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# A facile strategy to elute amoxicillin in a controlled way from hydroxyapatite-gelatin composite

**K. Sangeetha[1], Y. Yokogawa[2], E.K. Girija[1*]**

[1]*Department of Physics, Periyar University, Salem 636 011, India*

[2]*Graduate School of Engineering, Department of Intelligent Materials Engineering, Osaka City University, Osaka 5588585, Japan*

[*]Corresponding author. Tel: (+91) 94443-91733; E-mail: girijaeaswaradas@gmail.com

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## ABSTRACT

In recent decades bone infection is one of the most challenging issues encountered in biomedical field and local antibiotic delivery is a key strategy to overcome this issue. Hence developing bioactive materials in combination with antibiotics is much focused recently for bone substitutes. Here we report the fabrication of pristine and natural polymer (gelatin) composite matrices of hydroxyapatite (HA) by a facile wet precipitation method and their drug release behavior from directly loaded and *in situ* loaded matrices using amoxicillin as the model drug. The products thus obtained were analyzed by X-ray diffraction (XRD), Fourier transform infrared (FT-IR) spectroscopy, thermogravimetry (TG) and scanning electron microscopy (SEM) which confirmed the formation of HA and nanocomposite of HA with gelatin. It was observed that under physiological conditions, for sustained and prolonged release of the drug *in situ* loading in composite matrix is a favorable approach. 

**Keywords:** Drug delivery; nanocomposite; hydroxyapatite; gelatin.

## Introduction

Bone repair is a subject of intensive investigation in human health care in which bone infections continue to cause significant morbidity and chronic recurring infections **[1]**. Periodontitis is an oral disease that involves progressive alveolar bone loss around the teeth, the usual treatment is focused on stopping the destruction of the periodontal support of the teeth by eliminating the pathogenic bacteria in the inflamed pocket followed by tissue reconstruction via tissue regeneration **[2]**. The choice of antimicrobial agent must be based on the bacterial aetiology of the infection and there are many pathogens involved in the periodontitis disease. Among the several antibiotics used for the treatment of periodontitis ampicillin, amoxicillin, erythromycin, metronidazole, tetracycline are few **[3]**. Amoxicillin is a β-lactam antibiotic that is active against both gram-positive and gram-negative bacteria. The effective use of antibacterial agents for the treatment of periodontitis requires an adequate drug concentration at the site of action for a long period **[4]**.

Drugs administered systemically cause serious side effects to other healthy tissues, further achieving effective local antibiotic concentration at the site of infection is relatively difficult. When administered locally, they limit the adverse effects of systemic administration and there is an effective concentration of medication at the targeted site. Hence, it is necessary to develop drug carriers capable of providing sufficient concentration of antibiotic at the target site. Instead of having a drug delivery system and bone graft separately for treating the periodontitis, the bone graft itself can be made to function as the drug delivery system **[5, 6]**.

Synthetic hydroxyapatite (HA) is identical to the natural mineral of the hard tissues of bone and teeth. It has been proposed as a grafting material in many dental applications including repair of periodontal defects, augmentation of alveolar bone, sinus lifts, tooth replacement and repair of large bone defects caused by tumors. In addition it finds wide applications as carrier for drug, bone substitute for filling bone defects, a scaffold for bone tissue engineering and a coating on metallic implants owing to its ease of production and handling, excellent biocompatibility, osteoconductivity and bioactivity **[7, 8]**. On the other hand, its usage at high load bearing applications has been restricted because of its poor mechanical performance. When used as powders it may migrate from the implanted site with the fluids before a sufficient amount of the patient's own tissue is regenerated. In order to overcome these clinical problems, composite materials based on natural or synthetic polymers can be employed.

 

Increasing interest and research efforts has recently been centered on natural biodegradable polymers due to their outstanding properties such hydrophilicity, biocompatibility and excellent biodegradability which make them useful for biomedical applications **[9-15]**. A drug delivery system based on composite of HA with polymers such as poly(lactic-co-glycolic acid), ethyl cellulose, chitosan or a mixture of chitosan and beta-cyclodextrin has been reported as an efficient strategy to slow down the release of amoxicillin **[16, 17]**. Gelatin is one of the natural biopolymer which is derived from collagen and has proven to be a good candidate for the controlled release of several biologically active molecules such as TGF- h1, bFGF and BMP-2 **[18, 19]**. Also it is used in pharmaceuticals as adhesives and wound dressings, tissue engineering scaffolds and as drug carriers due to its bioaffinity and hydrogel characteristics. Therefore, HA-gelatin nanocomposite carrier may present several advantages over pristine HA and gelatin and made as a promising vehicle for drug entrapment and release. In addition, hydroxyapatite-gelatin nanocomposite can mimic the constituents of natural bone to some extent. However, a detailed study of the effect of processing conditions on controlled release of amoxicillin from HA-gelatin composites had not yet been studied. The aim of the present study is to synthesize drug loaded bioactive hydroxyapatite and bone like hydroxyapatite (HA) - gelatin nanocomposite by two different processing routes and investigate the influence of matrix preparative conditions on the release behavior of amoxicillin.

## Experimental

*Materials*

The chemicals used were amoxicillin (Himedia), calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$, 98 %), di-ammonium hydrogen phosphate ($(NH_4)_2HPO_4$, 99 %), gelatin, glutaraldehyde and ammonia solution ($NH_4OH$, 25 %) obtained from Merck. All the reagents were used without further purification. Deionized water was employed as the solvent.

*Composite formation*

0.5 M $Ca(NO_3)_2.4H_2O$ and 0.3 M $(NH_4)_2HPO_4$ were prepared in 1.5 % gelatin solution and their pH was maintained above 10 using ammonia. After vigorous stirring for 6 h Ca solution was titrated against P solution and the mixture was aged at room temperature for one day. The precipitate formed was in the form of foam which floated above the solution and crosslinking of the foam was done using 30 µl of 5 % glutaraldehyde solution. Then the foams were separated, washed and dried at room temperature and named as HG. For comparison hydroxyapatite was prepared without gelatin in the above experiment and the resulting precipitate was centrifuged and dried and named as HA.

*Characterization*

Analysis of the mineral phase present in the samples was done by powder X-ray diffraction using PANalytical X'Pert PRO diffractometer with CuKα radiation, operated at 40 kV and 30 mA with scan speed 2 °/min. The interaction between mineral and polymer matrix was analyzed by Fourier Transform Infrared Spectroscope (FT-IR, Perkin Elmer RXI1). The morphology of the samples was observed by Scanning electron microscope (JEOL JSM-6060 model, Japan). The amount of amoxicillin present in as-synthesized samples was analyzed using a thermogravimetry (TG) analyzer (Make: TA Instruments, Model: Q600).

*Cytocompatibility*

Cells and matrix seeding: Human osteoblast like cell MG-63 was used to assess the cellular response of the composite. This cell line has previously been used in biocompatibility studies, because it exhibits a number of features similar to those of typical human osteoblasts **[20]**. 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 µ/ml steptomycin. 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% $CO_2$. Maintained cultures were passaged every week and the culture medium was changed twice a week. 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 h in a $CO_2$ incubator. Samples at dosages of 25, 50 and 100 µg/ml were dispersed in DMEM and added to the cells. The plates were further incubated for 48 h in the $CO_2$ incubator.

MTT staining: The cell viability was evaluated by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, Sigma) colorimetric assay for 48 h. After that, 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 by using the following equation

$$\text{Cell viability (\%)} = (OD_{sample} / OD_{control}) \times 100$$

where, $OD_{sample}$ and $OD_{control}$ represent the optical density (OD) values of cells cultured with the sample and without the sample respectively.

*Drug loading and releasing*

Amoxicillin was incorporated in the matrix by two different ways, one is direct loading and the other is *in situ* loading. In the case of direct loading, 100 mg of HA/HG was ground and mixed thoroughly with 25 mg of amoxicillin and pressed into pellets of 8 mm diameter (AHA/AHG). 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 foams were separated, dried and was named as IHA (without gelatin) / IHG. Then 100 mg of IHA/IHG 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 and the encapsulation efficiency of HA and HG was calculated using the following equation

$$\text{Encapsulation Efficiency (\%)} = \frac{\text{Actual drug content}}{\text{Theoretical drug content}} \text{ X } 100$$

The assays were carried out in triplicate for each batch and the value was found to be 45.53 and 89.4 % respectively.

The *in vitro* release of amoxicillin was carried out by soaking the drug loaded pellets in 200 ml of phosphate-buffer solution (PBS, pH 7.4) at physiological temperature. All experiments were performed in triplicate.

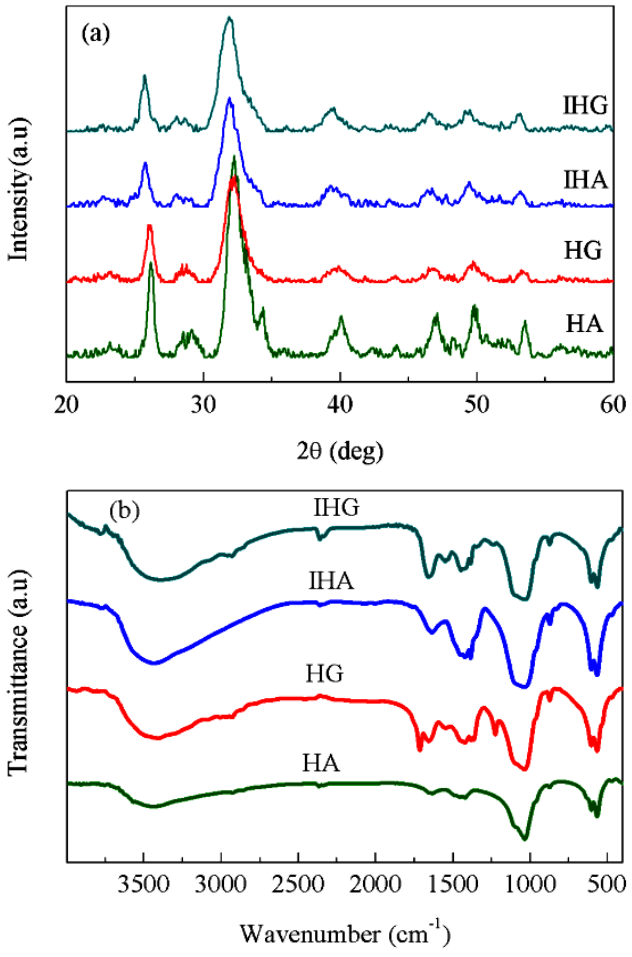


**Fig. 1.** (a) XRD and (b) FT-IR spectra of the samples HA, HG, IHA and IHG.

## Results and discussion

*XRD, FT-IR and SEM*

**Fig. 1(a)** shows the XRD patterns of as-prepared HA, HG and *in situ* drug loaded samples IHA, IHG. The mineral parts in the samples are found to be HA (JCPDS card no. 09-0432), addition of gelatin reduced the crystallinity of HA and the nature of composite is almost similar to that of natural bone. Amoxicillin did not affect the crystallinity and the average crystallite size of mineral phase in HA, HG, IHA and IHG was calculated to be 40, 31, 39 and 30 nm respectively. The FT-IR spectra of the samples are shown in **Fig. 1(b)**. The characteristic $PO_4$ ($\upsilon_4$) vibrations of HA are observed in all the spectra at 601 and 561 $cm^{-1}$. However, the $CO_3^{2-}$ absorption peak observed at 870 $cm^{-1}$ suggested that the obtained HA is carbonated apatite. The FT-IR spectra of HG and IHG show the typical amides I and II bands of gelatin at 1659 and 1550 $cm^{-1}$ respectively. In IHA and IHG, the bands of amoxicillin such as C-H bending and N-H stretching vibrations are situated at 963 $cm^{-1}$ and the other band observed at 1384 $cm^{-1}$ corresponds to the symmetric stretching of C-H vibrations **[21]**. Thus, FT-IR spectrum of both the samples IHA and IHG showed the characteristic peaks of all the constituents with no band shifts from their original positions revealing no obvious chemical reactions between the carrier and the drug.

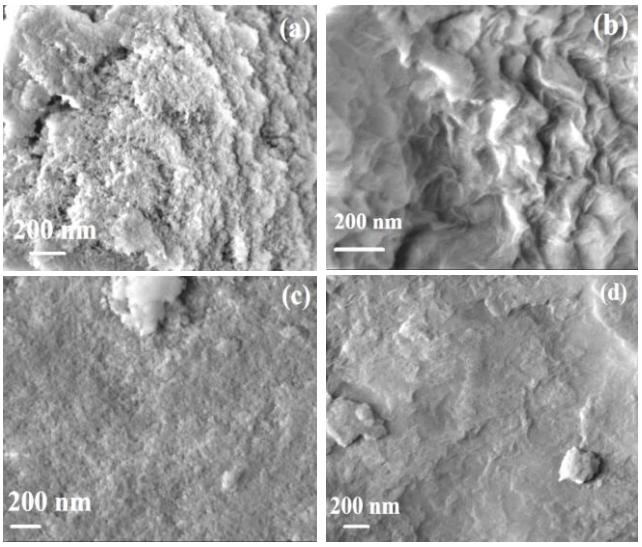


**Fig. 2.** Microstructure of (a) HA, (b) HG, (c) IHA and (d) IHG.

SEM image of HA (**Fig. 2(a)**) shows agglomerated clusters consisting large number of nanosized particles whereas in the composite, the HA and gelatin components indistinguishably merged together forming a homogeneous matrix. In IHA (*in situ* amoxicillin loaded) loosely aggregated HA particles was observed and in IHG amoxicillin has homogeneously merged with the matrix (**Fig. 2(c, d)**).

*Thermo gravimetric analysis (TG)*

TG curves of the as-synthesized samples are shown in **Fig. 3**. The weight loss observed up to 180 °C in all samples is due to desorption of physically adsorbed water molecules. With increasing temperature, there is no significant weight loss for HA whereas in the case of IHA, a weight loss of about 4 % was observed that is attributed to the removal of amoxicillin from the sample. In HG, a weight loss of about 25 % was observed between 180 °C to 500 °C due to decomposition of gelatin. On the other hand

 

an additional weight loss of about 7 % was observed between 180 and 600 °C in IHG due to the removal of amoxicillin loaded.

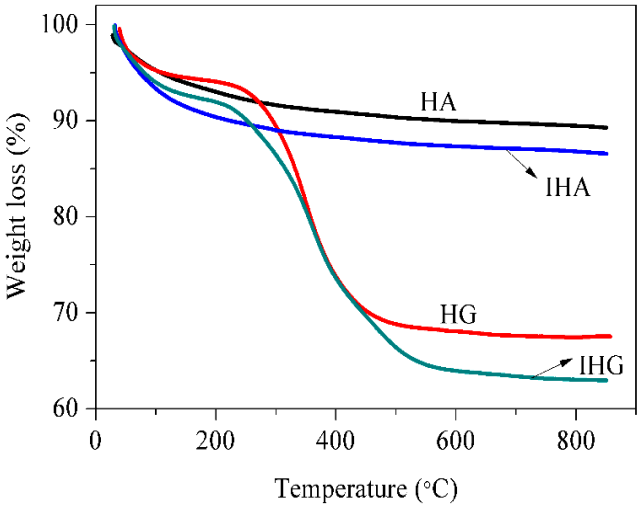


**Fig. 3.** TG curve of as-synthesized samples.

*Cytocompatibility with human osteoblast like MG-63 cells*

**Fig. 4(a)** shows the cell viability for different dosages of the composites for 48h incubation. It is evident that both the samples exhibit viability ranging between 83 % to 100 % for the dosages studied. According to biological evaluation of medical devices – Part 5: tests for in vitro cytotoxicity (ISO 10993-5: 2009), if cell viability of the material is less than 70 % then it has a cytotoxic potential **[22]**.

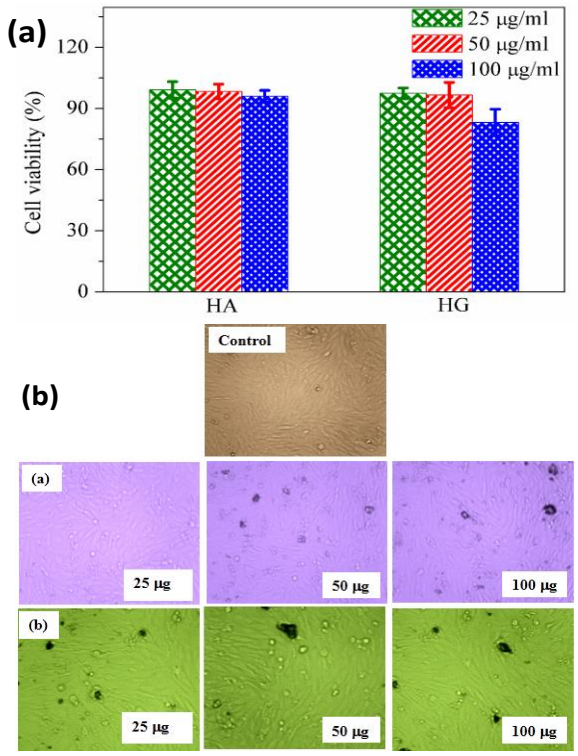


**Fig. 4.** (a) Cell viability of HA and HG with human osteoblast MG-63 cells. and (b) Optical images of human osteoblast like MG-63 cells and cells cultured with different dosages of samples (a) HA and (b) HG.

However HA and HG exhibit cell viability greater than 83 % indicating that they can be considered as biocompatible with human osteoblast like cells MG-63. Optical images of MG-63 cells cultured with different dosages of HA and HG are shown in **Fig. 4(b)**.

*In vitro drug release behavior*

The drug release profile of AHA exhibited three distinct phases which is shown in **Fig. 5(a)**. In the first one 60 % of drug was released in 5 h followed by a gradual release of 30 % in 24 h and the remaining was released in 48 h. On the other hand AHG exhibited burst release behavior releasing 94 % in 5 h which may be due to the mode of drug loading (direct mixing).

Drug release profiles of *in situ* drug loaded matrix are as shown in **Fig. 5(b)**. IHA exhibited a two stage release pattern with 60 % release in first 6 h followed by a slow release stage (about 27 % for last 114 h) and the release terminated at 87 %. On the other hand, the drug release profile of IHG 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 *in vitro* drug release from the direct drug loaded sample (HG) showed almost complete release in 5 h whereas *in situ* drug loaded sample (IHG) showed initial burst release followed by the sustained release for 312 h.

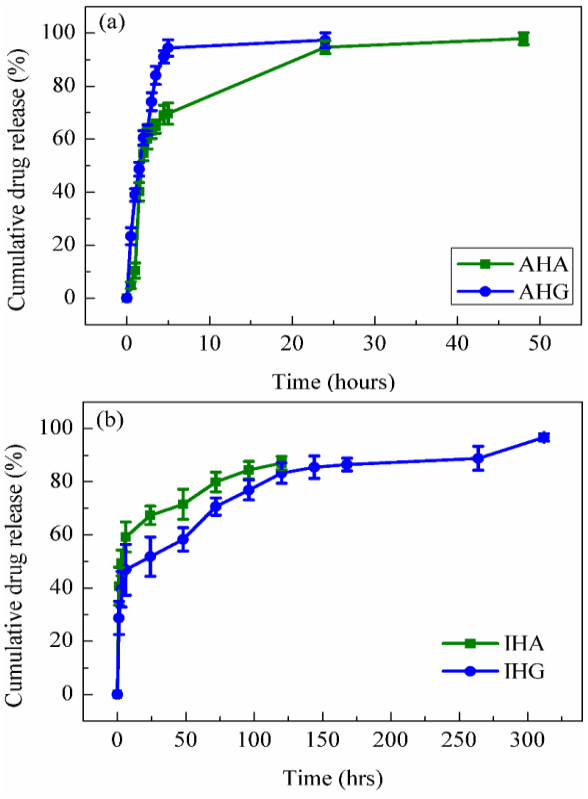


**Fig. 5(a, b).** Amoxicillin release from direct (AHA & AHG) and *in situ* loaded matrices (IHA & IHG).

*Drug release mechanism*

The drug release data of IHA and IHG was fitted to zero-order ($Q = K_0t$), first-order ($\ln Q = \ln Q_0 - K$) and Higuchi models ($Q = K_H t^{1/2}$) where $Q_0$ is the initial concentration of

drug and Q is the cumulative amount of drug release at time *t* and $K_0$, K and $K_H$ are Zero-order, First-order and Higuchi rate constants respectively. The data 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 (**Fig. 6(a)**). The diffusion mechanism of drug release was further confirmed by Korsmeyer-Peppas plots ($Q=Kt^n$) that showed fair linearity, with slope values less than 0.5, indicating that drug release mechanism from the selected IHA and IHG matrices was Fickian diffusion controlled (**Fig. 6(b)**).

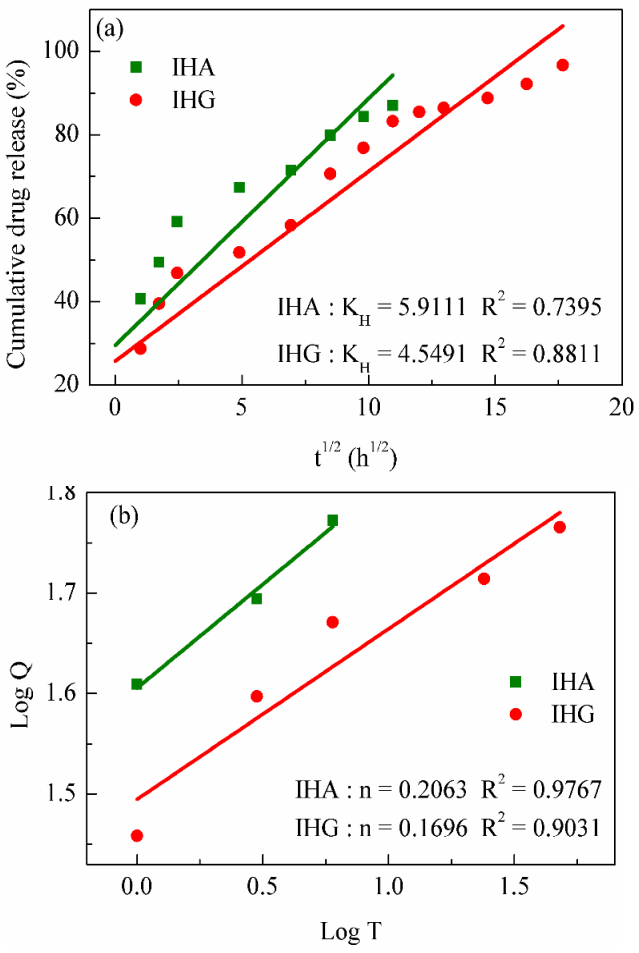


**Fig. 6 (**a, b**).** Higuchi and Korsemeyer plots of *in situ* loaded matrices (IHA & IHG), respectiely.

In direct loading, there is no interaction between the drug and the matrix (as the drug was physically entrapped) and which might be the reason for the burst release. In the case of *in situ* loading there is no chemical interaction between the drug and the matrix as observed from FT-IR. But the drug was present in molecular form in the synthesis medium during the fabrication of the matrix, hence the drug molecules might have homogeneously incorporated into the matrix. On exposure to the medium release of the drug molecules present on the surface of the matrices immediately lead to the burst release from both the matrices and then the release became controlled in both the cases. In IHA drug release terminates around 87 % which might be due to the limited accessibility of release medium to the core of the sample (pellet). On the contrary the composite sample IHG could release almost the entire encapsulated drug due to its swelling nature and the controlled release for extended period can be attributed to the presence of crosslinked polymer chains. The rapid delivery of drug during the initial burst release is required immediately after surgery for effective inhibition of microorganisms and a controlled release is needed to aid long term healing and to avoid the toxic and adverse systemic effects caused by high concentration of antibiotics.

## Conclusion

Amoxicillin loaded pristine HA and HA with gelatin composite matrices were prepared by two different approaches *viz*. direct loading and *in situ* loading. It is observed from that in the case of direct loading the drug release terminated or completed in 24 to 48 h. On the other hand *in situ* loading exhibited controlled and prolonged for 13 days through Fickian mechanism from the composite matrix, while pristine HA released the drug in 5 days. The entrapments of the drug at molecular level in the matrix during *in situ* loading and the presence of gelatin in the composite matrix which is capable of swelling might be the reason for the controlled and sustained release. These findings collectively exhibit that HA-gelatin composite matrix with *in situ* loaded amoxicillin could be a promising drug carrier and grafting material for the treatment of bone infection.

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