Research Article **Nanotechnolagy**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

ANTIBACTERIAL ACTIVITY OF ANTHRAQUINONE ENCAPSULATED CHITOSAN/POLY(LACTIC ACID) NANOPARTICLES

JEEVITHA D[*1], MALATHY BR[2], PRADEEP PS[3] AND SRIVIDYA S[4]

[1]Faculty of Science and Humanities (Life Sciences), [3]Faculty of Bioengineering (Life Sciences), Department of Biotechnology, Sathyabama University, Rajiv Gandhi Salai, Sholinganallur, Chennai – 600119, Tamilnadu, INDIA.
[2]Department of Microbiology, [4]Department of Biochemistry, Sathyabama University Dental College and Hospitals, Rajiv Gandhi Salai, Sholinganallur, Chennai – 600119, Tamilnadu, INDIA

ABSTRACT

The aim of this research is to evaluate antibacterial activities against multi drug resistance pathogens with the prepared anthraquinone (AQ) coated polymeric nanoparticles, chitosan (CS) and poly(lactic acid)(PLA) (CS/PLA-AQ NPs). The preliminary and cold stability study was determined for its drug loading capacity, encapsulation efficiency and *in vitro* drug release behaviour using UV spectrophotometer. The formulation (CS/PLA-AQ NPs) was tested for its antibacterial effect by disc diffusion method and Minimum bactericidal Concentration (MBC) on Gram negative bacteria's (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Proteus vulgaris*) which was found to have higher activity when compared to CS-PLA NPs and bare AQ. The results specified that the nanoparticles formulation is a possible applicant to be considered as an antimicrobial agent *in vitro*.

KEYWORDS: Antibacterial, Anthraquinone, Chitosan, MBC, Poly(lactic acid), Stability.

JEEVITHA D
Faculty of Science and Humanities (Life Sciences), Department of Biotechnology, Sathyabama University, Rajiv Gandhi salai, Sholinganallur, Chennai – 600119, Tamilnadu, INDIA.

***Corresponding author**

INTRODUCTION

Natural products have been rich causes of substances that have established numerous applications in the field of medicine. The advancement of medical machinery, expansion in antibiotics has been significantly enhanced to covenant with the growing drug resistance pathogens. Though, a monocomponent antibacterial agent doesn't meet the requirements for several unique circumstances. Consequently, it is compulsory to discover antibacterial agents to resolve this crisis [1-6]. Controlled drug delivery systems tenders several recompense over conventional dosages, together with less toxicity, better efficacy, and enhanced patient agreement, and can be used in the type of nanocarriers in drug delivery[7]. In microbiology, several plant-derived complexes have been recorded, including alkaloids, flavonoids, tannins, quinones, essential oils and other derivative metabolites for delivering antibiotics in the form of nanocarriers[8]. Among these compounds, anthraquinone derivatives have provoked unique concern because they have established prospective therapeutic uses as antiviral, antibacterial, antifungal agents and other biological activities[8-12]. Anthraquinones belongs to the class of tricyclic compounds produced by higher plants and fungi, present in human foods[13]. Both synthetic and naturally occurring AQ have been extensively used as colorants in foods, cosmetics, hair dyes, textiles and as laxative, sedatives and antimicrobics drugs[13,14]. Over the decades, the polymers which are biodegradable and biocompatible have shown a significant interest from both biological and biomedical perspectives[15]. Aliphatic polyesters are majority in biodegradable polymers such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(e-caprolactone) (PCL) and poly(3- hydroxybutyrate) (PHB), in which PLA were considered the most. Poly(lactic acid)(PLA) is the most extensively researched and an eco-friendly with excellent biocompatibility, processibility, and less energy reliance with feasible to replace conservative petrochemical-based polymers. Henceforth PLA is extensively used in medical application such as surgical implants[16], tissue culture[17], resorbable sutures[18], wound closure, and controlled release systems[19-22]. Apart from its beneficial features, PLA has drawbacks as well, which limit its use in certain applications due to its crystalline structure and hydrophobic nature. Hence, in this research we have combined the hydrophobic PLA with hydrophilic CS for the strong amino and hydroxyl bonding. Among the hydrophilic polymeric carriers used, chitosan has paying attention due to its unique properties such as biocompatibility, biodegradability[23,24] and the capacity to improve the paracellular transport of drugs[25].Chitosan, a class of nature polysaccharide, having structural characteristics comparable to glycosaminoglycans, and are non-toxic[26], which has delivered it widely, applicable in the field of biomedical and pharmaceutical[27-29]. An earlier report states the loading and sustainable release of protein using hydrophilic chitosan–polyethylene oxide nanoparticles as a carrier[30]. In this paper, we are illustrating the synthesis of anthraquinone coated CS-PLA NPs, preliminary analysis and cold stability of synthesized nanoparticles for drug loading, encapsulation efficiency and *in vitro* drug release and further, NPs is employed to evaluate the antibacterial effect on Gram negative multi drug resistant organisms.

MATERIALS AND METHODS

Chitosan Hydrochloride (83% deactylated), Poly (Lactic Acid) (Mw = 60 kDa) was purchased from Sigma Corporation. Anthraquinone was purchased from Fluka. Multi drug resistant *E. coli, P. aeruginosa, K. pneumoniae, P. vulgaris* strains were obtained from Sathyabama Dental College and General Hospitals, Chennai. Obtained cultures were sub-cultured in a nutrient agar slant. All other chemicals used were of analytical reagent grade and without further purification

1. *Preparation of Chitosan/PLA nanoparticles by dropping method*

CS–PLA nanoparticles were prepared as we discussed earlier in our report by mixing positively charged CS and negatively charged PLA by dropping method. Briefly, 1 ml of 0.02% CS solution (0.02 g of Chitosan hydrochloride in 100 ml of distilled water) was added dropwise into 5 ml 0.02% PLA aqueous solution, stirred under magnetic stirring, until an opalescent suspension was formed. The obtained suspension was then filtered by paper filter, the filtered suspension was incubated in a buffer solution of pH = 4.5 for 24 h using a dialysis membrane bag for characterization[31].

2. *Preparation of AQ loaded CS–PLA nanoparticles*.

The drug loaded nanoparticles were prepared by dissolving 50 mg of anthraquinone in 50 ml of CS–PLA solution prepared by dropping method and incubated for 48 h. Then, these nanoparticles were separated from the aqueous phase by ultracentrifugation with 50,000 rpm at 41 ◦C for 40 min. Then the obtained AQ loaded CS/PLA nanoparticles were washed by acetone 3 times, frozen by liquid nitrogen and lyophilized by freeze dryer to obtain dried AQ loaded CS/PLA nanoparticles[31]. The nanoparticles was analysed for their morphology, particle size, chemical interaction, preliminary drug loading and encapsulation efficiency which was reported in our previous study[31]. Additionally, in this study we have performed cold temperature stability studies with the obtained nanoparticles.

3. *In vitro* drug release

In vitro drug release was performed in phosphate buffered saline at pH 7 with slight modification in the protocol[32]. 5mg of drug loaded polymeric nanoparticles was suspended in 10 ml of PBS buffer at pH 7 and placed in a shaker for 72 hrs. At predetermined time intervals, medium was removed and replaced with the same amount of fresh saline which was monitored by UV spectrophotometer (Shimadzu) at 274 nm. The drug release can be determined by the following equation:*In vitro* drug release (%) = D(t) / D(0) x 100Where, D(0) is amount of drug loaded and D(t) is amount of drug released at a time, respectively.

4. *Stability study*

The stability of drug loaded nanoparticles was dogged in the terms of its drug content with slight modification in the protocol[33]. The firmness of nanoparticles was evaluated in PBS (pH-7). Prepared nanoparticles were incubated at 4° for a period of 2 months. After the particular time intervals, the suspension was centrifuged at 12,000 rpm for 1h, supernatant was removed and nanoparticles were dissolved in methane. Distilled water was added to about 5ml, the amount of drug was detected by UV spectrophotometer (Shimadzu) at 274nm for their drug loading, encapsulation and drug release in *in vitro.*

5. *Antibacterial activity by disc diffusion method*

The antibacterial activity of AQ encapsulated polymers was tested against the 24 h old cultures of *P. aeruginosa, K. pneumoniae, P. vulgaris and E. coli* was done by disc diffusion assay. The respective broth culture was uniformly spreaded with sterile cotton swabs on Mueller Hinton agar media plates. The sterile filter paper was made into 6mm discs and dipped with uncoated AQ, CS/PLA NPs and CS/PLA-AQ NPs of various concentrations (100-500µg/ml in PBS) was loaded into the wells. The plates were incubated at 37^{o}C for 24 h. The antibacterial activity was expressed as zone of inhibition in each concentration which was plotted as bar diagram.

6. *Confirmation of antibacterial effect with minimal bactericidal concentration of CS/PLA-AQ NPs*

To determine the MBC for CS/PLA-AQ NPs against *E. coli, P. aeruginosa, K. pneumoniae and P. vulgaris,* a fixed amount of isolates (1x10^{6} CFU in 100µl) was incubated with different concentrations of CS-PLA-AQ NPs (31.25–500µg/ml in PBS buffer) at 37°C for 5 hours in a 96 well plate. After incubation, the mixtures were diluted at 1:10 to 1:10^{5} with sterile PBS and 3µl of the diluted suspension was spotted on MHA agar plate for overnight incubation at 37°C. The isolates without nanosuspension were used as controls in the study. The lowest

concentration that killed bacterial growth was considered as MBC[34].

RESULTS AND DISCUSSION

Nanoparticles preparation allows cross-linking between the positively charged amine group of CS and negatively charged carboxyl groups of PLA and forms nanoparticles upon magnetic stirring. The size of polymeric nanoparticles is important because it has eternal application with the stability, cellular uptake, biodistribution and drug release of nanomedicines.

1. Drug loading and Encapsulation efficiency

Preliminary drug loading and encapsulation efficiencies were evaluated[31], showed maximum drug loading capacity of 23.8% and 91.4% encapsulation efficiency and minimum 2.8% and 5.6%. These efficiencies were also performed after the stability test for 60 days at 4^{o}C, which was shown very slight decrease in their maximum value, i.e. 21.72% and 89.38% respectively (Table 1). This might be due to shrinkage of particles or saturation of polymers when it's stored for a long time. The stability improvement of positively charged nanoparticles in the existence of natural cations and may be positive for various drugs payable to their interface with negatively charged biological membranes[35,36].

Table 1
Determination of drug and polymers

Formulations (mg) CS-PLA NPs	AQ	Preliminary Evaluation		Stability of NPs after 2 months	
		Drug Loading (%)	Encapsulation Efficiency (%)	Drug Loading (%)	Encapsulation Efficiency (%)
2	10	2.8	5.6	1.9	5.2
2	15	19.6	91.4	18.2	89.38
2	20	23.8	87.6	21.72	86.1

2. In vitro drug release study

Figure. 1 shows the drug release rate of the preliminary evaluation of drug loaded formulation and the stability evaluation of drug loaded formulation at pH7. Both the formulations gave almost similar controlled release. After 24h, preliminary drug loaded sample released 35.5% of the AQ from CS/PLA NPs (Figure 1a), whereas the stability showed the release of 33.2% of the drug (Figure 1b). After 48h, preliminary drug loaded sample released 72% (Figure 1a) of the AQ from CS/PLA NPs and stability drug release showed 71.34% (Figure 1b). After 72h the released concentration of drug attained around 93% and 92.1% in both the evaluations, respectively. A related outcome of continuous release was previously observed for anti-cancer drugs encapsulated PLGA NPs stabilized by PVA[37]. Furthermore, considering AQ as an acidic drug, its solubility can be enhanced significantly perhaps due to ionization into anions under the alkaline environment. Instead, the mechanisms of deliberated release may engage the CS-PLA NPs that had a firm and hydrophobic core[38].

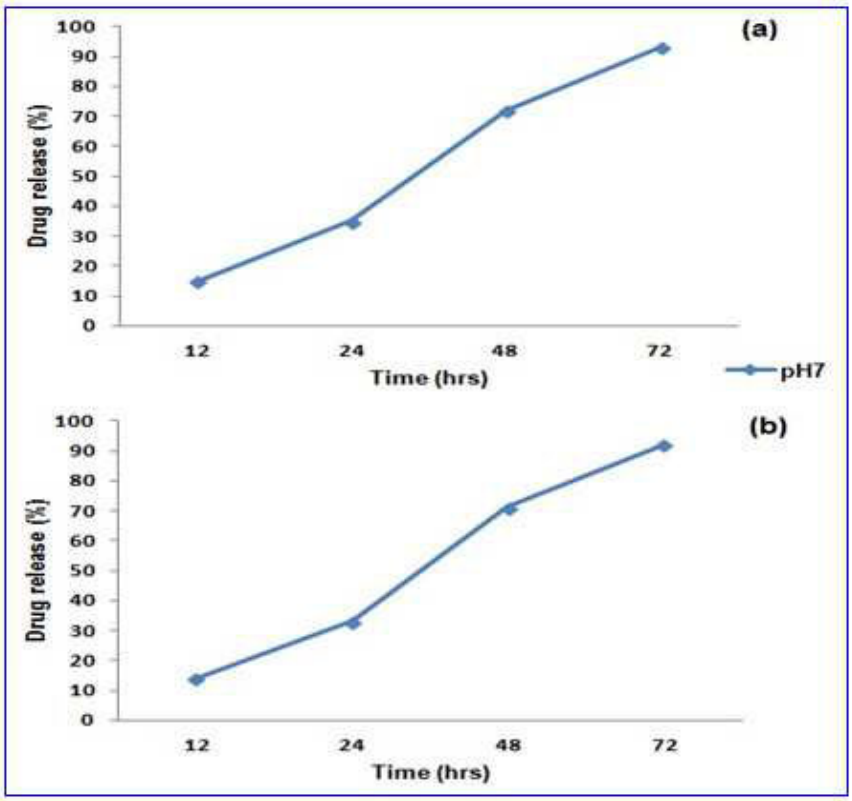

Figure 1

In vitro drug release behavior with respect to time intervals (a) release of AQ from CS/PLA NPs from preliminary evaluation (b) release of AQ from CS/PLA NPs after 2 months.

3. Antibacterial activity of CS/PLA-AQ NPs

CS/PLA-AQ NPs, CS-PLA NPs and bare AQ was tested against *E. coli* (Figure 2a), *K. pneumoniae* (Figure 2b), *P. aeruginosa* (Figure 2c), and *P. vulgaris* (Figure 2d). CS/PLA-AQ NPs showed the zone of inhibition from the concentration of 100µg/ml against *K. pneumoniae* and *P. vulgaris* whereas *E. coli* and *P. aeruginosa* in 300-500µg/ml. However, AQ showed the zone of inhibition in 300-500µg/ml in *E. coli, K. pneumoniae* and *P. vulgaris* whereas *P. aeruginosa* showed in 400 and 500µg/ml. CS-PLA NPs showed zone in 400 and 500µg/ml in *E. coli, K. pneumoniae* and *P. vulgaris* and 500µg/ml in *P. aeruginosa.* Due to the presence of lipopolysaccharide in the outer membrane of Gram-negative bacteria, by itself its acts as an endotoxin, thereby leads bacteria to undergoes a genetic change or acquires genetic material that confers resistance to most of the antibiotics. Hence, Gram negative is considered as resistant organisms compared to Gram positive[39].

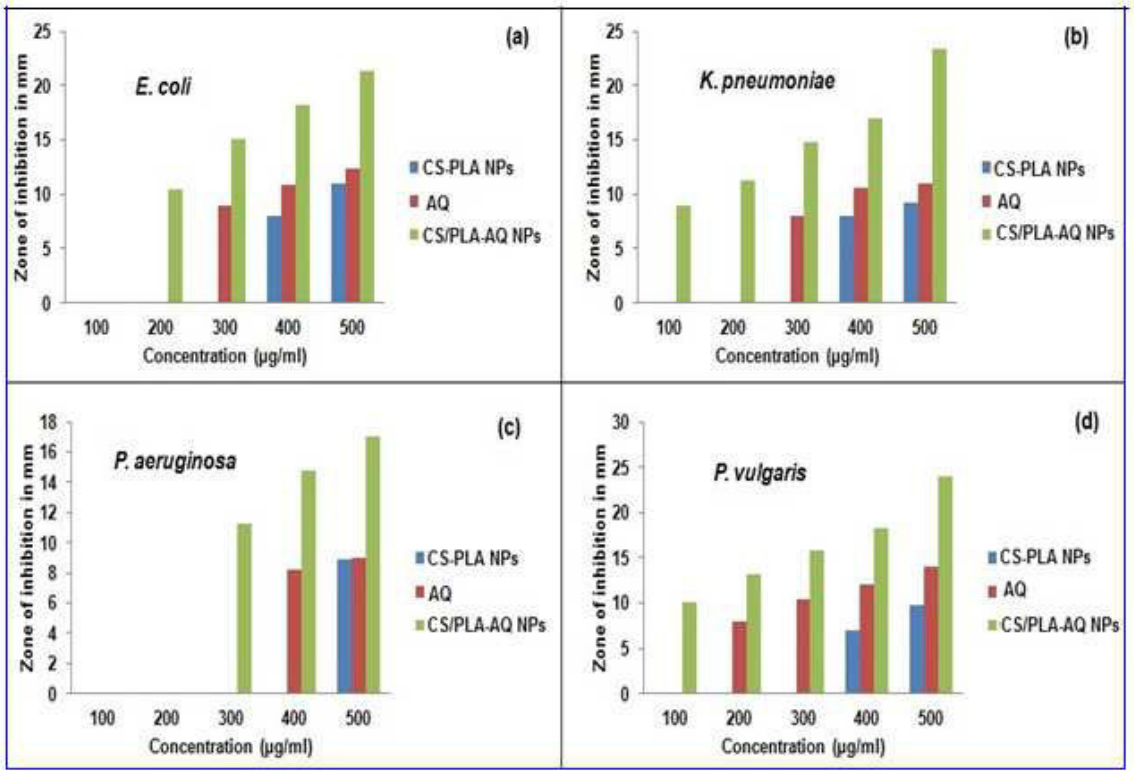

Figure 2

Antibacterial activity of CS/PLA-AQ NPs with the control of CS/PLA NPs and AQ against (a) Escherichia coli (b) Klebsiella pneumoniae (c) Pseudomonas aeruginosa (d) Proteus vulgaris

4. MBC determination of CS/PLA-AQ NPs

MBC was determined for CS/PLA-AQ NPs against *E. coli, K. pneumoniae, P. aeruginosa, and P. vulgaris*, respectively (Figure 3). CS/PLA-AQ NPs killed *E. coli* at 125 µg/ml, *K. pneumoniae* at 62.5µg/ml, *P. aeruginosa* at 250µg/ml and *P. vulgaris* at 62.5µg/ml. Reports say the efficacy of the vancomycin increases due to non-pegylated liposomes in sufficiently high concentrations (120-fold) inside the intracellular compartments of macrophages and showed increased bactericidal activity against methicillin resistant *S. aureus*[40].

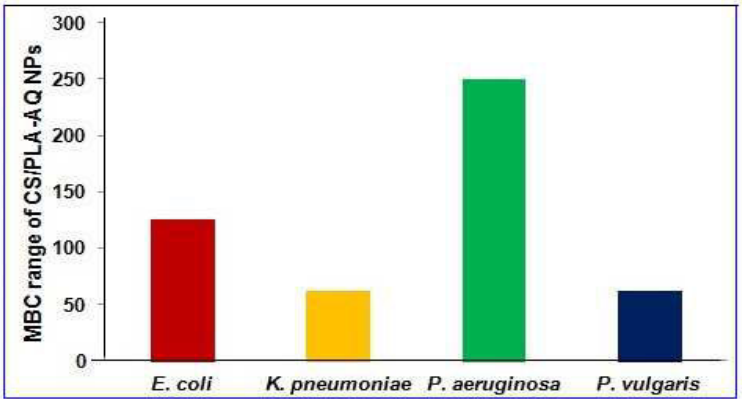

Figure 3

MBC determination of CS/PLA-AQ NPs ranged from 500-31.25 µg/ml

CONCLUSION

The present work confirms the stability of the anthraquinone loaded polymeric nanoparticles though it's stored at 4^{O}C. There was no immense change in drug loading, encapsulation efficiency and drug release *in vitro*. The CS/PLA-AQ NPs showed dominant antibacterial activity against multi drug resistant *E.coli, K. pneumoniae, P. aeruginosa* and *P. vulgaris* than CS-PLA NPs and bare AQ. *K. pneumoniae* and *P. vulgaris* showed the less MBC value compared to *E. coli and P. aeruginosa.* The use of this formulation as bactericidal agents needs supplementary evaluation with the group of organisms for the better use with humans.

Conflict of Interest

Conflict of interest declared none.

REFERENCES

1. Krajewska B, Application of chitin- and chitosan-based materials for enzyme immobilizations: A review. Enzyme Microb Technol, 35: 126–139, (2004).
2. Lei ZL and Bi SX, The silica-coated chitosan particle from a layer-by-layer approach for pectinase immobilization. Enzyme Microb Technol, 40: 1442–1447, (2007).
3. Liu H, Li H, Cheng WJ, Yang Y, Zhu MY and Zhou CR, Novel injectable calcium phosphate/chitosan composites for bone substitute materials. Acta Biomaterialia, 2: 557–565, (2006).
4. Petri DFS, Donega J, Benassi AM and Bocangel JAJS, Preliminary study on chitosan modified glass ionomer restoratives. Dental Materials, 23: 1004–1010, (2007).
5. Wu JM, Luan MM and Zhao JY, Trypsin immobilization by direct adsorption on metal ion chelated macroporous chitosan-silica gel beads. International Journal of Biological Macromolecules, 39: 185–191, (2006).
6. Xi FN and Wu JM, Preparation of macroporous chitosan layer coated on silica gel and its application to affinity chromatography for trypsin inhibitor purification. Reactive and Functional Polymers, 66: 682–688, (2006).
7. Majeti NV and Kumar R, Nano and microparticles as controlled drug delivery devices. J Pharm Sci, 3: 34-258, (2000).
8. Cowan MM, Plant Products as antimicrobial agents. Clin Microbiol Rev,12:564-582, (1999).
9. Wijnsma R and Verpoorte R, Anthraquinones in the Rubiaceae. In: Hill RA, Krebs HCh, Verpoorte R, Wijnsma R, Herz W, Grisebach H, Kirby GW, Tamm Ch, eds. Progress in the Chemistry of Organic Natural Products, Wien: Springer-Verlag, 49:79-149, (1986).
10. Rath G, Ndonzao M and Hostettmann K, Antifungal anthraquinones from Morinda lucida. Int. J. Pharmacognosy,33:107-114, (1995).
11. Ali AM, Ismail NH, Mackeen MM, Yazan LS, Mohamed SM, Ho ASH and Lajis NH, Antiviral, cytotoxic and antimicrobial activities of anthraquinones isolated from the roots of Morinda elliptica. Pharm Biol,38: 298-301 (2000).
12. Agarwal SK, Singh SS, Verma S and Kumar S, Antifungal activity of anthraquinone derivatives from Rheum emodi. J Ethnophamacol,72:43-46 (2000).
13. Brown JP, A review of the genetic effects of naturally occurring flavonoids, anthraquinones and related compounds, Mutat Res, 75: 243–277, (1980)
14. Sendelback LE, A review of the toxicity and carcinogenicity of anthraquinone derivatives, Toxicol, 57 227–240, (1989)
15. Ikada Y and Tsuji H, Biodegradable polyesters for medical and ecological Applications. Macromol Rapid Commun, 21:117-132, (2000).
16. Jain RA, The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. Biomater,21:2475-2490, (2000).
17. Mikos AG anLyman MD, Freed LE, Langer R. Wetting of poly (L-lactic acid) and poly (DL-lactic-co-glycolic acid)

foams for tissue culture. Biomater, 15:55-58, (1994).
18. Taylor MS, Daniels AU, Andriano KP and Heller JJ, Six bioabsorbable polymers: In vitro acute toxicity of accumulated degradation products. J Appl Biomater, 5:151-157, (1994).
19. Park TG, Cohen S and Langer R, Poly(L-lactic acid)/Pleuronic blends: Characterization of phase separation behavior, degradation, and morphology and use as protein-releasing matrices. Macromol, 25:116-122, (1992).
20. Kamath KR and Park K, Biodegradable hydrogels in drug delivery. Adv Drug Deliv Rev, 11:59-84, (1993).
21. Davis SS, Illum L and Stolnik S, Polymers in drug delivery. Curr Opin Colloid Interface Sci, 1:660-666, (1996).
22. Edlund U and Albertsson AC, Degradable polymer microspheres for controlled drug delivery. Adv Polym Sci,157:67-112, (2002).
23. Hirano S, Seino H, Akiyama Y and Nonaka I, Chitosan: a biocompatible material for oral and intravenous administrations. In: Progress in Biomedical Polymers. Plenum Press, New York, pp. 283–290, (1990).
24. Knapczyk J, Krowczynski L, Pawlik B and Liber Z, Pharmaceutical dosage forms with chitosan. In: Chitin and Chitosan: Sources, Chemistry, Biochemistry, Physical Properties and Applications. Elsevier, London, pp. 665–669, (1984).
25. Artursson P, Lindmark T, Davis SS and Illum L, Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2). Pharm Res, 11, 1358–1361, (1994).
26. Muzzarelli R, Baldassarre V, Conti F, Ferrara P, Biagini G, Gazzanelli S and Vasi V, Biological activity of chitosan: ultrastructural study. Biomater, 9:247–252, (1988).
27. Hirano S, Seino H, Akiyama Y and Nonaka I. Chitosan: a biocompatible material for oral and intravenous administration. In: Gebelein CG,Dunn RL,editors. Progress in biomedical polymers. New York: Plenum Press, p. 283–290, (1990).
28. Muzzarelli RAA. Biochemical significance at exogenous chitins and chitosans in animals and patients. Biomater, 20:7–16, (1993).
29. Lorenzo-Lamosa ML, Remunan-Lop ez C, Vila-Jato JL and Alonso MJ. Design of microencapsulated chitosan microspheres for colonic drug delivery. J Control Rel;52:109–118, (1998).
30. Calvo P, Remunan-L opez C,Vila-Jato JL, Alonso MJ. Novel Hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers. J Appl Polym Sci, 63:125–32, (1997).
31. Jeevitha D and Kanchana A, Chitosan/PLA nanoparticles as a novel carrier for the delivery of anthraquinone: Synthesis, characterization and in vitro cytotoxicity evaluation, Colloids Surfaces B: Biointerfaces, 101: 126– 134, (2013).
32. Ashish D, Binulal NS, Anitha A, Nair SV, Furuike T, Tamura H, Jayakumar R, Preparation of poly(lactic acid)/chitosan nanoparticles for anti-HIV drug delivery applications. Carbohyd Polym, 80: 833–838 (2010).
33. Ramteke S, Maheshwari RBV, Jain NK, Clarithromycin based oral sustained release nanoparticulate drug delivery system. Indian J Pharm Sci, 68: 479-484, (2006).
34. Chung-Ming H, Chao-Hsuan C, Dissaya P, Li Z, Michael C, Ming-Fa H and Liangfang Z, Eradication of drug resistant *Staphylococcus aureus* by liposomal oleic acids. Biomater, 32: 214-221, (2011).
35. Yong H, Xiqun J, Yin D, Haixiong G, Yuyan Y and Changzheng Y, Synthesis and characterization of chitosan-poly(acrylic acid) nanoparticles. Biomater, 23: 3193-3201, (2002).
36. Rahul MR, Amol VJ and Douglas EH, Poly lactic acid modifications. Prog Polym Sci, 35: 338-356, (2010).
37. Jaber E, Aida P, Hojat S, Jaleh V and Hamed H, Formulation and optimization of celecoxib-loaded PLGA nanoparticles by the Taguchi design and their *in vitro* cytotoxicity for lung cancer therapy. Pharmaceut develop Technol, (doi: 10.3109/10837450.2014.920360)(2014).

38. Xu-Bo Y, Yan-Bo Y, Wei J, Jie L and En-Jiang T, Preparation of rapamycin-loaded chitosan/PLA nanoparticles for immunosuppression in corneal transplantation. Int J Pharm, 349: 241-248, (2008).
39. Sivasakthi A and Vijayalakshmi M, Antibacterial activities of phytochemical extracts from the leaves of Justica gendarussa burm.F, 5:433-438, (2014).
40. Kanchana M, Malathy BR, Jeevitha D, Reema O and Sonia S. Synthesis and Evaluation of amoxicillin and cephalexin encapsulated chitosan nanoparticles against urinary tract infection causing *Escherichia coli* and *Klebsiella pneumoniae*, 3: 2512-2526, (2014)