Digest Journal of Nanomaterials and Biostructures Vol. 13, No. 3, July - September 2018, p. 685 - 692

EFFECT OF FIBROBLAST CELL LINES ON CIPROFLOXACIN LOADED COMPOSITE SCAFFOLD FOR BONE REGENERATION

R. NITHYA[a*], K. PORKUMARAN[a], N. M. SUNDARAM[b]
[a]*Department of Biomedical Engineering, Dr.N.G.P. Institute of Technology, Coimbatore-641048, Tamilnadu, India*
[b]*Department of Physics CA, LRG Government Arts College for Women - Tirupur, Tamil Nadu, India*

Osteomyelitis is a bone infection and inflammation caused by bacteria. Osteomyelitis often requires surgery and prolonged antibiotic for weeks or months. The aim of this proposed work is provide a cost-effective biomaterial that can be implanted at the target site to effectively eradicate the disease. One of the downside of this disease is the recurrence of the disease, even after treatment. The ciprofloxacin loaded hydroxyapatite/agarose scaffold is synthesized and characterized to selectively target the diseased area, to prevent the recurrence of the disease by providing local sustained antibiotic drug delivery which is considered advantageous over the conventional methods. The biomaterial is prepared in a way to make it biocompatible by using the materials like Hydroxyapatite (HAp), which mimics the natural bone composition; Agarose, which is a natural polymer that provides excellent mechanical strength and porous structure to help cell ingrowth and Ciprofloxacin, which is an anti-bacterial drug that helps to treat the disease. The prepared material is characterized using Fourier Transform Infra-Red spectroscopy (FT-IR) and X-Ray Diffraction (XRD). The biocompatibility test is done by MTT Assay to determine the morphology changes, cell viability and cell membrane integrity.

(Received January 11, 2018; Accepted July 27, 2018)

Keywords: Osteomyelitis, Hydroxyapatite, Agarose, Ciprofloxacin, Drug delivery

1. Introduction

Bone regeneration is a complete restoration of lost or injured tissue, and healing encompasses repairing some original structure. Regeneration is typical of tissues with high proliferation capacity by providing a scaffold. The scaffold assists cell attachment and subsequent proliferation and differentiation [1]. Osteomyelitis is an infection typical of bone and is caused by pyogenic bacteria. Typical microorganisms which are prevalent causative agents of the disease are Staphylococcus aureus. Osteomyelitis following grafting of prosthesis is commonly caused by Staphylococcus aureus. Antibiotic administration is essential in order to reduce infection risks during the grafting procedure and healing process or to treat pre-existing infections. Their direct injection into the damaged site is generally not effective because of their rapid diffusion from the injected site, as well as their enzymatic digestion and deactivation [2].

Treatment after initial surgical debridement often comprises the implantation and subsequent removal of antibiotic-impregnated beads in parallel with systemic broad-spectrum antibiotics before bone grafting can be carried out. This is a lengthy and costly process, with 3.7 % of infections unsuccessfully treated [3]. These issues can be overcome by combining scaffolds with a drug delivery system (DDS). The DDS can promote a prolonged drug release both directly and selectively at the implantation site, and it can protect growth factors and protein molecules from degradation.

*Corresponding author: nithya.rajendran07@gmail.com

Drug delivery can be achieved by incorporation of drugs into polymeric materials to control drug release at a predefined and reproducible rate for a prolonged period of time [4]. Bone substitutes are often required to replace damaged tissue due to disease, trauma, or surgery. Current bone substitutes do not exhibit the physiological or mechanical characteristics of true bone. While autogenic and allogeneic grafts can be used successfully under certain conditions, these grafts suffer from problems associated with additional harvesting costs, donor site morbidity, and graft availability. Metal implants cannot perform as well as healthy bone or remodel with time. To help address the need for better bone substitutes, tissue engineers seek to create synthetic, three dimensional scaffolds made from porous bioceramic and/or polymeric materials to induce the growth of normal bone tissue [5]. One example of a bioceramic used as a bone substitute is hydroxyapatite (HAp) [5,6,7]. Although HAp is osteogenic, it is brittle and difficult to process into complex shapes. Hence HAp is added with polymers to form bio-composites to strengthen their structure [8]. Natural polymer is developing the most promising therapeutic systems, namely drug delivery system which provides an effective therapy to the patients for prolonged periods. A natural polymer have lots of advantage over synthetic polymers as safe and non-toxic in nature, highly biocompatible and bio degradable very much environment and eco-friendly [9]. Agarose is one of the natural polymer obtained from seaweed. Agarose hydrogel scaffolds were engineered to stimulate and guide neuronal process extension in three dimensions in vitro. The extracellular matrix (ECM) protein laminin (LN) was covalently coupled to agarose hydrogel using the bifunctional cross-linking reagent 1, 1'-carbonyldiimidazole (CDI). Agarose gel is widely used in the application of tissue engineering because it supports cell growth [10]. The prepared scaffold is loaded with drug depending on the application. Since osteomyelitis is caused mostly by bacteria, Ciprofloxacin is one such. It is a fluroquinolone derivative, widely used in osteomyelitis because of its favourable penetration and bactericidal effect on all the probable osteomyelitis pathogens [12]. The minimum inhibitory concentration (MIC) of CIP is as low as 0.25–1 μg/mL for Staphylococcus (frequently found in osteomyelitis) [12]. So, our work basically involves combining these three factors to provide a better solution for osteomyelitis. Through this work, we are trying to achieve the following aspects. They are, Better biocompatibility, thus further not causing any more infections, Effective targeting of the drug, to avoid damages to surrounding cells, Accelerated healing process.

2. Experimental Setup

2.1. Materials

Calcium nitrate tetrahydrate, Diammonium phosphate and Agarose were purchased from Sigma Aldrich. Ammonia solution was purchased from Merck. All chemicals were analytical grade and used as received without further purification.

2.2. Synthesis of Hydroxyapatite

Scientific literature finds mention of several methods of preparation of Nano HAp[13,14]. The Precipitation technique is most commonly used method due to its simplicity, rapid preparation as well as easy control of particle size, composition and various possibilities to modify overall homogeneity of the product [13,14,15]. The first step involved with the mixing of Anion solution e.g. calcium source with cation solution e.g. phosphorous. Then the precipitated solution is dried and calcified at 700^{o}C for 3 hours in muffle furnace.

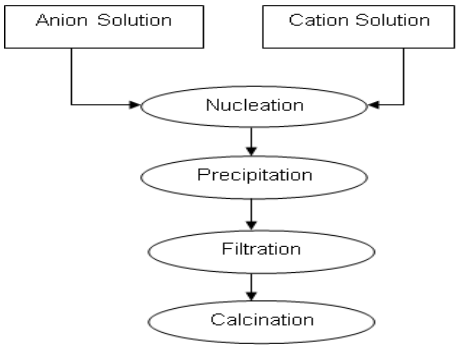

Fig. 1: Flowchart of Precipitation technique

2.3. Preparation of composite scaffold

Polymeric scaffold for tissue engineering can be prepared with the multitude of different techniques. Many diverse approaches have recently been under development [11,16]. At present, tissue engineering method generally require the use of porous scaffold that serves as a matrix for initial call attachment and subsequently for tissue in vitro and in vivo. Up to now the scaffolds made from biomaterial have temporarily substituted the extracellular matrix. Solvent evaporation is an effective method of composite preparation. In this process 6 w/v % of Agarose 9 w/v % of Hydroxyapatite and 3 w/v % of ciprofloxacin was mixed in distilled water at 70^0 C. The solvent evaporation is carried out by stirring this mixture at certain temperature using magnetic stirrer for 1 hr [17]. These mixtures are left undisturbed for a day in a petridish so that they form film like structure. Also the prepared mixtures are added with Nacl salt to obtain the porous film upon washing the scaffold with water.

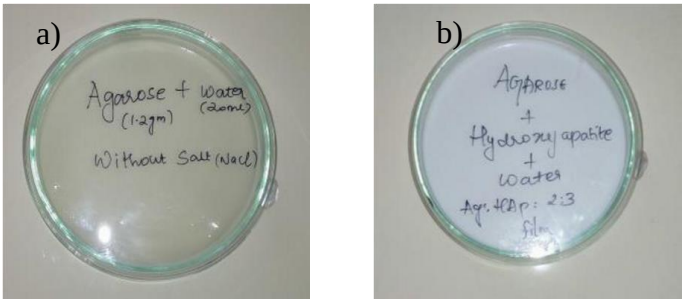

Fig. 2. Prepared scaffold a) Agarose film b) Composite film

3. Characterization

The XRD patterns were recorded using diffractometer system -XPERT-3. Fourier Transform-Infrared spectrometer (Schimadzu, Japan) was used to analyse the spectral assignments of the prepared samples. Fibroblast c ell line was purchased from National Centre for Cell Science (NCCS), Pune and cell membrane integrity with sample was monitored using 3-(4,4-dimethyl-2thiazol)-2,5-diphenyl-tetrazolium bromide(MTT) assay.

4. Results and Discussion

4.1. X-Ray Diffraction (XRD) analysis

XRD is a tool used for identifying the atomic and molecular structure of a crystal .It is a measuring instrument for analysing the structure of material from the scattering pattern produced when a beam of radiation or particles interacts with it primarily used for phase identification of a

crystalline material and can provide information on unit cell dimensions and for the identification of unknown materials include characterization of crystalline materials and measurement of sample purity and also make textural measurements such as the orientation of grains in a polycrystalline sample[15]. In this, the crystalline atoms cause a beam of incident X-rays to diffract into many specific directions. In figure 3, XRD pattern shows a strongest peak at 31.74 corresponding to (211) planes of hydroxyapatite. Other characteristic peak at (101), (200), (002), (112), (202), (310), (222), (303). The obtained samples were in good agreement with the XRD pattern of a hydroxyapatite standard available in JCPDS (09-0432).

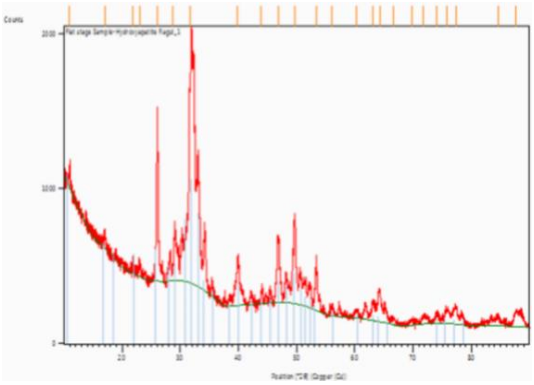

Fig. 3: X-Ray Diffraction of Hydroxyapatite

4.2 Fourier Transform- Infra Red Spectroscopy (FT-IR) analysis

FT-IR is a technique which is used to obtain an infrared spectrum of absorption or emission of a solid, liquid or gas. An FTIR spectrometer simultaneously collects high spectral resolution data over a wide spectral range [23]. It offers quantitative and qualitative analysis for organic and inorganic samples. It identifies chemical bonds in a molecule by producing an infrared absorption spectrum. FTIR spectroscopy has numerous advantages when used for chemical analysis of CaP products [24].

In figure 4 the most characteristic chemical groups of synthesized HAp are PO_4^{3-}, OH^-, CO_3^{2-}, as well as HPO_4^{2-} that characterize nonstoichiometric HAp. Similarly, in figure 5-8 the FTIR of prepared composite is shown.

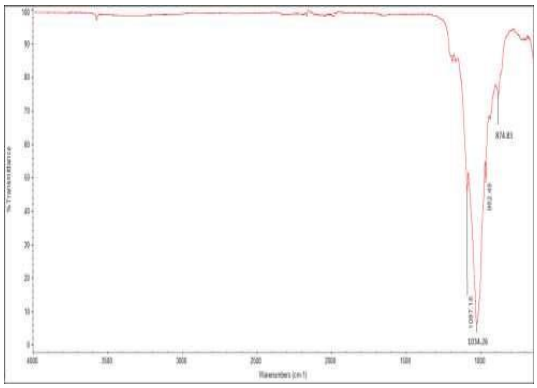

Fig. 4: FT-IR of Hap

Tabel 1. Prominent peaks of HAp

Standard Peak Values (cm^{-1})	**Obtained Peak Values (cm^{-1})**	**Peak Assignment**
1000-1100	1087.16,1034.26,962.49	PO_4^{3-}
1400-1450, 870-880	874.83	CO_3^{2-}
3497	3500	O-H
3000-3400	3000-3400	O-H Stretch Band

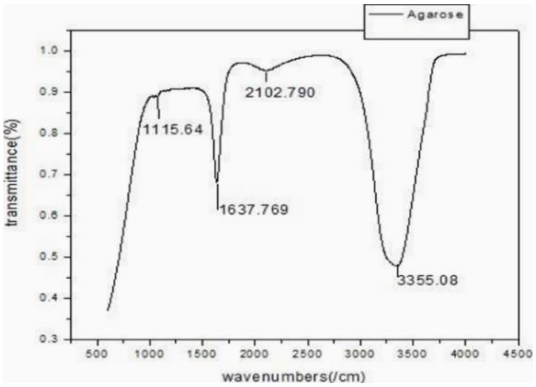

Fig. 5. FT-IR of Agarose and Water with salt

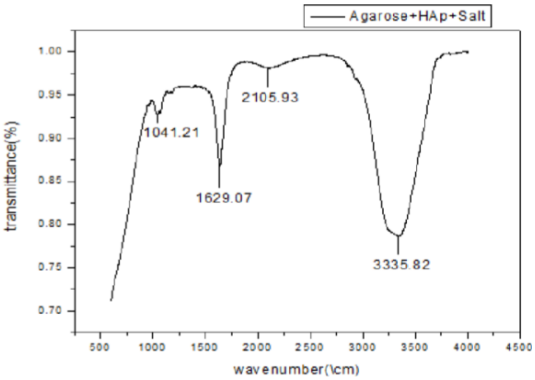

Fig. 6. FT-IR of Agarose, Hydroxyapatite and Water with salt

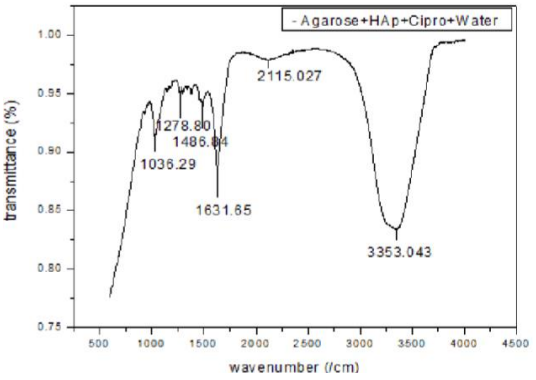

Fig. 7. Agarose, HAp, Ciprofloxacin and Water without salt

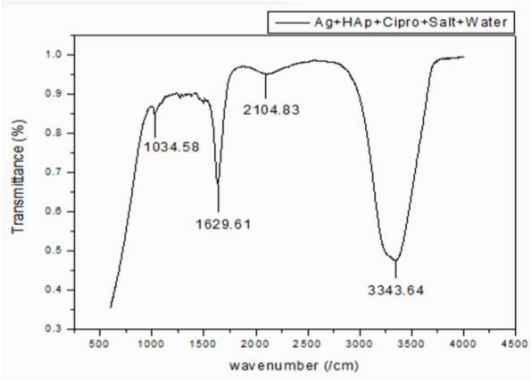

Fig.8. Agarose, HAp, Ciprofloxacin and Water with salt

Tabel 2. Prominent peaks of agarose and Ciprofloxacin

Standard Peak Values (cm^{-1})	**Obtained Peak Values (cm^{-1})**	**Peak Assignment**
1077 and 1152	1036 and 1034	Agarose
3000-1950, 1650-1600, 1450-1400, 1250-1200, 1050-1000	3353, 3343, 1631 and 1629, 1486, 1278, 1028	Ciprofloxacin

4.3 Biocompatibility Result

The MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) cleavage assay is a sensitive, quantitative and reliable colorimetric assay that measure viability, proliferation and activation of cells or, when metabolic events lead to apoptosis or necrosis, a reduction in cell viability. The assay is based on the capacity of the cellular mitochondrial dehydrogenase enzyme in living cells to reduce the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2yl) -2,5diphenyl tetrazolium bromide (MTT) into a dark blue/purple formazan product which is insoluble in water. After that a solubilization solution (DMSO) is added to dissolve the insoluble purple formazan product into a coloured solution. The amount of formazan produced is directly proportional to the cell number in a range of cells lines. Figures 9-12 shows the cell viability graph for different concentration of the sample.

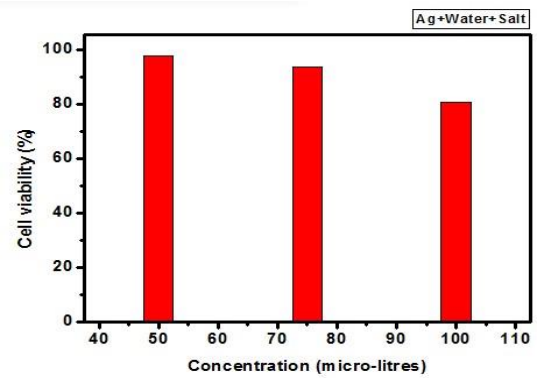

Fig. 9: Dose-response cell viability (Agarose+Water+Salt)

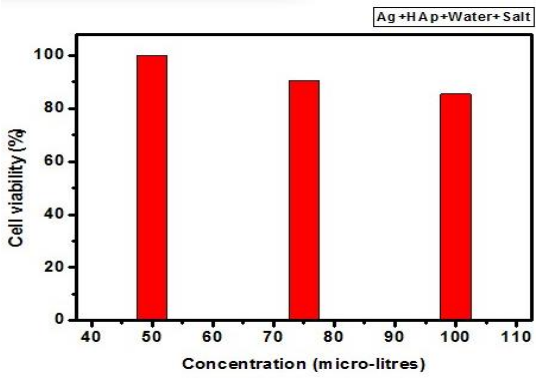

Fig.10. Dose-response cell viability (Agarose+HAp+Water+Salt)

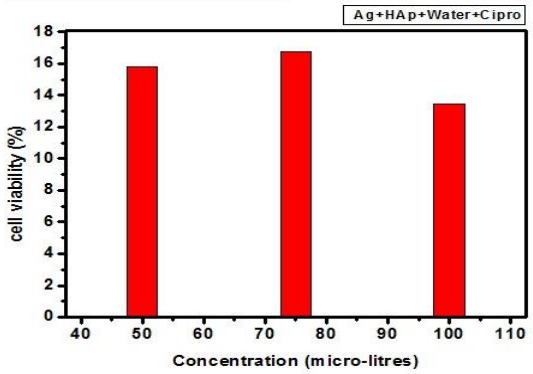

Fig. 11. Dose-response cell viability Agarose+HAp+Water+Ciprofloxacin)

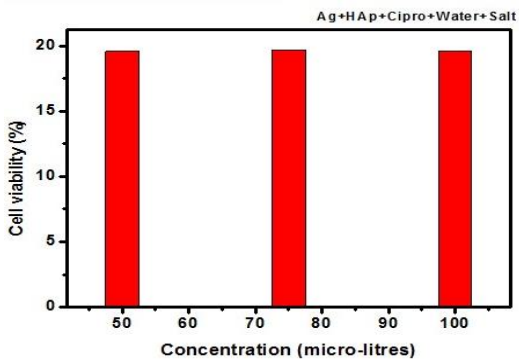

Fig. 12. Dose-response cell viability (Agarose +HAp+Water+Ciprofloxacin+Salt)

5. Conclusions

Thus from the results obtained, a novel biomaterial for osteomyelitis proves to be biocompatible. This work can be further improved by conducting clinical trials to know their effectiveness to fullest extent. Further tests like drug release study and biodegradability test will support the results obtained. From this work, it is evident that the methods followed here prove to be effective and convenient. The HAp synthesized by precipitation method provides the required porous structure, thus making it one of the easiest and simplest method for preparation of a drug delivery system. The scaffold preparation is done in such a way that the porous structure helps in cell ingrowth. The biocompatibility results show that the prepared materials are non-toxic to living

cells. From all the results obtained, the prepared material is considered an innovative step towards achieving the objectives of this work.

Acknowledgement

The authors would like to thank SITRA (South India Textile Research Association) who helped to carryout biocompatibility study and Dr. N. G. P. Institute of Technology, Department of Biomedical Engineering for providing facilities to carry out the research work.

References

[1] Rozalia Dimitriou, Elena Jones, Dennis McGonagle, Peter V Giannoudis. BMC Medicine **9**, 66 (2011).
[2] Rossella Dorati, Antonella DeTrizio, Tiziana Modena, Bice Conti, Francesco Benazzo, Giulia Gastaldi Ida Genta, Pharmaceuticals, **10**, 96 (2017).
[3] N.S. Motsitsi, Int. J. Care of Injured, **39**, 155 (2008).
[4] V. Mourino, Nanocomposites for Musculoskeletal Tissue Regeneration, 175–186 (2016).
[5] P. V. Giannoudis, H. Dinopoulos, Tsiridis E, Injury **36**, 20 (2005).
[6] E. M. Younger, M. W. Chapman, J. Orthop Trauma **3**(3),192 (1989).
[7] C. G. Finkemeier, J. Bone Joint Surg Am **84**(3), 454 (2002).
[8] V. Campana, G. Milano, J.Mat. Sci. **25**, 2445 (2014).
[9] Artoum Rakovsky, Irena Gotmann, Eugen Rabkin, Elazar Y. Gutmanas. J. Mechanical behavior of biomedical material **32**, 89 (2014).
[10] Soichi Iwai, Hidetaka Shimizu, Yoshika Suzawa, Mitsuru Akashi, Yoshiaki Yura, J. Oral and Maxillofacial Surgery **27**, 637 (2015).
[11] K. Senthilarasan, A. Ragu, P. Sakthivel. Int. Journal of Engineering Research and Applications **4**, 55 (2014).
[12] Diana Gomes, Margarida Pereira, Ana Francisca Bettencourt. Brazilian J. Pharmaceutical Sciences **49**, 13 (2013).
[13] Amit Kumar Nayak. International J. ChemTech Research, **2**(2), 903 (2010).
[14] Mehdi Sadat-Shojai, Mohammad-Taghi Khorasani, Ehsan Dinpanah-Khoshdargi, Ahmad Jamshidi, Acta Biomaterialia **9**, 7591 (2013).
[15] Syed Sibte Asghar; Qasim Murtaza; U.P.B. Sci. Bull, 75 (3) (2013).
[16] Hadeel Alobeedallaha, Jeffrey L. Ellis b, Ramin Rohanizadehc, Hans Costera, Fariba Dehghania, Trends Biomater. Artif. Organs **25**(1), 12 (2011).
[17] Elayaraja Kolanthai, V. Sivaraj Dikeshwar Colon, P. Abinaya Sindu, V. Sarath Chandra. RSC Adv **5**, 18301 (2015).
[18] Xiaohua Liu, Peter X. Ma. Annals of Biomedical Engineering **32**(3), 477 (2004).
[19] Nora T. Khanarian, Nora M. Haney, Rachel A. Burga, Helen H. Lu. Biomaterials, **33**(21), 5247 (2012).
[20] Yoshika Suzawa, Norihiko Kubo, Soichi Iwai, Yoshiaki Yura, Hajime Ohgushi, Mitsuru Akashi, Int. J. Mol. Sci. **6**(6), 14245 (2015).
[21] S. Sanchez-Salcedo, A. Nieto, M. Vallet, J. Chemical Engineering, **137**, 62 (2008).
[22] Thomas Weigel, Gregor Schinkel, Andreas Lendlein, Expert Rev. Med. Devices, 835-85 2006).
[23] Caibao Xue, Yingzhi Chen, Yongzhuo Huang, Peizhi Zhu, Nanoscale Research Letters **10**, 316 (2015).
[24] C.S. Ciobanu, S.L. Iconaru, I. Pasuk, B.S. Vasile, A.R. Lupu, A. Hermenean, A. Dinischiotu, D. Predoi, Materials Science and Engineering **33**, 1395 (2013).
[25] Combes, C. Rey. Acta Biomaterialia, **6**(9), 3362 (2010).