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

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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.

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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. 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. 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. 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. Anthraquinones belongs to the class of tricyclic compounds produced by higher plants and fungi, present in human foods. 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. Over the decades, the polymers which are biodegradable and biocompatible have shown a significant interest from both biological and biomedical perspectives. 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, tissue culture, resorbable sutures, wound closure, and controlled release systems. 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, and the capacity to improve the paracellular transport of drugs. Chitosan, a class of nature polysaccharide, having structural characteristics comparable to glycosaminoglycans, and are non-toxic, which has delivered it widely, applicable in the field of biomedical and pharmaceutical. An earlier report states the loading and sustainable release of protein using hydrophilic chitosan–polyethylene oxide nanoparticles as a carrier. 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.

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. The nanoparticles was analysed for their morphology, particle size, chemical interaction, preliminary drug loading and encapsulation efficiency which was reported in our previous study. 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. 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: \[ \text{In vitro drug release (\%) = } \frac{D(t)}{D(0)} \times 100 \] Where, 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. 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°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\(^{\circ}\)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>
<thead>
<tr>
<th>Formulations (mg)</th>
<th>Preliminary Evaluation</th>
<th>Stability of NPs after 2 months</th>
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<tbody>
<tr>
<td></td>
<td>Drug Loading (%)</td>
<td>Encapsulation Efficiency (%)</td>
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<tr>
<td>CS-PLA NPs AQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>2.8</td>
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<td>2</td>
<td>15</td>
<td>19.6</td>
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<td>2</td>
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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\(^{38}\).
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\[^{39}\].
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 62.5 µg/ml, *P. aeruginosa* at 250 µg/ml and *P. vulgaris* at 62.5 µg/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 meticillin resistant *S. aureus*.

**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°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.

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