

RESEARCH ARTICLE

Polymer Synergy in Action: Antimicrobial Properties of Chitosan-Alginate Complexes against Diverse Bacterial Strains

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ABSTRACT

Background: This study investigates the formation and antimicrobial properties of polyelectrolyte complexes (PECs) made from chitosan with sodium alginate (CS-SA) and CS-carboxylated SA (CS-CMA). The research compares their effectiveness against Gram-positive and Gram-negative bacteria, exploring how polymer modifications affect antimicrobial activity. These findings contribute to the development of tailored antimicrobial materials for various applications in medicine, food packaging, and water treatment.

Results: This study mainly focuses on the screening and comparison between two complexes PEC CS-SA and PEC CS-CMA, that is, formed by ionic interaction, CS with SA and CMA. Fourier transform infrared spectroscopy analysis confirmed the formation of complexes, while optical microscopy revealed irregular and fibrous surface morphologies of the PEC films compared to smooth individual polymer films. Antimicrobial activity was assessed against both Gram-positive bacteria (Gram [+]) and Gram-negative bacteria (Gram [–]) on solid agar media for both complexes. Results indicate that PEC of CS and SA shows broad spectrum activity (zone of inhibition [ZOI] Gram [+] 10.4 mm and for Gram [–] 10.3 mm). This is due to the presence of free amino group and electrostatic interaction as compared to the parent molecules. However, PEC of CS and CMA are highly active against Gram (–) bacteria (ZOI-14 mm) and ZOI-1.5 mm against Gram (+) bacteria. The reason may be the cell wall thickness (around 20–80 nm, peptidoglycan) and Gram (–) bacteria (around >10 nm, lipopolysaccharides), which is unable to penetrate due to less availability of free amine group ($-NH_2$). Other side COO^- group charge density is enhanced probably showing more activity against Gram (–) bacteria. These findings offer promising avenues for developing tailored antimicrobial materials in various fields, including medicine, food packaging, and water treatment. **Conclusion:** The ability to modify polymer complexes to target specific types of bacteria could lead to more effective and efficient antimicrobial strategies, potentially reducing the reliance on traditional antibiotics and addressing the growing concern of antimicrobial resistance.

Keywords: Antimicrobial activity, carboxylated-sodium alginate, chitosan, Gram (–) bacteria, Gram (+) bacteria, polyelectrolyte complexes, sodium alginate, zone of inhibition

INTRODUCTION

At present, the use of packaging in food products is considered as most important thing. Packaging is a protective shell of the product or foodstuff from physical impact and microbial contamination which in turn lengthens its shelf life.^[1] These

days, petroleum-based synthetic packaging is the most used packaging type. Nonetheless, the environment has suffered from its uncommon degradation into non-degradable litter. Initially, environment-friendly concerns, edible film-based packaging materials have been developed. Bio-composite materials have been developed and are being increased with the help of pectin. In addition, they could also serve as edible films, carrying out the delivery of the bonding of all the ingredients.

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First, the essential characteristic of the edible film is that it is resistant to food destruction triggered by microorganisms.^[2]

Chitosan (CS), derived from chitin, discovered in 1859 by Professor C. Rouget has antimicrobial properties useful in various fields.^[3] Sodium alginate (SA), from brown seaweed, first isolated by British chemist E.C.C. Stanford in 1881 is used for gelling, stabilizing, and antimicrobial properties in various industries.^[4] The antibacterial activities of CS are achieved through the polymer being positively charged as well as the bonded amine group. However, CS has a drawback of leaching under the conditions of high-water absorption and generalized acidity. This defect can be either removed with the making of polyelectrolyte complexes (PEC) or a PEC film.^[5-7]

PECs are typically formed using polycationic polymers like CS and polyanionic polymers such as alginate, κ -carrageenan,^[8] or sodium carboxymethyl cellulose.^[9] The synthesis process involves electrostatic interactions under specific pH conditions, with factors like polymer ratios, pH, and stirring speed significantly impacting PEC yield and properties. Characterization techniques including Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy, X-ray diffraction, and Ehlers-Danlos syndrome confirm PEC formation and reveal their structural properties.^[8,9] PEC films often exhibit irregular, fibrous surface structures, while aerogels demonstrate high specific surface areas (380–400 m²/g) and porous structures.^[8,10] A key feature of these PEC materials is their enhanced antibacterial activity against both Gram-positive bacteria (Gram [+]) and Gram-negative bacteria (Gram [–]), such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*.^[9,10] This property is attributed to the protonated amine groups (NH³⁺) in the PEC structure, which interact with bacterial cell membranes, increasing permeability and causing cell death.^[8,9] The studies highlight the advantages of using natural, non-toxic polymers and simple preparation methods, supporting the potential for large-scale application of PEC materials in various industries.^[8-11]

In this work, the fabrication of two PECs was made by a process in which the CS was used as polycationic

and formed the film with SA and carboxylated-SA which acts as a polyanionic. The CS-SA and CS-carboxylated-SA create electrostatic connections without physical crosslinking (NH₃⁺), resulting in a polymer electrolyte complex film that effectively blocks oxidation.^[12] Physical appearance confirmed that if there will be any changes in structure is there or not. FTIR confirms there is a formation of complex and antimicrobial study will confirm the efficacy of the complex's activity against Gram (+) and Gram (–) means broad spectrum or narrow spectrum. Hence, the ultimate goal is to develop an effective, environmentally friendly material with good antimicrobial properties for use in food preservation and packaging.

MATERIALS AND METHODS

Materials

CS of medium molecular weight 150–500 m. Pas, obtained from SRL chem laboratory, SA mol weight was obtained from LOBA chemie of 30 CPS viscosity (1% solution) laboratory, glacial acetic acid, NaOH, and deionized water are obtained from SRL chem laboratory, and solid agar media obtained from Hi Media Laboratories Limited and *E. coli* having stain number ATCC 25922 and *B. subtilis* having stain number ATCC 6633. Both strains were cultured and maintained under standard laboratory conditions to ensure viability and reproducibility in antimicrobial testing.

CS-SA Complex Preparation

CS-SA complex was prepared by mixing 1:1 molar ratio. 1% of CS was dissolved in 20 mL of glacial acetic acid while 4% of SA was dissolved in 20 mL of deionized water under magnetic stirrer (REMI 2MLH) at 600 rpm for 4 h at room temperature. Then previously dissolved CS solution was added to SA solution to obtain a white colored complex which then centrifuged, homogenized, and poured on a petri plate and then confirm of uniform thickness and dried at 55°C at Hot air Oven until a constant weight of that complex film was obtained. Then, it was preserved in a hermitically sealed container till further use.^[8]

Preparation of Carboxylated- SA

SA was derivatized to carboxylated-SA having substitution in the following method. In brief, the process of producing carboxylated-SA which comprises suspending required quantity of SA in an ice-cold deionized aqueous solution of not more than 60% w/v sodium hydroxide. Then, the dispersion was kept at 5–8°C for 1 h. After that the resulting solution was mixed with 1.5 g of monochloroacetic acid solution and the temperature was raised slowly to 15–18°C, after 30 min, the temperature was increased to 75°C and maintained for an additional 30 min. Wetted mass was collected and washed with 20 mL 80% methanol 3 times and maintained pH at neutral by glacial acetic acid and dried at 50–60°C, dried product was milled, washed, and redried.^[13]

$\text{R-OH (Alginate)} + \text{ClCH}_2\text{COONa} \rightarrow \text{R-OCH}_2\text{COONa (Carboxymethyl Alginate)} + \text{NaCl}$
Where R represents the alginate polymer backbone.

CS-Carboxylated SA (CMA) Film Preparation

CS CMA film was prepared with CS: CMA of 1:1 ratio. CS was dissolved in glacial acetic acid in a ratio following the same procedure as stated before of 1% and carboxylated- SA was dissolved in deionized water in a ratio of 4% with the help of magnetic stirrer at 600 rpm (REMI 2MLH) for 4 h at room temperature. Subsequently, centrifuged, homogenized, and poured on a petri plate and then confirm of uniform thickness and dried at 55°C at Hot air Oven until a constant weight of that complex film was obtained. Then, it was preserved in a hermitically sealed container till further use.^[8]

Physical Characteristic Test

Loss on drying

Weigh 1 g of each film sample (PEC-CS and SA, PEC-CS, and CMA) accurately. Now, place samples in a hot air oven at 60°C for 3 h. After that, put the samples in a desiccator and reweigh. Calculate the loss on drying using the formula (Ferreira *et al.*, 2022).

Loss on drying (%) = $\frac{[(\text{Initial weight} - \text{Final weight}) / \text{Initial weight}] \times 100$

Optical microscopy

Prepare thin sections of each film sample. Observe under an optical microscope at $\times 400$ magnification. Now, capture images and describe observed features.^[14]

Solubility

Cut 2 cm \times 2 cm squares of each film and weigh. Immerse samples in 50 mL of distilled water at room temperature for 24 h. Remove undissolved film, gently blot dry, and weigh. Calculate solubility using the formula:^[15]

Solubility (%) = $\frac{[(\text{Initial weight} - \text{Final weight}) / \text{Initial weight}] \times 100$

FTIR

IR spectra were recorded on a Bruker, FTIR spectrophotometer using KBr optics. Each sample was mixed with KBr and converted into disk at 100 kg pressure using a hydraulic press. The spectra were recorded within 4000–400 cm^{-1} wave numbers.

Nuclear Magnetic Resonance (NMR) Study of CMA

To confirm the carboxylation of SA, ^{13}C NMR spectroscopy analysis was conducted on a VARIAN 400 MHz NMR instrument using deuterium oxide (D_2O) as the solvent. The data comprise several important chemical shifts in parts per million (ppm), spanning from the aliphatic to the carboxylic regions, indicative of the structural components of the sample.

Antibacterial Activity Test

The antimicrobial properties of PEC films are evaluated through Agar plate method in which the Solid agar media is used to check the bacterial sensitivity which contains *B. subtilis* as Gram (+) and *E. coli* (Gram [–]) as a colony forming unit at a concentration of approximately 10^6 CFU/mL. Sterilized paper disks were carefully placed on the inoculated agar and dosed with 20 μL of the hydrosol film solution. As a positive control, 10 mg of amoxycillin was applied to another disk per

manufacturer's instructions while deionized water served as the negative control. Following incubation at 37°C for 24 h, the diameter of any observable inhibition zones surrounding each disk was measured. All experiments were conducted thrice to ensure the reliability and reproducibility of the findings.^[8]

RESULTS AND DISCUSSION

Formation of PEC Films

Formed film PEC

CS and SA yield 88.59% and PEC-CS and CMA yield 90%, all these yield values gained after drying till the constant weight is obtained.

Physical appearance

The film was initially pale yellow in colour, bumpy texture, and had a strong odour, which disappeared in the course of time. The film, upon drying, was 9 × 9 cm in diameter and was cut into 2 × 2 cm pieces for experimentation

FTIR Result Discussion

Comparative analysis of FTIR spectra of CS, SA and PEC of CS and SA

Based on the provided analyses, we can draw a comparative analysis between CS, SA, and the CS-based polyelectrolyte complex. All three compounds show broad bands in the 3600–3200 cm⁻¹ region, indicating the presence of O-H stretching, which is common in polysaccharides. However, CS and its polyelectrolyte complex also show N-H stretching in this region, a feature absent in SA. All three exhibit C-H stretching peaks around 2900 cm⁻¹, but with slight variations in exact positions. A key difference lies in the carbonyl region: CS shows amide I and II bands (1649 and 1595 cm⁻¹), SA displays carboxylate stretching (1649 and 1436 cm⁻¹), while the polyelectrolyte complex shows shifted amide bands and a new peak at 1515 cm⁻¹, indicating ionic interactions. All three compounds show peaks around 1000–1100 cm⁻¹, attributed to C-O-C stretching in their saccharide structures, but with variations in exact position and intensity

[Figure 1]. The polyelectrolyte complex spectrum shows features of CS but with notable shifts and new peaks, reflecting its more complex structure and interactions. These similarities and differences in spectral features reflect the structural similarities of these polysaccharide-based compounds while highlighting their distinct chemical characteristics and interactions.^[16,17]

Comparative Analysis of FTIR Spectra of CS, SA and PEC of CS and CMA

Comparing CS, CMA, and their polyelectrolyte complex reveals both similarities and distinct differences in their FTIR spectra. All three compounds exhibit broad bands in the 3600–3200 cm⁻¹ region, indicating O-H stretching common to polysaccharides, with CS and the complex also showing N-H stretching. They all display peaks around 1030–1091 cm⁻¹, attributed to C-O-C glycosidic bonds in their saccharide structures. However, key differences lie in their carbonyl regions: CS shows characteristic amide I and II bands (1649 and 1595 cm⁻¹), CMA exhibits strong carboxylate peaks (1590 and 1405 cm⁻¹), while the polyelectrolyte complex presents a combination of these features (peaks at 1694.55, 1640.20, and 1594.83 cm⁻¹), indicating the interaction between CS and alginate. CS uniquely shows a peak at 804 cm⁻¹ for N-H wagging, while alginate and the complex have more pronounced peaks in the 885–1095 cm⁻¹ fingerprint region [Figure 1]. The polyelectrolyte complex spectrum effectively combines features of both parent compounds, with shifts and new peaks reflecting their ionic interactions and the formation of a new, complex structure [Table 1]. These spectral differences arise from the distinct chemical structures of CS and alginate, and their electrostatic interactions in the complex, demonstrating how FTIR can effectively differentiate between these related polysaccharide materials.^[16]

Physical Characteristic Test

Loss on drying

The loss on drying was found to be [Table 1]

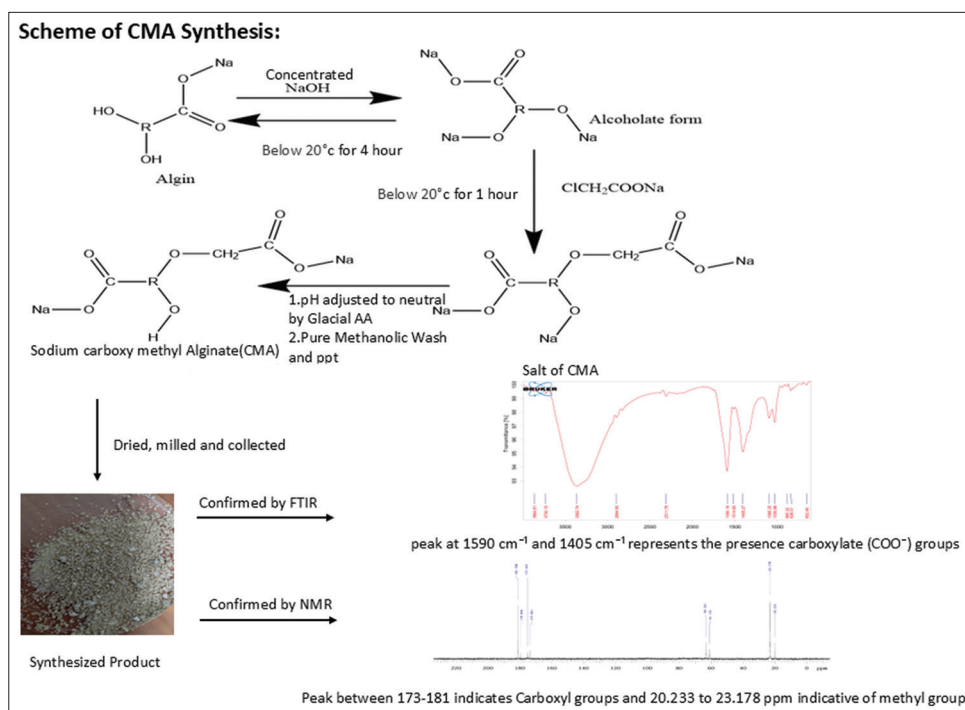


Figure 1: Scheme of carboxylated sodium alginate synthesis

- PEC-CS and SA- $9.7 \pm 0.3\%$
- PEC-CS and CMA- $9.5 \pm 0.2\%$

These results indicate the amount of moisture or volatile substances present in the polyelectrolyte complex films. The similar values suggest that both types of PEC films have comparable water retention properties. The relatively low values (around 9–10%) indicate that the films are fairly dry but still contain some moisture, which is typical for polysaccharide-based materials due to their hydrophilic nature.^[18]

Optical microscopy

CS is having smooth, uniform surface with minimal texture because CS typically forms smooth films due to its linear polymer structure and good film-forming properties. SA is also smooth surface with some visible striations because Alginate also forms relatively smooth films, but the striations may be due to the alignment of polymer chains during drying. CMA is having slightly rougher surface compared to SA, with visible granular structures because the carboxymethyl groups in CMA can lead to a more irregular packing of polymer chains, resulting in a rougher surface and granular appearance. PEC-CS and SA morphology indicates irregular surface

Table 1: Physical property study

| Polymer (complex) | Loss on drying (%) | Morphology | Solubility (%) |
|-------------------|--------------------|--|-----------------------------------|
| PEC-CS and SA | 9.7 ± 0.3 | Rough surface and pale yellow color | 62.4 ± 1.5 in distilled water |
| PEC-CS and CMA | 9.5 ± 0.2 | Irregular with fibrous structure and white color | 60.8 ± 1.3 in distilled water |

PEC-CS: Polyelectrolyte complexes-Chitosan, SA: Sodium alginate, CMA: Carboxylated sodium alginate

with fibrous structures visible PEC-CS and CMA's morphology similar to PEC-CS and SA but with more pronounced fibrous structures. The PECs show more irregular surfaces due to the interactions between the oppositely charged polymers [Table 1 and Figure 2]. The fibrous structures are likely a result of the complexation process, with the more pronounced structures in PEC-CS and CMA possibly due to stronger electrostatic interactions.^[19]

Solubility

The solubility of two complexes is given below PEC-CS and SA: $62.4 \pm 1.5\%$ PEC-CS and CMA: $60.8 \pm 1.3\%$. The PECs show intermediate solubility between CS and alginate. The electrostatic interactions between the polymers reduce overall solubility compared to pure alginate, but the presence of alginate increases solubility compared

to pure CS [Table 1]. The slightly lower solubility of PEC-CS and CMA might be due to stronger electrostatic interactions in this complex.^[18]

NMR Study Discussion

The presence of peaks in the range of 173–181 ppm aligns with the expected chemical shifts for carbonyl carbons in ester and carboxylate groups. This confirms the successful introduction of carboxyl groups into the SA structure.^[20] Peaks at 63.321 and 61.135 ppm are typical for carbohydrate-related carbon atoms, such as C2 and C3 in alginate, indicating that the primary structure of alginate is retained.^[21] The intensity of the peaks, particularly those corresponding to the carboxylate region, can be correlated to the degree of modification. The higher intensity of these peaks suggests that a substantial number of guluronic acid residues have been converted to their carboxylated forms. Peaks at lower chemical shifts (20.233–23.178 ppm) are indicative of methylene groups, which may relate to the backbone of the polysaccharide structure.^[21] The data suggest that carboxylation primarily affects the guluronic acid residues, while the alginate's backbone remains largely intact. The distribution of peaks indicates a relatively uniform

modification, essential for consistent material performance in applications like drug delivery or biodegradable packaging.^[22]

Antimicrobial Activity Test

Activity against Gram (–)

The activity of the PEC complex against Gram (–) is higher than the individual polymer of CS and SA. The average zone of inhibition shown by CS is 0.00 mm as it acts as a supporting layer to SA. On the other hand, the SA showed about 1.3 mm of inhibitory zone but the PEC shows significant improvement in the zone of inhibition activity which is about 10.3 mm [Figures 3-5, Table 2].^[23] On the other hand, CMA having major improvement of its parent polymer this is due to it has increased charge density due to additional carboxyl groups, which enhances interactions with positively charged microbial cells, leading to membrane disruption; improved solubility and diffusion allow it to interact more readily with microorganisms; the functional groups provide additional sites for interference with microbial metabolic processes; it can stabilize and enhance the bioavailability of antimicrobial agents; and the modification alters its viscosity and gel-forming properties, impacting

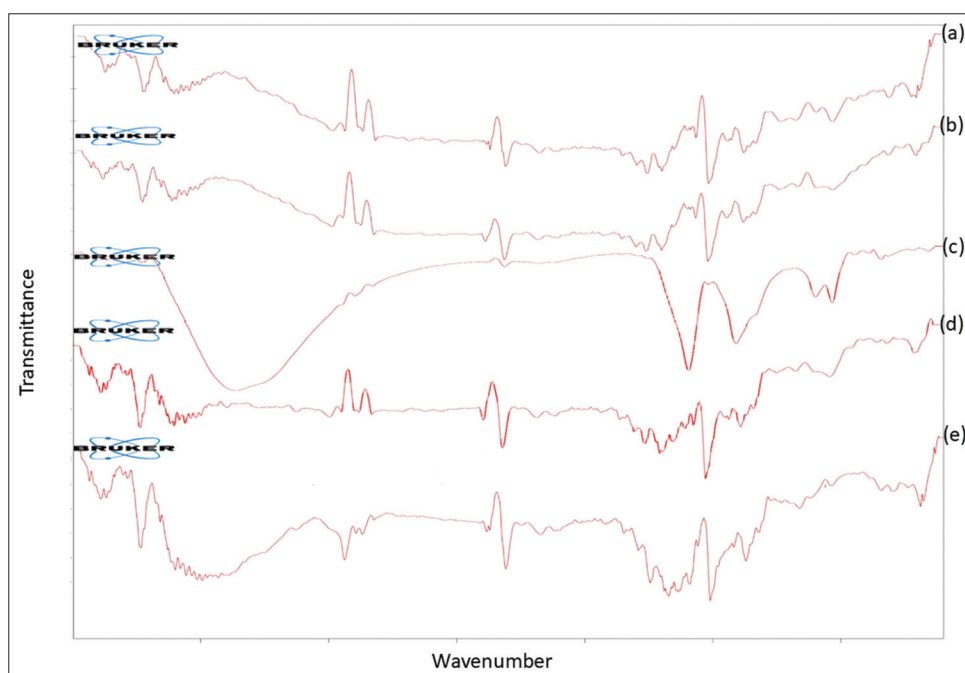


Figure 2: (a) Fourier transform infrared spectroscopy Spectra of chitosan (b) sodium alginate (SA) (c) carboxylated sodium alginate (CMA) (d) polyelectrolyte complexes- chitosan (PEC-CS) and SA, (e) PEC-CS and CMA

interactions with microbes and biofilms, making it a more effective antimicrobial agent overall. The zone of inhibition is shown about 14.3 mm which is the highest among all, while forming complex with CS there is no significant change in activity is shown, the zone of inhibition is around 14 mm [Figures 3-5, Table 2]. Among all the prepared PECs, they manifest their strongest antibacterial activity among their parent polymers. The $-NH_3^+$ group in PECs imparts a positive charge to PECs and thus the antibacterial activity of the PECs may be because of such a cationic charge that can interact electrostatically with the major components of positive and negative bacteria, namely anionic phospholipid dipalmitoyl phosphatidylglycerol. This interaction allowed protonated amino groups to react with anionic phospholipid of bacteria, thereby enhancing the permeability of cell wall membranes of both bacteria, stimulating release of nucleic acid, glucose, lactate dehydrogenase

from the cell and interfering transport of nutrient to the cells causing bacterial death. The higher effectiveness of PEC-CMA against Gram-negative bacteria can be attributed to its enhanced interaction with the outer membrane, increased charge density, chelating effects, synergistic action, favorable size and conformation, and improved pH responsiveness.^[8]

Activity against Gram (+)

The activity of the PEC complex against Gram (+) is higher than the individual polymer rather than the individual polymer of CS and SA. The average zone of inhibition shown by CS is 2 mm as it acts as a supporting layer to SA. On the other hand, the SA showed about 6.2 mm of inhibitory zone but the PEC shows significant improvement in the zone of inhibition activity which is about 10.4 mm [Figures 3 and 4, Table 2].

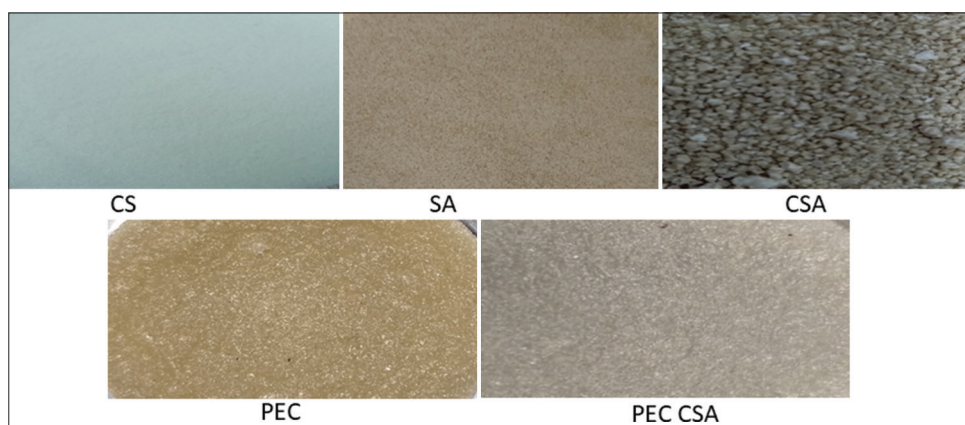


Figure 3: Surface morphology of polymer

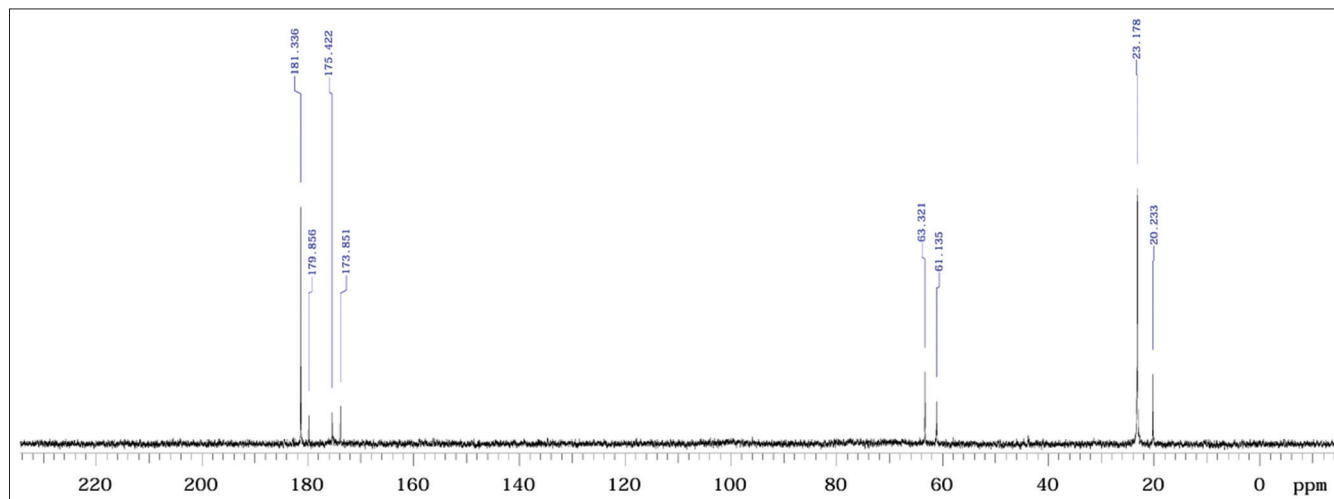


Figure 4: Nuclear magnetic resonance study of carboxylated sodium alginate

On the other hand, CMA may have limited intrinsic antimicrobial properties and might rely on other mechanisms, like acting as a carrier for other antimicrobial agents, rather than being directly effective or the interactions between the antimicrobial agent and the bacterial surface are crucial. Gram (+) bacteria might have cell

surface charges and structural features that do not favor strong binding or penetration by these complexes. One more reason that may be due to the cell wall of Gram (+) is of about 20–80 nm but Gram (–) bacteria having cell wall thickness of about >10 nm^[24] due to this reason it is unable to penetrate on Gram-positive cell wall.^[8]

Table 2: Table of antimicrobial study

| Polymer (complex) | Gram (–) ZOI (mm) | Gram (+) ZOI (mm) |
|-------------------|----------------------|----------------------|
| PEC-CS and SA | 14.3 | 10.4 |
| PEC-CS and CMA | 14.0 | 1.5 |

PEC-CS: Gram (–): Gram-negative bacteria, Gram (+): Gram-positive bacteria, ZOI: Zone of inhibition, CMA: Carboxylated sodium alginate

CONCLUSION

The study demonstrates the successful synthesis of PECs through ionic interactions between CS and SA or carboxylated- SA, which was

Table 3: Findings support complex formation and anti-bacterial activity

| PEC-CS and SA | PEC-CS and CMA | Findings support complex formation and anti-bacterial activity |
|--|--|--|
| - O-H and N-H stretching (3390-3263 cm ⁻¹) | - O-H and N-H stretching (3428.94 cm ⁻¹) | 1. The presence of free amino groups (N-H stretching at 3390–3263 cm ⁻¹) contributes to the antibacterial activity. |
| - C-H stretching (2989-2823 cm ⁻¹) | - C=O stretching (1694.55 cm ⁻¹) | 2. The new ionic interaction peak (1515 cm ⁻¹) indicates strong electrostatic interactions between CS and alginate, which may enhance the overall stability and effectiveness of the complex. |
| - Shifted amide I band (1694 cm ⁻¹) | - N-H bending (1640.20 cm ⁻¹) | 3. The shifted amide I band (1694 cm ⁻¹) suggests structural changes that might improve the complex's ability to interact with bacterial cell walls. |
| - Amide II band (1562 cm ⁻¹) | - Asymmetric COO ⁻ stretching (1594.83 cm ⁻¹) | 4. The higher wavenumber for O-H and N-H stretching (3428.94 cm ⁻¹) suggests weaker hydrogen bonding, potentially reducing the complex's stability and effectiveness. |
| - New ionic interaction peak (1515 cm ⁻¹) | | 5. The presence of asymmetric COO ⁻ stretching (1594.83 cm ⁻¹) indicates a higher degree of carboxylation, which might reduce the number of free amino groups available for antibacterial activity. |
| - C-O-C stretching (1029 cm ⁻¹) | | 6. The N-H bending peak (1640.20 cm ⁻¹) is more prominent, suggesting that more amino groups are involved in the complex formation, potentially reducing their availability for interaction with bacterial cell walls. |

PEC-CS: Polyelectrolyte complexe-chitosan, SA: Sodium alginate, CMA: Carboxylated sodium alginate

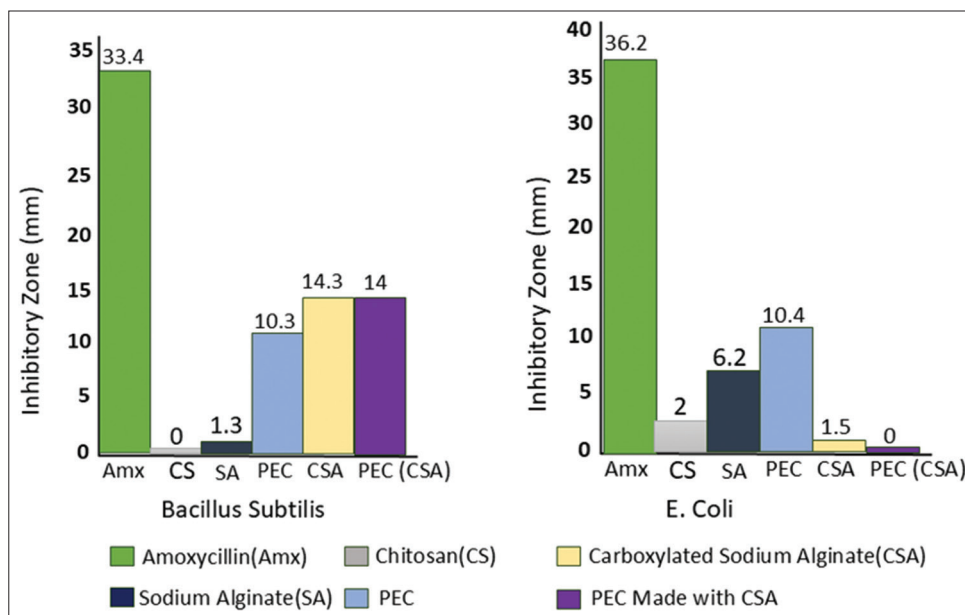


Figure 5: Comparison of antibacterial activity of polyelectrolyte complexes- chitosan (PEC-CS) and sodium alginate, PEC-CS and carboxylated sodium alginate with zone of inhibition

confirmed by physical appearance and supported via FTIR analysis. These PECs exhibit enhanced antibacterial properties compared to their parent polymers, particularly against *B. subtilis* and *E. coli*. The antimicrobial activity of PEC-CS and SA against Gram (+) bacteria was found to be ZOI of 10.4 mm and Gram (–) bacteria it was 10.3 mm. But PEC-CS and CMA showed very high Gram (–) activity with ZOI of 14 mm while it shows limited activity to Gram (–) bacteria with ZOI of 1.5 mm. The non-toxic, biodegradable, and edible nature of these complexes, combined with their improved antimicrobial activity, makes them promising candidates for use as edible films or coatings in food preservation. The electrostatic interactions between the polymers, confirmed by FTIR analysis, and the irregular, fibrous surface structure observed through optical microscope, contribute to the unique properties of these PECs. Overall, these findings suggest that PEC films have significant potential in preventing bacterial contamination and growth in food applications, offering a safe and effective solution for extending food shelf life and improving food safety.

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AUTHORS' CONTRIBUTIONS

Arindam Sarkar and Sanchita Mandal planned the experiment. Arindam Sarkar performed the experiment, carried out the calculations, interpreted results and discussion, and wrote the manuscript. Shila Barman contributed to the interpretation of the result. Sanchita Mandal contributed to the overall supervision and guided on manuscript writing, editing, and reviewing.

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AVAILABILITY OF DATA AND MATERIAL

Required data and Figures are included in the manuscript.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

Approved by all the authors.

CONSENT FOR PUBLICATION

Approved by all the authors.

COMPETING INTERESTS

The authors declare that they have no competing interests for the current work.

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