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FORMULATION AND EVALUATION OF CIPROFLOXACIN-COCKLE SHELLS DERIVED CALCIUM CARBONATE ARAGONITE NANOPARTICLES PHYSICOCHEMICAL PROPERTIES MEDIATED *IN VITRO* BACTERICIDAL ACTIVITY IN *SALMONELLA* TYPHIMURIUM

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ABSTRACT

Since the use of classical antibiotics in the management of resistant bacterial infections requires high dosage and regular administration for a lengthy duration, the enhanced delivery system that will ensure sustained release of antibiotic to the Site of action is important. We synthesized and formulated ciprofloxacin-cockle shell derived calcium carbonate aragonite nanoparticles (C-CSCCAN), appropriately analysed its physicochemical properties mediated antibacterial activity in *Salmonella* Typhimurium. The size of the formulated nanoparticles were in the range of 13.94 and 23.95 nm and Zeta potential was optimally negative. Diffraction pattern by X-ray powder diffraction (XRD) revealed strong crystallizations in all the formulations. Fourier-transform (FT-IR) spectra displayed evidence of interactions between the drug and nanoparticles at the molecular level and no change in peaks position was observed prior to and after the synthesis of the nanoparticles. Higher encapsulation (99.5) and loading capacity (5.9%) were attained at ciprofloxacin to nanoparticles ratio 1:17. No burst effect but a sustained drug release was observed from the formulation. C-CSCCAN suspension exhibited higher antibacterial activity than free ciprofloxacin. It was concluded that physicochemical properties of CSCCAN enhanced susceptibility of *Salmonella* Typhimurium, which could potentially improve the clinical efficacy of ciprofloxacin.

KEYWORDS: Antibacterial resistance, ciprofloxacin, calcium carbonate nanoparticles, physicochemical properties.

INTRODUCTION

Antibacterial resistance remains the universal health concern, threatens the potency of antibacterial therapies.[1] Ciprofloxacin is one of the frequently used antimicrobial of the fluoroquinolone groups available all over the world,[2] possessing strong bactericidal effect against a wide-range of medically important gram-negative and gram-positive bacteria.[2,4] Hence, it is required in several systemic diseases.[5] However, ciprofloxacin has been available only as conventional, immediate-release tablets, and has a biological half-life of about 3-5 hours for a single or repeated administration.[6,7] Accordingly, frequent administration of ciprofloxacin is associated with numerous side- effects and prolonged therapy is often associated with patient noncompliance, and incomplete treatment results in the development of resistance.[7,8,9]

The preparation or modification in antimicrobial compounds to further improve their bactericidal activities is often a top priority research area in the present day. Likewise, the development of drug delivery system capable of delivering therapeutic agents to sub-cellular levels is the promising strategy in overcoming microbial resistance.[10,11] Certainly, the sustained release formulation of ciprofloxacin will provide a new treatment option to clinicians that may enhance patient adherence/convenience and thereby enhances its antibacterial activity.[4]

Studies have highlighted that some nanoparticles work extremely well in combination therapy along with ciprofloxacin to minimize antibiotic resistance and improve the antibacterial activity of the drug against microorganism *in vitro*. The nanoparticles can indeed

obstruct the different proteins associated with antibiotic resistance or enhance the drugs intracellular retention, since nanoparticles are not liable substrate of the efflux pump proteins.[12,13,14] Cockle shells calcium carbonate aragonite nanoparticles (CSCCAN) are a promising drug delivery carriers because of their several notable characteristics. These characteristics include: ease of laboratory preparation, precise tailoring for the required physical and chemical properties, ability to entrap large amount of pharmaceutical compounds[15,16] as well as availability in large quantity and can easily be employed to satisfy the growing desire of biomaterials due to their low cost.[17,18,19] These observations among others prompted us to develop a delivery device for an antibiotic with a broad spectrum antibacterial activity, such as ciprofloxacin. Thus, the encapsulation of ciprofloxacin within CSCCAN was proposed in order to enhence release and improve the clinical efficacy of ciprofloxacin in the treatment of drug-resistant bacterial infections.

MATERIALS AND METHOD

Materials

Polysorbate-(Tween 80) (Thermo Fisher Scientific, Waltham, MA USA), glyceride (Sigma-Aldrich Co., St. Louis, United State), ciprofloxacin (LKT Laboratories, St. Paul, MN, USA), mueller–hinton agar (Difco Becton Dickinson, Sparks, MD), mueller–hinton broth (Difco Becton Dickinson, Sparks, MD), phosphate buffer saline (Sigma-Aldrich Co., St. Louis, United State), dimethylsulfoxide (Sigma-Aldrich Co., St. Louis, United State).

Bacterial Strain

Salmonella Typhimurium ATCC 14208 was obtained from American Type Culture Collection (ATCC, Rockville, MD-USA). S. Typhimurium ATCC 14028 was grown and maintained on Tryptic Soya agar (TSA) (Sigma-Aldrich Co., St. Louis, United State).

Preparation of Cockle Shells Calcium Carbonate Powder

The micronisized cockle shells calcium carbonate powders (CSCCP) were prepared according to the procedure described by Islam *et al.*[20] The cockle shells were initially scrubbed to remove dirt, boiled for 10 minutes and then allowed to cooled at room temperature. The shells were again scrubbed thoroughly with distilled water and dried in an oven for seven days at 50◦C. The shells were crushed and finely grounded using a rotary-type pulverizing machine (model: RT 08, Tainan, Taiwan). The powders were sieved using a stainless laboratory test sieve with an aperture size of 90 µm and subsequently 75 μm (Endecott Ltd., London, England) to obtain micronisized powders, approximately 5 µm to 75 µm in diameter. The CSCCP were then packed into a polyethylene plastic bag for further analyses.

Top down Synthesis of Cockle Shells Calcium Carbonate Aragonite Nanoparticles

The method for the synthesis of cockle shells calcium carbonate aragonite nanoparticles (CSCCAN) was based on the use of a microemulsion system using a high pressure homogenizer (HPH) (Avestin, Emulsiflex-C50 GmbH Wienheimer, Germany). Briefly, surfactants, Tween-80 and glycerol, in different molar ratios, were accurately weighed into a long necked quick fit round-bottom flask and dissolved in 15 mL weight/volume oil by means of sonication for 15 minutes. The resulting emulsion was carefully transferred into a flask containing 65mL weight/volume deionized water and agitated until it became transparent. This was followed by sonication in a water bath for 5 minutes. Top down synthesis of the nanoparticles was performed by suspending 2 g of dry, micronized CSCCP in the formulated microemulsion, moderately stirred for 10 minutes at 1000 rpm to form a CSCCP suspension. The colloidal suspension was pre-milled at a low pressure of 200 and 500 bars for three cycles each. After pre-milling, the suspension was again subjected to a high pressure of 1500 bars for 25 homogenizing cycles to get the desired fine particles. The final suspension was oven dried at 95^{o}C for 24 hours.[15]

Analysis of Ciprofloxacin Loading and Encapsulation Efficiency

Ciprofloxacin external calibration curve was constructed by plotting the drug peak areas versus the known concentrations of the drug.[21] This was used to determine percentage drug loading content and concentration of released ciprofloxacin. The curve (data not provided), represents known concentration of ciprofloxacin standard solution (100-3.10 µg/ml) against the absorbance from a micro-titer plate reader (TECAN Safire, Tecan Austria GmbH, Grödig, Austria). Linear correlation coefficient of the curve (equations for regressed straight line) between the ciprofloxacin concentrations and absorbance were given below: R^2=0.9823 and y = 0.0091x - 1.4894 respectively.

To load the drug, aqueous solution of ciprofloxacin of varying concentrations (1 mg/mL, 2 mg/mL and 3 mg/mL) were mixed by sonication for 10 minutes. To each mixture, 50 mg/mL nanoparticles was dissolved to obtain drug: nanoparticles ratios of 1:50, 1:25 and 1:17 for C-CSCCAN 1, C-CSCCAN 2 and C-CSCCAN 3, respectively. The formulations were mechanically stirred overnight at 200 ± 1 rpm at room temperature using a systematic multi-hotplate stirrer (Witeg,Wise Stir SMHS, Witeg Labortechnik GmbH, Wertheim Deutschland). The ciprofloxacin-nanosuspension was afterward centrifuged at 15,000 rpm for 15 minutes. The resulting ciprofloxacin-nanoparticles formulation were freeze-dried.[16]

The percentage loading and encapsulation efficiency were analyzed by calculating the difference between the total drugs fed (Wt) and the free encapsulated drug (Wf)

concentrations in the nanoparticles supernatant per nanoparticles weight. The residual quantity of free encapsulated ciprofloxacin remaining in the supernatant was determined by computing the optical density at 291 nm[7,22,23] on a micro-titer plate reader.[16,24] Data were given as an average measurement of three independent values. The percentage drug loading capacity (LC%) and encapsulation efficiency (EE%) were calculated as described by Yaod *et al*.[25]

Physicochemical Characterizations

The morphology and particles size of the nanoparticles formulations were analysed using transmission electron microscope (TEM, Hitachi H-7100, Japan) and field-emission scanning electron microscope (FESEM, model 100, Perkin Elmer, 710 Bridge port Avenue, Shelton, CI USA). The TEM measurements were carried out at 150 kilovolts. The samples were mixed with absolute alcohol, sonicated for 30 minutes, and the colloidal solution was dropped on to a carbon-covered copper grid, placed on a filter paper, and dried at room temperature for 1 hour. The FESEM observations were performed at 200 kilovolts. Samples were prepared on aluminium stubs and coated with gold under argon atmosphere using a sputter coater.

The particles surface charge were analyzed at pH 7.4 using Malvern Zetasizer Nano (Malvern Instruments, UK), which measures the electrophoretic mobility of particles in an electrical field, which is then converted into zeta potential. The measurement was performed by injecting the samples into cells of the zetasizer and run at room temperature. The process was repeated three times and the average was taken to determine the zeta potentials.

The purity and crystalline properties of the samples were investigated using X-ray Powder Diffraction (XRD) (Shimadzu XRD-6000, Yokohama, Kanagawa, Japan). Cross-section of the samples were taken and place on a quartz plate for exposure to Cu Kα radiation of wavelength (λ) of 1.5406 Å. The samples were then examined at room temperature over a 2θ range of 4–50◦, with sampling intervals of 0.02◦ 2θ and a scanning rate of 0.6◦/min.

The chemical analyses were done using Fourier-transform infrared spectroscopy (FT-IR, model 100, Perkin Elmer, 710 Bridge port Avenue, Shelton, CI USA), in a range of 280 to 4000cm^{-1} at a resolution of 2cm^{-1} and at scan speed of 64/second. The Pellets of the samples were mixed individually in a weight proportion of 1wt % in potassium bromide powder, and analyses were performed.

In Vitro Drug Release

In vitro releases were determined in phosphate buffer (pH 7.4) according to Prasad *et al.*[26] with little modification. A 10 mg C-CSCCAN was suspended into an Eppendorf tube containing three millilitres of phosphate buffer medium. The tube were put into orbital shaker incubator (TU-400) at 37 ◦C with a stirring rate of 120 rpm. At scheduled time intervals (0, 0.5, 1, 3, 6, 9, 12, 24, 48, 72, 96, 120, 144, 216, 312 408, and 504 hours), the tube were taken and centrifuged at 13200 × rpm for 15 minutes. One and a half millilitre sample were withdrawn and replaced with fresh medium to maintain sink condition of the system. Ciprofloxacin released was evaluated by measuring the optical density at a wavelength λ_{max} = 291[7,22] on a micro-titer plate reader.[24] The release study was conducted until there was apparently total release of the drug. Optical densities were interpreted according to the ciprofloxacin calibration standard curve described earlier. Data were given as mean ± standard deviation based on the measurements of the samples from three independent replicates and represented on a graph of percentage (%) drug release versus time intervals.

In Vitro Antibacterial Test

The bacteria stock solution were diluted according to the method in Clinical and Laboratory Standards Institute (CLSI).[27]

Preparation of drugs stock solutions

A stock solution of C-CSCCAN suspensions and ciprofloxacin dispersion (pH 7.4) at concentration of 1 mg/mL was prepared in 10% DMSO.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC was determined by a 2 fold-serial dilutions of the suspensions in a final concentration ranging from 100 to 1.6 µg/mL using 96-wells polystyrene microtiter plates. The microtiter plate wells were inoculated with 100 µL/well of initial bacterial cell suspension grows in Muller Hinton broth (MHB) at a final concentration of 1 × 10^6 CFU/mL. The dilutions were performed in decreasing order from the wells in column 12 down to column 3 of the microtiter plate. Column 2 containing the medium and inoculum, was taken as the positive control for all samples, and column 1 containing only medium, was taken as the negative control. Following overnight incubation at 37 °C, the plates were examined for visible bacterial growth as evidenced by turbidity.[28] The MIC is defined as the lowest concentration of antimicrobial agents that completely inhibits visible growth of microorganisms.[28] All experiments were performed in triplicate and the average values were reported as MICs.

The MBC was determined in a second step after the MIC evaluation, according to the guidelines recommended by CLSI.[28] Aliquots of 10 µl from all the wells in which no visible bacterial growth was observed were seeded in Muller Hinton agar (MHA) plates supplemented with neither ciprofloxacin nor C-CSCCAN suspension and were incubated for 24 h at 370C. The MBC end point is defined as the lowest concentration of antimicrobial agent that kills 100% of the initial bacterial population.

Statistical Analysis

All statistical analyses were performed using Minitab statistical software (Minitab Inc, State College, PA, USA). All experiment was performed three times. Values were expressed as mean ± standard deviation. Comparison of treatment effects and statistical significant differences between groups were determined using student's independence t-test. A value of $P < 0.05$ was regarded significant unless indicated otherwise.

RESULTS AND DISCUSSION

Top-down preparation of nanoparticles

The top-down synthesis of the CSCCAN using a HPH is greatly promising and easy-to-perform, environmentally friendly and a low-cost method. It involved a simple mechanical stirring of CSCCP in the presence of oil-in-water microemulsion followed by the application of a HPH. In this system, the particles sizes were virtually reduced after leaving the homogenizing gap by cavitations, particles collisions, and shear forces reported previously.[15,29,30] Figure 1 shows the representative FESEM and TEM micrographs of the nanoparticles that were formed by the HPH approach. Only spherical CSCCAN are seen in the TEM micrographs (Figure 1b). The spherical particles are fairly uniform and range in size from 11.93 and 22.12 nm. It is interesting to note from (Figure 1a), that the particles surface are fairly rough and porous.

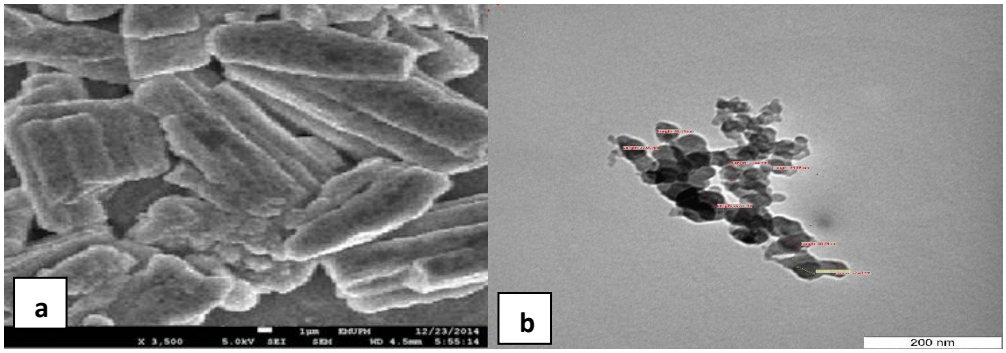

Figure 1: FESEM micrograph showing the pore structured of the micron-size cockle shells calcium carbonate powder (a), TEM micrographs showing synthesized, nanoscale, spherical-shaped cockle shells calcium carbonate (aragonite) nanoparticles (b)

However, the preparation parameters, such as the concentration of surfactants, sonication and homogenization time, were regularly optimized for the formulation and during the nanoparticles synthesis. This convenient approach allows for obtaining homogenized nanoparticles and is a good alternative to chemical method which uses dodecyl dimethyl betaine (BS-12). It also add no impurities to the final products but produced very small- sized particles in a reproducible fashion, a criteria which is strongly desired in industrial set up.[15]

Ciprofloxacin Loading

The percentage drug loading and encapsulation efficiency of ciprofloxacin from the nanoparticles was determined using a calibration curve, as described earlier. Ciprofloxacin has been encapsulated via various formulations and techniques.[5,21,31] Some of these techniques result in the incorporation of a small fraction of the drug compare to the present techniques and the nanoparticles formulation. The C-CSCCAN was successfully prepared in three different drugs: nanoparticles ratios 1:50, 1:25 and 1:17 for C-$CSCCAN_1$, C-$CSCCAN_2$ and C-$CSCCAN_3$, respectively. The encapsulation efficiency and loading content percentages, shown in Table 1, varied for all formulations. However, the encapsulation efficiency was affected by the amount of drug used and between different drug-nanoparticles ratios. An increase in the amount of the drug resulted in an increase in the drug encapsulation. The high Percentage encapsulation efficiency of ciprofloxacin in the prepared nanoparticles demonstrated minimal loss of drug during loading process. The small molecular size of ciprofloxacin,[32] and the long overnight loading period was presumed to influence the loading content. Along with the increase of the feeding drug concentrations, the increase of the loading capacity could also be induced by the porous nature of the nanoparticles (Figure 1a). The immense improvement in the present method is that it involves an uncomplicated and effective procedure. The obtained formulation with high encapsulation efficiency (99%) and drug-nanoparticles ratio (1:17) was selected for further analysis.

Table: 1. Loading content, encapsulation efficiency and zeta potential of ciprofloxacin conjugated cockle shells calcium carbonate aragonite nanoparticles (n = 3).

Formulations	Weight of nanoparticles	Weight of Drug	Loading contents (%)	Encapsulation efficiency (%)
C-$CSCCAP_1$	50mg	1mg	1.9	99.1
C-$CSCCAP_2$	50mg	2mg	3.9	99.3
C-$CSCCAP_3$	50mg	3mg	5.9	99.4

Ciprofloxacin-nanoparticles– Physicochemical Analysis

The Transmission electron microscopy (TEM) and field emission scanning electron microscopy (FESEM) were used to investigate the morphology and size of the nanoparticles. The particle sizes revealed by TEM (Figure 2a) were between 13.94 and 23.95 nm. While the FESEM further examined the surface structure of individual particles. The micrograph image showed dispersed nanoparticles with a smooth surface and spherical shaped (Figure 2b), this result is in agreement with results attained from TEM. Whereas the particle size revealed by TEM image averaged about 18 nm, the FESEM micrograph exhibited smaller particle size.

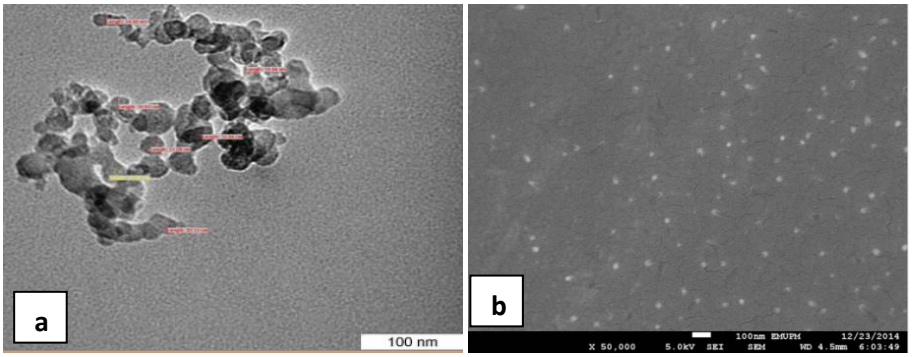

Figure 2: TEM micrograph showing homogenized, spherical shaped ciprofloxacin- cockle shells calcium carbonate (aragonite) nanoparticles (a), and FESEM micrographs showing dispersed and homogenized spherical shaped ciprofloxacin- cockle shells calcium carbonate (aragonite) nanoparticles (b)

It was clearly observed that increased agitation (sonication) in alcohol at amplitude of 50% for 1 hour significantly decreased the particles size and provided the dispersed particles. Das *et al.*[33] observed that sonication displayed huge effect on particle size. This is important since particle size could influence its antibacterial activities.[34]

The measurement of zeta potential was performed to confirm the surface charge property of the nanoparticles formulations. The zeta potentials of the formulations at pH 7.4 exhibited a slightly negative charge: -15.3±1.6 mV (Figure 3a). and -11.8±1.7 mV (Figure 3b) for the nude CSCCAN and C-CSCCAN, respectively.

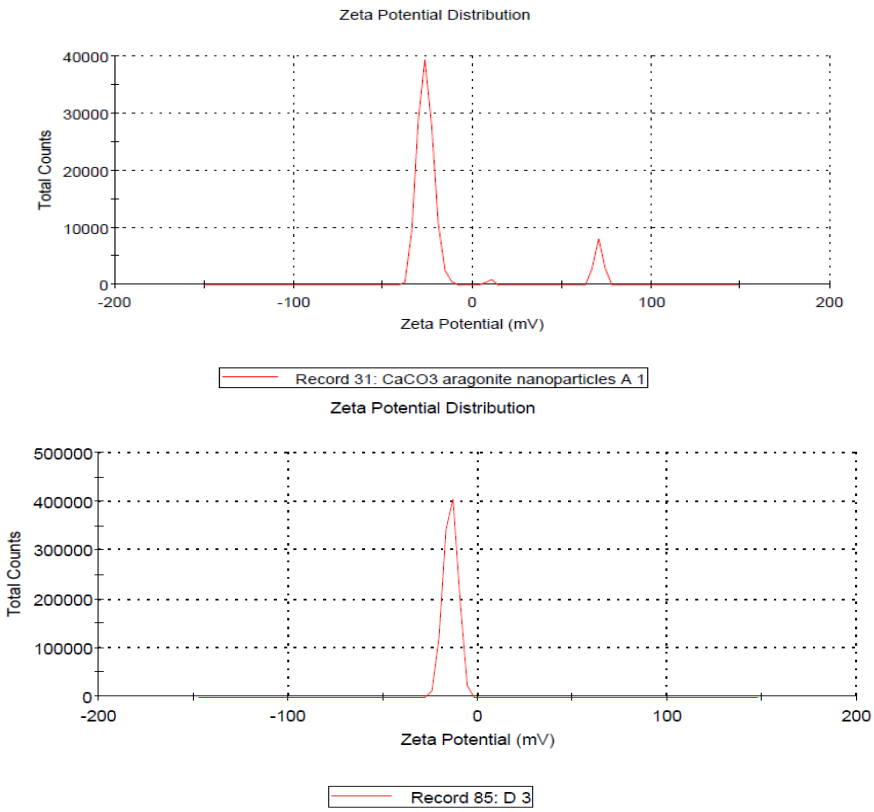

Figure: 3. Zeta potential distribution of cockle shells calcium carbonate (aragonite) nanoparticles (a), and ciprofloxacin- cockle shells calcium carbonate (aragonite) nanoparticles (b).

Being a molecule that can be found in four different forms: anion, zwitterion, neutral and cation,[35] the conjugation of the nanoparticles with ciprofloxacin resulted in lowering of the zeta potential values in comparison with the blank nanoparticles. At neutral pH, ciprofloxacin is zwitterion or neutral forms.[35] This analysis further showed that the drug was encapsulated in the nanoparticles matrix rather than physically adsorbed on the nanoparticle surface. Molecules as well as particles that are adequately small, which has a high zeta potential (negative or positive), maintains a stable system.[36] Considering all the above-mentioned information, the negative potential values obtained in this study was significant, which indicated stability and electrostatic repulsion that protects the particles from aggregation to some extent.

To determine the characteristic crystalline phases present and purity of the sample, XRD studies were conducted. The graphs depicted in Figure 3 (a, b c and c) show the XRD patterns of micron-size CSCCAP, nude CSCCAN, C-CSCCAN and the free ciprofloxacin respectively. The data revealed strong crystallizations in all the formulation including the free ciprofloxacin. Dominant XRD crystal peaks were observed at diffraction angles of 2θ = 26.2, 27.3, 33.1, 38.7, 38.8, 46.1, 48.3, and 52.8 in all the formulated samples (Figure 3a, b and c).

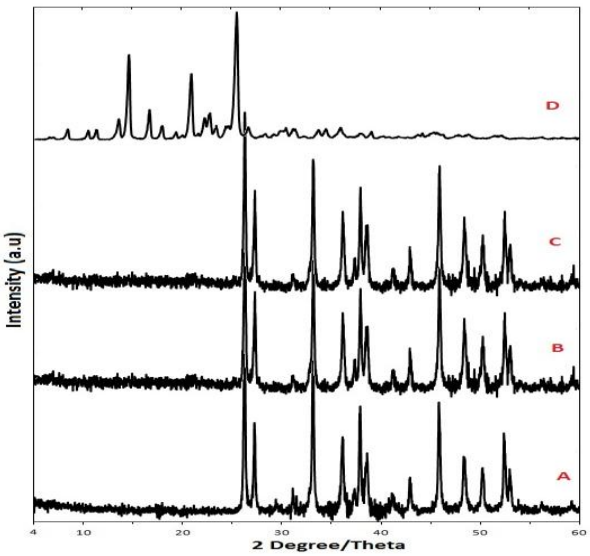

Figure 3: XRD spectra for micron-size cockle shells calcium carbonate aragonite powder (a), cockle shells calcium carbonate (aragonite) nanoparticles (b), ciprofloxacin-conjugated cockle shells calcium carbonate (aragonite) nanoparticles (c), and free ciprofloxacin (d) showing crystalline phases and purity

The graph clearly demonstrates that preparation processes have no effects on the nanoparticles crystalline properties and the formulation is free from impurities. Similarly, the original crystal natures of nude CSCCAP Did not change or disappear when compared to the loaded nanoparticles. This observation was supported by finding of Kamba *et al.*[16] Ciprofloxacin showed its specific crystal peaks around 2θ = 14.1, 21.1, and 25.1 (Figure 3d) which is similar to that obtained previously by Jeong and co-workers.[4]

FT-IR is an appropriate technique to study the chemical absorption or chemical interaction. The FT-IR absorption spectra of micron-size CSCCAP, nude CSCCAN, C-CSCCAN, and free ciprofloxacin were presented in Figure 4 (a, b, c and d, respectively). The presence of ciprofloxacin in the nanoparticles was confirmed by the peak at 1619, 1031, 940, 662, and 497 cm-^{1} (Figure 4c) resulting in broadening of the cited peaks compared with micron-size particles and unloaded nanoparticles spectrums.

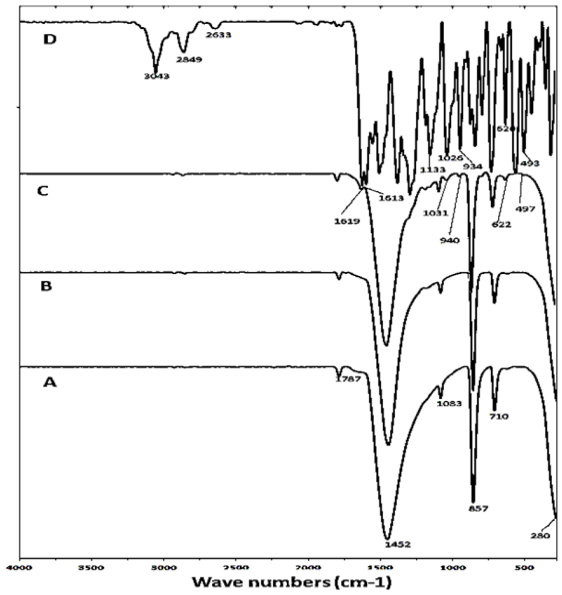

Figure 4: FT-IR spectra for micron-size cockle shells calcium carbonate aragonite powder (a), cockle shells calcium carbonate (aragonite) nanoparticles (b), ciprofloxacin-conjugated cockle shells calcium carbonate (aragonite) nanoparticles (c), and free ciprofloxacin (d) depicting the samples absorption or molecular interaction.

These data suggest that appreciable interaction exists between the drug and nanoparticles at molecular levels during the preparation of nanoparticles. Therefore, it can be deduced that attachment or loading of ciprofloxacin in the nanoparticles was effectively accomplished. Alike, the peaks at 1787, 1452, 1083, 857, 710, and 280 cm-^{1} were described as the universal distinguishing band features of the CO_3^{2-} in $CaCO_3$.[37] Figure 4 (a, b and c) exhibited characteristics of symmetric carbonate stretching vibration at 1083 cm^{-1}, a carbonate out-of-plane bending vibration at 857 cm^{-1} and a peak at 710 cm^{-1}, demonstrating formation of aragonite. These observations were supported by Naka *et al.*[38,16] No positional shifts of these peaks were observed before and after the synthesis of the nanoparticles.

***In vitro* Ciprofloxacin Release**

The ultimate and deliberate use of the CSCCAN as drug delivery vehicles of ciprofloxacin in this study, was to ensure prolong release of the antibiotic across cell membranes. The release profile of ciprofloxacin from CSCCAN was displayed in Figure 5. The release of ciprofloxacin from the nanoparticles involved an initial rapid release phase, which was followed by a slow sustained release phase. In the initial first-order release, approximately 70% of the drug were released in the first 144 hours, followed by a slow-sustained release profile for 504 hours where approximately 80% of the drug were released. However, this finding was consistent with the release mechanism described by Kamba *et al.*,[16] in which approximately 80% of the drug were released from aragonite calcium carbonate nanoparticles within 43 hours.

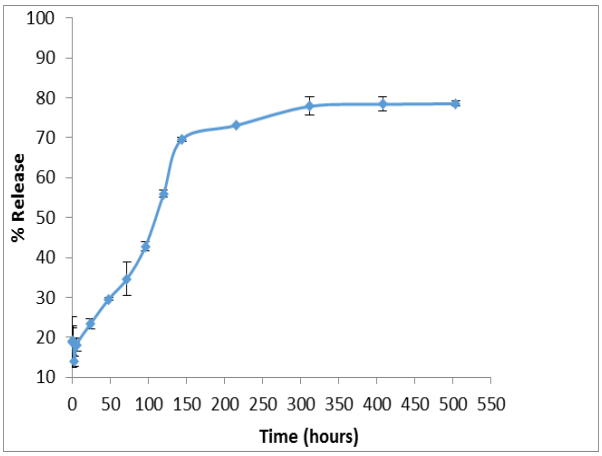

Figure 5: *In vitro* ciprofloxacin release profile of cockle shell calcium carbonate aragonite nanoparticles. Each bar represents mean ± standard deviation (n=3) of three independent experiments.

The data on drug release suggest that the system is very stable, and ciprofloxacin is well retained and/or localized in the core of the nanoparticles. Association of drug molecules with nanoparticles could occur in three different ways: at the surface of the nanoparticles, in the core as a reversible complex, or in the core as irreversible complex.[39] The drug release generally engaged different mechanism. The initial release may be attributed to the dissolution of the fraction of drug that is poorly entrapped or weakly bound to the nanoparticles large surface area. While the continuous slow release may be credited to the encapsulation and localization of the drug in the inner core of the nanoparticles.[5] Valizadeh and co-workers,[40] stated that the rate of drug release from any solid or semi-solid delivery system is usually controlled by dissolution and/or diffusion mechanisms.

High loading content in inorganic hybrids could result to release of high quantity of drugs, given that the diffusion driving force of the concentration gradient is enhanced. Accordingly the therapeutic efficiency will also be improved, since therapeutic efficacy is directly related to the amount of drug release from the carrier system.[41] This fact might be implicated in the most successful mechanism that improve ciprofloxacin efficacy (as shown in Table 2 below) and this strategy coupled with increased stability of the system at pH 7.4 might help to reduce drug toxicity.

Antibacterial Activity of Ciprofloxacin- CSCCAN nanoparticles Suspensions

The antimicrobial activities of free ciprofloxacin and C-CSCCAN formulations against *S.* Typhimurium were presented in Table 3. The free ciprofloxacin exhibits an *in vitro* MIC of 0.39µg/mL, whereas the MIC for C-CSCCAN suspensions was 0.19µg/mL. The MIC was reduced from 0.39 to 0.19 µg/mL, meaning the MIC value decreased more than 50% ($p < 0.05$). The MBC of C-CSCCAN suspensions and free ciprofloxacin were found to be 6.25µg/mL and 50 µg/mL, respectively. The former were about 8-fold more effective compare to the later in an in vitro model, considering that the MBC for the ciprofloxacin-nanoparticles formulations were approximately 8 times lower than that of free ciprofloxacin. In this case, the MBC value was decreased more than 80% ($p < 0.05$). Thus, the outcomes of the analysis were considered statistically significant. However, this study clearly suggest that the nude CSCCAN have no considerable inhibitory effect on bacterial growth of *S.* Typhimurium, indicating that aragonite polymorph of calcium carbonate do not have a cytotoxic effect against the bacteria tested. The same concentrations of nude CSCCAN, C-CSCCAN and the free ciprofloxacin were chosen to guarantee that the effect produced was as a result of the combination.

Table 3: Minimum inhibitory concentration and Minimum bactericidal concentration of ciprofloxacin-conjugated cockle shells calcium carbonate (aragonite) nanoparticles and free ciprofloxacin

Tested Bacterium	C-CSCCAN		Ciprofloxacin		CSCCAN	
	MIC	MBC	MIC	MBC	MIC	MBC
S. Typhimurium	0.19 µg/mL	6.25 µg/mL	0.39 µg/mL	50µg/mL	NI	NI

The values represent mean ± standard deviation (n=3); P>0.05 compared with ciprofloxacin. No inhibition (NI); ciprofloxacin-conjugated cockle shells calcium carbonate aragonite nanoparticles (C-CSCCAN); cockle shells calcium carbonate aragonite nanoparticles (CSCCAN).

The data obtained in the present study may declare that the formulations were successfully prepared and ciprofloxacin was continuously release from nanoparticles for a longer period, thus significantly extended the drug half-life compare to the free ciprofloxacin. The higher antibacterial effect of

ciprofloxacin nanoparticles may have resulted from nonspecific adsorption of the nanoparticles on the cell surface leading to higher diffusion of the particles through the bacteria. The nanoparticles once inside the cells might have specifically interfered with pumping activity of AcrAB-TolC efflux pump by depositing high dose of the antibiotic, in a sustained manner to practically outride the efflux pumps mechanism and suppressing the resistance of the bacteria. Since delivery systems like CSCCAN are not substrates of the AcrAB-TolC efflux pump transporter protein, the AcrAB-TolC over expression that acts synergistically with the outer membrane mutation was indicated to increase quinolones efflux from bacterial cell.[42] Similarly, time dependent studies and sustained mechanisms have demonstrated improvements in antimicrobial activity of ciprofloxacin encapsulated in carrier system, shortened the sterilization period.[32,43] The outcome of this study indicates that the effective dose of ciprofloxacin can be reduced against *S.* Typhimurium hence the side effects of the drug.

CONCLUSION

The potentials of ciprofloxacin encapsulation systems to provide controlled delivery of the drug and sustained activity against resistance bacterial infections have been reviewed in literatures. The physicochemical properties, drug loading and release properties of CSCCAN were studied. This study also established that the CSCCAN physicochemical properties mediated a sustained release which eventually influenced the antibacterial performance and increased susceptibility of *S.* Typhimurium. The encapsulation of ciprofloxacin may possibly modify the drug pharmacokinetics *in vivo*, by increasing permeation, serum half-life and reduce efflux so as to allow maximum tolerated dose. The MIC/MBC results bear significant implications, because reduction in dose frequency due to sustained release would certainly enhance patient compliance and the safety of treatment, subsequently, it may offer a great opportunity to overcome the emerging problem of antibiotic resistance among ranges of disease causing bacteria.

Conflict of Interests

The authors declare no conflict of interests.

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