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Fabrication of divalent ions substituted hydroxyapatite/gelatin nanocomposite coating on
electron beam treated titanium: mechanical, anticorrosive, antibacterial and bioactive
evaluations

A. Karthika\textsuperscript{a}, L. Kavitha\textsuperscript{b}, M. Surendiran\textsuperscript{a}, S. Kannan\textsuperscript{c}, D. Gopi\textsuperscript{ad*}

\textsuperscript{a}Department of Chemistry, Periyar University, Salem 636011, India
\textsuperscript{b}Department of Physics, School of Basic and Applied Sciences, Central University of Tamilnadu, Thiruvarur 610101, India
\textsuperscript{c}Department of Zoology, School of Life Sciences, Periyar University, Salem-636 011, India
\textsuperscript{d}Centre for Nanoscience and Nanotechnology, Periyar University, Salem 636011, India

* Corresponding author. Tel.: +91 427 2345766; fax: +91 427 2345124.
E-mail address: dhanaraj_gopi@yahoo.com (D. Gopi).

Abstract

The key property of the fabrication of a biomaterial is to facilitate the replacement and/or
regeneration of damaged tissues and organs. To obtain such a biomaterial, we fabricated triple
minerals (strontium, magnesium and zinc) substituted hydroxyapatite/gelatin (M-HAP/Gel)
nanocomposite coating in electron beam treated titanium (Ti) metal. The influence of gelatin
concentrations in M-HAP was studied with its effect on the morphological changes, crystallinity,
mechanical and anticorrosion properties. The M-HAP/Gel nanocomposite coating (with 3 wt% gelatin) on treated Ti resulted in better mechanical and anticorrosion properties as a consequence of electron beam treatment of Ti. A reduced amount of bacterial colonies was observed for the M-HAP/Gel composite against \textit{Staphylococcus aureus} (\textit{S. aureus}) and \textit{Escherichia coli} (\textit{E. coli}) microbes which evidences the less chance for the implant failure after implantation. Moreover, the cell proliferation assay, live/dead staining of MT3C3-E1 cells and cell viability of fibroblast stem cells on the resultant nanocomposite revealed that the M-HAP/Gel composite will definitely be an effective implant material for better cell growth in the orthopedic applications.
1. Introduction

Over the last decades, bioceramics have attracted more attention among the researchers because of their intensifying applications in treating damaged and infectious bone. The main requirement for a bioceramic material to replace the traumatized, damaged, or lose bone is that it should mimic the properties of the natural bone.\(^1\) Recently, hydroxyapatite (HAP, \(\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2\)) has been broadly used as a biomaterial due to similar characteristics as that of the natural bone.\(^2\) The HAP structure can accommodate a great variety of other substituents. Thus it can readily accept the minerals to mimic as bone like material. Among many mineral ions, strontium (Sr) is an important trace element in human body and enhances preosteoblasts proliferation and decreases bone resorption.\(^3\) In addition, Sr-HAP can exhibit better mechanical properties than pure HAP.\(^4-7\) Zinc (Zn) is required for human as an essential element and its requirement is estimated to be 15 mg day\(^1\).\(^8\) Also, Zn has antibacterial activity, which minimizes the bacterial load on the implant surface after orthopedic implantation and improves the mechanical strength.\(^9,10\) Similarly, magnesium (Mg) directly stimulates the osteoblast proliferation and helps for the mineralization of calcified tissues which indirectly controls the mineral metabolism.\(^11-13\) Previously, Gopi et al., have developed minerals substituted HAP coating over titanium alloy by pulsed electrodeposition method.\(^14\)

In order to fasten/improve the biological interactions with the surrounding tissues, nowadays, biopolymers such as chitosan, gelatin, etc., has been used. In particular, gelatin, a natural polymer, is composed of unique sequence of amino acids such as glycine, proline and hydroxyproline that motivate wound healing and intensify the osteointegration as well as three dimensional tissue regeneration.\(^15,16\) Moreover, gelatin is obtained by a controlled hydrolysis of fibrous insoluble protein, collagen, which is the major constituent of skin, bones and tissues.\(^17\)
Gelatin is currently used in pharmaceuticals, wound dressings and adhesives in clinics due to its good cytocompatibility, non-antigenity, plasticity and adhesiveness. Furthermore, it is completely resorbable in vivo, and its presence with bioceramic material can resemble bone due to the organic and inorganic phases. The addition of gelatin to the bioceramic not only can improve the biological interactions, but also expected to be enhancing the mechanical properties of the composite. Accounting the importances of these mineral ions, Sr, Mg and Zn are substituted in HAP and made as a composite with gelatin for better bioactivity for osteoblast cells in vitro and to improve the biocompatibility along with good mechanical properties.

The growth of bioceramic coating on implant material with good adhesion strength becomes a major issue in the field of biomaterials field. Also, the achievement of osseointegration is significantly affected by the surface nature of implants. So, the surface of the implants such as titanium (Ti) and its alloys, 316L stainless steel, etc., was modified using some modification techniques, which has resulted better adhesion strength along with good corrosion resistance. Among the various surface modification techniques, electron beam treatment has been accepted as a better technique for the surface modification of implant materials. Moreover, the electron beam treatment has improved the physical, chemical and mechanical properties of metals and was reported by Dong et al., and Proskurovsky et al. Here, we employed the high energy low current electron beam treatment on Ti metal for obtaining an increased roughness of the metal surface, that could lead to high adhesion strength for the coating since it has more advantages. Also, Lee et al., have fabricated the Zr-based bulk metallic glass (BMG)/Ti surface composites by high-energy electron beam irradiation and reported that the BMG composite has excellent bonding strength as well as very high hardness and wear resistance.
A composite biomaterial of HAP and gelatin is expected to show improved osteoconductivity and biodegradation along with mechanical strength for orthopedic usage.\textsuperscript{3} A nano-structured composite could show better adhesion of osteoblast cell at the implant tissue interface. Also, the nanostructured composites have mechanical properties similar to bone, since bone itself is a nanostructured composite.\textsuperscript{28} Zandi et al.,\textsuperscript{29} have evaluated the biocompatibility of the gelatin coated nano-HAP scaffold using mesenchymal stem cells. They reported that the prepared scaffolds possessed desirable biocompatibility, high bioactivity with better cell attachment and proliferation and sufficient mechanical strength in comparison with noncoated HAP sample. Recently, Liu et al.,\textsuperscript{30} fabricated gelatin functionalized graphene oxide for mineralization of HAP, which reported in detail about the MC3T3-E1 cells osteogenic activity in terms of the cell spreading, growth, and alkaline phosphatase activity.

Chang et al.,\textsuperscript{31} have developed HAP-gelatin system and they have proved the chemical binding between hydroxyapatite and gelatin molecule, which mimics natural bone microstructure. Moreover, the bioactivity and corrosion performances of the strontium substituted calcium phosphate and gelatin composite were studied by Huang et al.,\textsuperscript{32} and it has been reported that the resultant coating exhibited better cytocompatibility than Ca-P coating. Though they developed the coating, they reported only 5 MPa as bond strength which is insufficient for load bearing applications. Though the developed composite coating can exhibit good biological activities, the interaction between the bioceramic and implant material has to reach appreciable adhesion strength. For that reason, the implant material could be subjected to the electron beam irradiation for obtaining roughened surface, which leads to an adhesive coating.
The pulsed electrodeposition (PED) technique has been employed for the development of composite coating since it has good advantages for obtaining better coating than other deposition methods. The authors employed the PED technique for the development of M-HAP/Gel composite on the Ti implant material. The objective of the present work is to develop a more adhesive M-HAP/Gel nanocomposite coating on HELCDEB treated Ti surface and to study the effect of gelatin concentration in the M-HAP/Gel nanocomposite for various properties and to be a better bioimplant material. The in vitro corrosion behavior of the M-HAP/Gel nanocomposite coating on HELCDEB treated Ti has been evaluated in simulated body fluid and the mechanical property of the nanocomposite was studied in the name of adhesion strength. The antibacterial ability of the nanocomposite was tested against S. aureus (Gram-positive bacterium) and E. coli (Gram-negative bacterium). The MT3C3-E1 preosteoblast cells were cultured on the M-HAP/Gel nanocomposite coating to evaluate the viability and proliferation by MTT and live/dead cell assay. The apatite growth ability that is the bioactivity over the composite surface was investigated through the SBF immersion study for various days.

2. Materials and methods

2.1 Preparation of Ti substrate

The substrates were pure Ti metals (99.99 %) with a size of 10×10×3 mm³. These substrates were polished thoroughly with 400–1200 silicon carbide sheets, then cleaned ultrasonically with acetone to remove the residual particles from grits and dried in air at room temperature. After this process, the substrates were surface treated using a 700 keV DC accelerator with an electron beam of current 1.5 mA at an energy of 700 keV to enhance the mechanical properties and corrosion resistance of the Ti metal. The detailed procedure of the electron beam treatment was provided in our previous report.
2.2 Preparation of M-HAP/Gel composite coatings

Analytical grade Ca(NO$_3$)$_3$.6H$_2$O (0.294 M), Sr(NO$_3$)$_2$.6H$_2$O (0.042 M), Mg(NO$_3$)$_2$.6H$_2$O (0.042 M) and Zn(NO$_3$)$_2$ (0.042 M) were dissolved separately in deionized (DI) water. The (NH$_4$)$_2$HPO$_4$ (0.25 M) was dissolved in DI water and the solutions were mixed in the molar ratio of 1.67. Then, the gelatin solutions of 1, 2, 3 and 4 wt% were added in the electrolytes in the presence of nitrogen surroundings and maintained the pH of the solutions at 4.5. The electrolytes were stirred uniformly by keeping the temperature at 65 °C using the magnetic stirrer and the thermostat. Then, the resultant electrolytes were used for further processes.

A regular three electrode system was used for the pulsed electrodeposition of M-HAP/Gel composite using CHI 760C (CH Instruments, USA), where HELCDEB treated Ti substrate served as working electrode, platinum electrode as counter electrode and saturated calomel electrode (SCE) act as reference electrode. The deposition was performed for 1 h on HELCDEB treated Ti sample in a galvanostatic mode with a current density of 1.0 mA cm$^{-2}$. As per our previous study,$^{26}$ the optimum condition of 1 s pulse on time and 4 s pulse off time with respect to the current density of 1.0 mA cm$^{-2}$ and 0 mA cm$^{-2}$. While starting the deposition process, 2000 ppm of H$_2$O$_2$ was added into the electrolyte solutions to reduce the H$_2$ gas bubbles. After the coating process, the specimens were removed from the electrolyte and washed with deionized water, then dried the samples. The in situ formation of M-HAP/gelatin nanocomposite has been illustrated in Scheme 1.

2.3 Characterization of the composite coatings

The back bone of the coating material was analyzed using Fourier transform infrared spectroscopy (FT–IR) using Bruker Tensor 27. The FT–IR spectrum was recorded in the 4000–400 cm$^{-1}$ region with 4 cm$^{-1}$ resolution by using KBr pellet technique. The phases were characterized by powder X-ray diffraction (PANalytical) with CuKα radiation in the 2θ range.
20–60°. The surface topography and actual composition of the as-developed composite samples were observed by FEI Quanta FEG 200 - high resolution scanning electron microscope (HRSEM) equipped with energy dispersive X-ray (EDX) spectrometry.

2.4 Adhesion strength

The M-HAP/Gel composite coatings on HELCDEB treated Ti specimens were tested for the adhesion strength by pull-out test according to the American Society for Testing Materials (ASTM) international standard F1044-05 and the experiments were repeated for five times for every coated material. The specimens were exposed to tests at a constant cross-head speed using a universal testing machine (Model 5569, Instron).

2.5 Electrochemical studies

In this study, potentiodynamic polarization measurement was carried out for the HELCDEB treated Ti at 700 keV and M-HAP/Gel composite coatings on HELCDEB treated Ti specimens in simulated body fluid. The ion concentrations and pH of the simulated body fluid were maintained nearly equal to that of human blood plasma. The SBF solution is prepared by using Kokubo’s procedure. The polarization study was performed in the CHI 760C (CH Instruments, USA) in which HELCDEB treated Ti at 700 keV with an exposed surface area of 1 cm² was used as the working electrode. Potentiodynamic polarization was performed in a potential range of -1.2 to 1.2 V vs SCE at a scan rate of 0.001 V/s. The electrochemical impedance spectroscopic (EIS) measurements were carried out with the frequency ranging from $10^{-2}$ to $10^{5}$ Hz. Nyquist and Bode plots were obtained after the specimens were immersed in the SBF solution for 1 h. The potentiodynamic polarization and EIS were repeated at least three times.
2.6 Antibacterial activity

The antibacterial activity of the M-HAP/Gel composite coating was tested against *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) by colony count quantitative method. The inoculums of microorganism was prepared from fresh overnight broth (peptone broth) incubated at 37 °C. The nanocomposite was mixed with molten MacConkey agar at varying final concentrations (25, 50 and 100 µg/mL). Serial dilution (1/10⁴) of late log phase bacteria (OD₆₀₀ = 2.0) were then plated onto solidified nanocomposite agar plates and incubated at 37 °C for 24 h. Error bars were calculated for colony count based on the standard errors by t-test. To determine the antibacterial property of the material, the antibacterial reduction percentage was calculated using the formula

\[ R = \frac{(C_0 - C)}{C_0} \times 100 \]  

where, “R” is the microbial reduction percentage, “C₀” is the number of microbial colonies on the control plate (without nanocomposite) and “C” is the number of microbial colonies on the M-HAP/Gel nanocomposite samples.

2.7 Cell proliferation assay and Live/dead staining

MC3T3-E1 subclone 4 mouse osteoblast cell lines (ATCC) were cultured in Eagles minimum essential medium (α-MEM, Invitrogen), supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen), 10 µg/mL Streptomycin (Invitrogen), 10 µg/mL Penicillin and culture was incubated at 37°C in a humidified atmosphere with 5% CO₂. The medium was changed every two days until 80% to 90% confluency then sub-culture the cells. The cells monolayer was washed twice with PBS solution and detached from their culture flask by incubating with 0.25% Trypsin-EDTA (Gibco) solution for 3 min. Detached MC3T3-E1 cells of 1 x 10⁴ (Passage 16) were seeded in each well of 48 well tissue culture plates.
The cell proliferation was studied on the M-HAP/Gel nanocomposite sample and control sample (without nanocomposite), using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium Bromide) assay on 1, 4 and 7 days. Each time, 400 µL of MTT reagent (1 mg/mL) was added to each well and incubated for 4 h at the same condition. Finally, MTT reagent was removed and 400 µL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich) was added for dissolving the formasan crystals and the absorbance was measured at 570 nm on a spectrophotometric microplate reader. The proliferation rate of cells was quantified by measuring the optical density (OD).

After each culture, cells on the M-HAP/Gel nanocomposite sample were stained using live/dead assay kit (Molecular Probes) and the results are compared with control samples, containing calcein AM and ethidium homodimer. Every time, the media was discarded and the samples were washed with PBS, then live/dead solution containing 2 mM calcein AM and 4 mM ethidium homodimer was added to each well and were incubated at 37 °C for about 20 min. The stained cultures were viewed using fluorescent microscope Nickon Eclipse 80i.

2.8. Fibroblast-stem cells

An MTT assay test was used to determine the viability and proliferation of fibroblast-like stem cells on M-HAP/Gel nanocomposite coating on HELCDEB treated Ti. The cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco), which consisted of a minimal essential medium (Hi Media Laboratories), supplemented with 10% fetal bovine serum (FBS, Biowest, France) in 96-well tissue culture plates. The cell culture was incubated at 37 °C under a humidified atmosphere of 5% CO₂ and 95% air for one day. The dual layer coated samples were sterilized in an autoclave at 120 °C during 2 h and then placed in 96-well tissue culture plates. The cells were seeded onto the M-HAP/Gel nanocomposite coatings and were maintained at
37 °C under 5% CO₂. The culture media in each well were replaced with immersion extracts supplemented with 10% FBS, and incubated at 37 °C in an atmosphere of 5% CO₂ and 95% every day. MTT assay was carried out as a function of incubation time for 1, 4 and 7 days. The MTT (10 mL) solution containing 5 mg of thiazolyl blue tetrazolium bromide powder was then added into each well on day 1, 4 and 7. After an incubation period, 100 mL of 10% sodium dodecyl sulphate (Sigma-Aldrich, USA) in 0.01 M HCl (Sigma-Aldrich, UK) was added into each well and incubated at 37 °C in an atmosphere of 5% CO₂ and 95% air for 24 h. To determine the cell viability of the samples, the absorbance was measured using an ELISA microplate reader at 570 nm wavelength. Cell viability (%) was calculated with respect to control wells (without nanocomposite) using the formula as follows.40

% Cell viability = [A] Test / [A] Control x 100.  (2)

2.9 Statistical Analysis

In this study all the experimental groups were carried out in triplicate and the results are presented as average ± standard deviation and were analyzed using a one factor ANOVA statistical study. The differences were considered statistically significant if p < 0.05.

2.9 Immersion study in SBF solution

The apatite forming ability of the M-HAP/Gel composite coating (at an optimum concentration of gelatin) on HELCDEB treated Ti sample can be evaluated by immersing in simulated body fluid at 37 °C for 1, 7 and 14 days. As mentioned earlier, the composition and preparation of SBF solution was according to the Kokubo’s protocol.35 In the fresh SBF solution (40 ml) containing beaker, the resultant composite coated samples were soaked with an airtight lid for 1, 7 and 14 days and the temperature was sustained at 37 °C in an incubator. The SBF solutions were changed every two days to maintain the cationic concentration in the
test solution. After appropriate days of immersion, the samples were removed from SBF solution, then gently washed with DI water and the formation of apatite growth on the composite coated HELCDEB treated Ti samples was evaluated by microscopic study (HRSEM).

3. Results and discussion

3.1 Mechanism of M-HAP and gelatin composite

The possible mechanism of M-HAP/Gel composite is shown in Fig. 1. From the figure, it can be well evident that the complexation of M$^{2+}$ ions (Ca$^{2+}$, Sr$^{2+}$, Mg$^{2+}$ and Zn$^{2+}$) of M-HAP and gelatin takes place thereby forming a partial bond between the M$^{2+}$ cation and gelatin moiety. Then, the as-formed complex (M$^{2+}$ and gelatin) molecules interact with PO$_4^{3-}$ ions to form M-HAP/Gel composite. During this formation process, the carboxylate (COO$^-$) and amino (-NH-) groups in gelatin can form bond with P-O and OH groups of M-HAP. Thus, it can be evident that the above said process might take place in the electrolyte solution while depositing on the HELCDEB treated Ti surface.

3.2 FT–IR analysis of M-HAP/Gel composite coatings

The FT–IR spectra of pure gelatin and M-HAP/Gel composite coating are shown in Fig. 2. The data of the M-HAP/Gel composite coating (Fig. 2b) is compared with that of pure gelatin (Fig. 2a). The absorption peaks of phosphate ions are observed at 563, 602, 1030 and 1081 cm$^{-1}$. The small peak at 496 cm$^{-1}$ is attributed to the PO$_4^{3-}$ bending vibration. The broad bands that appeared at 3403 and 667 cm$^{-1}$ are mainly due to the stretching and bending modes of the hydrogen-bonded H$_2$O molecules. The characteristic –OH peaks of HAP at around 3540 cm$^{-1}$ corresponds to the stretching mode. The peaks at 2923 and 2852 cm$^{-1}$ are attributed to the presence of C-H band in the gelatin present in the M-HAP/Gel composite, which indicates that gelatin interacts with M-HAP. In addition, two bands are identified at 985 and 937 cm$^{-1}$. 


This may be observed due to the P-O-C aliphatic stretching and P-N-C stretching, which evidence for the binding of phosphate ions with gelatin.\textsuperscript{41} At the same time, the peak at 1642 cm\textsuperscript{-1} corresponds to the carboxyl group of gelatin and composite, which is similar to the presence of type-I collagen in biological tissue.\textsuperscript{42} The appearance of sharp peaks at 1531 and 1727 cm\textsuperscript{-1} represents to the N-H peak in the composite. Thus the obtained FT–IR spectra confirmed the formation of M-HAP/Gel composite coating and no other impurities were identified.

3.3 X-ray diffraction studies

Figure 3 shows the X-ray diffraction patterns of pure gelatin and M-HAP/Gel composite coating. For pure gelatin, the characteristic broad peak around 20° was observed, which is similar to the observation by Kim et al.,\textsuperscript{43} and is also in good agreement with ICDD card no. 09-432. The 2ϴ values for the HAP peaks were found to be 25.83, 31.54, 32.28, 32.80, 34.06, 39.89, 46.69, 48.27, 49.26, 50.42, 52.11 and 53.11, respectively and the planes corresponding to the 2ϴ values were (002), (211), (112), (300), (202), (310), (222), (312), (213), (321), (402) and (004).\textsuperscript{26} These peaks experienced a shift towards the lower diffraction angle which may be occurred due to the influence of the mineral ions substitution such as Sr\textsuperscript{2+} (radius, 0.112 nm), Mg\textsuperscript{2+} (0.072 nm) and Zn\textsuperscript{2+} (0.074 nm) in HAP (Ca\textsuperscript{2+} 0.100 nm), that leads to the distortion in the HAP lattice.\textsuperscript{44,45} In particular, the atomic radius of Sr ion is higher than Ca, that mainly induce a shift in the XRD pattern while substituting it at the place of Ca. Along with other peaks, a small peak was observed at 20°, which is evidenced for the binding of gelatin with HAP moiety. Moreover, the high intense peaks of M-HAP/Gel composite exhibit the crystalline nature of the coating material. This proves the fact that gelatin concentration does not influence the
crystallinity of the HAP when compared with the previous report.\textsuperscript{46} No other impurity peaks were observed, indicating that the purity of the M-HAP/Gel composite is appreciable.

3.4 Morphological results of composite coatings

The surface topographic images of the 700 keV HELCDEB treated Ti and M-HAP/Gel composite coating on HELCDEB treated Ti are displayed is Fig. 4(a-e). The Fig. 4a shows the morphology of electron beam irradiated Ti. The eruption seen on the surface of irradiated Ti might be obtained due to the surface changes that are occurring by superfast heating, melting and solidification of the Ti metal. This modified surface will provide improved physicochemical properties and bonding strength to the Ti substrate. The composite coatings on irradiated Ti result in the typical morphologies of M-HAP/Gel composite (with different concentrations of gelatin). The M-HAP/Gel composite (with 1 wt% gelatin) exhibited a novel dumbbell shaped structure on the HELCDEB treated Ti surface (Fig. 4b) with some heterogeneous surface coverage, which is clearly indicating that the nucleation of the composite was not completed with the minimum concentration of the gelatin (i.e. 1 wt\%) in M-HAP. On increasing the gelatin concentration to 2 wt\%, the dumbbell shaped composite was changed into irregularly arranged petals, which indicates that the rods/petals of the dumbbell shaped composite began to separate and were unevenly distributed (Fig. 4c). Interestingly, at 3 wt\% gelatin concentration in the composite (Fig. 4d), the morphology of uniformly organized flower petals of nanosized (~ 20 nm) was obtained and it was compactly packed on the irradiated surface without any voids. The texture and uniform coverage of the composite coating may also be resulted due to the deposition condition (i.e.) the prolonged pulse off time. This coating on HELCDEB treated Ti may result in a better anticorrosion performance due to the complete coverage of the material without any cracks or pores on the surface. As the concentration of gelatin was increased (Fig. 4e) to 4 wt\%
in M-HAP/Gel composite, irregular petal structure of composite with agglomeration was obtained. Moreover, the surface of the coating was observed with pores on the HELCDEB treated Ti surface. The morphology of the composite differs due to the higher concentration of the gelatin which greatly influenced the texture and coverage of the coating. Fig. 4f shows the elemental analysis results (EDX) of M-HAP/Gel nanocomposite coating (at 3 wt% of gelatin concentration) which exhibited the presence of Sr, Mg, Zn, Ca, P, N and C (which is the back bone of the gelatin), thereby supporting the formation of M-HAP/Gel composite coating.

3.5 Adhesion strength of the composite coatings

The adhesion between the coating and substrate is the most important factor for better mechanical interlocking and chemical bonding of the implant in the physiological solution. Thus, the pulsed electrodeposited M-HAP/Gel composite coatings were tested for their adhesion strength. The M-HAP/Gel composite-I (1 wt%) coating on HELCDEB treated Ti exhibited good adhesion strength of 22.8\(\pm\)0.9 MPa. The adhesion strengths of the M-HAP/Gel composite-II (2 wt%) and M-HAP/Gel composite-III (nanocomposite) (3 wt%) coatings exhibited 23.0\(\pm\)0.8 MPa and 23.8\(\pm\)0.6 MPa, respectively. Whereas, the adhesion strength for the M-HAP/Gel composite-IV (4 wt%) coating was found to be as 23.1\(\pm\)0.7 MPa, which possessed less adhesion value compared with the M-HAP/Gel composite-III coating. The M-HAP/Gel composite-III coating has good adhesion strength which will help for the long term orthopedic applications.

3.6 Potentiodynamic polarization measurements

The corrosion potential (\(E_{\text{corr}}\)) and the corrosion current density (\(I_{\text{corr}}\)) of the HELCDEB treated Ti and M-HAP/Gel composite coatings (at different concentrations of gelatin) were determined from the polarization curves and is shown in Fig. 5. As evidenced from the figure, the \(E_{\text{corr}}\) and \(I_{\text{corr}}\) values of the HELCDEB treated Ti were found to be -0.09\(\pm\)0.003 V and
0.08±0.003 µA/cm², respectively. The M-HAP/Gel composite-I coating on HELCDEB treated Ti showed a positive \(E_{\text{corr}}\) value (0.235V±0.002) with the \(I_{\text{corr}}\) of 0.032±0.005 µA/cm², whereas the M-HAP/Gel composite-II coating exhibited \(E_{\text{corr}}\) of 0.237±0.001 V and \(I_{\text{corr}}\) of 0.03±0.004 µA/cm² values (the corresponding curve is not provided). The values of \(E_{\text{corr}}\) and \(I_{\text{corr}}\) of the M-HAP/Gel composite-III and M-HAP/Gel composite-IV coatings were 0.324±0.002 V, 0.01±0.007 µA/cm², and 0.269±0.003 V, 0.027±0.002 µA/cm², respectively. The \(E_{\text{corr}}\) of the composite coated materials were higher than that of the HELCDEB treated Ti, which showed the protective nature of the coatings. In particular, the M-HAP/Gel composite-III coating on HELCDEB treated Ti exhibited a maximum positive shift towards the noble direction. The potentiodynamic polarization studies showed that the M-HAP/Gel composite-III coating on HELCDEB treated Ti had more corrosion potential, which implies the significant anticorrosion behavior of the composite coating.

The Nyquist plots of the HELCDEB treated Ti and M-HAP/Gel composite coated on HELCDEB treated Ti specimens at different concentrations of gelatin are shown in Fig. 6a. In comparison, the acquired polarization resistance (\(R_p\)) value for the M-HAP/Gel composite-I has shown high corrosion resistance as 180.2±0.02 kΩ cm² than that obtained for the HELCDEB treated Ti (114.3±0.07 kΩ cm²) and untreated Ti (52.4±0.03 kΩ cm²). The \(R_p\) value for the M-HAP/Gel composite-II was found at 182.8±0.01 kΩ cm² (not provided the curve) and M-HAP/Gel composite-III was obtained at 216.7±0.02 kΩ cm². For M-HAP/Gel composite-IV, the \(R_p\) value was observed to be 195±0.05 kΩ cm². A highest \(R_p\) value was obtained for the M-HAP/Gel composite-III on HELCDEB treated Ti sample, indicating the better and significant corrosion resistance of the coating.
Similarly the Bode plot for the M-HAP/Gel composite coating on HELCDEB treated Ti at different concentrations of gelatin exhibits maximum total impedance ($|Z|$) than the HELCDEB treated (174±0.03 kΩ cm$^2$) and untreated Ti (65±0.03 kΩ cm$^2$). The $|Z|$ values for the M-HAP/Gel composite-I and composite-II were found to be 230±0.05 kΩ cm$^2$ and 234.6±0.04 kΩ cm$^2$, respectively. The M-HAP/Gel composite-III exhibited around 425±0.07 kΩ cm$^2$ for $|Z|$ which was higher than the $|Z|$ of M-HAP/Gel composite-IV (270±0.03 kΩ cm$^2$) as shown in Fig. 6b. While comparing all the samples, the M-HAP/Gel composite-III has shown a shift towards the higher frequency range than the other coated samples and metal specimens.

The Bode phase angle also shows that the M-HAP/Gel composite-III has a high resistivity in simulated body fluid. The phase angle for the HELCDEB treated Ti was obtained at low frequency region and also the uncoated Ti attributed at lesser frequency region than the other coated samples as shown in Fig. 6c. Hence, based on the above results, it is clarified that the M-HAP/Gel nanocomposite coating with 3 wt% of gelatin resulted in a better anticorrosion performance in the SBF solution.

3.7 Antibacterial activity

The antibacterial efficacy of the M-HAP/Gel nanocomposite coating at various concentrations was tested against the microbes like *S. aureus* (Gram-positive bacterium) and *E. coli* (Gram-negative bacterium) by supplementing MacConkey agar (Fig. 7). The presence of M-HAP/Gel composite in the Mac Conkey agar plates was able to inhibit the formation of bacterial colonies for both the stains *S. aureus* and *E. coli*, while compared with the control (without composite material) samples. As shown in the Fig. 7a and b, the number of bacterial colonies was reduced on the increase of nanocomposite concentration from 25 µg mL$^{-1}$ to 100 µg mL$^{-1}$. The noticeable antibacterial activity was obtained for *S. aureus* (Fig. 7a) compared to *E. coli* (Fig. 7 b), as the nanocomposite was able to substantially reduce the number of bacterial colonies.
colonies at a final concentration of 100 µg mL\(^{-1}\). This study clearly evidenced that the M-HAP/Gel nanocomposite can affect the bacterial growth much more effectively. In addition, the Fig. 7c clearly indicates the quantitative reduction in colony count with different concentrations of nanocomposite, which displays a maximum bacterial reduction at the 100 µg mL\(^{-1}\) of nanocomposite. Thus, the results strongly revealed the higher antibacterial colonies reduction by the multifaceted composite coating which makes it possible for the prevention of implant failures.

3.8 Cytocompatibility

The cell proliferation on the M-HAP/Gel nanocomposite coating was evaluated against the MT3C3-E1 cells by MTT assay for 1, 4 and 7 days and the results are shown in Fig. 8. On day 1, the cells were started to spread on the control and composite surface (Fig. 8a). The MT3C3-E1 cells on the composite coating were spread well through cytoskeletal processes on day 4, when compared with control. For 7 days of culture, the multiplicity of cells and amount of cells on the nanocomposite sample (Fig. 8a) was higher than that on the control sample. The optical images suggest that the composite material has compatibility with osteoblast cells and improves the cell growth behavior. Moreover, the MT3C3-E1 cells on the nanocomposite coating spreads well through the cytoskeletal processes at all days.\(^{44}\) The cellular viability (cell proliferation numbers) was represented based on the absorbance value from MTT assay, and the results are shown in Fig. 8b. The M-HAP/Gel nanocomposite showed statistically significant increase in the cellular viability on day 7 compared with control (p<0.05), which suggests a strong positive effect on the cell proliferation of MC3T3-E1 cells due to the presence of Zn ions in the nanocomposite coating. This result is a significant evidence for the beneficial effect of biocompatibility of the composite coating material.
3.9 Live/dead cell assay

Fig. 9 shows the live/dead cells on the M-HAP/Gel nanocomposite coating at different days of incubation by fluorescent microscopy. The cells started to spread on the nanocomposite surface which is evidenced from Fig. 9a. The viable cells were present in more numbers on the composite for day 1 of incubation and no dead cells were detected when compared with control. While prolonging the incubation period to 4 and 7 days, the pronounced cells have been observed. From the image, it can be observed that the MC3T3-E1 cells proliferated well and were spread at 7 days of incubation. From the morphology of the cells it is well evident that cells seemed to prefer expanding on the nanocomposite surface, which actually enhances the metabolic condition of the cells (Fig. 9a). Figure 9b shows proliferation data of the cells seeded on the nanocomposite at different days such as 1, 4 and 7 days of incubation, which showed more favorable adhesion and higher proliferation rate of MC3T3-E1 cells. The cell proliferation on the nanocomposite was significantly (p < 0.05) higher compared with that on control (without nanocomposite) at 7 days of cell culture, which is mainly due to the presence of mineral ions and gelatin in the nanocomposite.

3.10 Fibroblast stem cells

The viability of fibroblast stem cells on nanocomposite coating was determined by MTT assay. The absorbance at a wavelength of 570 nm is directly proportional to the number of live cells in fibroblast stem cells culture medium. The % of viable cells of 125 mg mL⁻¹ nanocomposite coating was calculated with respect to the control for 4 and 7 days (Fig. 10b). From the optical images, it is evident that the % of viable fibroblast stem cells are more for 4 and 7 days of incubation. At 7 days of incubation, the nanocomposite coating exhibited higher/greater cell viability (99.7%). This result is further confirmed with the optical microscopic
images (Fig. 10a) for control and nanocomposite coating at 1, 4 and 7 days of incubation. The figures evidenced that the more number of cells were found to be viable in the nanocomposite coating. The nanocomposite coating exhibited cell morphology similar to that of the control group on day 4 and 7 of incubation and the cells were completely spread out, which revealed the biocompatibility of the nanocomposite coating. Thus, it is proved that the nanocomposite coating greatly improvises the bioactivity and promotes the cell growth and hence the nanocomposite coated sample is believed to be used as an orthopedic implant with better osteoinductive properties.

3.11 Apatite forming ability of the nanocomposite coating

The bioactivity of the M-HAP/Gel nanocomposite coating was confirmed by the SBF immersion study. The resulting apatite growth was evaluated after immersing the coated sample in SBF for various days such as 1, 7 and 14 days of immersion. The growth of apatite on the nanocomposite coating differs at each day. At day 1, the apatite started to grow on the surface of flower petal as shown in Fig. 11a. The growth of spongy apatite was formed on the nanocomposite at 7 days of immersion (Fig. 11b). On increasing the immersion period to 14 days, some spongy flower structure was observed as a result of apatite growth (Fig. 11c). Usually, the apatite formation of the sample takes place through a sequence of chemical reactions. When the coated samples are soaked in the SBF solution, the negative ions (PO$_4^{3-}$ and OH$^-$) can easily attract the positive ions (Ca$^{2+}$) and form Ca-rich amorphous calcium phosphate. Then, the surface gains the positively charged ions from the SBF solution, which began to attract ions such as PO$_4^{3-}$ and OH$^-$ and forming Ca-poor amorphous calcium phosphate. This process leads to the formation of bone-like apatite growth on the nanocomposite surface. Thus, the micrographs displayed the apatite mineralization ability at 1, 7 and 14 days of immersion and revealed that the resultant nanocomposite coating improves the bioactivity in SBF solution.
4. Conclusions

The development of M-HAP/Gel composite coating (with different concentration of gelatin) on HELCDEB treated Ti was successfully achieved by pulsed electrodeposition method. The presence of functional groups and phase purity of M-HAP/Gel composite were confirmed by the FT–IR and XRD observations, respectively. The typical nanostructured coating morphology with complete and uniform surface coverage was achieved at 3 wt% gelatin. The M-HAP/Gel nanocomposite coating on HELCDEB treated Ti exhibited better adhesion strength (23.8±0.6 MPa), when compared to that on the untreated Ti, which envisioned that the HELCDEB treatment on Ti surface helps to improve the adhesion strength of the coating material. Moreover, the electrochemical studies revealed that the resultant composite coating exhibited excellent anticorrosion property in simulated body fluid. The bacteriocidal ability of the nanocomposite resulted in more bacterial colony reduction at 100 µg/mL for both bacteria (S. aureus and E. coli). The cell proliferation assay, live/dead staining of MT3C3-E1 cells and cell viability of fibroblast stem cells on the resultant nanocomposite coating showed better biocompatibility and bioactivity at 7 days of incubation. Furthermore, the mineralization study of the M-HAP/Gel nanocomposite performed at 14 days, demonstrated the excellent apatite growth with bone mimicking character. Thus, the M-HAP/Gel nanocomposite coating on HELCDEB treated Ti can be an alternative implant material with improved properties in orthopedic application.

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**Figure captions**

**Fig. 1** Schematic representation of the formation of M-HAP/Gel composite.

**Fig. 2** FT-IR spectra of (a) pure gelatin and (b) M-HAP/Gel nanocomposite coating.

**Fig. 3** X-ray diffraction patterns of (a) pure gelatin and (b) M-HAP/Gel nanocomposite coating.

**Fig. 4** Morphological features of (a) 700 keV treated Ti, M-HAP/Gel composite coating on 700 keV treated Ti at different gelatin concentrations of (b) 1 wt% (c) 2 wt% (d) 3 wt% (e) 4 wt% and EDS spectrum of M-HAP/Gel composite coating with 3wt% of gelatin.

**Fig. 5** Potentiodynamic polarization curves of untreated, 700 keV treated Ti and M-HAP/Gel composite coatings on 700 keV treated Ti at various gelatin concentrations.

**Fig. 6** Impedance spectra of untreated, 700 keV treated Ti and M-HAP/Gel composite coatings on 700 keV treated Ti at various gelatin concentrations in simulated body fluid: (a) Nyquist, (b) Bode impedance and (c) Bode phase plots.

**Fig. 7** Petri dishes with MacConkey agar inoculated with (a) *S. aureus* and (b) *E. coli*, showing variable numbers of colonies when supplemented with different amounts of M-HAP/Gel nanocomposite and (c) Quantitative bacterial colony count of M-HAP/Gel nanocomposite against *S. aureus* and *E. coli*.

**Fig. 8** (a) Optical microscopic images showing proliferation of MC3T3-E1 cells on control and M-HAP/Gel nanocomposite coating at (a) day 1, (b) day 4, (c) day 7 and (b) Optical
density measurement illustrating MC3T3-E1 cell proliferation on the M-HAP/Gel nanocomposite coating and control. Asterisk denotes a significant difference compared with control (P < 0.05).

**Fig. 9** (a) Fluorescence micrographs of osteoblasts cultured on M-HAP/Gel nanocomposite coating for 1, 4 and 7 days and (b) MTT assay of osteoblasts incubated on M-HAP/Gel nanocomposite for 1, 4 and 7 days (Asterisk denotes a significant difference compared with control, P < 0.05).

**Fig. 10** (a) Optical microscopic images showing the viability of fibroblast stem cells on control and M-HAP/Gel nanocomposite at 4 and 7 days of incubation and (b) % viability of fibroblast stem cells on M-HAP/Gel nanocomposite at 4 and 7 days of incubation (Asterisk denotes a significant difference compared with control, P < 0.05).

**Fig. 11** SEM images of apatite growth on M-HAP/Gel nanocomposite after (a) 1 days (b) 4 days and (c) 14 days of immersion in SBF solution.

**Scheme 1**

*In situ* pulsed electrodeposition of M-HAP/Gel nanocomposite on HELCDEB treated Ti
Gelatin

\[ \text{Gelatin} \]

\[ \text{M}^{2+} - \text{Ca}^{2+}, \text{Sr}^{2+}, \text{Mg}^{2+}, \text{Zn}^{2+} \]

Pulsed electrodeposited M-HAP/Gel composite

M-HAP/Gel composite
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

(c)

E. coli

S. aureus

Bacterial colony count %

Concentrations (µL)

control

25µg/mL

50µg/mL

100µg/mL

Figure 7
Figure 8

(b) Control
M-HAP/Gel composite

OD value

1.5
1.0
0.5
0.0

1 day
4 days
7 days

Incubation time (days)

Figure 8
Figure 9

(b)

Absorbance at 560 nm

Control
M-HAP/Gel composite

Culture time (days)

1 day, 4 days, 7 days

*
(a) Control M-HAP/gel composite

(b) Control M-HAP/Gel composite

Figure 10
Scheme 1

HEL CDEB treated Ti → Pulsed electrodeposition → M-HAP/gelatin composite formation → M-HAP and gelatin nanocomposite → Pulsed electrodeposited M-HAP/gelatin nano composite