Defence Science Journal, Vol. 66, No. 4, July 2016, pp. 434-438, DOI : 10.14429/dsj.66.10218

RESEARCH ARTICLE

Bioactive Hybrid Composite Membrane with Enhanced Antimicrobial Properties for Biomedical Applications

Abin Davis[#] and Balasubramanian K.[*]

[#]*Centre for Biopolymer science and Technology, a unit CIPET, Cochin - 680 102, India*
[*]*Departmentof Materials Engineering, DIAT (DU), Pune - 411 025, India*
[*]*E-mail: meetkbs@gmail.com*

ABSTRACT

Azadirachta indica extract has been explored as an antibiotic in hygienic chitosan matrix system to enhance antimicrobial and medicinal property in a cost effective manner. The hygienic composite system has been successfully fabricated via solvent casting. The antibacterial activities of the hybrid system were examined by agar diffusion method against gram positive *S.aureus* and gram negative *K. pneumoniae.* From conventional antibacterial test for 24 h, the system exhibited an excellent antimicrobial activity against both bacterial strains in ranges of 1.2 cm - 1.5 cm for *S. aureus* and 1.8 cm - 2.3 cm for *K. pneumoniae* Fourier transform infrared spectroscopy revealed successfully embedded A. indica on the chitosan substrate via weak electrostatic interaction, resulting in the easy release of the additive. Moreover, atomic force microscopy showed a membrane roughness of 0.084 nm which confirms the uniform distribution of the additive throughout the membrane. These hybrid membranes have potential applications in skin tissue engineering, wound healing and as coatings for implantable scaffold material.

Keywords: *A. indica*, chitosan, antibacterial, wound healing

1. INTRODUCTION

The lush flora bestowed around us by the nature, is invariably valuable beyond measure on account of the medicinal value they posses. Over the period of time the medical arenas are witnessing a gradual transition from dependence on the chemical drugs to natural compositions as they prove to be effective and devoid of side effects[1,2]. A series of research, has been directed towards the formulation of such functional biomaterial, setting a stage to the biomedical revolution.

Chitosan is a cationic aminopolysaccharide of randomly distributed β-linked D-glucosamine and N-acetyl-D-glucosamine (NAG), derived from the chitin by means of deacetylation[3,4]. The commercial and laboratory chitosan products are not completely deacetylated and tend to be copolymers of N-acetyl glucosamine (NAG) and N-glucosamine[3-5]. The ratio of these two repeating units depends upon the source and method of preparation of chitosan but glucosamine unit predominates[6,7]. The cationic nature of the chitosan enhances the activity of inflammatory cells such as poly-morphonuclear leukocytes, macrophages and fibroblast without any toxic nature, thereby accelerating epithelisation and regeneration of normal skin[8].

The protonated amine cluster in chitosan exerts the pronounced antimicrobial nature to the system, which gets bound to the bacterial membrane and disrupts the membrane permeability resulting in protein leaching which causes cell death[9]. Additionally, chitosan mediated chelation of metal ions such as Ni, Zn, Cu, Fe, Mg necessary for the pathogen growth has also been suggested as a mode of antimicrobial activity[10]. Based on the facts, such as biodegradability, non-cytotoxicity, healing art and antimicrobial nature makes the chitosan an illustrious nominee for pharmaceutical and biomedical application[11]. The activities exerted by the bioactive chitosan can be modified by the incorporation of the other active ingredients likes therapeutical herbal species from the nature[12].

Azadirachta indica (neem), which belongs to the family of Meliaceae, is a therapeutical herbal species used in Ayurveda and homoeopathic systems of medicine, by virtue of its antioxidant, antimutagenic, anti-inflammatory, antidiabetic and contraceptive properties[13,14]. *A. indica* contains a numerous active ingredients, among them the active components are azadirachtin, nimbin, nimbidin which exerts the admirable properties to the herbal species[15,16]. Generally, these compounds have higher molecular-mass with significant amount of oxygen resulting in complex structure. These oxygen active centres in the pharmacophore operate as a free radical scavenger that inhibits the oxidation reaction in the cell[16-18].

The present work is based on the formulation of a hybrid system through the combination of two bioactive elements into a single matrix and evaluating the medicinal characteristics of the adduct. In this research, the bioactive elements viz. *A. indica* and hygienic chitosan have been united to cultivate a hybrid system with enhanced antimicrobial properties. As the germicidal chitosan degrades, the *A. indica* diffused out and enhancing the antimicrobial properties drastically. The *A. indica* extracts merged with a bioactive chitosan matrix

Received : 08 May 2016, Revised : 17 May 2016
Accepted : 02 June 2016, Online published : 28 June 2016

by means of solvent casting technique making the fabrication more cost effective and simpler. The antibacterial behavior of the chitosan embedded with *A. indica* is investigated by in vitro antimicrobial test against gram negative (*K. pneumoniae*) and gram positive bacteria (*S. aureus*). The topographical features of the hybrid system was determined by the atomic force microscopy (AFM) and the interaction between chitosan and *A. indica* has been studied by fourier transform infrared spectroscopy (FTIR).

2. EXPERIMENTAL SECTION

2.1 Materials

Chitosan (CS) of molecular-weight 100000-150000 Da), Formic acid, Ethanol n-Hexane were purchased from Sigma Aldrich Co Ltd (United States of America). The Neem seeds were collected from the Vanashree Agriculture Pvt. Ltd, Pune. Bacterial strains of *Staphylococcus aureus* (MTCC-96) and *Klebshiella pneumoniae* (NCIM-5432) were secluded from a infectious trauma (Department of Chemistry (Biochemistry), Pune). The mouse embryonic fibroblasts cell line (NIH=3T3) was obtained from National Centre for Cell Science (NCCS), Pune.The complete laboratory work has been carried out with deionised-water obtained by Millipore Mill-Q system.

2.2 Preparation of the *Azadirachta indica* Extracts

The neem seeds were collected, among them fresh seeds are apprehended for the oil extraction. Initially, seeds were decortified and rinsed with deionised water, dried (50 ^{0}C) in seed dryer till it achieved constant moisture content, followed by crushing and grinding using pulveriser made into powdered form. About 200 gm of the powdered seeds were extracted by means of a bisolvent mixture of hexane and ethanol in the ratio 1:5 in an overhead stirrer. Centrifuging and filtration polished off the solid particles from the extracts which further get concentrated by employing vacuum evaporator[19].

2.3 Preparation of the Chitosan and *Azadirachta indica* Membrane

Chitosan/*A. indica* thin membranes were fabricated by solvent casting method. Briefly, 2wt% chitosan solution was prepared by dissolving chitosan in 0.2 M formic acid and stirred for 90 min at room temperature using a magnetic stirrer. To the mixture of chitosan, 2 ml of *A. indica* extract was added

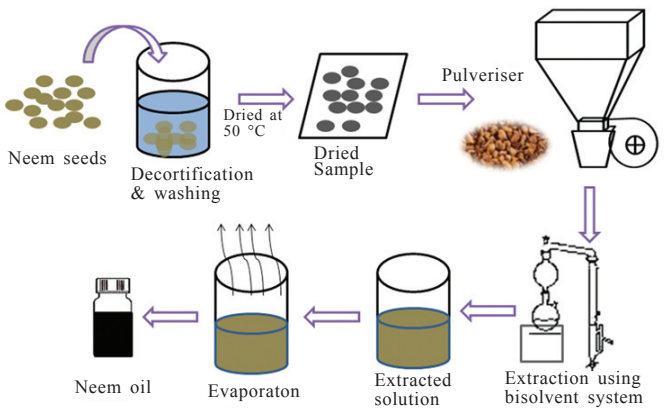


Figure 1. Extraction of the neem oil.

(more than 2 ml resulting in aggregates formation) and the solution was stirred again for 90 min to obtain a homogenous viscous mixture, which was then casted in a glass petridish and vacuum dried for 12 h at temperature of 37 ^{o}C and stored in a vacuum desiccators[20].

2.4 Characterisation

Antimicrobial activity was significantly evaluated against gram negative and gram positive bacterial strains of *S. aureus* and *K. pneumoniae* respectively, by disc diffusion method. Single colony of each bacterium was selected and cultured in a MH agar plates loaded with 15 mm of the sample and incubated at 37 °C for 24 h. After the incubation period, the zone of inhibition was measured by means of optical microscopy[21]. Surface morphology of the adduct system was imaged and studied using atomic force microscopy (AFM, Asylum research, UK) and ingredient's interaction was confirmed by fourier transform infra red spectroscopy (FTIR, Perkin-Elmer, USA).

3. RESULT AND DISCUSSION

3.1 Microstructure of Chitosan/ *Azadirachta indica* Film

The surface morphology of the system was investigated by means of the high resolution scanning probe microscopy test viz. atomic force microscopy. The results revealed chitosan *A. indica* film illustrating distinct surface morphology as observed from AFM micrographs. Fig. 2 shows the membrane surface as seen by the AFM ,clearly indicating the porous surface of the hybrid membrane, and confirms that the system possesses bioadhesive nature for tissue engineering[22]. The Fig. 2(a) indicates the 2D micrograph of the membrane which plainly indicates the porous morphology of the chitosan/*A. indica* membrane, also confirming the homogenous distribution of the additive in the system. The Fig. 2 (b) shows the 3D view of membrane, depicts a cluster of hollow peaks, with varying height, explains the characteristic membrane roughness of 0.084 nm, computed by SPIP 6.0.12 software. The hollow peak structure reflects the incorporation of additive called *A. indica's* advancing the rough surface of the membrane. The cluster of the hollow peak will resulting in the surface roughness will slightly improves the hydrophobic nature of hybrid substrate[22]. The higher roughness enhances the antibacterial nature of the hybrid system, which means rough surface provide a viable surface for additive to form a bond with the negative charged bacterial cell wall resulting in the inhibition of the microbial growth[23]. Moreover, the porosity and hydrophilicity of the of hybrid membrane can aid to the easy release of the active ingredients of the *A. indica* which exerts the antimicrobial and healing properties to the lesion site.

3.2 Compositional Analysis

The spectrum (Fig. 3(c)) characterizes the functional group and structural changes in the chitosan matrix by the insertion of the *A. indica* additive and it substantiates the evolution of the hybrid composite system. Pure Chitosan constitutes numerous -(NH) and -(OH) groups in their structure, which exhibits a broad peak at 3750 cm^{-1} - 3000 cm^{-1} attributed to the

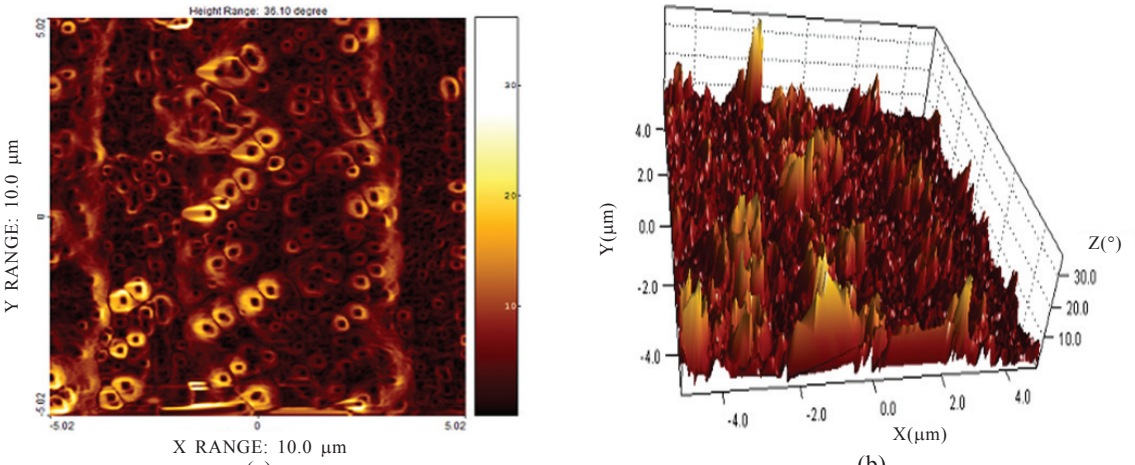


Figure 2. AFM images of membrane (a) 2d image (b) 3d image.

stretching vibration of –(NH_2) and –(OH) as well as inter and intra molecular hydrogen bonding. This is the characteristic band for chitosan which overlaps the stretching vibrations of -(NH), -(CH) bond in -(CH_2) and -(CH_3) groups[25]. Additionally, appearance of 1560 cm^{-1} peak corresponds to the bending vibration of primary amine groups in chitosan (–NH_2), whereas the absorption in the range of 1694 cm^{-1} corresponds to the -(C=O) stretching. In Fig. 3(b) shows the FT-IR spectrum of the *A. indica* depicted as a series of characteristics absorption bands at 1642 cm^{-1}, 1449 cm^{-1}, 1084 cm^{-1} representing the stretching of the -(C=O) group, deformation of the asymmetric -(CH_2) and vibrational stretching of the -(C-C) groups, respectively. The other notable peaks were at 3326cm^{-1} reflecting the stretching of the -(OH) group and at 2978cm^{-1} illustrating -(CH_2) stretching[26-27]. FT-IR spectrum of hybrid chitosan system indicates that characteristic peak for chitosan/*A. indica* film appears between 3500 cm^{-1} - 3000 cm^{-1} and 1500 cm^{-1} - 500 cm^{-1}. However, weak electrostatic interaction between chitosan -(NH_3) and negatively charge component of *A. indica* extract can be predicted from slight shift in the band corresponding to -(NH_3) (1587 cm^{-1})[28]. Peak broadening and shift in the band attributed to -(OH) and overlapping -(NH) explains the hydrogen bonding between chitosan and *A. indica* extract. It is evident from the absence of the additional peak that the additive *A. indica* is embedded on the chitosan matrix only by means of physical interaction, which aids the easy release of drug from the matrix and makes it a better system with antimicrobial and medicinal properties.

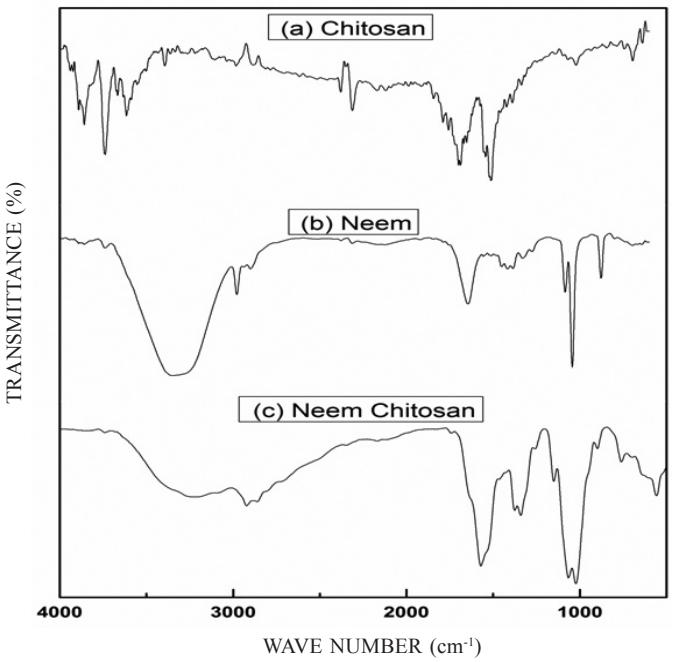
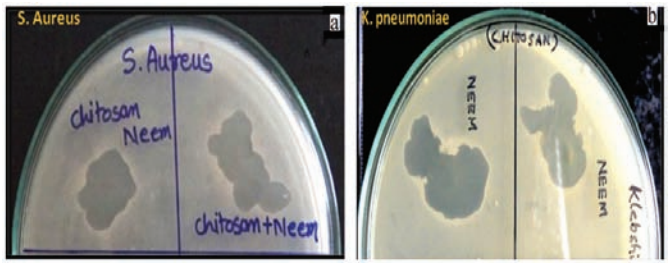


Figure 3. FT-IR spectrum of the hybrid system.

3.3 Antibacterial Efficiency

The novel chitosan hybrid membranes were tested on their antibacterial competence against two bacteria where one is Gram positive bacteria of *S. aureus* and Gram negative bacteria of *K. pneumonia*. Antibacterial activity was measured in terms of diameter of zone of inhibition created around chitosan/*A. indica* disc and the obtained antibacterial effects of the film are presented Fig. 4. The enriched antibacterial efficiency has been demonstrated by the obtained uneven zone of inhibition in a range of 1.2 cm - 1.5 cm for *K. pneumoniae* and 1.8 cm - 2.3 cm for *S. Aureus*. Ram[29], *et al.* has demonstrated antibacterial properties of PVA hybrid membrane with *A. indica* with an even zone of inhibition range, in our system an uneven zone due to the complete oozing out of the additive occurs. The bacterial action of the chitosan is based on the interaction of the protonated amine group with the negative elements in the bacterial cells, disrupts the membrane permeability culminating in bacterial death. While incorporating the *A. indica* extract in the germicidal chitosan substrate, the antibacterial efficiency



(a) (b)

Figure 4. Antibacterial efficiency of hybrid membrane against (a) *S. aureus* and (b) *K. pneumoniae*

gets pronounced by the action of the azadirachtin, nimbidin and nimbin that are effective components of the *A. indica* which detecting the uneven zone of inhibition in the agar medium after a period of 24 h. The hydrophilic and porous nature of the chitosan polymer results in degradation of matrix over the period of time enhancing the easy leaching of the herbal extract and leading to the formation of uneven zone of inhibition. The antibacterial activity of the crossbreed chitosan membrane was more efficient in bacterial strain of *K. pneumoniae* than *S. aureus*. This effectiveness may due to the large negative charge density possessed by the bacterial surface of the gram negative bacteria when compared with gram positive bacteria, so it favours the formation of the complex with reactive sites of the chitosan hybrid system[30]. A significant antibacterial results were obtained by the novel hybrid system against both the species which is relevant for its medicinal applications.

4. CONCLUSIONS

A novel chitosan hybrid membrane with therapeutic *A. indica* extracts nanoparticles, was successively amalgamated by solvent casting method and studied for potential applications as a biomaterial system. *A. indica* modified chitosan membrane has been put forward as a substitute for existing membranes with chemical drug. The additive is embedded in the germicidal chitosan network by means of weak electrostatic interaction, confirmed by fourier transform infrared spectroscopy which results in the easy release of herbal extracts. The AFM micrograph of the hybrid membrane indicates structure with porous nature and rough surface of value of 0.084 nm, also embellishing the distribution of the extract. The porous and rough surface favours the tissue adhesion, thereby providing a viable environment for tissue regeneration and healing nature. The antibacterial assessment of membrane has been carried out by disc diffusion method which results in a uneven zone of inhibition with a ranges of 1.2 cm - 1.5 cm against *S.aureus* and 1.8 cm - 2.3 cm against *K. pneumoniae*. It can be concluded that chitosan/*A. indica* membrane, a blend of two natural therapeutic components with excellent antimicrobial have a promising medical applications in skin tissue engineering as scaffolds or as coatings, wound healing patches etc.

REFERENCES

1. Sha, B.; Gao, W.; Wang, S.; Xu, F.; Lu, T. Cytotoxicity of titanium dioxide nanoparticles differs in four liver cells from human and rat. *Composites: Part B.* 2011, **42**, 2136-2144. doi: 10.1016/j.compositesb.2011.05.009
2. Nowack, B. & Bucheli, T.D. Occurrence, behavior and effects of nanoparticles in the environment. *Environmental Pollution.* 2007, **150**, 5-22. doi: 10.1016/j.envpol.2007.06.006
3. Aider, M. Chitosan application for active bio-based films production and potential in the food industry: Review. *LWT - Food Sci. Technol.,* 2012, **43**(6), 837-842.
4. Elsabee, M.Z. & Abdou, E.S. Chitosan based edible films and coatings: a review. *Mater. Sci. Eng. C. Mater. Biol. Appl.,* 2013, **33**(4), 1819-1841. doi: 10.1016/j.msec.2013.01.010
5. Bulwan, M.; Antosiak-Iwańska, M.; Godlewska, E.; Granicka, L.; Zapotoczny, S. & Nowakowska, M. Chitosan-based nanocoatings for hypothermic storage of living cells. *Macromol Biosci,* 2013, **13**(11), 1610-1620. doi: 10.1002/mabi.201300258
6. Majeti, N.V. & Kumar, R. A review of chitin and chitosan applications. *Reactive Functional Pol.,* 2000, **46**, 1-27. doi: 10.1016/S1381-5148(00)00038-9
7. Croisier, F. & Jerome, C. Chitosan-based biomaterials for tissue engineering. *European Pol. J.,* 2013, **49**(4), 780-792. doi: 10.1016/j.eurpolymj.2012.12.009
8. Dai, T.; Tanaka, M.; Huang, Y. & Hamblin, M. R. Chitosan preparations for wounds and burns: antimicrobial and wound-healing effects. *Expert Rev. Anti Infect. Ther. (NIH),* 2011, **9**(7), 857-879. doi: 10.1586/eri.11.59
9. Hafdani, F.N. & Sadeghinia, N. A Review on Application of Chitosan as a Natural Antimicrobial. *World Acad. Sci. Engineering Technol.,* 2011, **5**, 225-229.
10. Kong, M.; Chen, X.G.; Xing, K. & Park, H. J. Antimicrobial properties of chitosan and mode of action: a state of the art review. *Int. J. Food Microbio*., 2010, **144**, 51-63. doi: 10.1016/j.ijfoodmicro.2010.09.012
11. Sarasam, A.R.; Krishnaswamy, R.K. & Madihally, S.V. Blending Chitosan with Polycaprolactone: Effects on Physicochemical and Antibacterial Properties. *Biomacromolecules.,* 2007, **7**, 1131-1138. doi: 10.1021/bm050935d
12. Wang, L.; Liu, F.; Jiang, Y.; Chai, Z.; Li, P.; Cheng, Y.; Jing, H. & Leng, X. Synergistic antimicrobial activities of natural essential oils with chitosan films. *J. Agric. Food Chem*., 2011, **59**, 12411–12419. doi: 10.1021/jf203165k
13. Subapriya, R.; Velmurugan, B. & Nagini, S. Modulation of xenobiotic-metabolizing enzymes by ethanolic neem leaf extract during hamster buccal pouch carcinogenesis. *J. Exp. Clin. Cancer Res*., 2005, **24**(2), 223-230.
14. Bharati , S.; Rishi, P. & Koul, A. Azadirachta indica Modulates electrical properties and type of cell death in NDEA-induced hepatic tumors. *Cell Biochem Biophys.,* 2014. doi: 10.1007/s12013-014-9923-6
15. Sidhu, O.P.; Kumar, V. & Behl, H.M. Variability in triterpenoids (nimbin and salanin) composition of neem among different provenances of India. *Ind. Crops Prod.,* 2004, **19**, 69-75. doi: 10.1016/j.indcrop.2003.07.002
16. Valek, L. & Martinez, S. Copper corrosion inhibition by Azadirachta indica leaves extract in 0.5 M sulphuric acid. *Mater. Lett*., 2007, **61**, 148-151. doi: 10.1016/j.matlet.2006.04.024
17. Martinez, S. & Štern, I. Inhibitory mechanism of low-carbon steel corrosion by mimosa tannin in sulphuric acid solutions. *J. Appl. Electrochem*., 2001, **31**(9), 973-978. doi: 10.1023/A:1017989510605
18. Saleh, R.M.; Ismail, A.A. & El Hosary, A.A. Corrosion inhibition by naturally occurring substances. vii. the effect

of aqueous extracts of some leaves and fruit peels on the corrosion of steel, Al, Zn and Cu in acids. *Br. Corros. J.*, 1982, **17**, 131-135. doi: 10.1179/000705982798274345

19. Manikandan, P.; Letchoumy, P. V.; Gopalakrishnan, M. & Nagini, S. Evaluation of Azadirachta indica leaf fractions for in vitro antioxidant potential and in vivo modulation of biomarkers of chemoprevention in the hamster buccal pouch carcinogenesis model. *Food Chem. Toxicol.*, 2008, **46**, 2332-2343. doi: 10.1016/j.fct.2008.03.013
20. Balasubramanian, K. & Yadav, R. Polymer membrane and process for preparing the same. Indian patent [601/MUM/2013].
21. Pant, H.R.; Bajgai, M.P.; Namd, K.T.; Seo, Y.A.; Pandeya, D.R.; Hong, S.T. & Kim, H.Y. Electrospun nylon-6 spider-net like nanofiber mat containing TiO_2 nanoparticles: A multifunctional nanocomposite textile material. *J. Hazard. Mater*., 2010, **185**, 124-130. doi: 10.1016/j.jhazmat.2010.09.006
22. Sahoo, B. N.; Kandasubramanian, B.& Sabarish, B. Controlled anisotropic wetting behaviour of multi-scale slippery surface structure of non fluoropolymer composite. *Express Polym. Lett*., 2013, **7**, 900-909. doi: 10.3144/expresspolymlett.2013.88
23. A. Hadjizadeh and D. Mohebbi-Kalhori, Porous hollow membrane sheet for tissue engineering applications. *J. Biomed. Mater. Res. A.*, 2010, **93**, 1140-1150.
24. Ramdayal & Balasubramanian, K. Antibacterial application of polyvinylalcohol-nanogold composite membranes. *Colloids and Surfaces A: Physicochem. Eng. Aspects.*, 2014, **455**, 174-178. doi: 10.1016/j.colsurfa.2014.04.050
25. Liang, S.; Liu, L.; Huang, Q. & Yam, K.L. Preparation of single or double-network chitosan/poly(vinyl alcohol) gel films through selectively cross-linking method. *Carbohydr. Polym.*, 2009, **77**, 718-724. doi: 10.1016/j.carbpol.2009.02.007
26. Devi, N. & Maji, T.K. Study of complex coacervation of gelatin a with sodium carboxymethyl cellulose: Microencapsulation of Neem (Azadirachta indica A. Juss.) Seed Oil (NSO). *Int. J. Polymer. Mater.*, 2011, **60**, 1091-1105. doi: 10.1080/00914037.2011.553851
27. Sundrarajan, M.; Selvam, S. & Ramanujam, K. Synthesis of sulfated b-Cyclodextrin/Cotton/ZnO Nano composite for improve the antibacterial activity and dyeability with Azadirachta Indica. *J. Appl. Polym. Sci*., 2013, **128**, 108-114. doi: 10.1002/app.38127
28. Premika, G.; Balasubramanian, K. & Kisan, M.K. Molecular interactions and antimicrobial activity of Curcumin 1 (*Curcuma longa*) loaded polyacrylonitrile films. *Mater. Chem. Phy.*. doi: 10.1016/j.matchemphys.2014.06.040
29. Yadav, R. & Kandasubramanian, B. Egg albumin PVA hybrid membranes for antibacterial application. *Mater. Lett*., 2013, **110**, 130-133. doi: 10.1016/j.matlet.2013.07.109
30. McCoy, C.P.; Craig, R.A.; McGlinchey, S.M.; Carson, L.; Jones, D.S. & Gorman, S.P. Surface localisation of photosensitisers on intraocular lens biomaterials for prevention of infectious endophthalmitis and retinal protection. *Biomaterials.*, 2012 **33**, 7952-7958. doi: 10.1016/j.biomaterials.2012.07.052

ACKNOWLEDGEMENTS

The Authors would like to thank Dr Prahlada, former Vice Chancellor, DIAT-DU for contiuous support and encouragement. We are also grateful to Ram Dayal, Madhura Hudlikar, Kisan M Kodam (University of Pune), Dr T.O. Varghese (CBPST, Kochi) and Nitesh Singh for technical support.

CONTRIBUTORS

Mr Abin Davis received his BSc (Chemistry) from Mahatma Gandhi University, Kottayam, Kerala and MSc (Biopolymer Science) from Centre for Biopolymer Science and Technology, Kochi. Currently working as Assistant Technical Officer at Central Institute of Plastics Engineering and Technology, Kochi. His current research interests includes: Biopolymers, biomaterials and polymers for biomedical applications.

Prof Balasubramanian K. is Dean, Professor and Head of Department of Materials Engineering at Defence Institute of Advance Technology (DU), India since 2010. Received the most prestigious *Hind Rattan Award*, 2010 for outstanding achievements and contribution internationally in the areas of Science and Technology, Ministry of Heavy Industries and Public Enterprises, Government of India. Having rich experience in evaluation of DTI (UK), TSB (UK), FP6 and FP 7 European research programmes, DST, Brussels, Belgium. He was awarded the *Best Researcher of the Year*, 2014 by the Ministry of Defence, Government of India, for outstanding contribution and development of "Novel Hybrid Ablative Composite as Thermal; Protection Systems for Re-entry Vehicles".