Chapter-2

Fabrication of hydroxyapatite-gelatin nanocomposites as a functional bone graft for orthopedic applications

2.1. Introduction

Bone substitutes are needed for the repair of segmental defects created upon the removal of infected tissue or tumor of bone. The limited availability of live, normal bone for surgical repairs (autograft and allograft) drive orthopedic surgeons to look for synthetic alternatives [41,158]. In order to mimic the constituents and the organization of the natural bone investigations are focused on various polymers, ceramics and their mixtures [39,202,203]. The synthetic bone substitutes based on the simple physical mixture of polymer and ceramic may result in the composition close to that of the major component of natural bone but their organization is extremely different [135]. Therefore, a plethora of research activity is focused to find suitable methods for in situ formation of the inorganic phase during the synthesis of the composite.

Bone tissue is a viscoelastic composite made up of long collagen fibrils threading with crystalline HA minerals having highly hierarchical structure. Gelatin is a high molecular weight polypeptide, a denatured collagen, chemically and structurally close to collagen [82,160]. In synthetic bone graft, collagen is often replaced by gelatin, due to various reasons like lower cost, commercial availability, solubility in aqueous systems, etc. As mentioned earlier, considerable efforts are being focused on the in situ formation of HA-gelatin composite [121,197–200].

Structurally, an implant material should have an open and interconnected porous architecture to facilitate fluid flow and thus the exchange of gases, homogenous cell distribution, cell access to nutrients, etc [121,201,202]. Furthermore, functionality of the bone graft can be enhanced by combining antibiotics with bone implant materials that offers a multitude of advantages like the delivery of antibiotic at the desired site and a bone scaffold to guide its repair [203–
Hence this chapter focusses on a novel in situ method of preparation of HA-gelatin nanocomposite bone substitute material by wet precipitation. The results on bioactivity, dissolution, mechanical property and cytotoxicity evolution are presented. In addition to this, the potential of the HA-gelatin nanocomposite as a drug delivery matrix has been investigated using rifampicin (RIF), indomethacin (IND) and amoxicillin antibiotics by appropriate drug loading methods with respect to their release behaviour.

2.2. Experimental

2.2.1. Composite preparation

Calcium nitrate tetrahydrate [Ca(NO$_3$)$_2$.4H$_2$O, 98%], di-ammonium hydrogen phosphate [(NH$_4$)$_2$HPO$_4$, 99%], ammonia solution [NH$_4$OH, 25%], gelatin purified, sodium chloride [NaCl], sodium bicarbonate [NaHCO$_3$], potassium chloride [KCl], di-sodium hydrogen phosphate dihydrate [Na$_2$HPO$_4$.2H$_2$O], magnesium chloride hexahydrate [MgCl$_2$.6H$_2$O], calcium chloride dihydrate [CaCl$_2$.2H$_2$O], sodium sulphate [Na$_2$SO$_4$], tris buffer [(CH$_2$OH)$_3$CNH$_2$] and 5% solution of glutaraldehyde [C$_5$H$_8$O$_2$] were procured from Merck. The drugs, IND, RIF and amoxicillin were obtained from Himedia. All the reagents were used without any further purification. Experiments were carried out using deionized water.

HA-gelatin nanocomposite preparation was done by employing wet precipitation method and the flowchart for the same is shown in Fig. 11. 0.5M Ca(NO$_3$)$_2$.4H$_2$O and 0.3M (NH$_4$)$_2$HPO$_4$ were prepared in gelatin (1.5, 5 and 8 wt%) solution and their pH was maintained above 10 using ammonia. After vigorous stirring for 6 h Ca solution was titrated against P solution while maintaining the pH above 10 and the mixture was aged at room temperature for 24 h. The precipitate formed was in the form of a foam which floated on the solution and crosslinking of the foam was done using 30 µl of 5% glutaraldehyde solution. Then the foams were separated, washed and freeze dried for 3 h. The composite with gelatin concentrations 1.5, 5 and 8 wt% were named as HGA, HGB and HGC respectively.
2.2.2. Characterization

The synthesized samples were subjected to powder x-ray diffraction (XRD) using PANalytical X’Pert PRO diffractometer with monochromatic CuKα radiation, operated at 40 kV and 30 mA with a scan step of 0.02° in the 2θ range 20° to 60°. Crystallographic phase identification of the samples was accomplished by comparing the experimental XRD pattern with the standard data compiled by the International Center for Diffraction Data (ICDD). The average crystallite size of the synthesized samples was calculated from the full width at half maximum (FWHM) of the (002) plane of the XRD patterns using Scherrer’s approximation [206].

Fig. 11. Flowchart for the composite preparation
\[ D = \frac{K\lambda}{(\beta \cos \theta)} \]  

Where \( D \) is the average crystallite size as calculated for the \((hkl)\) reflection, \( \lambda \) is the wavelength of CuK\(\alpha \) radiation \( (\lambda = 1.5406 \text{ Å}) \), \( \beta \) is the full width at half maximum (in radian), \( \theta \) is the diffraction angle (in degree) and \( K \) is the broadening constant.

Fourier transform infrared (FT-IR) spectra of the samples were recorded using a Perkin Elmer spectrum RX1 by KBr pellet technique in the range 4000 to 400 cm\(^{-1}\) with 4 cm\(^{-1}\) resolution in transmission mode. The morphology of the samples was observed by scanning electron microscopy (SEM, JEOL JSM-6060 model, Japan) at an accelerating voltage of 20 kV after gold coating, in order to avoid the accumulation of electrons on the surface of the sample which leads to charging effect. The size and shape of HA on the polymer matrix were investigated using Philips-CM200 analytical transmission electron microscope (TEM) operated at 200 kV. The sample was dispersed in acetone followed by ultrasonication for 15 min, a small drop of the supernatant was dispersed and dried on carbon coated copper grids. Thermogravimetric analysis of the composites was carried out using a Perkin Elmer TGA 7 instrument. Samples were placed in alumina crucibles and an empty alumina crucible was used as the reference. Samples were heated from ambient temperature to 1000 °C in a (50 ml/min) flow of N\(_2\) at heating rates of 20 °C min\(^{-1}\). Continuous recordings of sample temperature and sample weight were performed.

### 2.2.3. Drug loading

RIF and IND loaded samples were prepared by a direct mixing mode and the samples are referred as RHGA, RHGB, RHGC and IHGA, IHGB, IHGC respectively. Discs of 8 mm diameter and 1 mm thick were made by thoroughly mixing 75 mg of sample with 25 mg of the drug and were subjected to uniaxial pressing under 100 MPa. This direct mixing mode resulted in burst release in the case of amoxicillin (AHGA-D). In order to achieve a control on the release, an attempt was made with \textit{in situ} amoxicillin loading strategy using the sample HGA.
For **in situ** loading 0.1 g of amoxicillin was dissolved in the gelatin solution which was used as the solvent for the HA synthesis. The remaining steps were repeated as mentioned in the experimental method and then the drug loaded foam was separated, dried and was named as AHGA-I. Then 100 mg of AHGA-I was pressed into a pellet of 8 mm diameter for studying the drug release behavior. The drug encapsulated in the matrix was estimated by UV-Vis spectrophotometer ($\lambda_{\text{max}} = 273$ nm) and the encapsulation efficiency of AHGA-I was calculated using the following equation

\[
\text{Encapsulation Efficiency (\%)} = \frac{\text{Actual drug content}}{\text{Theoretical drug content}} \times 100 \quad \text{(2)}
\]

The assay was carried out in triplicate and the value was found to be 89.4%.

### 2.2.4. *In vitro* drug release

*In vitro* release of RIF, IND and amoxicillin was carried out by soaking the drug loaded pellet in 200 ml of phosphate buffered saline (PBS, pH=7.4) contained in a conical flask, which was placed in a shaker incubator maintained at a temperature 37 °C (close to the body temperature) and the shaking speed was 100 rpm. 1 ml of PBS solution was withdrawn and replaced by fresh medium at predetermined time intervals. The concentration of drug released in the buffer solution was estimated spectrophotometrically using Perkin Elmer UV/Vis spectrometer at 319, 473 and 273 nm ($\lambda_{\text{max}}$) for RIF, IND and amoxicillin respectively. Drug release test was performed in triplicate for each formulation.

### 2.2.5. Apatite forming ability in SBF

The ability of the composites to induce apatite formation in a body fluid environment was studied by *in vitro* bioactivity test using simulated body fluid (SBF). 100 mg of the powdered sample was weighed and pressed into discs of about 8 mm diameter and 1 mm thickness. Bioactivity studies were carried out by immersing HGA, HGB and HGC discs in 30 ml of SBF in an airtight container at 37 °C. SBF is an acellular aqueous solution with inorganic ionic concentration similar to human blood plasma and buffered to physiological pH 7.4. Table 1 lists
the ionic concentrations of human blood plasma and that of the SBF [207]. The SBF was prepared by dissolving appropriate amount of NaCl, NaHCO₃, KCl, Na₂HPO₄·2H₂O, MgCl₂·6H₂O, CaCl₂·2H₂O, Na₂SO₄ and (CH₂OH)₃CNH₂ (Tris) in deionized water and 1M HCl was used to maintain the physiological pH [207]. The solution was renewed once in every three days for a period of 21 days. After 21 days, the pellets were taken out from the SBF solution and washed then allowed to air dry at 37 ± 0.1 °C for analysing the formation of apatite layer on the surface by scanning electron microscopy.

Table 1. Ion concentrations (mM/l) of human blood plasma and SBF

<table>
<thead>
<tr>
<th>Ion</th>
<th>Human blood plasma</th>
<th>SBF</th>
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<tr>
<td>Na⁺</td>
<td>142.0</td>
<td>142.0</td>
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<tr>
<td>Cl⁻</td>
<td>103.0</td>
<td>125.0</td>
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<tr>
<td>HCO₃⁻</td>
<td>27.0</td>
<td>27.0</td>
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<tr>
<td>K⁺</td>
<td>5.0</td>
<td>5.0</td>
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<tr>
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<tr>
<td>HPO₄²⁻</td>
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<td>1.0</td>
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<tr>
<td>SO₄²⁻</td>
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2.2.6. Cytocompatibility

Cells and matrix seeding

Human osteoblast like MG-63 cell was used to assess the cellular response of the composite. This cell line has generally been used in biocompatibility studies, because it exhibits a number of features similar to those of typical human osteoblasts [208]. The cells were obtained from National Centre for Cell Sciences (NCCS), Pune, India and were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/ml penicillin and 100 U/ml streptomycin. The cells were cultured in 25cm x 25cm x 25cm sized tissue culture flask at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Maintained cultures were passaged every week and the culture medium was changed twice a
When the cell density in culture flask reached 70-80% confluence, they were harvested by trypsinization and seeded in 96-well plates in the density of 2.5 X 10^3 cells per well in 100 μl and incubated for 24 hours in a CO_2 incubator. The powdered sample at dosages of 50, 100 and 200 μg/ml were dispersed in DMEM and added to the cells. The plates were further incubated for 24, 48 and 72 hours in the CO_2 incubator.

**MTT staining**

Ample number of assays are available to measure the cytotoxicity of the biomaterials, such as MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma), WSTs (Water Soluble Tetrazolium salts) and LDH (Lactate dehydrogenase) assays. We have used the MTT assay to measure the biocompatibility of the sample which is based on the ability of cellular mitochondrial dehydrogenase to reduce the yellow coloured tetrazolium salt to blue coloured formazan crystals using human osteoblast like cells (MG-63).

The cell viability was evaluated by MTT colorimetric assay for three growth periods _viz._, 24, 48 and 72 h. After each period, 50 μl of MTT solution at 5 mg/ml in PBS was pipetted out into each well to achieve 1 mg/ml as final concentration and the plate was further incubated for 150 min. Then the medium was carefully decanted and the blue formazan crystals were air dried in dark place and dissolved in 100 μl dimethyl sulfoxide (DMSO) and the plates were mildly shaked at room temperature and the optical density was measured using Synergy H4 micro plate reader at 570 nm. All tests were performed in triplicate and the results are expressed with the statistical error bars. The cell viability was calculated using the following equation

\[
\text{Cell viability (\%)} = \left( \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \right) \times 100 \quad \text{--------} \quad (3)
\]

where \(\text{OD}_{\text{sample}}\) and \(\text{OD}_{\text{control}}\) represent the optical density (OD) values of cells cultured with the sample and without the sample respectively.
2.3. Results and discussion

2.3.1. Mechanism of composite formation

Ceramic-polymer composite preparation is usually done either by dispersing the preformed ceramic phase in the polymer matrix or by in situ process [121,159]. The wet precipitation method usually results in a product which gets settled at the bottom of the flask. But in the present case, existence of gelatin along with calcium phosphate mixture prevented the settling of the HA precipitates. Also the entire mixture did not transform into a gel during the sol-gel transition as it occurs in thermally induced phase separation instead the product (composite) floated as a highly stable foam on the surface of the liquid medium. The process of the product formation here can be described as a method of separation using foaming technique [209].

![Diagram of HA-gelatin nanocomposite formation](image)

**Fig. 12. Schematic representation of the HA-gelatin composite formation**

It has been reported that near or above isoelectric point (IEP), the surface activity of protein increases [210]. The accumulation of surface active agents at the
liquid/gas interface leads to the reduction of liquid phase surface tension and hence an enhancement of viscosity and elasticity resulting in the formation of stable foams. Though the calcium phosphate phase formed in the existing conditions is not a surface active agent, it is capable of associating with the surface active agent gelatin and hence it gets separated along with the gelatin from the mixture as the composite. A schematic of this is shown in Fig.12.

2.3.2. XRD, FT-IR, SEM

XRD pattern of the samples confirmed the mineral phase in the prepared composite to be HA (JCPDS card No. 04-0932) (Fig.13). With increasing concentration of gelatin, we found that the peaks of HA broadened and the intensity of the peaks also got reduced. This indicates the reduction in the crystallinity of the mineral phase as is usually observed in the organic mediated crystallization of apatite [211]. The pattern of apatite with broader bands are very similar to those of minerals in human bone and dentin. The crystallite size of the samples was calculated to be 48, 33 and 30 nm for HGA, HGB and HGC respectively.
Fig. 14. FT-IR spectra of the composites (a) HGA (b) HGB and (c) HGC

The FT-IR spectrum (Fig. 14) of all the samples exhibited the characteristic $PO_4$ ($v_4$) vibrations of HA at 566 & 605 cm$^{-1}$ and the broad band around 1040 cm$^{-1}$ that belongs to the bending vibrational mode of $PO_4$ ($v_3$). In HGB and HGC, the $v_4$ peaks of $PO_4$ around 566 and 605 cm$^{-1}$ cannot be detected individually, instead a broad peak around ~560 cm$^{-1}$ was observed with increasing gelatin content. The bands around 875 cm$^{-1}$ appeared for all the samples indicating that carbonation of inorganic phase occurs during the nucleation of HA. Moreover, the carbonation results in B-type carbonated apatite which closely resembles that of the natural bone tissue [212–214].

The typical amide I band of gelatin around 1655 cm$^{-1}$ (peptide bond C=O stretch) indicates that the composite has a predominantly $\alpha$-helical configuration and this is further confirmed by the presence of amide II band at ~1551 cm$^{-1}$ (mixed C-N stretch and N-H in-plane bending) [215,216]. These amide bands of gelatin resemble that of the collagen bands in the human cortical bone [217]. Also N-H deformation at ~1224 cm$^{-1}$ for the amide III bands is observed. The peak at 1375 cm$^{-1}$ indicates the chelation of Ca$^{2+}$ ions by the carboxylic group of gelatin.
When gelatin content was increased, amide band at 1636 cm\(^{-1}\) revealed a gradual red shift in wavenumber from 1659 cm\(^{-1}\) to 1634 cm\(^{-1}\) which confirmed the chemical interaction between the mineral phase and the organic matrix. In addition the red shift from 1448 cm\(^{-1}\) to 1424 cm\(^{-1}\) corresponding to amino acids of gelatin indicates the chemical interaction between the gelatin amino acids and the calcium and phosphate ionic groups of HA.

![SEM images of the composites](image_url)

**Fig. 15. SEM images of the composites (a) HGA (b) HGB and (c) HGC**

SEM images of the prepared composites at various gelatin concentrations are shown in Fig.15. The composite formed with low gelatin content exhibit an inhomogeneous matrix with distinct gelatin fibres and HA agglomerates (Fig. 15(a)). As the content of gelatin increased the homogeneity of the composite improved with indistinguishable polymer and ceramic phases (Fig. 15 (b &c)). Also it resulted in
the formation of a three dimensional interconnected porous structure with pore sizes ranging between 5-15 μm.

Fig.16 &17 depicts the FT-IR spectra of RIF and IND loaded composites. The characteristic peaks of HA were observed without any shift for both the drugs. RIF loaded samples showed a gradual shift in wave numbers from 1646 to 1657 cm\(^{-1}\) and 1531 to 1558 cm\(^{-1}\) which indicates furanone C=O and amide C=O groups of RIF overlapped with the amide groups of polymer matrix with increasing gelatin content [220]. Whereas in IND loaded samples, the characteristic peaks of drug 1261 cm\(^{-1}\) (asymmetric aromatic O-C stretching), 1086 cm\(^{-1}\) (symmetric aromatic O-H stretching) and the characteristic bands of HA and polymer were observed without any shift indicating that IND got loaded into the composites without any interaction.

![FT-IR spectra of RIF loaded composites](image)

**Fig. 16.** FT-IR spectra of the RIF loaded composites (a) RHGA (b) RHGB and (c) RHGC
2.3.3. TEM

A more detailed analysis of the composite morphology was carried out using TEM and the images are shown in Fig. 18. It was observed that when the concentration of gelatin was increased, the alignment of the apatite crystals changed considerably. The sample with the minimum gelatin content (HGA) exhibited randomly aligned rod shaped HA crystals. Whereas HGB that contains more gelatin than HGA, formed HA whiskers aligned sparsely along the gelatin matrix. With further increased gelatin content, that is in HGC, densely packed and aligned HA whiskers were observed. Analysis of the SAED patterns revealed a typical HA polycrystalline structure with apparent (002) and (112) planes. Apatite crystals grown in collagen based organics normally have an elongated shape due to the mediation of the amino acid backbone in mineralization of apatite, which leads to their preferential growth along $c$-axis. It is well known that in real bone, the HA crystals grow in parallel with the collagenous fibers along the $c$-axis.
2.3.4. Thermogravimetric analysis (TG)

TG curves of the prepared samples are shown in Fig. 19. All samples exhibited approximately 5% weight loss up to 225 °C due to desorption of physically adsorbed water molecules. With increasing temperature, a weight loss of about 20%, 28% and 40% is observed for samples HGA, HGB and HGC respectively between 225 and 425 °C due to gelatin decomposition. Above 425 °C the observed weight loss of about 14% (HGA) and 17% (HGB & HGC) could be attributed to the combustion of the residual organic components. The total weight loss of 39%, 50% and 62% observed for HGA, HGB and HGC respectively clearly indicates the
increasing concentration of polymer that got incorporated into the composite as the content of gelatin was increased during the synthesis of the composite.

Fig. 19. TG curve of the prepared samples (a) HGA (b) HGB and (c) HGC

Fig. 20 shows the SEM image of the surfaces of HGA, HGB and HGC discs before (a, b, c) and after (d, e, f) soaking in SBF for 21 days. Before soaking in SBF, all the samples exhibited a plain surface whereas after soaking in SBF, the surface revealed the formation of apatite deposits. Although HGA sample surface showed the formation of aggregated apatite deposits the coverage of the surface was less. Conversely, the entire surface of HGB and HGC was covered by large amount of apatite deposits. At pH greater than 7 the RCOO$^-$ groups present in gelatin gets largely ionized and offer binding sites for Ca$^{2+}$. These ion complexes further interact with (PO$_4$)$_3^{3-}$ present in SBF and owing to its supersaturation with respect to calcium phosphate and results in the formation of decisive size nuclei and subsequent growth as HA particles on the composite surfaces. It is to be mentioned here that the HA content present in all the three samples before immersion was the same. Hence, the increased bioactivity of HGB and HGC can be attributed to the presence of higher gelatin content in them.
2.3.5. *In vitro* bioactivity

Fig. 20. SEM images of the surface of the samples before (a) HGA (b) HGB & (c) HGC and after (d) HGA (e) HGB & (f) HGC immersion in SBF for 21 days
2.3.6. Cytocompatibility

A common objective in orthopedic and dental fields is the design of biomaterials that support cell and tissue growth while improving fracture healing and bone defect filling. Cell viability and function on a bone graft are closely related to the physical, chemical and biological characteristics of the materials used. The toxicity and biocompatibility of the prepared composites are the important concerns before proceeding to *in vivo* study. Among the three samples prepared the sample with higher gelatin content which exhibited a good 3D porous structure was chosen for the cytotoxicity study. Cell viability and optical images of osteoblast like MG-63 cells were studied with different dosages of HGC for different incubation periods (Fig. 21 & 22).

![Cell viability of HGC nanocomposite with MG-63 cells](image)

It is evident that the sample showed good cell viability ranging between 88% to 100% for 72 h for the various concentrations of the sample studied. According to biological evaluation for *in vitro* cytocompatibility (ISO 10993 - 5: 2009) if the cell viability of the material is less than 70% obviously it has a toxic potential. Since the
studied nanocomposite HGC did not exhibit cell viability less than 70% it can be considered as biocompatible with human osteoblast like MG-63 cells.

Fig. 22. Optical images of HGC nanocomposite with MG-63 cells (72 h incubation - 20X mag)

2.3.7. *In vitro* drug release

The *in vitro* drug release from the three composite matrices was measured with different immersion periods by maintaining the physiological conditions. It is obvious from Fig. 23 that there is a sudden increase of RIF release in the first 5 h in all the samples. The cumulative drug release at 5 h was found to be 25%, 16% and 21% respectively. From then on the release was found to be linear in HGB and HGC until 48 h and gets terminated at 168 h but HGC showed a controlled release of RIF up to 240 h releasing almost 95% of the loaded drug.
Fig. 23. RIF release from (a) RHGA (b) RHGB and (c) RHGC

Fig. 24. IND release from (a) IHGA (b) IHGB and (c) IHGC
Release of IND followed almost the same trend for HGB and HGC releasing about 30% of IND in less than 5 h (Fig. 24), and about 55 to 65% in 48 h from all the three samples. Then the release became slow and lasted up to 216 h. Both drug release patterns are characterized by a typical triphasic drug release that is normally exhibited by biodegradable polymers [236]. The initial burst is due to the drug molecules present on the surface of the matrices which gets released immediately after exposure to the release medium. The second slow phase is governed by swelling of gelatin that caused drug release by diffusion in low dose and this phase continues to about 2-3 days. The third stage is characterised by the matrix erosion, in which polymer degradation occurred.

The drug release data was fitted to zero-order (Q = K₀t), first-order (lnQ = lnQ₀-Kt), Higuchi (Q= Kₜ½) and Korsmeyer peppas (Q=Ktⁿ) models, where Q₀ is the initial concentration of drug and Q is the cumulative amount of drug released at time t and K₀, K and Kₜ are Zero-order, First-order and Higuchi rate constants respectively and n is the release exponent. The best fit with higher correlation value was found with the Higuchi’s equation and the release of both drugs from the matrices was diffusion controlled (Fig. 25 & 26). Diffusion controlled release of the drug loaded samples are intimately related to the molecular transport of drugs through the polymeric matrices.

In order to understand the type of transport phenomenon, first 60% drug release data were fitted using Korsmeyer peppas model. The value of n characterizes the release mechanism of the drug where when n ≤ 0.45 corresponds to a Fickian diffusion mechanism and 0.45 < n < 0.89 to non-Fickian transport [237,238]. Korsemeyer-peppas fits of the experimental data for RIF and IND are shown in Fig. 27 & 28. The value of n was determined to lie in between 0.48 to 0.84 and these values indicate an anomalous (non-Fickian) diffusional mechanism suggesting that the drug release involves more than one mechanism such as diffusion, swelling, erosion, etc.
Fig. 25. Higuchi plots of RIF release of (a) RHGA (b) RHGB and (c) RHGC

Fig. 26. Higuchi plots of IND release of (a) IHGA (b) IHGB and (c) IHGC
Fig. 27. Korsemeyer-peppas plots of RIF release of (a) RHGA (b) RHGB and (c) RHGC

Fig. 28. Korsemeyer-peppas plots of IND release of (a) IHGA (b) IHGB and (c) IHGC
2.3.8. Characterization of *in situ* amoxicillin loaded composite

The XRD pattern, FT-IR spectrum, SEM image and TG curve of amoxicillin loaded composite (AHGA-I) are shown in Fig. 29 (a, b, c & d). The mineral part in the sample is found to be HA (JCPDS card no. 09-0432) and the nature of composite is almost similar to that of natural bone. Amoxicillin did not affect the crystallinity of the mineral phase. The average crystallite size of the mineral phase in AHGA-I is calculated to be 30 nm.

![XRD pattern of the in situ amoxicillin loaded composite](image)

**Fig. 29 (a) XRD pattern of the *in situ* amoxicillin loaded composite**

The characteristic FT-IR bands of HA and gelatin are observed along with the bands of amoxicillin. The band situated at 963 cm\(^{-1}\) corresponds to C-H bending & N-H stretching vibrations and the other band at 1384 cm\(^{-1}\) corresponds to the symmetric stretching of C-H vibrations [159]. FT-IR did not reveal any obvious chemical interactions between the carrier and the drug. SEM image of AHGA-I shows the formation of a homogeneous matrix with HA and gelatin components indistinguishably merged together.
Fig. 29 (b) FT-IR spectrum of the \textit{in situ} amoxicillin loaded composite

Fig. 29 (c) SEM image of the \textit{in situ} amoxicillin loaded composite
TG curves of the synthesized (HGA) and in situ amoxicillin loaded samples (AHGA-I) are given in Fig. 29 (d). The weight loss observed up to 180 °C in both the samples is due to the desorption of physically adsorbed water molecules. A weight loss of about 25% observed between 180 °C to 500 °C is due to the decomposition of gelatin in both the samples. On the other hand, an additional weight loss of about 7% is observed between 180 and 600 °C in AHGA-I due to the removal of amoxicillin.

2.3.9. In vitro amoxicillin release behavior

Fig. 30 shows the amoxicillin release from the direct and in situ loaded matrix. The drug release of AHGA-D exhibited burst release behavior releasing 94% in 5 h (inset in Fig. 30). This burst release feature may be attributed to the mode of drug loading (direct mixing). On the other hand, the drug release of AHGA-I comprises an initial burst release (about 47% within 6 h) followed by a sustained release over a period of 10 days (about 27% in 306 h).

The drug release data of AHGA-I did not follow zero-order and first order release kinetics and the best fit with higher correlation value was found for the
Higuchi’s equation indicating the release of amoxicillin from the matrices was governed by diffusion process. The diffusion mechanism of drug release was further confirmed by Korsmeyer-Peppas plots that showed fair linearity and the slope value was determined to be 0.17 indicating that drug release mechanism from the selected AHGA-I matrix was Fickian diffusion controlled (graphs are not given for brevity).

![Graph showing drug release over time](image)

**Fig. 30. Amoxicillin release from *in situ* loaded matrix (AHGA-I); inset plot: release from direct (AHGA-D) loaded matrix**

In the case of direct loading of amoxicillin there is no interaction between the drug and the matrix (as the drug was physically entrapped) and this might be the reason for the burst release. In the case of *in situ* loading also there is no obvious chemical interaction between the drug and the matrix as observed from FT-IR. But the release behavior was highly controlled that can be attributed to the incorporation of the drug at the molecular level in the matrix when the mode of loading was *in situ*. When exposed to PBS medium the swelling induced pores in the cross linked matrix which are large enough to favour the diffusion of the drug molecules entrapped inside the matrix lead to a controlled release.
2.4. Conclusions

Engineered bioactive and biocompatible bone substitute materials incorporating antibiotics are promising functional materials for repair of bone defects and fractures. We prepared HA-gelatin nanocomposites by wet precipitation method with varying gelatin content. It is observed from the present study that the alignment of the apatite whiskers changes greatly with increase in the gelatin content. The nanocomposite exhibited good apatite forming ability and cell viability with human osteoblast like MG-63 cells. Drug release potential of the composites was studied with RIF, IND and amoxicillin by direct mixing method. RIF and IND exhibited sustained release whereas amoxicillin got released completely in 5 h. On the other hand in situ amoxicillin loading exhibited controlled and prolonged release for 13 days through Fickian mechanism from the composite matrix. These findings collectively exhibit that HA-gelatin composite matrix could be a promising drug carrier if appropriate drug loading strategy is adopted and also be a good candidate for bone graft substitutes.