five days. Finally the Ce, La-HAP composite coating on titanium alloy for recovering the bone defect diseases and superb biocompatibility.

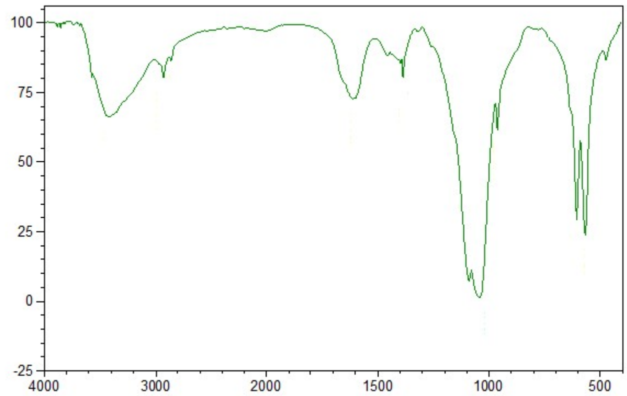

Figure 1: FT-IR spectra of Ce, La-HAP composite coated on Ti alloy

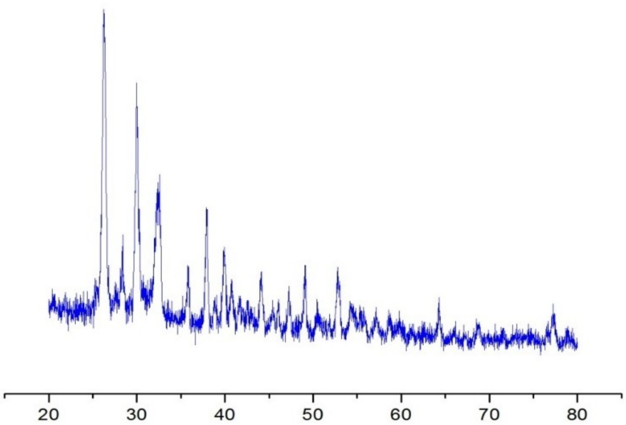

Figure 2: XRD spectra of Ce, La-HAP coated on Ti alloy

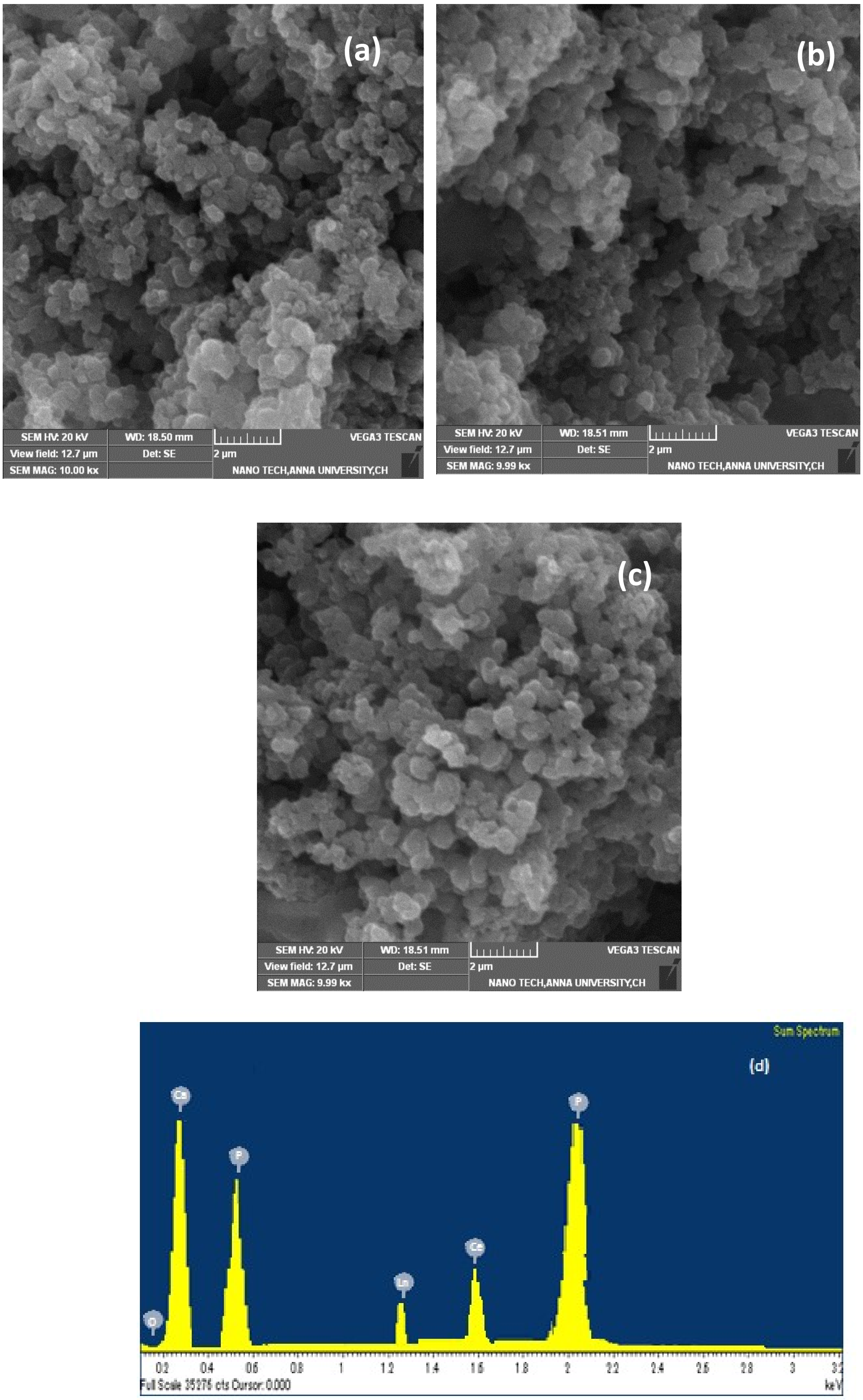

Figure 3: SEM micrographs of different weight percentages at (a) Ce, La-HAP coating on 1wt% and (b) Ce, La-HAP coating on 2 wt%, (c) Ce, La-HAP coatingon 3 wt% (d) EDAX spectrum of Ce, La-HAP coating on Ti alloy

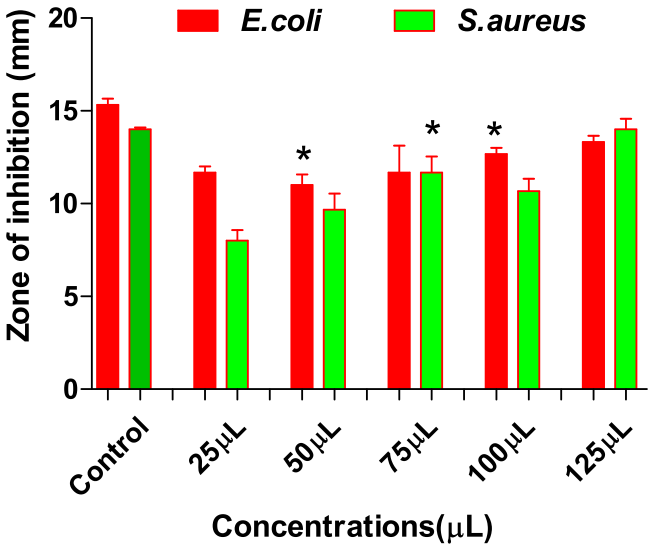

Figure 4: Antibacterial activities of Ce, La-HAP coating at different concentrations against pathogenic bacteria *E.coli* and *S.aureus* (*) denotes a significant difference compare to the control (P≤ 0.05)

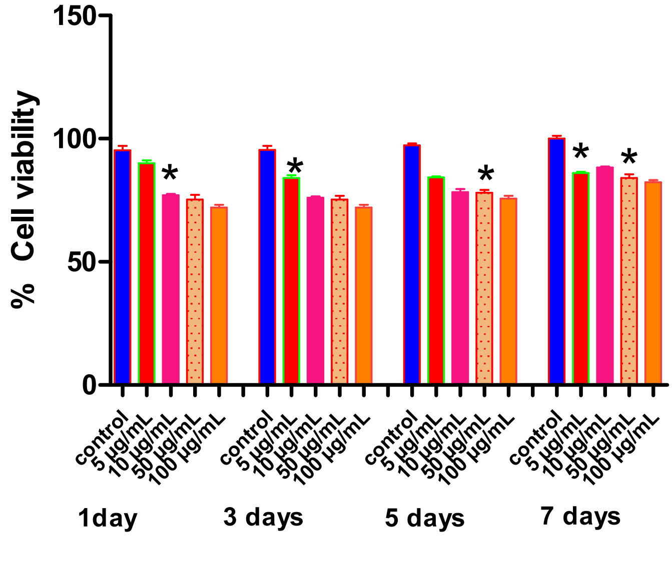

Figure 5: Cell viability of Ce, La-HAP composite coating on MC3T3-E1 cells for 1,3,5 and 7 days (*) denotes a significant difference compare to the control (P≤ 0.05)

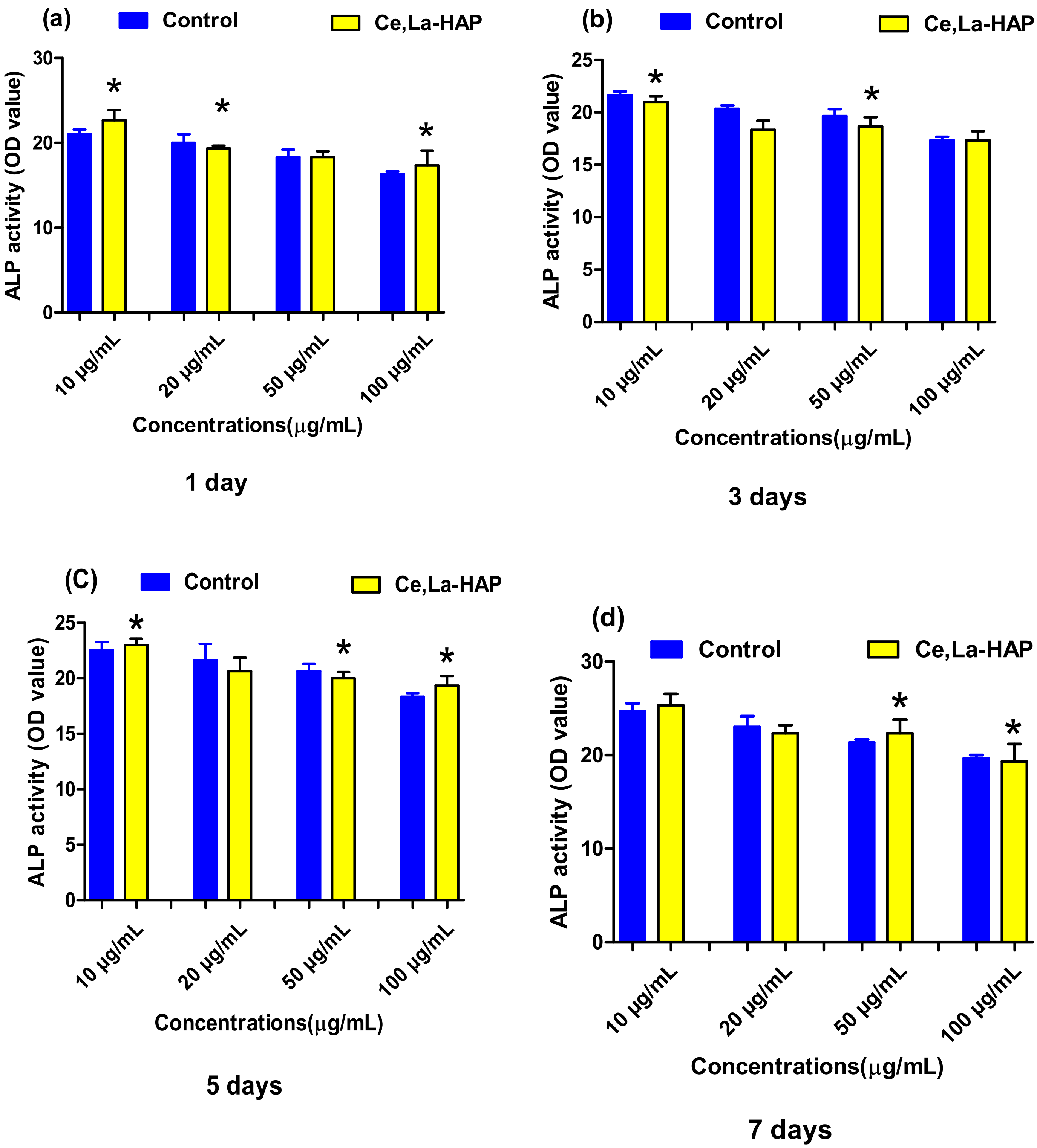

Figure 6: Alkaline Phosphate activity of Ce, La-HAP coating on MC3T3-E1 cells for 1,3,5 and 7 days (*) denotes a significant difference compare to the control (P≤ 0.05)

CONCLUSION

The Ce, La-HAP composite coating on titanium alloy was successfully done by electrode position technique. The physical investigations of structural study and stability of nature analysis were used in FT-IR and XRD analysis confirmed the formation of Ce, La-HAP composite coating on titanium alloy. Hence, the surface morphology investigation of Ce, La-HAP composite coated on titanium alloy revealed the formation of uniform porous like spherical surface morphology and improvement of the human cells. Since, the antibacterial ability achieved on disc diffusion method using by gram positive bacteria and gram negative bacteria while the effects undoubtedly showed on superior zone of inhibition against both prokaryotic strains. However, cell proliferation evidences presented that Ce, La-HAP composite coating better cell development and without toxic nature of MC3T3-E1 cell lines. The alkaline phosphate investigations demonstrated the superb phosphate improvement. Therefore, Ce, La-HAP composite coating on titanium alloy implants will serve as a suitable applications of orthopedic field.

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