Original Research Article

Journal of Biological Research

& Biotechnology

Bio-Research Vol. 18 No.2; pp. 1147-1153 (2020). ISSN (print): 1596-7409; eISSN (online):9876-5432

Chitosan-based nanoparticles sustenance and potentiation of the antibacterial effect of ampicillin against drug resistance among strains of *Escherichia coli*

§Onuigbo EB, Anozie-Ikeanyi C, Edeh NE, Eze CO and Gugu TH

Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, 410001, Enugu State

§Corresponding Author: Email/Phone: ebele.onuigbo@unn.edu.ng; +234-806-299-5712

Abstract

The study seeks to evaluate nanoparticles based on chitosan for enhanced delivery of ampicillin in plasmid-mediated drug resistance. Serial dilutions of a mixed population of *E. coli* was plated on nutrient agar and streaked on Replica-plate 25 random colonies using MacConkey agar with or without ampicillin (100 µg/ml) daily for 96 h. Nanoparticles were prepared by cross-linking chitosan with sodium tripolyphosphate with ampicillin trihydrate adsorbed. Three different batches were prepared for optimization. The nanoparticles were optimized based on encapsulation efficiency, *in vitro* drug release, pH stability and microbiological assay using two laboratory strains of *E. coli*. Increased resistance to ampicillin due to possible plasmid transfer was established *in vitro* after 96 h. The encapsulation efficiency of the three batches was between 21-57 %. The drug release showed a burst effect and slow extended release over 8 h and reached a peak of about 19 % release at the 6 and 7 h in Batch A, B and C. The pH of the particles was stable over a period of 6 d. The nanoparticles containing only 0.075 mg of ampicillin dropped in an agar well plate inoculated with 1 ml of *E. coli* J62 *lac pro trp his*spF*lac*::Tn*3* (Amp^R) gave an IZD of ≥ 25 mm. Chitosan nanoparticles holds good potentials in potentiating the antibacterial effect of ampicillin against possible plasmid-mediated drug resistance.

Keywords: Chitosan, Nanoparticles, Plasmid, Resistance, Ampicillin, *E. coli*

https://dx.doi.org/10.4314/br.v18i2.3 Open Access article distributed under the terms of the Creative Commons License [CC BY-NC-ND 4.0] http://creativecommons.org/licenses/by-nc-nd/4.0.
Journal Homepage: ***http://www.bioresearch.com.ng.***
Publisher***: Faculty of Biological Sciences, University of Nigeria, Nsukka, Nigeria.***

INTRODUCTION

The emergence of antibiotic resistance worldwide has become an old but unsolved problem. In some bacteria, resistance is encoded by conjugative plasmids expressing sex-pili that can readily spread resistance through bacterial populations (Colom *et al*., 2019). Pathogenic *Escherichia coli* causes enteric diseases such as diarrhoea, urinary tract infections and meningitis. Ampicillin (AMP), an extended spectrum penicillin, is widely used to treat human and livestock *E. coli* infections, but recently its resistance rate has increased (Li *et al*., 2019). It is effective against Gram-positive and Gram-negative bacteria. Ampicillin works on the active replicating stage of bacteria, inhibiting the synthesis of bacterial cell wall (Li *et al*., 2019). Pharmaceutical companies are already daunted with the rapid spread of resistance to multiple and unrelated drugs. Development of new drugs to combat the global threat seems to have become an unnecessary course to follow since there is that high probability of microbial resistance after some years. The search for alternatives has led researchers to look at drug delivery systems with antibacterial properties such as chitosan. Chitosan is biodegradable, mucoadhesive, non-toxic and used widely as an antibacterial agent (Kong *et al*., 2010). It has been used in potentiating the biocidal efficacy of antimicrobial compounds. The spectrum of activity of chitosan includes fungi, yeast, Gram-negative and Gram-positive bacteria (Raafat and Sahl, 2009). The mechanism of action is the interaction between positively-charged chitosan molecules and negatively-charged microbial cell membranes. There is an electrostatic force between the protonated NH_3^+ groups on the chitosan molecules dissolved in acetic acid and the negative residues presumably by competing with Ca^{2+} for electronegative sites on the membrane surface (Goy *et al*., 2009). In this study, we used a highly self-transmissible F*lac* plasmid tagged with Tn3 (Amp^R) in a laboratory *E. coli* strain. The transfer rate of this Flac plasmid is 95.7 % ± 3.5 (Colom *et al*., 2019). The plasmid has AMR genes. So, we sought in this study to evaluate the potentiation of the antibacterial effect of ampicillin by chitosan polymer against this *E. coli* J62 *lac pro trp his*spF*lac*::Tn*3* (Amp^R)

MATERIALS AND METHODS

Microorganisms

Laboratory model of *E. coli* J62 *lac pro trp his*spF*lac*::Tn*3* (Amp^R). Donation from Professor Paul Barrow, University of Nottingham, UK.

Methods

Preparation of nanoparticles

Chitosan particles were prepared by solubilizing 250 mg of chitosan (Sigma Aldrich, Darmstadt, Germany) in 2 % v/v acetic acid (Sigma Aldrich, Darmstadt, Germany). Chitosan nanoparticles were formed by dropwise addition of 1 % w/v sodium tripolyphosphate (Sigma Aldrich, Darmstadt, Germany) to 2.5 %w/v chitosan particles using a 23-gauge needle. This was done on a magnetic stirrer (Julabo, Allentown, PA, USA) at 150 rpm for 30 min. The chitosan nanoparticles were incubated with 2.5 % w/v of ampicillin trihydrate (Elbe Pharma, Lagos, Nigeria). This was done in three batches namely Type A, Type B and Type C as shown in Table 1 (Onuigbo *et al*., 2016).

Table 1. Experimental design including the variables with different ratios of the polymers

Batch	Chitosan (ml)	TPP (ml)	Drug (ml) (2.5 %w/v)	Stirring speed (rpm)	Stirring time (min)
A	7	2	1	150	15
B	6	3	1	200	20
C	5	4	1	300	30

Determination of Percent encapsulation efficiency

To determine encapsulation efficiency, the different formulations (Batch A, Batch B and Batch C) were centrifuged at 4000 rpm for 30 min and 2 ml of the supernatant was withdrawn using a syringe. Appropriate dilution was prepared and the solutions analyzed by UV-spectrophotometer at a wavelength of 245 nm. The absorbances of the supernatant were recorded. From the absorbances, concentrations of the supernatant were obtained using the calculated molar absorptivity (from the Beer's plot). The encapsulation efficiency was calculated using the formula below:

$$EE = \frac{Encapsulated\ drug}{Total\ drug\ used} \times 100$$ ……..equation 1

pH assessment of the formulations

The different batches of nanoparticles were stored at room temperature for 6 d. The pH of the solutions was determined using a digital pH meter (JENWAY, Eaton Socon, UK). This was repeated three times and the average pH taken to determine its stability on storage.

In-vitro Drug release study

The dissolution medium consisted of 100 mL of freshly prepared PBS maintained at 37 ± 1℃ using a heater/magnetic stirrer. The polycarbonate dialysis membrane used was pre-treated by soaking it in the dissolution media for 24 h. For the study, 2 mL of the formulated chitosan nanoparticles was placed in the dialysis membrane securely tied to a thermo-resistant thread and then immersed in the dissolution medium under agitation provided by the magnetic stirrer at 150 rpm. At pre-determined time intervals, 2 mL portions of the dissolution medium were withdrawn, filtered and analysed spectrophotometrically (UV/VIS spectrophotometer (Spectrumlab 752S, California, USA) at 245 nm. For each sample withdrawn, an equivalent volume (2 mL) of phosphate buffer maintained at the same temperature was added to the contents of the dissolution medium to maintain sink conditions throughout the release period. The amount of drug released at each time interval was determined with reference to the standard Beer's plot for ampicillin in phosphate buffer (Umeyor et al., 2012)

$$\%\ \text{Drug released} = \frac{\text{Conc (from Beer's plot)}}{\text{Conc of drug in formulation}} \times 100$$……
……equation 2

Percent plasmid transfer

An overnight culture of a mixed population of *E. coli* containing *E. coli* J62 *lac pro trp his*pF*lac*::Tn*3* (Amp^R) was prepared in 10 ml of sterile nutrient broth (Lab M, Lancashire, UK). This was diluted 1/100 with nutrient broth and then incubated for 2 h at 37 ℃ (log phase). Decimal dilutions to 10^{-6} was prepared on nutrient agar and incubated for 24 h at 37 ℃, then original and diluted cultures were counted. Replica-plated 25 random colonies on MacConkey agar (Lab M, Lancashire, UK) with or without ampicillin (100 µg/mL) (control) were prepared. This procedure was repeated daily for 3 days with the 1/100 broth dilution and percentage resistance recorded (Colom et al., 2019).

Microbiological assay on different bacterial strains

Molten MacConkey agar (19 mL) was inoculated with 1 mL of *Escherichia coli* and *E. coli* Flac∴Tn3 broth cultures, respectively. It was mixed thoroughly, poured into Petri dishes, and rotated to ensure even distribution of the organism. The agar plates were allowed to set and a sterile cork-borer (8 mm diameter) was used to cut three wells in each of the agar medium plate representing the three isolates. The three different batches of nanoparticles were prepared and used as follows: 2 drops of the formulations were added into the different holes in each of the plates using sterile Pasteur pipettes. The plates were allowed to stand at room temperature for 15 min to enable the samples diffuse into the medium before incubating at 37 ℃ for 24 h. The inhibition zone diameters were carefully measured and recorded.

RESULTS AND DISCUSSION

pH

The pH of the nanoparticles ranged from 4.29 to 4.34 in Batch A, 4.29 to 4.33 in Batch B and then in Batch C, it was between 4.36 and 4.39 as seen in Table 2. The variation in pH for the three groups was minimal. The minimal pH variation showed that the method produced

particles of good stability. There was also no discoloration or odour or aggregation of the particles under storage conditions. Modification of pH has been shown to have effect on encapsulation efficiency and release of drug from chitosan microparticles. Low pH of sodium tripolyphosphate (TPP) resulted in slower felodipine release from microparticles. (Yongmei and Yumin, 2003). Chitosan is poorly water soluble at neutral or alkaline pH. Ionization of chitosan decreases with increase in pH leading to weaker cross-linking density (Khan *et al*., 2010). It is only soluble at mild acidic pH hence the dissolution in 2 % acetic acid. Upon dissolution it is protonated with a free -NH_3^+ site which has a low pH. Sodium tripolyphosphate ($Na_5P_3O_{10}$) dissociates in water to produce both tripolyphosphoric acid ($H_5P_3O_{10}$) which is a weak polyprotic acid and OH-. The cross-linking of chitosan with sodium tripolyphosphate is called ionic gelation. The $–OH^-$ or tripolyphosphoric ions competitively react ionically with the $–NH_3^+$ binding site by deprotonation and covalent bonds respectively (Shihui *et al*., 2011). Prabaharan and Mano (2005) reported that high porosity of the chitosan microspheres was obtained with cross-linking with TPP solution of pH 8.6. The porous microspheres could be modified chemically by adding additives like carboxyl, hydrophobic acyl and quaternary ammonium groups) acting as hydrophobic barrier on the surface of the nanoparticles hindering leaching or penetration of water (Saeed *et al.*, 2020). Basic conditions deprotonate chitosan particles forcing it to lose stability and form aggregates (Saeed *et al*., 2020).

Table 2. Time-dependent pH stability studies of the formulations

Formulation Types	1st Day	2nd Day	6th Day
A	4.29	4.30	4.34
B	4.29	4.30	4.34
C	4.39	4.39	4.36

Percent encapsulation efficiency (PEE)

In the percent encapsulation efficiency, Batch C had the highest PEE followed by Batch B and least was Batch A. Batch B had a PEE > 50 %. This formulation was set at a stirring speed of 300 rpm and stirring time of 30 min. The high PEE could be attributed to the higher amount of TPP which produced a strong and closer network with the chitosan in a hydrogen bonding (Yongmei and Yumin, 2003). TPP is used in cross-linking chitosan thereby improving the mechanical strength of the gel. Sodium tripolyphosphate has a high negative charge density and the more the amount added, the stronger the matrix (Bangun *et al*., 2018) and more resistant to erosion (Saeed *et al*., 2020). Concentration of cross-linking agent, stirring time and speed are important factors in entrapment of drug (Razak *et al*., 2017). This high drug encapsulation is an indication of the efficiency of the ionic gelation method and the effect of chitosan:TPP ratio on preparation process. It has been observed that the microparticles do not form spherical shapes at higher stirring speed like 1500 rpm but at lower stirring speeds. Tilkan and Ozdemir (2018) observed that the optimum microsphere formation was a stirring speed of 100 rpm. He also observed that the duration of cross-linking time for microparticle formation was 30 min. High encapsulation efficiency close to 100 % has been achieved with ionotropic gelation method when interactions between chitosan and the drug are optimal (Pedroso-Santana and Fleitas-Salazar, 2020).

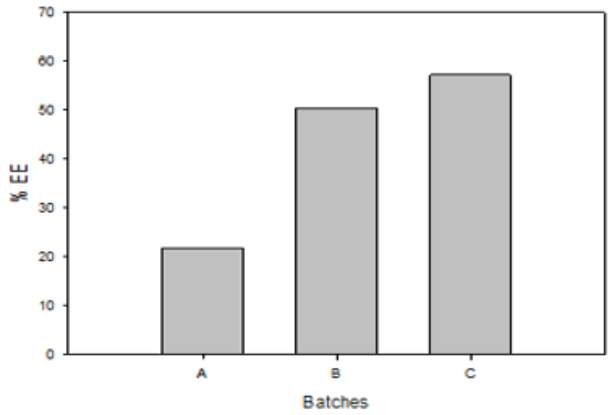

Figure 1: Percent drug encapsulation efficiency (%EE) of the three batches of chitosan nanoparticles

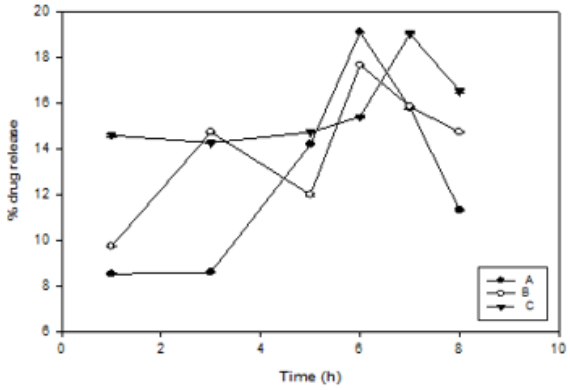

Figure 2: Release profile of ampicillin encapsulated in chitosan nanoparticles

In-vitro drug release

Batch C had a burst release in the first hour of release which became stable for the next 6 h which may be due to gradual penetration of dissolution medium into the matrices followed by a rise at the 7 h and then decrease which could be associated with degradation of the cross-linker. This result also correlates with the percent encapsulation efficiency with Batch C > B > A. Batch B had a lower release than A at the first hour and then a rise and fall which peaked at the 6 h releasing the remaining drug entrenched inside the matrices. Batch C had the lowest release at the first hour which was sustained for the next two hours and then rose and peaked at the 6 h with an equal percent release as Batch C and then a decrease. For Batch C, the percentage release oscillates between 14 and 19 %. For Batch B, the percent release is between 10 and 17 % while for Batch A, the percent release is between 8 and 19 %.

Percent plasmid transfer

The antimicrobial resistance test showed 100 % growth of the mixed population of *E. coli* in all the 25 replica random control plates for four consecutive days. In the plates containing 100 µg/ml of the pure ampicillin drug, there was 16 % ampicillin-resistance on day 1, 40 % ampicillin-resistance on day 2, 56 % ampicillin-resistance on day 3 and 84 % ampicillin-resistance on day 4. From the result, there was increasing resistance over time. The Amp^R transfer efficiency was high. The highly self-transmissible F*lac* plasmid tagged with Tn3 (Amp^R) in a laboratory *E. coli* strain has a transfer rate of 95.7 % ± 3.5 (Colom *et al*., 2019). The plasmid has AMR genes. The increasing resistance could be as a result of conjugative transfer of the plasmid containing ampicillin resistance gene to other *E. coli* sensitive strains and the increasing fitness of the strain carrying Amp^R. In an unpublished data, *E. coli* J62 *lac pro trp his*spF*lac*::Tn*3* (Amp^R) which served as positive control had about 96 % growth in the ampicillin plate

Table 3: Percent plasmid transfer in ampicillin and ampicillin-free plates

Plate	Duration (hr)	% growth in ampicillin-free plate	% resistance in ampicillin plate
A	24	100	16
B	48	100	40
C	72	100	56
D	96	100	84

Microbiological assays

From the results, the nanoparticles were able to sustain and potentiate the antibacterial effect of ampicillin. We can rightly assert to a synergy between the drug and the chitosan particles (Saha et al., 2010, Porras-Comez *et al*., 2018). Chitosan, having a net positive charge could have induced alterations in the zeta potential of the cell membrane of the organisms' permeability to the drug. Permeability could also have increased because of the mucoadhesive nature of chitosan. It has been reported that both chitosan and tripolyphosphate when combined with b-lactam drugs form nanostructures with antibacterial activity against *S. aureus* with different degrees of resistance. (Ciro *et al*., 2019). We can also see that increasing the concentration of TPP increased the antibacterial effect of the chitosan with Batch C having the highest IZD against all the organsisms. CS-TPP nanoparticles have a larger surface area so that nanoparticles can be tightly absorbed onto the surface of the bacteria cells so as to disrupt the membrane, which would lead to the leakage of intracellular components, thus killing the bacteria cells. The IZD in all the batches showed sensitivity to the ampicillin-loaded chitosan nanoparticles.

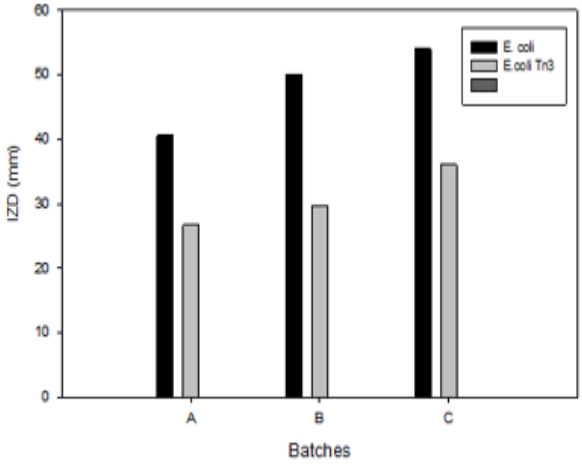

Figure 3: Sensitivity of two different strains of laboratory *E. coli* to the three batches of chitosan nanoparticles

CONCLUSION

The chitosan nanoparticles had relatively good encapsulation efficiency and displayed slow drug release with good potentials to potentiate the antibacterial effect of ampicillin against *E. coli* J62 *lac pro trp his*pF*lac*::Tn*3* (Amp^R).

Conflict of Interest

Authors have no conflict of interest to declare.

REFERENCES

Bangun, H., Tandiono, S. and Arianto, A. (2018). Preparation and evaluation of chitosan-tripolyphosphate nanoparticles suspension as an antibacterial agent. *J Appl. Pharm. Sci.*8(12): 147-156.

Ciro, Y., Rojas, J., Onate-Garzon, J and Salamanca, C.H. (2019). Synthesis, characterisation and biological evaluation of ampicillin–chitosan–polyanion nanoparticles produced by ionic gelation and polyelectrolyte complexation assisted by high-intensity sonication. *Polym.* 11(11):1758

Colom, J., Batista, D., Baig A., Tang Y., Liu S. *et al*. (2019). Sex pilus specific bacteriophage to drive bacterial population towards antibiotic sensitivity. *Sci Rep*. 9: 12616.

Goy, R.C., de Britto, D. and Assis, O.B.G. (2009). A review of the antimicrobial activity of chitosan. *Polimeros*. 19(3).

Khan, M.S., Sridhar, B.K. and Srinatha, A. (2010). Development and evaluation of pH- dependent micro beads for colon targeting. *Indian J Pharm. Sci*. 72(1): 18-23.

Kong, M., GuangChen, X., Xing, K. and JinPark, H. (2010). Antimicrobial properties of chitosan and mode of action: A state of the art review. *Int. J Food Micro.* 144(1): 51-63.

Li, M., Liu, Q., Teng, Y., Qu, L., Xi, Y., Chen, S. and Duan, G. (2019). The resistance mechanism of *Escherichia coli* induced by ampicillin in laboratory. *Infect Drug Resist*. 12: 2853-2863.

Onuigbo, E.B., Onugwu, A., Nwocha, M., Odiase, A. and Attama, A.A. (2016). Preparation and *in-vitro* evaluation of amoxicillin encapsulated in alginate-coated chitosan microparticles. *Trop J Pharm. Res.* 15(1):2303-2309.

Pedroso-Santana, S and Fleitas-Salazar, N. (2020). Ionotropic gelation method in the

synthesis of nanoparticles/microparticles for biomedical purposes. Polym. Int. 69(5): 443-447.

Porras-Gómez, M. Vega-Baudrit, J. and Núñez- Corrales, S. (2018). Ampicillin-loaded chitosan nanoparticles for *in-vitro* antimicrobial screening on *Escherichia coli*. doi: 10.5772/intechopen.76034.

Prabaharan, M. and Mano, J.F. (2005). Chitosan-based particles as controlled drug delivery systems. *Drug Del*. 14:41-57.

Raafat, D. and Sahl, H. (2009). Chitosan and its antimicrobial potential – a critical literature survey. *Microb. Biotechnol.*2(2): 186-201.

Razak, F., Chyzna, V., Morris, N., Murphy, A. and Kennedy, J. (2017). An investigation into the effects of pH and material concentration on the morphology of chitosan-alginate microspheres prepared using an ionic gelation technique. *Int. J Adv. Sci. Res. Manag*. 2(10): 2455-6378.

Saeed M.R., Dmour, I. and Taha, M.O. (2020). Stable chitosan-based nanoparticles using polyphosphoric acid or hexametaphosphate for tandem ionotropic/covalent crosslinking and subsequent investigation as novel vehicles for drug delivery. *Front. Bioeng. Biotechnol.* https://doi.org/10.3389/fbioe.2020.00004

Saha, P, Goyal, A.K. and Rath, G. (2010). Formulation and evaluation of chitosan-based ampicillin trihydrate nanoparticles. *Trop. J Pharm. Res.* 9(5): 483-488.

Sihui Wu, S., Tao, Y., Zhang, H and Su, Z. (2011). Preparation and characterization of water-soluble chitosan microparticles loaded with insulin using the polyelectrolyte complexation method. *J Nanomat*. https://doi.org/10.1155/2011/404523.

Tilkan, M.G. and Özdemir, N. (2018). Investigation of the parameters affecting the release of flurbiprofen from chitosan microspheres. *Braz. J Pharm. Sci*. 53(4):e00242.

Umeyor, E. C., Kenechukwu, F.C., Ogbonna, J.D., Chime, A.S., Attama, A.A. (2012). Preparation of novel solid lipid microparticles loaded with gentamicin and its evaluation *in vitro* and *in vivo*. *J Microencapsul*. 29(3): 296-307.

Yongmei, X and Yumin, D. (2003). Effect of molecular structure of chitosan on protein delivery properties of chitosan nanoparticles. *Int. J Pharm*. 250: 215-226.