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Research Article

Antimicrobial and Antibiofilm Potential of PEGylated Chitosan-Based Nano-Antibiotics against Multidrug-Resistant *E. coli* Strains

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Abstract

Antibiotic resistance is a major public health burden in the contemporary world, imposing high levels of mortality, morbidity, and financial losses annually. In the current study, chitosan (CS) and polyethylene glycol (PEG) based hybrid nanosystems were developed and their intrinsic and synergistic (with antibiotics) antibacterial activity was assessed against multidrug-resistant *Escherichia coli* strains, a widespread food and waterborne pathogen causing severe ailments. Enteropathogenic (EPEC) and enterotoxigenic (ETEC) strains of *E. coli* exhibited resistance against multiple classes of antibiotics. PEGylated CS nanosystems demonstrated higher encapsulation efficiency (EE) for ciprofloxacin (61.3 ± 0.76%), compared to ceftriaxone encapsulation (49.4 ± 0.52%). Scanning electron microscopy revealed a smooth surface and homogenous distribution of void and loaded nanosystems. Fourier Transform Infrared Spectroscopy (FTIR) spectra indicated no new chemical bonding and change in functional groups suggesting antibiotics were successfully incorporated into the nanosystems by electrostatic interactions. Growth kinetics and colony forming unit (CFU) assay revealed a restored activity of antibiotics encapsulated in hybrid PEGylated CS nano-conjugates against resistant *E. coli* strains. Furthermore, PEGylated CS hybrid nanosystems with intrinsic activity effectively curbed the biofilm formation in EPEC and ETEC strains. For future biopharmaceutical manufacturing, we propose that PEGylated-CS hybrid nanosystems can be a potential therapy against resistant *E. coli* and biofilm-associated chronic illnesses.

Keywords: Chitosan, hybrid nanosystems, nano-antibiotics, antimicrobial resistance, *E. coli*

1. Introduction

Antimicrobial resistance is a property exhibited by bacteria to grow and survive amid antimicrobial pressure causing increased morbidity and mortality in comparison to susceptible bacteria (Ambreen et al. 2023). The phenomenon of resistance has been seen against almost all antimicrobials including antimicrobials of last resort particularly utilized in severe life-threatening infections (Boolchandani, D'Souza, and Dantas 2019). It has been estimated that over 700,000 infected individuals die all over the world from antimicrobial resistance (Hayat et al.). If left unattended, it is feared that the death toll will rise to 10 million by 2050, which would culminate in a reduction of 2.5% in GDP worldwide (Ghosh et al. 2019). The evolution of resistant bacteria along with declining antibiotic development is claimed to be the commencement of the post-antibiotic era (Fair and Tor 2014). Furthermore, antibiotic resistance poses a serious threat to treating bacterial infections and performing procedures like chemotherapy, transplants, and surgery, which involve effective antibiotic prophylaxis (Sommer et al. 2017). There are several

mechanisms of resistance acquired by bacteria to counter antibiotic action, including impermeable barriers, genetic mutation (Poirel et al. 2010), inactivation of antibiotics (Wilson 2014), multi-drug efflux pumps, surface remodeling (Guilhelmelli et al. 2013), target mutation and modification (Crofts, Gasparrini, and Dantas 2017), manipulation of host cell pathways, and biofilm formation (Bhavsar, Guttman, and Finlay 2007).

Food and waterborne diseases are a continuous threat to public health globally. According to the WHO report, approximately 2.2 million deaths are caused per year by waterborne diseases (Singh et al. 2017) (Ramírez-Castillo et al. 2015). The burden of foodborne diseases is highest in the African population followed by the South East Asia region, and Eastern Mediterranean region (Cissé 2019). Moreover, children and the elderly are the most affected group by infectious diseases and acute diarrheal disease. This phenomenon continues to be the second leading contributor to mortality among children around the world (Palacio-Mejía et al. 2022). It is estimated that, after pneumonia, diarrheal diseases are the second most common cause of mortality in children under 5 years of age, which accounts for 19% of deaths approximately (Levine et al. 2020).

Among the diarrheal diseases, enteropathogenic *E. coli*, and *Vibrio cholera* are the major pathogens in low-income areas (Organization 2015). *E. coli* is a gram-negative bacteria that is present as a commensal in the gastrointestinal tract of humans and animals. It causes serious clinical ailments and foodborne disease outbreaks (Ye et al. 2017). It is estimated that it resulted in more than 2 million deaths per year by both intestinal and extra-intestinal diseases (Abia et al. 2017). Based on the mechanism of pathogenesis and the presence of virulent factors, *E. coli* is divided into four intestinal pathogens; *enteropathogenic* (EPEC), *enterotoxigenic* (ETEC), *enteroaggregative* (EAEC), *enteroinvasive* (EIEC). In addition, extraintestinal pathogens of *E. coli* include uropathogenic *E.coli* (UPEC), and Neonatal meningitis *E. coli* (NMEC) (Lu et al. 2016). In developing countries, children under the age of five are primarily affected by diarrheagenic *E. coli*, with the most common prototype being EPEC, ETEC, and EAEC(Alfinete 2020). The most common causes of travelers' diarrhea in adults are ETEC and EPEC. This can prove to be fatal for afflicted children under the age of 5 (Cabrera-Sosa and Ochoa 2020). The pathogens belonging to the EPEC family form lesions on the surface of epithelial cells of an intestine. The attached pathogen destroys the microvilli and destabilizes and rearranges the actin of the host cell to form pedestals below the site of attachment (Kalman et al. 1999). Additionally, ETEC attaches to epithelial cells of the small intestine, which is mediated through colonization factors (Nataro and Kaper 1998).

Nano-antibiotics are emerging as promising alternative medicines, capable of eradicating multi-drug resistant, and biofilm-forming pathogens (Pelgrift and Friedman 2013). The most prevalent strategy in this regard is the physical entrapment of antibiotics into a polymeric system to formulate antibiotic-loaded biodegradable polymeric nanosystems. This approach substantially enhances the solubility of hydrophobic antibiotics and perhaps antibacterial activity. Furthermore, owing to the versatile design of de novo synthesized polymers, they can respond to microenvironment stimuli i.e. pH, temperature, and enzymes from infected sites, and manifest multimodal response (Ding et al. 2019). Notably, controlled release, enhanced durability, increased absorption, better circulation, and targeted delivery are some of the other advantages that nano-antibiotics have over conventional antibiotics (Ovais et al. 2019).

Polymeric nanosystems include nanospheres and nanocapsules that can be prepared from one or more types of polymers as a single or hybrid system. They constitute different sizes from 10 to 1000 nm (Rao and Geckeler 2011). Moreover, polymers should be biocompatible with host cells,

non-toxic, non-immunogenic, and biodegradable in physiological conditions. Biodegradation inside the body plays a major role in the nanocarrier's pharmacokinetic profile, it affects controlled release properties and compatibility with other cells and tissues (Lerch et al. 2013).

Hybrid nanosystems have emerged as multifunctional drug carrier systems, combining the mechanical benefits of a polymeric core with the bio-stimulatory advantages of the shell into a single platform (Mandal et al. 2013). Hybrid nanosystems exhibit higher encapsulation efficiency (EE), increased level of cellular uptake due to nano-size with a sustained delivery option, and better penetration (Hadinoto, Sundaresan, and Cheow 2013, Rahman and Khalil 2023).

The objective of the present study was to develop potent PEGylated chitosan (CS) hybrid nanosystems loaded with antibiotics to overcome resistant food and waterborne pathogens. In addition, antimicrobial activity against multi-drug resistant *E.coli* and antibiofilm activity was also investigated in this study. We think that this system can control the resistance in multi-drug resistant pathogens and can prove to be a very promising approach for drug delivery of antimicrobial drugs.

2. Material and Methods

2.1 Materials

Growth media (Muller Hinton Nutrient broth and Nutrient ager) for strains' growth and preservation were purchased from Sigma-Aldrich. CS powder with 75-85% deacetylation, 200-800cP viscosity of 1% w/v in 1% acetic acid was procured from Sigma-Aldrich. Tripolyphosphate (TPP) was also purchased from Sigma-Aldrich. Acetic acid was ordered from Riedel-de Haen and all antibiotic discs were obtained from Oxoid, UK. Ciprofloxacin drug powder was obtained from Global Pharmaceuticals (Pvt) Ltd., and ceftriaxone was purchased from a local pharmacy.

2.2 Collection of Multi-Drug Resistant Strains

Samples were collected from the Microbiology and Public Health Laboratory, COMSATS University Islamabad, Pakistan. These strains were cultured on nutrient agar media and were stored at -80º in 20% glycerol stock. Two diarrheagenic strains of *E. coli* (EPEC, ETEC) were selected for experimentation, which were characterized for their identification and resistance. *E.coli* is a rod-shaped, gram-negative, oxidase-negative bacterium, which belongs to the *Enterobacteriaceae* family. It plays a major role in diarrhea-related mortality and morbidity in developing countries, resulting from both diarrheal outbreaks and sporadic cases (Saka et al. 2019). *E.coli* is also involved in the formation of biofilms on food and contact surfaces, biofilms are more resistant to antimicrobials, disinfectants, and environmental conditions (Dávila-Aviña et al. 2020).

2.3 Resistance profiling of *E. coli* strain

Standard Kirby Buyer disk diffusion method was used for the analysis of resistance profiling of selected pathogens (Nain, Islam, and Minnatul Karim 2019). EPEC and ETEC strains were subjected to growth on Muller Hinton media and tested antibiotic disks were placed on growth media. Agar plates were incubated overnight, and zone of inhibitions (ZOIs) were measured after 24 hours. The resistance pattern of pathogens against different classes of antibiotics was identified. The antibiotic disks included Minocycline (MH30), Amoxicillin-Clavulanic acid (AMC30), Levofloxacin (LEV5), Aztreonam (ATM30), Ciprofloxacin (CIP5), Cephazolin (KZ30), Sulpha-methoxazole (SXT25), Cefoxitin (FOX30), Azithromycin (AZM15), Erythromycin (E15), Doxycycline (DO30), and Ceftriaxone (CRO). All bacterial strains were marked resistant or sensitive based on their ZOI (Table S1, supplementary material), according to quality control data obtained by the Clinical & Laboratory Standards Institute (CLSI) guidelines (Weinstein and Lewis 2020).

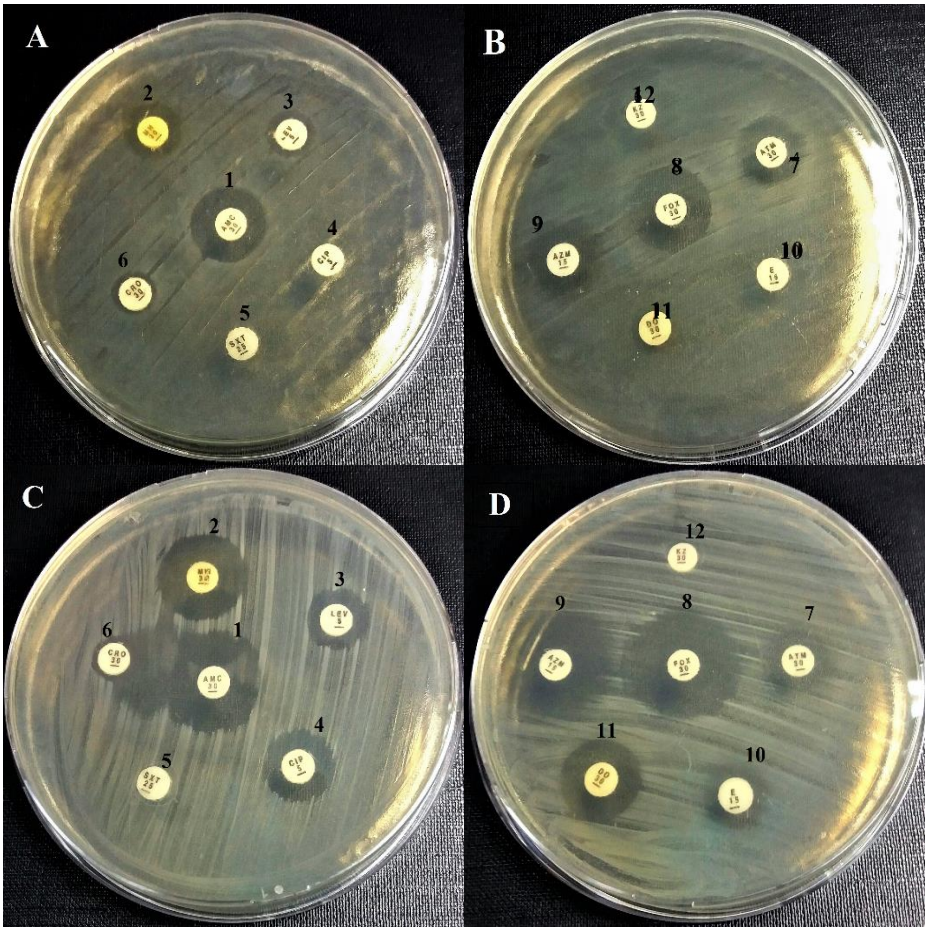

Figure 1: Multi-drug resistance profiling of E.coli strains, enterotoxigenic E.coli (ETEC) (A, B), and enteropathogenic E.coli (EPEC) (C, D) against tested antibiotics. The antibiotic discs of different classes in sequence with numbering include 1 (AMC), 2 (MH 30), 3 (LEV5), 4 (CIP5), 5 (SXT25), 6 (CRO), 7 (ATM30), 8 (FOX30), 9 (AZM15), 10 (E15), 11 (DO30), 12 (KZ30).

2.4 Extended Spectrum β-Lactamase Detection

One of the mechanisms of resistance adopted by bacteria is the formation of extended-spectrum β-lactamase (ESBL) enzymes, produced as a result of mutation. This enzyme equips bacteria against third-generation cephalosporins, extended-spectrum penicillin, and monobactam antibiotics (McDanel et al. 2017). These enzymes are particularly found in the members of the Enterobacteriaceae family including *E. coli*, which is a clinically significant ESBL-producing pathogen (Malande et al. 2019). The method employed for ESBL testing was a double-disc synergy test. Test strains were cultured in Muller-Hinton media, and an amoxicillin-clavulanic acid disc was placed in the center. Third-generation cephalosporin discs were placed 20 mm apart from the central disc. After overnight incubation, a keyhole phenomenon was detected due to clavulanic acid activity (Ejaz 2013).

2.5 Determination of Minimum Inhibitory Concentration (MIC)

Estimation of MIC of ciprofloxacin and ceftriaxone antibiotics was carried out by agar-well diffusion assay. Stock solution was prepared for both antibiotics with a concentration of 20 mg/mL. Subsequently, antibiotic dilutions were prepared from this stock solution. Petri plates with inoculated medium were prepared, and after solidification of inoculated media wells were

bored with a sterile borer. Afterward, 20 µL of each antibiotic dilution was poured into the respective wells. After overnight incubation, zones of inhibition (ZOI) were observed for each well. The lowest concentration with a ZOI after 24 hours is the MIC of the particular drug.

2.6 Quantification of Antibiotics

Quantification of antibiotics was carried out by UV-Visable spectrophotometer. Volumetric dilution of antibiotics ceftriaxone, and ciprofloxacin were prepared and poured symmetrically in a 96-well plate. Using a UV spectrophotometer, a wave scan was carried out at 200-900 nm range to obtain λ_{max}. The highest and most clear visible peak in the graph indicates λ_{max}, and dilutions were run in triplicate and all the readings were taken at λ_{max}. Moreover, data was plotted in MS Excel and analyzed with the help of a graph. By using the average absorption of three replicates graph was plotted and a calibration curve was obtained by Bradford assay (Niaz, Shabbir, Noor, Rahman, et al. 2018). By linear regression, a trend line was obtained along with a standard equation, and the value of the coefficient of determination (r^2) was calculated.

2.7 Fabrication of PEGylated-Chitosan Hybrid Nanosystems

PEGylated CS nanosystems were fabricated through a modified ionic gelation method. To begin, 2% (w/v) CS was dissolved in 1% (v/v) acetic acid solution. This solution was stirred overnight for complete dissolution. On the next day, 1% TPP solution was prepared and added drop-wise to the CS solution (Niaz, Nasir, et al. 2016), and antibiotics were directly dissolved in the TPP solution. The resulting solution was subjected to stirring for 30 minutes, followed by sonication for 10 minutes at 25,000 rpm. Furthermore, 2% (w/v) PEG solution, which was stirred overnight, was added to sonicated CS solution drop-wise and magnetically stirred for 2 hours approximately. After stirring, the PEGylated CS solution was again sonicated for 25 minutes at 25,000 rpm. Pressure waves are created by the mechanical vibration of the sonicator, as a result, shear agitation is generated. This shear action results in the conversion of large particles into nanoparticles.

2.8 Scanning Electron Microscopy (SEM)

For SEM analysis, a single drop of nanosuspension was placed on a 1×1 cm clean glass slide and dried completely. It was subjected to gold coating to a maximum of 250 Å for 6 seconds, using a Jeol Quick Auto Coater (JFC-1500) ion sputtering device. Moreover, the anodic current and anodic voltage used during the process were 7mA and 1Kv. The scanning electron microscope used was Jeol JSM 6490 A, with a magnification of 3000-50,000 and a resolution of 20kV (Niaz, Shabbir, et al. 2019).

2.9 Fourier Transform Infrared Spectrum (FTIR)

FTIR analysis is a type of spectrophotometry used for qualitative analysis of organic compounds, providing information about molecular structure, chemical bonding, and molecular environment. Moreover, it also relays detailed information about the mechanisms of molecular reactions (Mohamed et al. 2017). The liquid sample of PEGylated-CS hybrid nanosystems was analyzed by placing a drop on the glass plate. However, the drug samples were prepared by grinding the powder and their subsequent blending with KBr, with a 1:5 ratio of sample to KBr, respectively. Furthermore, it was compressed to form discs. Spectra were recorded in the 4000-400cm^{-1} range, resolution was 4 cm^{-1}, with the absorbance mode of 8-128 scans at room temperature (Niaz, Ihsan, et al. 2019).

2.10 Encapsulation Efficiency of Antibiotics in Hybrid Nanosystems

To evaluate the EE of drug-loaded nanosystems, an indirect method was applied. After fabrication of nanosystems loaded with antibiotics, they were subjected to centrifugation at 12,000 rpm for 20 minutes. The supernatant obtained after centrifugation was picked and the pallet was discarded. Afterward, the free drug-containing

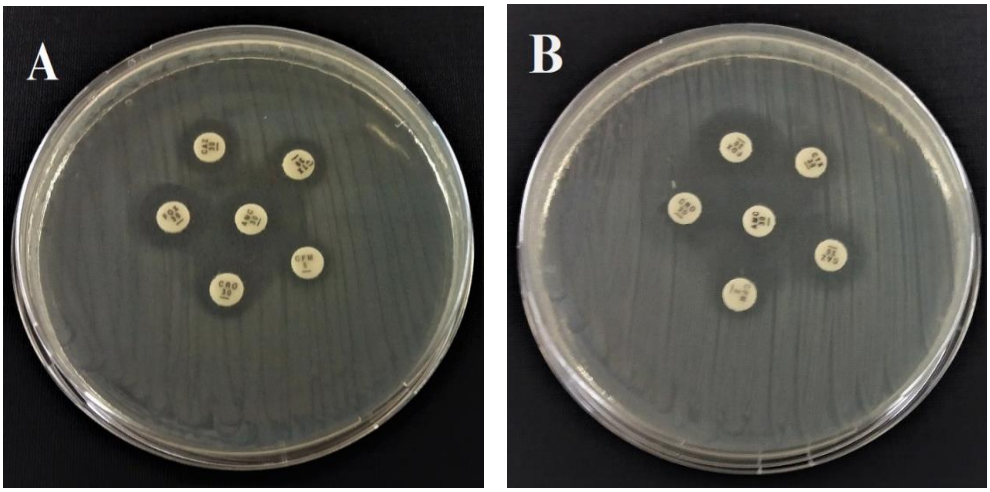

Figure 2: Detection of ESBL phenomenon in two resistant strains of E.coli, ETEC (A) and EPEC (B).

supernatant was quantified by UV-spectrophotometer at the optimum wavelength (556 nm) obtained from the wave scan. EE values were estimated by adding OD values to the standard curve equation. Using the formula for EE, the percentage of encapsulated drug was calculated by triplicate analysis.

$$EE\% = \frac{Total\ amount\ of\ drug - Unencapsulated\ drug}{Total\ drug} \times 100$$

2.11 Antimicrobial Potential of PEGylated Chitosan Nanosystems

The antimicrobial action of nanosystems and antibiotics was evaluated through a micro-dilution broth assay. Fresh colonies of *E. coli* were obtained from a solid nutrient agar medium. Afterward, the nutrient broth was inoculated with *E.coli* strains (EPEC, ETEC), and was compared to Mac-Farland solution, and incubated overnight at 37°C. Inoculated broth was poured into titers of 96 well plates and 100µL of blank nanosystems, loaded PEGylated CS nanosystems, and antibiotic dilutions (ciprofloxacin, ceftriaxone) were added to the wells along with positive and negative controls. After this, plates were placed in an incubator at 37 °C, and OD values were measured at 595 nm at different intervals for 5-7 days.

2.12: Colony Forming Unit Assay

A colony-forming unit (CFU) assay was performed to determine the number of viable colonies in the presence of different concentrations of the antibacterial agent and nano-encapsulated antibiotics. For this purpose, bacterial cultures were inoculated with different concentrations of free and encapsulated antibiotics. The bacterial suspensions were serially diluted and plated on a nutrient agar plate and incubated at 37 °C. CFU was calculated by multiplying the number of countable colonies with a dilution factor.

2.13 Anti-biofilm Assay

This assay was done to assess the anti-biofilm activity of ciprofloxacin and ceftriaxone-loaded PEGylated CS nanosystems using a modified protocol reported previously. Moreover, a 96-well plate was used in the process and results were obtained by ELISA plate reader (Stepanović et al. 2000). From freshly cultured pathogens on a Petri plate, the inoculum was prepared by using a sterilized cotton swab. Its turbidity was compared to McFarland's solution. Additionally, the nutrient broth was added to a 96-well plate, with both positive and negative control. An equal amount of drug solution and nanosystems were added to the growth medium in each well. Then the plate was incubated for 48 hours in the incubator at 37^0C. After 48 hours, planktonic cells were removed

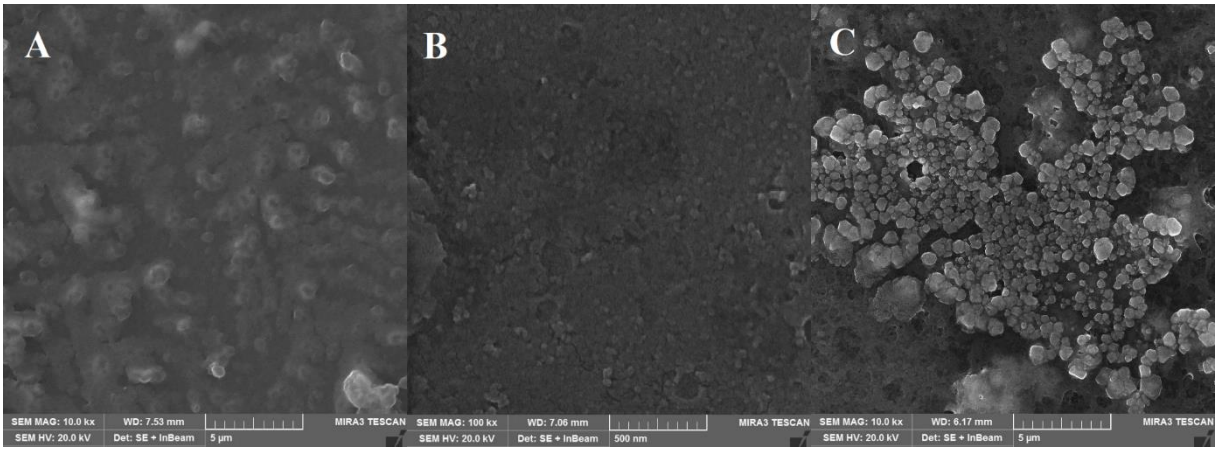

Figure 3: Scanning electron microscopic visualization of void/blank PEG chitosan nanosystems at 5 µm (A), ciprofloxacin-loaded nanosystems at 500 nm (B), and ceftriaxone loaded nanosystems at the scale of 5 µm (C).

through extraction by micropipette, and washing was done properly. Afterwards, 200 µL of ethanol was added for washing, and discarded after 15 minutes. Subsequently, 200 µL of 0.1% crystal violet was then added to the wells (Kart et al. 2017) After 15 minutes, crystal violet was discarded and 33% (v/v) acetic acid was added in wells as a decolorizer, which completely solubilized the crystal violet. The 96-well plate was again incubated at room temperature for 15 minutes. This solution was transferred to another plate and absorbance was taken in a Multiskan Go ELISA plate reader at 595 nm.

$$Biomass\ inhibition\ \% = 100 - \left[\frac{Optical\ density\ of\ sample}{Optical\ density\ of\ control} \times 100\right]$$

3. Results and Discussion

3.1 Resistance Profiling

Resistance profiling for multi-drug-resistant *E. coli* strains was carried out by using commercially available antibiotic disks from different classes of antibiotics. ZOIs were compared to CLSI guidelines to determine their resistance to different classes of antibiotics (Table S2 supplementary material).

ETEC was resistant to multiple classes, even against next-generation antibiotics, like second-generation fluoroquinolones i.e. ciprofloxacin, and third-generation cephalosporin, like ceftriaxone, as well as macrolides. Only cefoxitin and azithromycin showed sensitivity against ETEC (*Figure 1A, B).* Whereas, EPEC was also resistant to second-generation fluoroquinolones, and third-generation cephalosporin i.e. ceftriaxone (*Figure 1C, D*). It was sensitive only to azithromycin, cefoxitin, doxycycline, and ampicillin while showing resistance to all other antibiotics. The results showed the high level of resistance the food and waterborne pathogens have acquired, even to the third generation of antibiotics. These results corroborate the resistance reports of *E. coli* strains in different regional references. In this study, third-generation cephalosporin i.e. ceftriaxone, and second-generation fluoroquinolone, ciprofloxacin, having complete resistance against selected strains, were selected for incorporation into PEGylated CS nanosystems. A study indicated that a 20% resistance rate of *E.coli* to ciprofloxacin, through an aggregate resistance database of in-patients, led to treatment failures (Hitzenbichler et al. 2018). In a study conducted in Pakistan, 87.6% of clinical samples received were resistant to ciprofloxacin, CTX-M type β-lactamase is the most prevalent enzyme in the Pakistani population causing

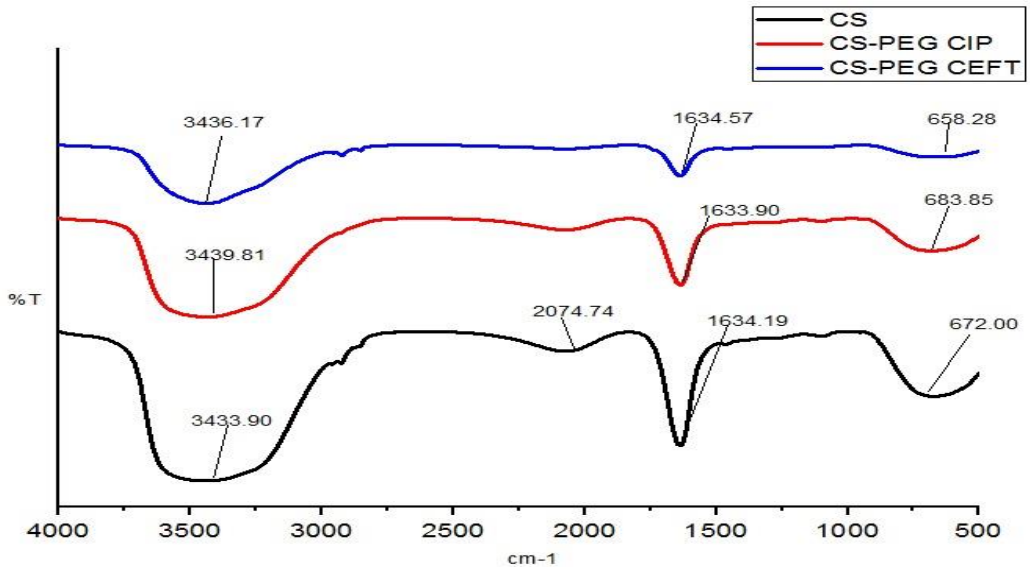

Figure 4: Comparative FTIR spectrum of void PEG chitosan hybrid nanosystems, ciprofloxacin-loaded nanosystems, and ceftriaxone-loaded nanosystems.

resistance with a prevalence of 57.7% (Hussain et al. 2011).

3.2 Extended Spectrum β-Lactamase Phenomenon Observation

The most reported antibiotic resistance mechanism is the production of β-lactamase enzymes, against widely used β-lactam antibiotics (Rogers, Sidjabat, and Paterson 2011). Notably, ESBL presence in pathogens is increasing worldwide, including in Asia, which causes resistance against broad-spectrum β-lactam antibiotics. In addition to resistance, they also serve as virulence determinants, responsible for extraintestinal infections (Sidjabat and Paterson 2015).

E. coli pathogenic strains in this study ETEC and EPEC, tested positive for ESBL. Double-disc synergy test and CLSI guidelines confirmed the presence of ESBL in both strains. In addition, the lock and key model was also observed and there was a clear indication of the presence of extended-spectrum β-lactamases (*Figure 2A, B*).

E. coli is the major ESBL-producing bacteria, causing more severe infections as compared to non-ESBL-producing strains. The broad spectrum β-lactams contain rings known as β- β-lactam rings in their structure that inhibit the synthesis of cell walls in bacteria. ESBL-producing strains of *E. coli* hydrolyze the β-lactam ring, rendering the antibiotics ineffective, and limiting the choice of drugs. ESBL genes are primarily transferred to other bacteria by horizontal gene transfer by plasmids, and sometimes by conjugation mechanism(Sah and Hemalatha 2015).

3.3 Estimation of minimum inhibitory concentration (MIC)

ETEC showed a high value of MIC of 5000 µg/mL, for both ciprofloxacin and ceftriaxone, which indicates a very high resistance of ETEC strain against third-generation cephalosporin (ceftriaxone) and fluoroquinolones (ciprofloxacin). ETEC is a primary cause of traveler's diarrhea and it is among the most prevalent infections among travelers, particularly during a visit to low or middle-income countries (Kantele et al. 2015). Ceftriaxone, a third-generation cephalosporin, is used in diarrheal infection; unfortunately, it shows a very high value of MIC. Moreover, fluoroquinolones are frequently prescribed antibiotics for traveler's diarrhea, and it is also used as preventive medicine to avoid infection in

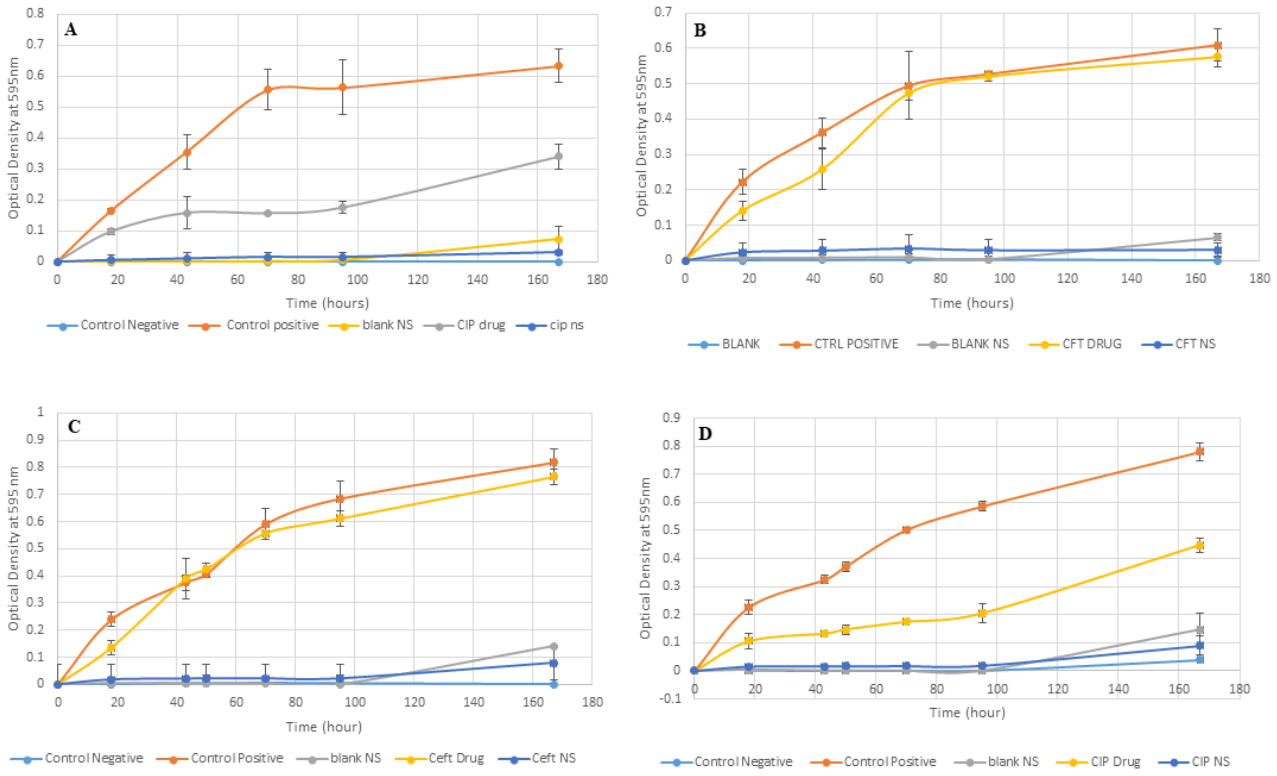

Figure 5: Qualitative antibacterial analysis of ciprofloxacin, void and ciprofloxacin encapsulated nanosystems (A), ceftriaxone, void/blank and ceftriaxone encapsulated nanosystems (B) against enterotoxigenic E.coli (ETEC), similar formulations were tested against resistant enteropathogenic E.coli (EPEC) (C) (D), respectively.

immunocompromised patients while traveling. Studies suggest ciprofloxacin exhibits resistance up to 62.5% in ETEC strains (Guiral et al. 2019).

EPEC strain also showed resistance to both the antibiotics ciprofloxacin and ceftriaxone. In the present investigation, ciprofloxacin and ceftriaxone had a MIC of 100 µg/mL and 2500 µg/mL, respectively. This indicates a very high level of resistance, even at a very large concentration of third-generation antibiotics.

Enteropathogenic *E. coli*, is considered a primary cause of diarrhea in developing countries (Aslani and Alikhani 2009). Commonly used antibiotics like cephalosporins, and fluoroquinolones are now becoming increasingly ineffective against EPEC strains as elucidated by the results above. Mutation induced in pathogens, perhaps due to the frequent use of third-generation cephalosporins has contributed to the emergence of ESBL strains (Karami et al. 2017). The result of resistance in third-generation cephalosporins and fluoroquinolones is manifested in the form of high mortality, economic burden, and prolonged hospital stay (Vogt et al. 2014).

3.4 Scanning Electron Microscopic Evaluation

Morphological characterization of void/blank CS-PEG system (*Figure 3A*) and antibiotic-loaded nanosystems with ciprofloxacin (*Figure 3B*) and ceftriaxone (*Figure 3C*), respectively, was done by SEM. Nanosystems were spherical, and well-formed, with regular and smooth surfaces. The size of the nanoparticles was appropriate and within the range. Nanosystems, loaded with ciprofloxacin, were observed to be more homogeneous and smaller as compared to the void ones. Here, it can be concluded that the drug might act as a surfactant, which increases the dispersity. On the other hand, ceftriaxone-loaded

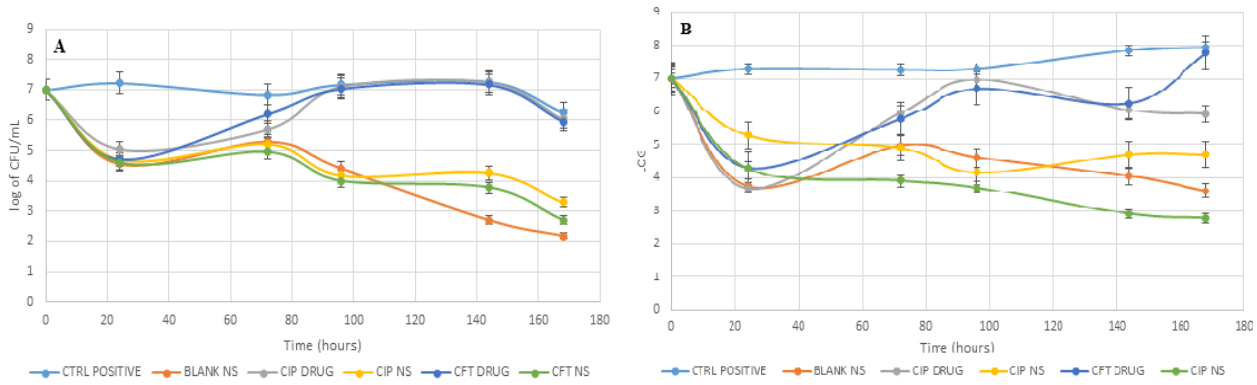

Figure 6: Graphical representation of colony forming unit assay of void/blank nanosystems along with void and ciprofloxacin or ceftriaxone encapsulated nanosystems against ETEC (A), and EPEC (B).

nanosystems were more uniform, bigger in size, dense, and concentrated. There was no detection of any crystalline structure in the images obtained which indicates that the drugs were well encapsulated inside the nanosystems.

3.5. FTIR Spectroscopy

FTIR Spectroscopy was performed to determine the interaction between antibiotics and CS biopolymers in nanosystems (Figure 4). In FTIR analysis of the nanosystems, four major characteristic peaks were obtained at 3433, 2074, 1634, and 672 cm^{-1}. Peaks obtained are attributed to different bonds, 3433 represents (N-H) bond, 2073 (C-C) bond, 1615 ($C-NH_2$).

These results illustrate the interaction between the phosphate group of TPP and the amino group of CS. In addition, cross-linking between CS and PEG polymer showed no molecular reaction, as evidenced by insignificant differences in peaks. Peaks have emerged because of the inter- and intra-molecular action of PEGylated CS systems. Therefore, the FTIR study of the loaded system showed no significant chemical interaction after the encapsulation of antibiotics in nanocarrier systems. Interestingly, peaks for both encapsulated and void nanosystems were substantially similar. It is indicative of the fact that the drug was encapsulated inside nanosystems and it was not chemically bound with any other component of the nanosystems. Moreover, it indicated that the drug was not attached to the surface, hence their chemical structures are unaltered and the surface chemistry remained the same (Niaz, Shabbir, Noor, Abbasi, et al. 2018).

3.6 Determination of Encapsulation Efficiency

The two antibiotics, ciprofloxacin, and ceftriaxone, were selected based on availability and cost-effectiveness. EE for ciprofloxacin-loaded nanosystems was found to be (61.3 ± 0.76%), whereas ceftriaxone was found to be (49.4 ± 0.52%). Notably, EE for ciprofloxacin was relatively higher as compared to ceftriaxone but overall, both showed average encapsulation within the nanosystems. This may be attributed to positive charges of both drugs and CS that resulted in repulsion, hence reduced encapsulation (Sobhani et al. 2017). The difference in the entrapment depends primarily on the surface charge, size, and polymeric composition of the nanosystems, as well as the hydrophobicity of the drug entrapped inside the nanosystems (Niaz, Shabbir, et al. 2016) (Niaz, Nasir, et al. 2016). Since hybrid nanosystems are formulated by PEG and CS, therefore, cross-linking between two polymers and their interaction is also a major determinant of EE.

3.7 Antimicrobial Assay

The growth kinetics of pathogens, under the influence of PEGylated CS-based nano-antibiotics, were compared to free drug, leading to significant

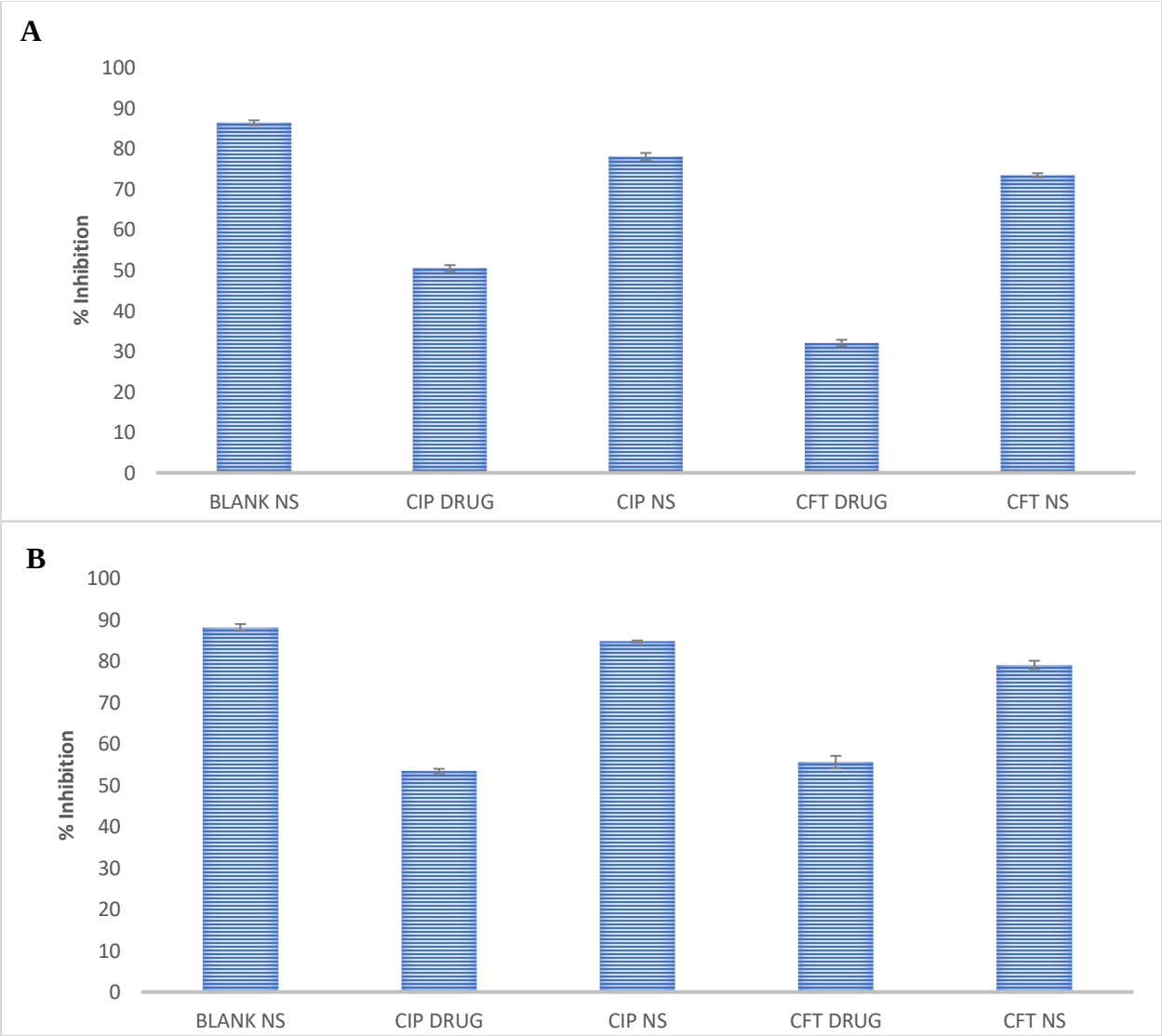

Figure 7: Antibiofilm analysis of nano-formulations, percent inhibition by void/blank nanosystems with void and ciprofloxacin or ceftriaxone encapsulated nanosystems against ETEC (A), and EPEC (B).

results. Prepared nanosystems were able to substantially reduce bacterial growth, in comparison to the unencapsulated antibiotics. There is a significant delta range of antimicrobial efficacy between ciprofloxacin-loaded nanosystems and free ciprofloxacin drug. Furthermore, loaded nanosystems showed remarkable activity and bacterial growth was almost completely inhibited. Similarly, void nanosystems also controlled the growth of resistant pathogens; however, after 150 hours, bacterial growth was observed with void nanosystems, while drug-loaded nanosystems were able to thwart the growth of ETEC (*Figure 5A, B*). This indicated the sustained-release activity of dug-loaded nanosystems, even after 150 hours. ETEC was completely resistant to ceftriaxone, whereas encapsulated ceftriaxone and void nanosystems effectively controlled the growth, almost suppressing it. Similarly, a substantial activity of the nanosystems was observed against EPEC strain. Encapsulated drug and void nanosystems showed remarkably good activity

and control as compared to unencapsulated drug (*Figure 5C, D*).

Earlier studies also suggest the activity of encapsulated ciprofloxacin nanoparticles against gram-negative *E. coli*. The presence of cell walls in gram-negative bacteria can enhance the resistance due to an extra barrier to the entry of antibiotics. CS with encapsulated drug enhances the potency of antibiotics with low or no efficacy at all, resulting in increased penetration of antibiotics into the bacterial cell, enhanced drug delivery to the site of action, and better stability of encapsulated drug into the nanosystems (Sobhani et al. 2017). Moreover, the activity of CS nanoparticles with loaded ceftriaxone is also reported against *E. coli*, reinforcing the results obtained in this study (Mushtaq et al. 2017). PEGylated CS nanosystems were used for the first time in this study against food and waterborne pathogens. Also, PEG provides additional qualities to nanosystems, as mentioned in previous studies. For many years, PEGylation has been successfully adopted for improvement in drug delivery systems. There can be multiple factors behind improved delivery with PEG incorporation in nanosystems. For example, hydrophilic chains of PEG (around 2000-13,000 Da) create a buffer zone around the nanosystems, reducing the interaction with plasma proteins and uptake by macrophages (Amoozgar and Yeo 2012). Furthermore, high hydrophilicity attained on the surface results in low immunogenic response and antibody detection of the nanosystems. Overall, PEGylation leads to improved bioavailability by increasing the circulatory time of the nanosystems and also changes in drug release rate (Eslami, Rossi, and Fedeli 2019).

3.8 Colony Forming Unit (CFU) Assay

To further confirm the data obtained by growth kinetics, a CFU assay was performed in which viable colonies were counted. There was more than 4 log units decrease in the growth of ETEC, while, EPEC exhibited a decrease of 5 log units, with the nanosystems loaded with antibiotics. For the ETEC strain, during the first 20 hours, both unencapsulated and encapsulated ciprofloxacin and ceftriaxone drugs controlled the bacterial activity (*Figure 6A*) but gradually free antibiotics lost their activity and bacteria grew exponentially. However, void nanosystems and drug-loaded nanosystems effectively controlled bacterial growth. For the EPEC strain, the free ceftriaxone drug completely failed to control the bacterial growth (*Figure 6B*). Whereas ciprofloxacin in unencapsulated form showed some activity, it was significantly low as compared to the encapsulated drug. Notably, void nanosystems were also able to control the bacterial growth showing intrinsic antimicrobial potential that should be further studied. CS polymer is widely used in the formulation of nano-antibiotics, owing to its biocompatibility and biostability, whereas, PEG provides stealth properties inside the body. Studies have shown that the PEGylation on the surface delays the elimination by the reticuloendothelial system, which is beneficial for the delivery of small molecule drugs effectively. Polymeric nanoparticles have the capability of drug release through hydrolysis (Abeylath et al. 2009) (Huh and Kwon 2011). CS has inherent antimicrobial activity, as proven in the earlier literature. The synergistic effect of CS and antibiotics released from nanosystems evading resistance mechanisms prove to be potent against multi-drug resistant *E.coli* and the microbial defense system is overwhelmed (Labreure, Sona, and Turos 2019).

3.9 Anti-biofilm Assay

Biofilm-forming bacteria are a major cause of prosthetic device-induced infections, leading to serious medical issues (Moteeb 2008). Preventive biofilm assay was performed by inoculation of bacteria with different formulations simultaneously. Anti-biofilm formation by EPEC and ETEC was sufficiently reduced, up to 80% by using nano-antibiotics, in contrast to free antibiotics, which almost failed to control biofilm

formation. The superior activity of loaded nanosystems can be observed in comparison to unloaded antibiotics, against ETEC biofilm *(Figure 7A).* Therefore, the loaded system is showing higher activity against ETEC biofilm and can be effective in the removal, and prevention of biofilm in medical, and surgical applications. In *(Figure 7B),* the activity against EPEC strain is shown, where loaded nanosystems have exhibited around 80% to 85% inhibition, which is significantly high. In contrast, both antibiotics showed a percentage inhibition of almost 50% which is significantly low. Additionally, blank nanosystems also showed impressive activity by thwarting biofilm growth, pertaining to the inherent antimicrobial activity of CS.

CS is a copolymer of glucosamine and N-acetyl glucosamine and has potential for biomedical applications due to its intrinsic antimicrobial potential. It is biocompatible and biodegradable, making it suitable for in-vivo applications (Fahmy et al. 2022) (Dutta, Dutta, and Tripathi 2004). The poly-cationic surface of CS interacts with the negatively charged membrane of pathogens, disturbing the properties of their barrier, hence, causing leakage of intracellular components and also preventing nutrient entry into the bacterial cell (Ruiz and Corrales 2017). Moreover, PEG is a non-toxic polymer with having amphiphilic nature, as it can dissolve into both water and organic solvents. Biologically active materials when linked to PEG, can have enhanced properties of pharmacodynamics because of the amphiphilic nature of PEG (Pintér et al. 2009). Surface modification with PEG-like PEGylated conjugates provides better stability, enhanced controlled release, low immunogenic response, and prolonged systemic circulation time (Ghitman et al. 2018).

CS nanosystems can effectively penetrate into bacterial biofilm and damage it, this can be attributed to the positive charge of CS that can disrupt negatively charged bacterial membrane, altering its permeability (Tan et al. 2018). Subsequently, it binds to DNA and inhibits its replication, causing cell death. Another proposed mechanism is that it acts as a chelating agent, binds to certain trace metal elements, and produces toxins, which stops bacterial growth (Divya et al. 2017). Biofilms help bacterial resistance development to conventional antibiotics and antimicrobials via metabolic dormancy and molecular persistence programs. In addition, cell density also plays a part in imparting resistance (Koo et al. 2017). Biofilm matrix serves as a barrier to entry of antibiotics. Recent studies have established the activity of CS-based nanosystems against biofilms. Moreover, antibiotics encapsulated in CS nanosystems were also able to overcome resistance (Khan et al. 2019). Nanosystems can penetrate the biofilm and halt t bacterial growth by hitting multiple targets, which include metabolism, biofilm matrix components, and quorum sensing system. However, unencapsulated antibiotics were unable to get into the biofilm and biofilm formation couldn't be controlled.

4. Conclusions

Food and waterborne *E.coli* are widespread throughout the world and the evolution of such common and prevalent pathogens into resistant strains can play havoc in our lives. As the post-antibiotic era is fast approaching, there is a dire need to formulate new effective treatment strategies to overcome bacterial resistance. Biodegradable polymers hold great potential for future therapies. PEGylated-CS hybrid systems formulated in this study have proven to be very potent against multi-drug-resistant *E. coli*. Moreover, hybrid nanosystems loaded with conventional antibiotics effectively managed to overcome resistance in multi-drug resistant *E. coli*, as well as biofilm formation was actively thwarted. Based on the promising observations in this study, PEGylated-CS nano-conjugates-based nano-antibiotics can be further tested for in-vivo efficacy along with assessing its toxicity profile.

Conflict of Interest

The authors declare that they have no competing interests.

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Study Approval

This stdy was approved by the COMSATS University Islamabad, Pakistan.

Consent Forms

NA.

Authors Contribution

SA and AS carried out all the data collection, bench work, and manuscript writing. ON helped in data collection and statistical analysis. AI and MI conceptualized and supervised the study.

Data Availability

Data is available upon reasonable request from the corresponding author.

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