Synergistic antibacterial effect of streptomycin-loaded starch nanocrystals with antibiotics Ciprofloxacin and Polymyxin B

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Abstract

Multi-drug resistance among various bacteria is a major challenge in the medical field. In the present study, we used streptomycin-loaded starch nanocrystals (SSNCs) to assess its synergistic antibacterial effect with two antibiotics, ciprofloxacin and polymyxin B to combat drug-resistant bacteria. The streptomycin-loaded starch nanocrystals were structurally characterized using FTIR and SEM to confirm their improved properties. FTIR analysis revealed the possible interaction between streptomycin and starch nanocrystals by measuring its absorbance, thereby confirming that the streptomycin could effectively load onto starch nanocrystals. The SEM images of the modified starch nanocrystals revealed the aggregated topography of streptomycin-loaded starch nanocrystals.

Additionally, SSNCs could prolong the sustained release of streptomycin for more than one week. The synergistic antimicrobial activity due to the combination of SSNCs with two antibiotics, polymyxin and ciprofloxacin, emphasized the role of starch nanocrystals as a nanocarrier for different antibiotics and this combination approach will enable the reduction of their effective doses. Therefore, the present study on using a combination of SSNCs and antibiotics suggests its potential use as a strategy to combat different pathogens.

Keywords: Ciprofloxacin, Polymyxin B, Starch nanocrystals, Streptomycin, Synergistic activity.

Introduction

The continuous enhancement in multi-drug resistance among bacteria is one of the critical issues in global public health. In this context, developing new effective antibiotics/antimicrobials, as well as new drug delivery and targeting strategies, gains prime importance. Nanomaterials are endowed with various unique size-dependent physico-chemical features including high surface-to-volume ratio, tunable size, shape, biocompatibility, drug loading, releasing and targeting capacity. Taking advantage of these improved properties, the nanomaterials can act as a promising solution to combat multi-drug resistant bacteria to a greater extent and can also act as a nanocarrier for various drugs including antibiotics and antimicrobials. The combination of antimicrobials with nanoparticles helps to address toxicity issues, to adverse side effects due to high systemic drug concentrations and frequent dosing[10].

Furthermore, in this strategy, coupling of nanoparticles with the antimicrobials enables the inhibition of the activity of bacterial efflux pumps, inhibits the formation of biofilms, prevents quorum sensing and causes plasmid curing which in turn helps to combat multi-drug resistant bacteria[5]. Polymeric nanoparticles with unique physico-chemical properties and exceptionally high surface-to-volume ratio make them ideal for sustained or controlled delivery of drugs, nutraceuticals, cosmetics etc. Starch is the second most abundant biomass material in nature and most plants use it as a source of energy. It is one of the most commonly used biopolymers in various products because of its total biodegradability, biocompatibility, stability in storage, cost-effectiveness and simple fabrication method. Its unique semicrystalline structure favours the production of biobased elements like starch nanocrystals or starch nanoparticles.

Several research activities are being carried out to utilize the potential of starch nanocrystals that make them appealing in various applications like drug delivery and food packaging and research works are being carried out to exploit the different physical assets of starch nanocrystals. Different biocomposites used in food packaging and other medical applications were developed by blending starch nanocrystals with different biopolymeric matrices, thereby improving their mechanical and barrier properties. The most exciting properties of starch lay in its semicrystalline and gelatinization ability. The latter is exploited to produce plasticized starch that can be used to make biobased films (e.g. such as those developed for biodegradable garbage or shopping bags).

The starch crystal's platelet-like morphology and its inherent rigidity, powerful interfacial interactions and percolation network contribute to their optimized thermal and mechanical properties, solvent absorption and excellent barrier properties. It was also noted that developing high-performance nanostructured systems like starch nanomaterials for food applications would help to enhance food safety. Considering the widespread impact of these polymer nanoparticles in food packaging, a large number of nanotechnology-based products have been launched in the market in recent years, claiming enhanced functionality based on their nanoscale features[13]. This work envisages a new dimension in applying starch nanocrystals which can be industrially scaled up.

Here, particular emphasis will be given to the synthesis, characterization and improved properties of starch nanocrystals like drug loading capacity, synergistic antimicrobial activity etc. The functional modification of starch nanocrystals under optimum conditions will improve its practical utility in the future.

Material and Methods

Fabrication and characterization of streptomycin-loaded starch nanocrystals (SSNCs): Regeneration methods were adopted for the synthesis of starch nanocrystals. Here, the co-crystallization of starch solutions results in the development of starch nanocrystals.

Fourier Transform Infrared Analysis (FTIR): The FTIR absorption spectrum of a compound gives information about the type of bond in the molecule and thus, it is often used for the direct identification of certain specific functional groups constituting organic molecules. The width of the IR band gives information about the strength and nature of the molecular interaction. The spectrum interpretation process is simplified because the bands, which apparently can be assigned to a particular part of the molecules, produce group frequencies. The constant of the position of the group frequency forms the basis for the structural analysis of the compound. Here, the vibrational mode in nanoparticles was studied using a Shimadzu FTIR spectrometer in the mid-IR from 350 - 4000 cm^{-1} by KBr (Potassium bromide) techniques. The samples were thoroughly mixed with KBr to record the IR spectra, keeping the nanoparticle sample volume low and analyzing it in transmission mode.

Scanning Electron Microscope (SEM): The morphology and distribution of the starch nanocrystals and streptomycin-loaded starch nanocrystals were analyzed by SEM images using the Nova Nanosem 450 instrument.

Determination of swelling behavior of SSNCs: The swelling behavior of starch nanocrystals was investigated by a simple gravimetric technique. Here, the amount of water absorbed is analysed by weighing the swollen nanoparticles[3]. The swelling nature of nanocrystals depends on the solvent and polymer's nature[4]. For the study of the water sorption capacity of the starch nanocrystals, pre-weighed (10 mg) nanocrystals were immersed in 10 mL 1X phosphate buffer saline (pH 7.4) for swelling at room temperature (30 ± 2 ˚C). After pre-determined time intervals, the nanocrystals were filtered, gently passed through the filter papers to remove excess solvent and weighed. The process was repeated every ten minutes until the equilibrium swelling was achieved (after one and half hour).

The swelling ratio of the nanoparticles was calculated by using the following equation:

$$\text{Swelling ratio} = \frac{\text{Weight of swollen SSNCs} - \text{Dry weight of SSNCs}}{\text{The dry weight of SSNCs}} \quad (1)$$

Release profile of SSNCs: The release of streptomycin from starch nanocrystals was performed under shaking conditions (120 rpm). Here the starch nanocrystals were maintained in a definite volume (10 mL) of release medium (1X PBS, pH 7.4) for a pre-determined period. After fixed time intervals (at one h interval), the suspension was centrifuged at 12,000 rpm for 15 min and the supernatants were withdrawn from the solution and were analyzed for the remaining concentration of drug-using UV/VIS spectrophotometer (Shimadzu - UV-1700 Pharma spec, Japan). Streptomycin concentration was estimated at fixed intervals by monitoring the absorbance at 195 nm. Then, an equal volume of fresh 1X PBS (pH-7.4) solution was added to maintain a constant volume[15]. The experiments were carried out in triplicate and standard deviations were calculated.

Tested microorganisms and preparation of test cultures: Bacterial stock cultures of *Pseudomonas aeruginosa* (MTCC 424) and *Staphylococcus aureus* (ATCC 25923) were obtained from the Microbial Type Culture Collection (MTCC), The Institute of Microbial Technology, Chandigarh, India and American Type Culture Collection respectively. The stock cultures were maintained at 4 ˚C on nutrient agar by regular subculture at the Department of Biotechnology, University of Kerala. Overnight-grown cultures were adjusted to 0.5 McFarland turbidity standards and were used for testing antibacterial activity[16].

Preparation of culture media: The nutrient agar was used to regularly maintain test culture, peptone water for inoculum preparation and Mueller Hinton agar (MHA) for plate preparation.

Synergistic interaction between antibiotics (Ciprofloxacin and Polymyxin B) and SSNCs: The disc diffusion method was used to assay the synergistic effect of antibiotics (ciprofloxacin and polymyxin B) with SSNCs for bactericidal activity against different test strains on Muller Hinton agar plates. The standard antibiotic discs (Ciprofloxacin and Polymyxin B) were obtained from HiMedia (Mumbai, India). The stock cultures were inoculated in nutrient broth and incubated overnight. Then, the inocula were prepared by diluting these overnight cultures with 0.9% NaCl to a 0.5 McFarland standard and were applied to the plates along with the standard and prepared discs containing different concentrations of SSNCs with the antibiotics (Ciprofloxacin and Polymyxin B). The inhibition zones were measured after incubation at 37 ºC for 24 - 48 h[7].

Results and Discussion

Fourier Transform Infrared Analysis (FTIR): The Fourier transform infrared (FTIR) analysis was conducted to verify the possibility of interaction of chemical bonds between drug (Streptomycin) and starch nanomaterial (inert polymer nanomaterial) i.e. the FTIR spectra of pure streptomycin, starch nanocrystals (S-NCs), drug and streptomycin-loaded starch nanocrystals (SSNCs) gave

insight into the intra- and intermolecular interactions of the formation of S-NCs and SSNCs. The FTIR analysis of S-NCs reveals the peaks at 597.93 cm^{-1}, 862.18 cm^{-1} and 1149.57 cm^{-1}, mainly due to the functional groups of alkyl halides. Other peaks of S-NCs are at 1456.25 cm^{-1}, 2347.36 cm^{-1} and 2493.95 cm^{-1}, corresponding to the vibrations C-H bend, N-stretch and O-H stretch respectively. The peak at 1687 cm^{-1} of S-NCs represents the alkene group.

The comparative study of FTIR of streptomycin and streptomycin-loaded starch nanocrystals (SSNCs) was also performed and its analysis manifested that the antibiotic streptomycin exhibited some strong peaks at 1118.71 cm^{-1}, 1668 cm^{-1}, 2360.87 cm^{-1}, which represent C-N stretch, N-H bending and N-H stretch respectively. At the same time, other peaks like 1367.53 cm^{-1}, 3323.34 cm^{-1} and 3853.7 cm^{-1} correspond to N-O asymmetric stretch, N-H stretch and O-H stretch respectively. The peak 623.007 cm^{-1} represents the functional group alkyl halides (Fig. 1a).

The FTIR spectra of streptomycin-loaded starch nanocrystals are shown in figure 1b and the closer evaluation of these spectra revealed that the presence of peaks at 1127.44 cm^{-1} (C-N stretch), 1677.17cm^{-1}(N-H bending), 2361.93 cm^{-1} (N-H stretch) and 3737.24 cm^{-1} (O-H stretch) indicates the possible incorporation of streptomycin into starch nanocrystals. These results were in agreement with a previous report where the incorporation of streptomycin sulfate into solid lipid nanoparticles was confirmed by its FTIR analysis[11]. Other peaks observed in SSNCs are 1458.247 cm^{-1} (C-H bends) and 623.03 cm^{-1}. These peaks represent alkanes and alkyl halides respectively.

Here the peaks observed at 3323.34 cm^{-1}, 3853.7 cm^{-1} for streptomycin and 3737.24 cm^{-1} for SSNCs represent the extent of the formation of inter-and intra-molecular hydrogen bonds, which in turn contributed to its peak intensity[6]. Peaks also provided information about the position of O-H stretching vibrations[1]. In all the above-mentioned cases, the absorption in the infrared region is mainly because of the changes that occurred due to the vibrational energy. The essential requirement for a substance to absorb in these regions is the vibrations in the molecule resulting in unsymmetrical charge distribution. Here, the region 1400-650 cm^{-1} is known as the fingerprint region. Therefore, this region is usually checked for the identification of the functional groups. It is also associated with vibrational (and rotational) energy changes in the molecular skeleton and is a characteristic of the compound under study.

In the case of streptomycin, the peaks observed at 1118.71 cm^{-1}, 1668 cm^{-1} and 2360.87 cm^{-1} correspond to C-N stretch, N-H bending and N-H stretch respectively. At the same time, the streptomycin-loaded starch nanocrystals (Fig.1b) revealed the presence of peaks at 1127.44 cm^{-1} (C-N stretch), 1677.17cm^{-1} (N-H bending) and 2361.93 cm^{-1} (N-H stretch). Here, it was clear that streptomycin-loaded starch nanocrystals retained almost all the functional groups of streptomycin with a slight difference in the intensity and peak position of the bands. These peaks indicate the possible incorporation of streptomycin into starch nanocrystals. The variation in the intensity and the peak position should be due to the presence of strong inter and intramolecular hydrogen bonds[8].

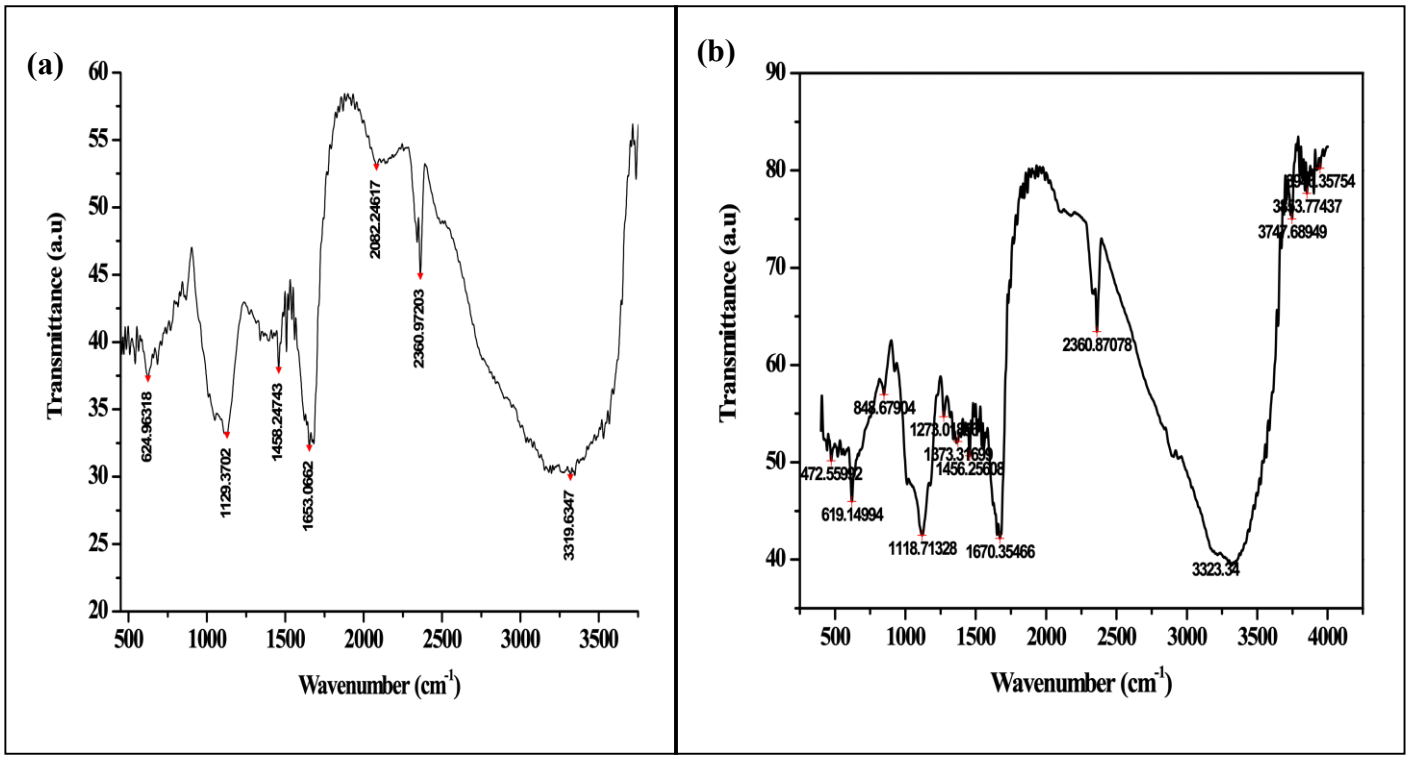

Figure 1: (a) FTIR Profile of Streptomycin and (b) FTIR profile of Streptomycin loaded starch Nanocrystals.

Scanning Electron Microscope (SEM): The morphology of the S-NCs and SSNCs was analyzed at the magnification of 12,000 X. Like previous reports, the starch nanocrystals seem smooth externally. Fig. 2a and fig. 2b depict the images of S-NCs and SSNCs. The formation of nanocrystals (S-NCs and SS-NCs), as well as their size distribution and morphology, were confirmed by SEM analysis. In both cases, it was found to show the presence of uniform nanocrystals with a particle size of 44.3 nm - 65.7 nm. However, streptomycin-loaded starch nanocrystals showed a slight aggregation. This may occur due to the presence of adsorbed streptomycin on the surface of the starch nanocrystals.

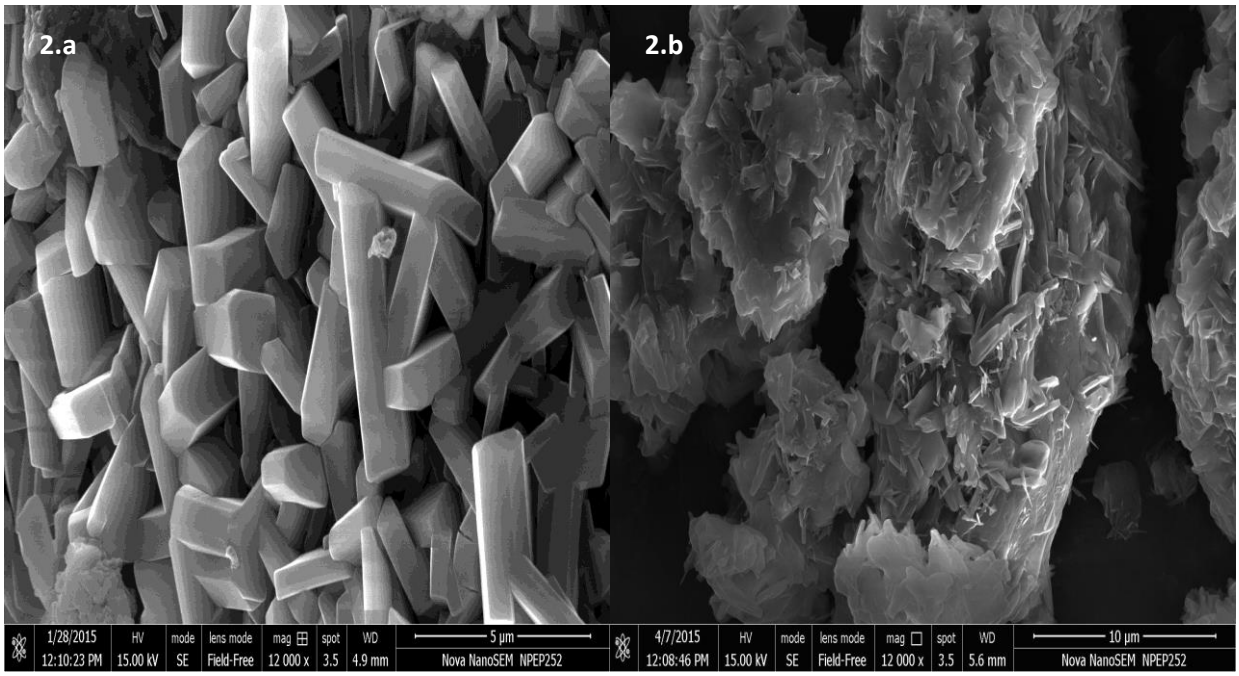

Figure 2: (a) SEM images of starch nanocrystals (S-NCs) and (b) SEM image of streptomycin loaded starch nanocrystals, scale bar: 5 µm and 10 µm respectively

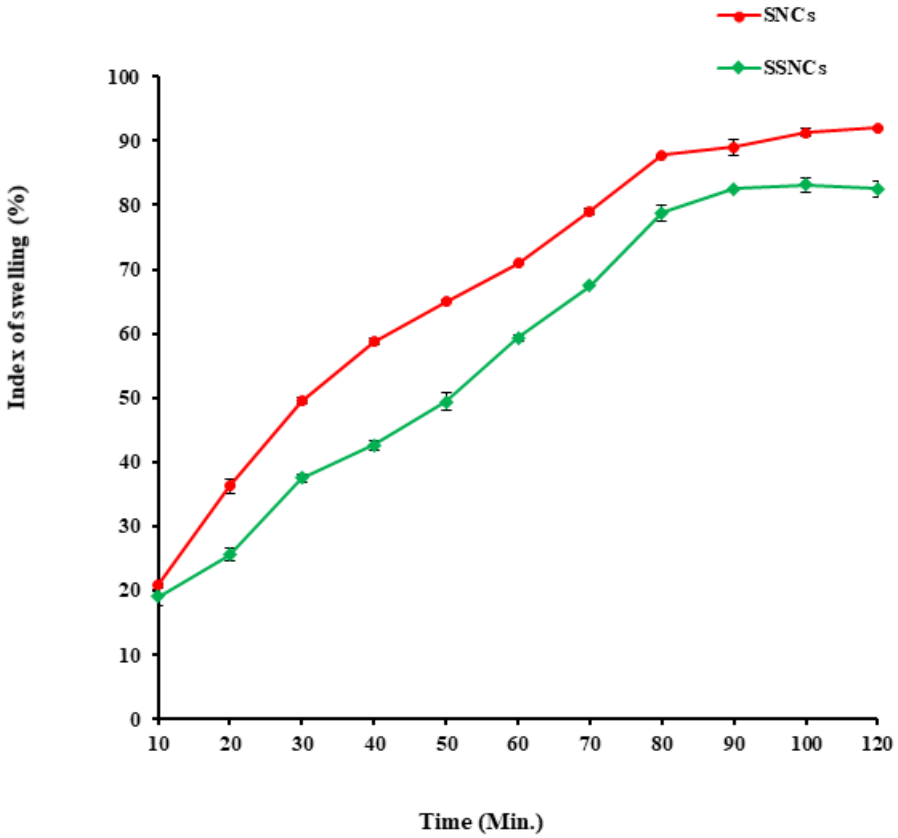

Figure 3: Swelling behavior of starch nanocrystals and streptomycin-loaded starch nanocrystals

Swelling behavior of S-NCs and SSNCs: The swelling capacity of starch nanocrystals is a function of the water-holding ability of these nanomaterials. Herein, the effect of S-NCs and SSNCs on water sorption capacity has been investigated. The swelling capacity of the starch nanocrystals was found to be very high compared to that of streptomycin-loaded starch nanocrystals (SSNCs). It was found that SSNCs showed a comparatively reduced swelling index as compared to that of S-NCs i.e. 82.5% swelling index for SSNCs at 120th min, while S-NCs showed 92% swelling at 120th min (Fig. 3). The swelling capacity of starch nanocrystals significantly decreases after the loading of streptomycin into it.

The significant increase in the swelling index of starch nanocrystals can be related to an increase in the surface area of these nanomaterials. Furthermore, it was observed that enhanced hydrophilicity of the starch nanomaterials and the amount of polymer present in the nanomaterials, would greatly influence the swelling behavior of the starch nanoparticles[14]. The swelling studies showed that the swelling of starch nanocrystals increases as its amount increases in composition. The observed decrease in swelling of the modified starch nanocrystals (SSNCs) may be because the nanocrystals become more integrated and compact due to the incorporation of streptomycin, allowing a smaller number of water molecules to enter the nanoparticle network.

Release profile of streptomycin from starch nanocrystals: The prepared SSNCs were suspended in 1X PBS (pH = 7.4) to release streptomycin, detected by a UV spectrophotometer at 219 nm at a different time interval (Fig. 4). The particles showed a burst release within the first 1 h; this initial burst release of the drug, regardless of the temperature difference, results from drug molecules adsorbed to the nanomaterial surface. It was followed by a release of ≈28.5% over the next 24 h; then, it was observed that the SSNCs showed maximum release at its 36th hour i.e. 67.1% (Fig. 4)

The above-mentioned swelling property of the starch and modified starch nanocrystals has greatly influenced the drug-releasing properties of starch nanocrystals. Herein, the *in vitro* release of streptomycin from starch nanocrystals was carried out in 1 X PBS (pH -7.4) and it was observed that the release occurred in two stages. In the initial stage, a rapid release occurred with an increase in time (for the first 48 h). This abrupt release may be due to the diffusion of adsorbed streptomycin on the surface of starch nanocrystals[9]. In the second stage, SSNCs showed a slight decrease in their release followed by a controlled/sustained release of streptomycin (58.7%) from starch nanocrystals. This sustained release may be due to the self-biodegradability and the starch nanocrystals' improved swelling properties. This kind of continuous and slow release has been reported earlier for drugs such as acetylsalicylic acid, probucol etc.[2]

The drug release mechanism may involve swelling-based release followed by release due to either drug molecule diffusion or polymer matrix degradation[17]. Furthermore, streptomycin can diffuse easily through the surface or pores of nanomaterials quickly possibly due to its smaller size. The streptomycin was observed to release out from starch nanocrystals in a sustained manner under physiological pH over 12 days.

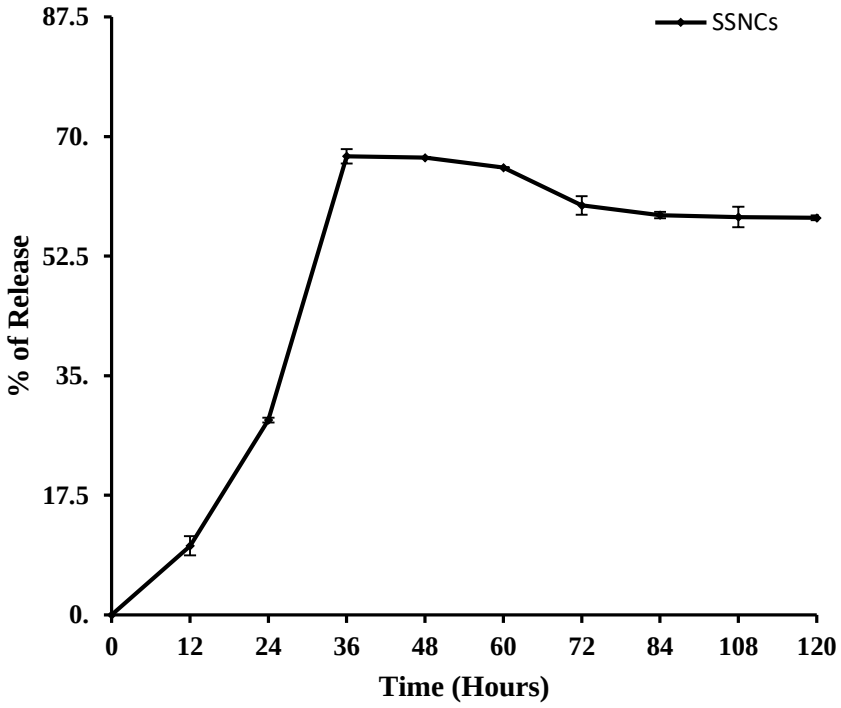

Figure 4: The release profile of streptomycin from starch nanocrystals

Synergistic interaction between antibiotics and SSNCs: The enhanced antimicrobial activity of the modified starch nanocrystals was confirmed by investigating the synergistic interaction between the antibiotics and SSNCs. The zone (mm) of inhibition of different antibiotic discs with and without SSNCs against test strains was measured (Fig. 7). Both antibiotics (polymyxin B and ciprofloxacin) showed a specific extent-fold increase of antibacterial activity combined with different concentrations (20 µg/mL, 10 µg/mL, 5 µg/mL) of SSNCs against *P. aeruginosa* and *S. aureus* respectively. Ciprofloxacin is a broad-spectrum antibiotic of the fluoroquinolone class (Fig. 5), but it is active against Gram-positive and Gram-negative bacteria.

It functions by inhibiting DNA gyrase and a type II topoisomerase, topoisomerase IV, necessary to separate bacterial DNA, thereby inhibiting cell division. In the case of ciprofloxacin, streptomycin-loaded starch nanocrystals (20 µg/mL) together with ciprofloxacin showed a maximum of 16%-fold increase against Gram-positive *S. aureus* at 20 µg/mL of SSNCs. It also showed a fold increase of 8.3% against the same microorganisms at the lowest concentration (5 µg/mL) whereas ciprofloxacin with SSNCs showed a fold increase (maximum) of 10.34% against *P. aeruginosa* at 20 µg/mL of SSNCS and a 3.4%-fold increase at 5 µg/mL of SSNCs as shown in table 1.

The second antibiotic used for the study was polymyxin B. Polymyxin B (Fig. 6) is an antibiotic used mainly for resistant Gram-negative infections. It is derived from the bacterium *Bacillus polymyxa.* Here also, an enhanced synergistic activity was observed in the presence of SSNCs against different pathogens (*P. aeruginosa* and *S. aureus*). This combination exhibited its maximum synergistic activity of 16.7%-fold increase against Gram-positive *S. aureus* at 20 µg/mL of SSNCs and 8.3%-fold increase for 10 µg/mL and 5 µg/mL of SSNCs (Table 2).

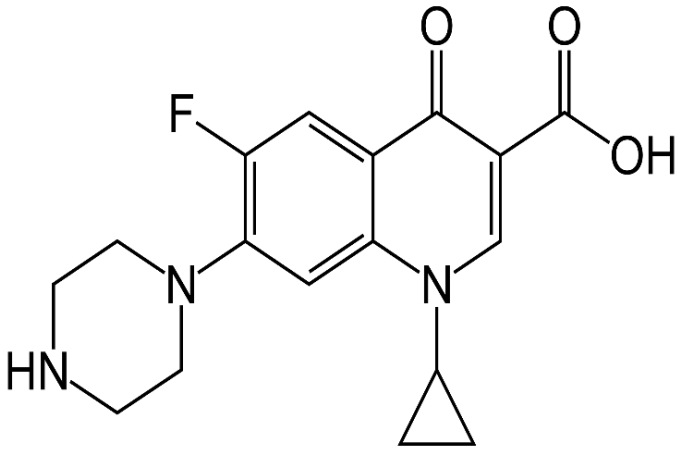

Figure 5: Chemical structure of ciprofloxacin

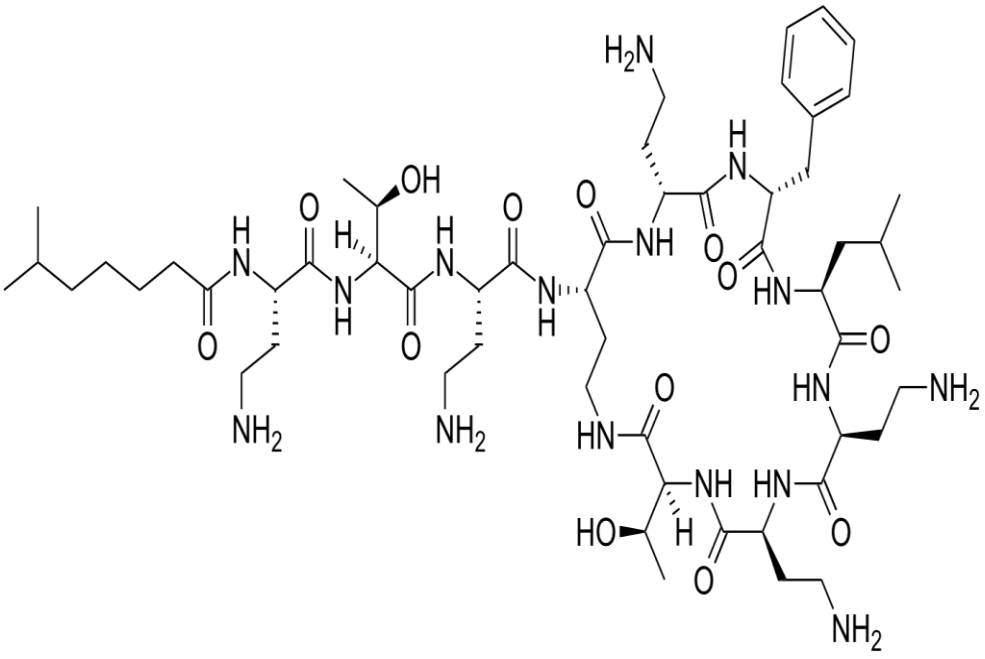

Figure 6: Chemical structure of Polymyxin B

Table 1
Synergistic activity of SSNCs with Ciprofloxacin against different pathogens

Micro organism	Ciprofloxacin Zone (mm)	SSNCs (20µg/ disc)	SSNCs + Ciprofloxacin		
			Fold increase (%) 20 µg of SSNCs	Fold increase (%) 10 µg of SSNCs	Fold increase (%) 5 µg of SSNCs
S. aureus	24	7	**16**	12.5	8.3
P. aeruginosa	29	11	**10.34**	6.89	3.4

Table 2
Synergistic activity of SSNCs with Polymyxin B against different pathogens

Micro organism	Polymyxin B Zone (mm)	SSNCs (20µg/ disc)	SSNCs + Polymyxin B		
			Fold increase (%) 20 µg of SSNCs	Fold increase (%) 10 µg of SSNCs	Fold increase (%) 5 µg of SSNCs
S. aureus	12	9	16.7	8.3	8.3
P. aeruginosa	14	10	7.1	7.1	14.28

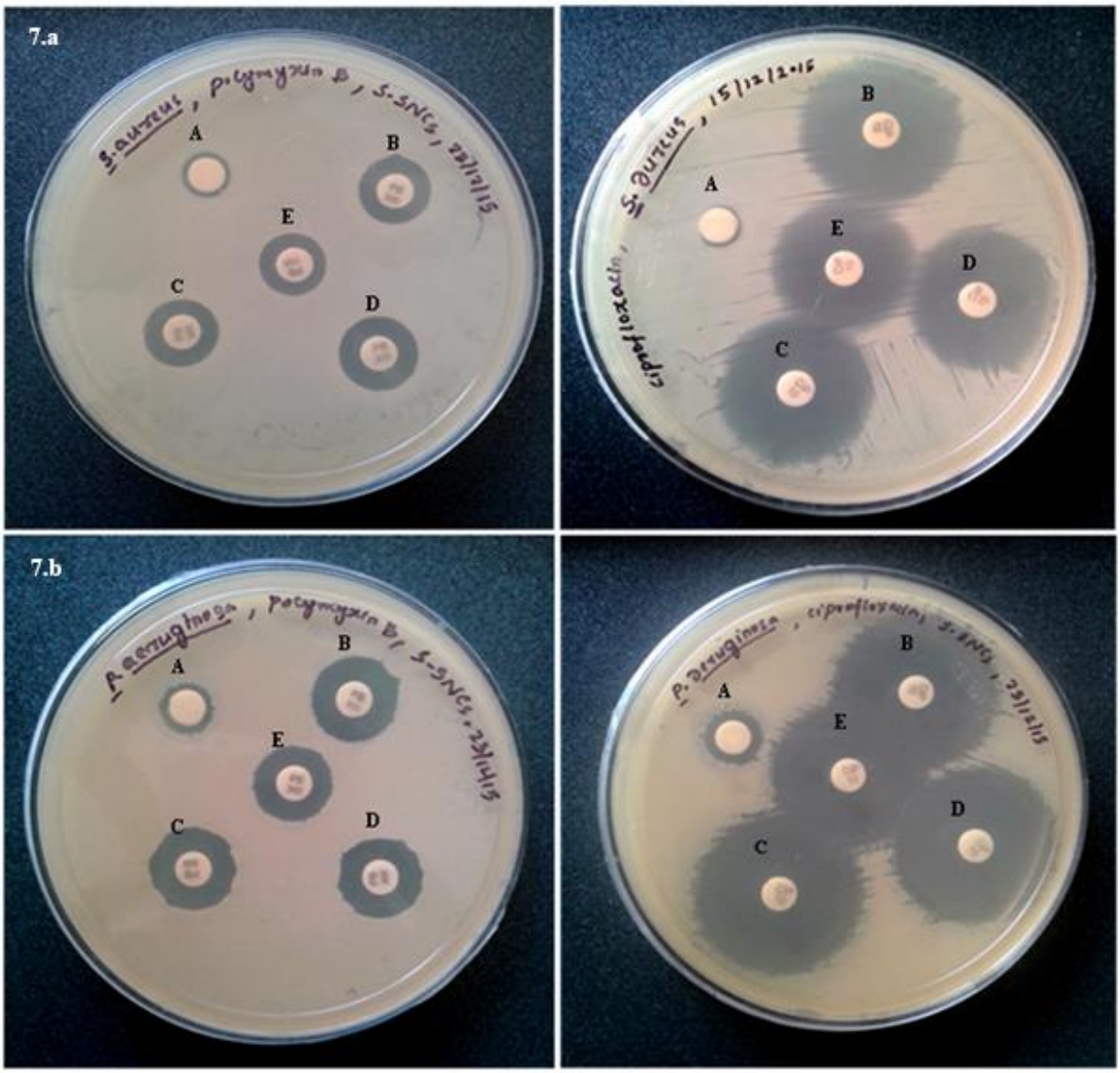

Figure 7: (A) Synergistic activity of polymyxin B and ciprofloxacin with SSNCs (20 µg/mL - B, 10 µg/mL-C, 5 µg/mL - D, SSNCs Control - A, Antibiotic control - B) against *S. aureus* (B) Synergistic activity of polymyxin B and ciprofloxacin with SSNCs (20 µg/mL-B, 10 µg/mL-C, 5 µg/mL-D, SSNCs Control - A, Antibiotic control - B) against *P. aeruginosa.*

Against Gram-negative bacteria (*P. aeruginosa*), it showed a maximum fold increase (14.28%) at a lower concentration (5 µg/mL) of SSNCs. The mechanism of antibacterial activity of polymyxin B antibiotics is that the outer membrane permeability of the bacteria gets altered by binding to the negatively charged site of the lipopolysaccharide layer, resulting in a destabilized outer membrane. Since the fatty acid portion dissolves in the hydrophobic region of the cytoplasmic membrane, it disrupts membrane integrity and causes the leakage of cellular molecules followed by the inhibition of cellular respiration, leading to bacterial death.

This study clearly indicates that the combination of ciprofloxacin and polymyxin B with SSNCs shows synergistic antimicrobial activity[12] and this antibiotic activity increases with an increased concentration of SSNCs. The combination of SSNCs with polymyxin B showed maximum synergistic activity against Gram-negative bacteria at the lowest concentration of 5µg/mL as compared to the combination of SSNCs and ciprofloxacin. Here, we tried to propose a possible mechanism of synergistic antimicrobial activity of SSNCs with antibiotic polymyxin B against Gram-negative bacteria (Fig. 8) i.e. Due to high surface-to-volume ratio, streptomycin-loaded starch nanocrystals can easily accommodate polymyxin B followed by its interaction with the microbial cell membrane.

Polymyxin B is known to permeabilize bacterial cell membranes, especially for gram-negative bacteria and it helps SSNCs access internal target sites. The antibacterial activity of streptomycin is mainly due to the inhibition of protein synthesis by binding to bacterial 30S ribosomal subunit, causing misreading of t-RNA. Herein, the binding of SSNCs to different cell organelles, especially ribosomes may eventually result in the disruption of various cell functions such as permeability, protein synthesis, respiration and finally, cell death.

Conclusion

The antibiotic streptomycin was successfully loaded onto starch nanocrystals using a simple nanoprecipitation technique in a microemulsion system. This drug-loaded starch nanocrystal demonstrated improved structural characteristics and a sustained drug-release property. Streptomycin-loaded starch nanocrystals were found to be more effective when combined with ciprofloxacin or polymyxin B against various bacterial strains rather than alone.

Therefore, the synergistic interaction of streptomycin-loaded starch nanocrystals with the antibiotics (Ciprofloxacin and Polymyxin B) makes this nanosystem an effective antimicrobial candidate against various bacterial strains. Although further investigations are needed, the synergistic antimicrobial effect exhibited by the combination of SSNCs and the antibiotics (Ciprofloxacin and Polymyxin B) holds a promising platform for treating infectious diseases by bacteria.

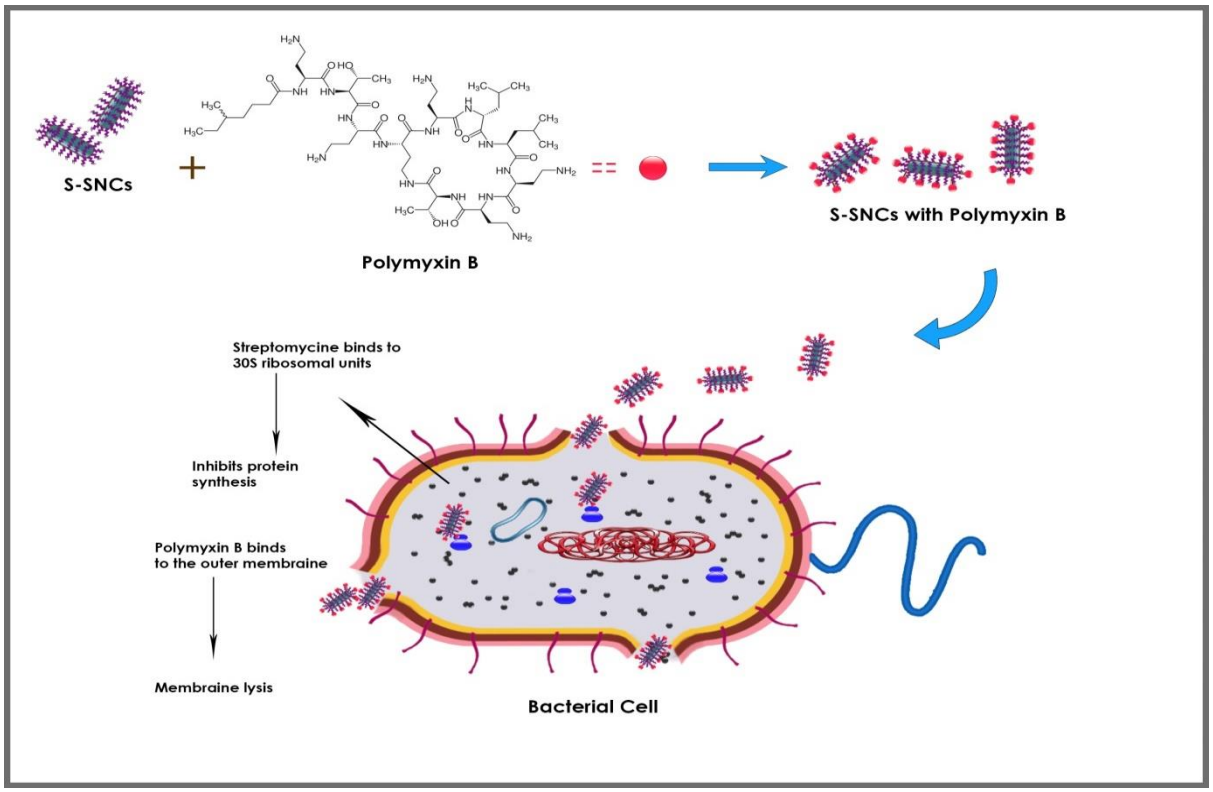

Figure 8: Synergistic antibacterial pathway of SSNCs (Streptomycin loaded starch nanocrystals) with Polymyxin B against Gram-negative bacteria. The binding of Polymyxin B to the outer membrane of bacteria and the interaction of streptomycin with the 30S ribosomal subunit leads to cell death.

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