Materialia 16 (2021) 101082

Contents lists available at ScienceDirect

Materialia

journal homepage: www.elsevier.com/locate/mtla

Full Length Article

Chitosan-terephthaldehyde hydrogels – Effect of concentration of cross-linker on structural, swelling, thermal and antimicrobial properties

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ARTICLE INFO

Keywords:
Chitosan
Terephthaldehyde
Cross-linking
Swelling
Antimicrobial activity

ABSTRACT

Chitosan properties can be finely tuned for various bio-applications by cross-linking with suitable cross-linkers and the extent of its cross-linking. The effect of concentration of cross-linker on the biopolymer would exhibit different physiochemical properties, surface charges, thermal stability, morphology and their swelling behavior. In this study, chitosan has been cross-linked with varying concentration of hydrophilic terephthaldehyde (TAL) as cross-linker and characterized using FT-IR, XRD and thermogravimetric analysis (TGA). The effect of concentration of the cross-linker on the morphology, surface charge, pH dependent swelling was studied. The formation of imine bond between chitosan and TAL was confirmed by using FT-IR. The XRD analysis shows increase in crystallinity upon increasing the cross-linker concentration of TAL in comparison with the pure chitosan. The thermal stability of the hydrogels was also studied. With increase in the concentration of TAL, the hydrogels show a polyphasic structure and the surface charge become more positive. The TAL cross-linked chitosan exhibited both highest and lowest in swelling at the physiological pH with varying concentration of the cross-linker. The chitosan-terephthaldehyde hydrogels exhibits good antimicrobial response towards gram negative bacteria. The results indicate chitosan-terephthaldehyde hydrogels (**CST**) to have potential applications in various biomedical applications at physiological pH.

1. Introduction

Different methodologies are emerging to develop materials having antimicrobial activity against infections [1]. In recent years, researchers have given considerable attention in developing materials that are biocompatible, biodegradable and possess antimicrobial properties [2]. Studies suggested that the polymeric hydrogels serves as an effective platform to engineer antimicrobial materials. They are formed either by non-covalent cross-linking process or hydrogen bonding or hydrophobic attractions and shown to exhibit high water absorbing and retaining capability without getting dissolved [3].

Designing a suitable biopolymer with mechanical robustness and antimicrobial activity has been a major objective in developing materials for various biomedical applications. Chitosan is the second most abundant natural biopolymer after cellulose. Chitosan, obtained from chitin by alkaline deacetylation process,owing to its biocompatibility, biodegradability, biological activity, and non-toxicity [4], are promising biomaterials for tissue engineering, pharmaceuticals, and drug delivery applications [5].

Recently, there has been a continuous interest in modifying chitosan in order to improve its biological activity and mechanical strength and hence widen its applications [6]. Quaternized chitosan has been reported to show bioadhesive properties, permeation enhancing effects and high efficacy against bacteria and fungi even at neutral conditions [7]. Chemical cross-linking is an effective method in preparing mechanically durable chitosan hydrogels that can be rolled out as sheets and films. Covalent cross-linking treatments have been used to modulate the physical properties and biofunctions of chitosan hydrogels [8]. Usually, the hydrophilicity-hydrophobicity, swelling behavior, biodegradation performance, mechanical properties, and biocompatibilities of crossed linked chitosan have a good relationship with the network structure [9]. The extend of cross-linking has shown to influence a lot on the pH dependent swelling, surface charge, thermal stability and morphology of the chitosan [10].

The amino groups of the glucosamine residue within chitosan chains serves as cross-linking sites, for example, by reacting with glutaraldehyde, glyoxal, proanthocyanidin, genipin to form cross-linking between linear chitosan chains and leads to gel formation [11]. Glutaraldehyde is well known cross-linker for chitosan and various researches have been done, but in comparison to terephthaldehyde (TAL) as cross-linker, there are lot of differences. The notable ones are glutaraldehyde, which is an aliphatic di-aldehyde, and TAL which is an aromatic di-aldehyde.

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https://doi.org/10.1016/j.mtla.2021.101082
Received 8 February 2021; Accepted 25 March 2021
Available online 2 April 2021

Glutaraldehyde is a hydrophobic cross-linker while TAL is hydrophilic in nature. Aliphatic moieties are non planar whereas the aromatic systems are planar. Such vital differences in their structural form keeps the glutaraldehyde cross-linked chitosan and TAL cross-linked chitosan far apart based on their properties like crystallinity, swelling and thermal stability [12,13].Terephthaldehyde (TAL), reported earlier as a cross-linker for polyvinyl alcohol and chitosan polymer that reacts with the amine functional groups to form an imines bond [14]. The innocuousness of TAL lies in the absence of alpha hydrogen on the aldehydic functional group, which eliminates the possibility of TAL to undergo aldol reaction, as in the case of glutaraldehyde, this has been attributed for the safe and non-volatility of TAL [15]. Moreover due to its hydrophilic nature TAL is expected to have higher swelling ability in aqueous and organic media, and with greater positive charge density, it exhibits better antimicrobial activity upon cross linking with chitosan [16]. In addition, the ability to tailor the physical properties such as permeability, fluid content, and mechanical strength of chitosan hydrogels polymers is an integral part of biopolymer design, as these properties have been shown to influence drug release, as well as cellular growth and function [17].

The present work aims on the preparation and characterization of chitosan-terephthaldehyde hydrogels (**CST**) and attempts to investigate the effect of cross linker concentration on the thermal stability, structural changes, pH dependent swelling, and antimicrobial activity of the **CST**.

2. Materials and methods

2.1. Materials

Chitosan (48700 Da) with a degree of deacetylation (DD) of 80% was purchased from Matsyafed chitosan plant, Kerala, India. Terephthaldehyde, ethanol and glacial acetic acid were purchased from Avra synthesis Pvt. Ltd and were used without further purification. Acetone was purchased from Avra synthesis Pvt. Ltd and distilled before use.

2.2. Preparation of chitosan-terephthaldehyde hydrogels

1 g of chitosan (**CS**) was dissolved in 50 mL of 1% aqueous acetic acid under continuous magnetic stirring at room temperature for 16 h. Terephthaldehyde (TAL) of various concentrations, 10 mg, 25 mg,50 mg, and75 mg (**CST-I, CST-II, CST-III,** and **CST-IV**, respectively) were dissolved in 5 mL of ethanol. TAL solution was charged to the chitosan solution drop by drop and stirred until the chitosan solution turned into a more viscous gel. The prepared hydrogels were washed with acetone thrice. The products were dried under vacuum at room temperature [14].

2.3. Characterization of hydrogels

Fourier transform infrared (FTIR) spectra were recorded on Nicolet IS-5, Thermo fisher spectrophotometer, USA. The XRD patterns of chitosan and the hydrogels were recorded using Bruker X8 KAPPA APEX II single crystal X-ray diffractometer, USA. The surface morphology of chitosan and the hydrogels were studied using FEI Quanta 450 Scanning electron microscope, USA. Thermogravimetric Analysis (TGA) was recorded using TGA Q500 V6.7 Build 203 TA Instruments USA. It was recorded at 10.00 °C/min to 350 °C.The surface potential was recorded using Zetasizer 3000 Malvern Instruments, UK.

2.4. Determination of Amine Content

To determine the extent of cross-linking the amine content present in the chitosan and the hydrogels were calculated by acid-base titration method [18]. 0.1 g of chitosan and the hydrogels were added in a flask containing 30 mL of 0.05 N HCl solution and kept for equilibrium for 15 h, the solution was then titrated against 0.05 M NaOH with phenolphthalein as indicator.

2.5. Swelling analysis

To determine the swelling capability, chitosan hydrogels were kept into aqueous solutions of varying pH between 1.0 and 14.0 at room temperature for a required period of time [19]. The wet weight of the swollen samples were determined by blotting the samples with filter paper to remove water from the surface and then weighed immediately. The swelling percentages of the prepared hydrogels in the media were calculated as follows:

$$E_{sw} = \left[(W_e - W_0)/W_0\right]100$$

Where, E_{sw} is the swelling percentage of the gel at equilibrium.

W_e denotes the weight of the gel when the swelling equilibrium is reached and

W_0 is the initial weight of the gel.

Each swelling experiment was repeated thrice and the average value was taken for calculating the swelling percentage.

2.6. Antimicrobial studies

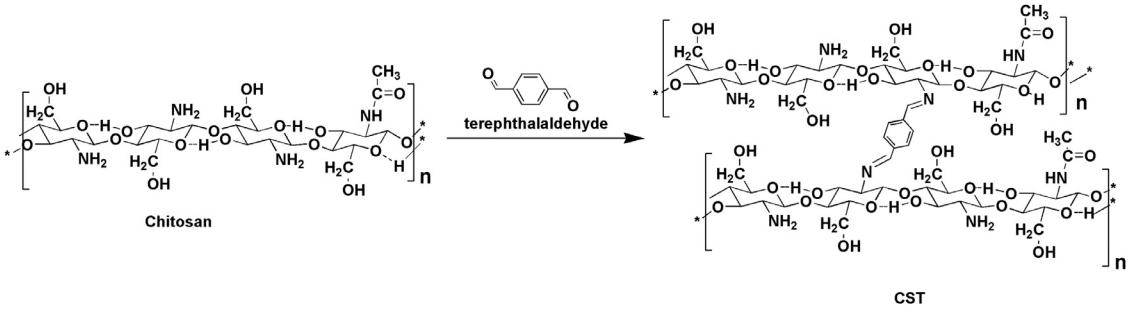
The antimicrobial studies were done by well diffusion method using two gram positive and gram negative bacteria as indicator organisms (*S. mutans, S. aureus, P. vulgaris and K. pneumonia*). The samples were dissolved in Dimethyl sulphoxide (DMSO). A cork borer (4 mm) was sterilized by autoclaving or disinfecting it by rinsing in alcohol followed by sterile water. In the nutrient agar plates, 4 mm holes are punched aseptically in the agar using the cork borer. The undersides of the petri plates were labeled using a wax pencil. The indicator organisms were aseptically swabbed onto the nutrient agar plates in three directions to ensure complete plate coverage. The plates were left to stand for 5 min. 10 μL, 20 μL and 30 μL of the sample were filled in the appropriate wells. The plates were incubated at 35 °C for 24–48 h. The zones of inhibition were then measured using a ruler on the undersides of the plates and recorded [20].

3. Results and discussion

Chitosan is mainly made up of two monomer units namely glucosamine and *N*-acetyl glucosamine. The presence of amino groups of chitosan may exhibit polycationic properties which is responsible for the electrostatic interactions with anionic systems. This property has been found to be quite valuable in using chitosan as an antibacterial agent [21]. The presence of high content of amino and hydroxyl groups allow easy and selective modifications of chitosan to improve the solubility and biocompatible properties. Chitosan undergoes Schiff's base reaction with terephthaldehyde as shown in Scheme 1. The obtained chitosan-terephthaldehyde gels (**CST I-IV**) were characterized using FT-IR, XRD, SEM, and TGA.

4. Fourier-transform infrared spectroscopy analysis

Fig. 1 represents the FT-IR spectra of chitosan (**CS**) and chitosan-terephthaldehyde gels (**CST**). The broad peaks exhibited around ~3400 cm^{-1} are due to the amine N–H symmetrical and O-H vibration. The peaks centered between 1630 cm^{-1} and 1645 cm^{-1} in the FTIR spectra of **CST**s are assignable to $\nu_{C=N}$ stretching vibration of the imine bond, which is absent in the spectrum of uncross-linked chitosan **CS** [22]. This confirms the formation of imine bonds present in **CSTs** due to the cross-linking of TAL with chitosan.



Scheme 1. Cross-linking of Chitosan with TAL.

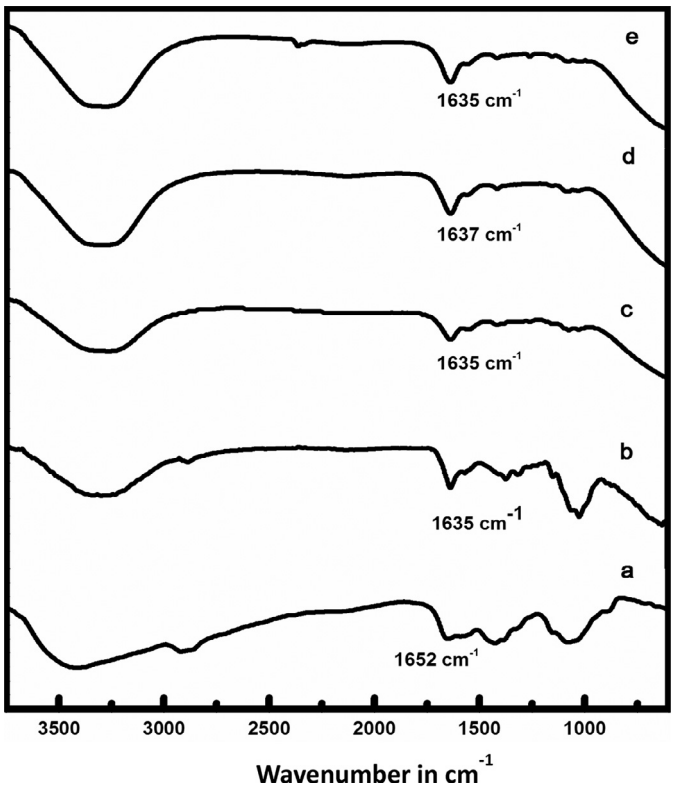


Fig. 1. FT-IR spectra of pure chitosan a) (**CS**) and with different % of cross linker**TAL** (b-e) (**CST 1-V**).

Table 1
The amine content present in Chitosan and CST.

Sample	Amine content in mmol/g
CS	510
CST-I	180
CST-II	120
CST-III	90
CST-IV	90

5. Estimation of Amine Content

The amine content present in the chitosan and chitosan-terephthaldehyde derivatives were calculated by volumetric method and the values are given in the Table 1. Upon addition of increasing amount of terephthaldehyde to chitosan, the concentration of free amine functional group present in the chitosan decreases, due to cross-linking of terephthaldehyde with chitosan. For **CST-III** and **CST-IV** a constant value of 90 mmol/g of amine content was observed. At higher concentration of TAL, due to the non- flexible nature of the terephthaldehyde system, it is unable to cross link with available free -NH_2 groups present in the chitosan [23].

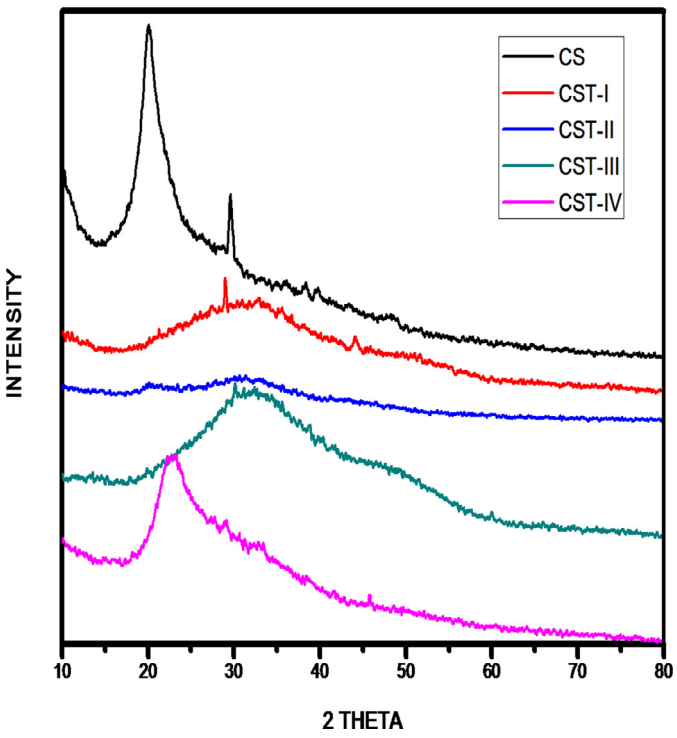


Fig. 2. XRD patterns of **CS** and **CST (I-IV)**.

Table 2
X-ray Diffraction data's of **CS** and **CS-(I-IV)**.

Sample	2 Theta	Crystallinity index
CS	9.77°, 20.19°	0.97
CST-I	25.18°	0.83
CST-II	20.5°, 29.99°	0.56
CST-III	26.16°	0.91
CST-IV	6.35°,20.31°	0.94

6. X-ray diffraction analysis

The XRD patterns of the **CS** and **CST** gels are shown in Fig. 2. The XRD of pure chitosan shows a weak peak around $2\theta = 9.77°$ and a main crystalline peak at $2\theta = 20.19°$ (Fig. 2 and Table 2).The absence of weak peak and shifting of crystalline peak from ~20 to 25–29° with increasing broadness, indicates the amorphous nature of these **CSTs (I-II)** than **CS**

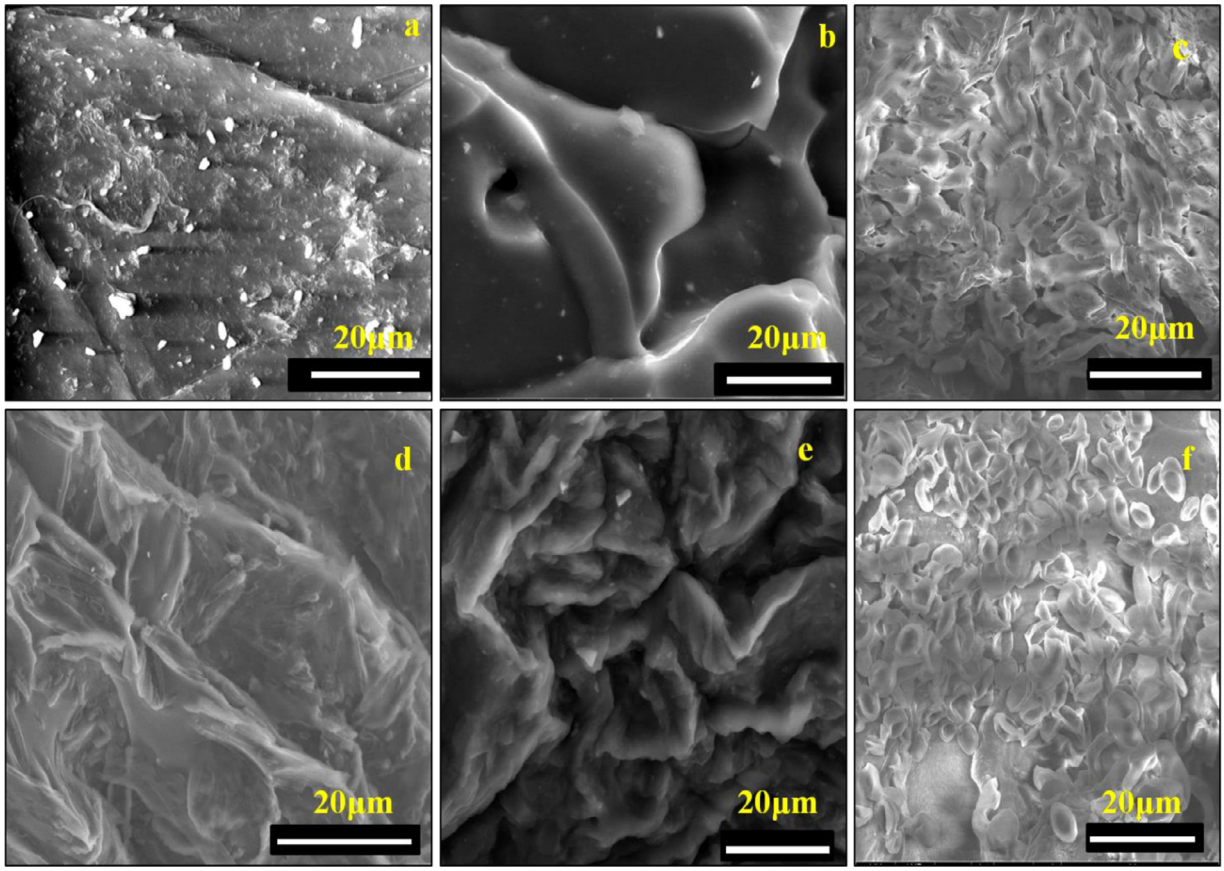


Fig. 3. SEM images of **CS** and **CS-(I-V)**.

[24]. **CST-II** show high degree of amorphous nature, seen by the lower intensity of the peaks. The further increase in the amount of concentration of terephthaldehyde, in **CST-III** and **CST-IV** leads to reappearance of two peaks around, 26.16° and 6.35°, 20.31°, respectively, signifying the semi crystalline nature of **CST**s. Further the crystallinity index (CI) were also calculated [25], it was observed that the CI of pure chitosan was 0.97, initially upon cross-linking a decrease in crystallinity index was observed for **CST-I** and **CST-II** with CI of 0.83 and 0.56, respectively, as the concentration of cross-linker increases the crystallinity index becomes higher with a CI of 0.91 and 0.94 for **CST-III** and **CST-IV**. This is due to the structural rigidity offered by the planar aromatic cross-linker, terephthaldehyde, with symmetrically placed aldehyde group, which builds a tighter structure in the chitosan as a result of high degree of cross linking [26].

7. Scanning electron microscopy analysis

The SEM images of native chitosan exhibits a porous, non-smooth membranous phase. When chitosan is cross-linked with terephthaldehyde, the gels exhibits a polyphasic structure (Fig. 3). At lower degree of cross-linking, a flat lamellar structure was observed; on increasing the degree of cross-linking further, an edge folded lamellar structures have evolved [27]. Further the structure is manifested into cup like shapes, arising due to the maximized folding of the lamellar edges. A morphological evolution is observed in **CST**s with increase in degree of cross-linking of chitosan [28].

8. Thermo Gravimetric analysis

The comparative TGA study of chitosan with those of **CST**s hydrogels confirms the cross linking and thermal stability of the hydrogels (Fig. 4). In chitosan, the weight loss takes place in two steps as a result of water evaporation and degradation. The initial weight loss of 11 % at 44.01 °C to 83.86 °C corresponds to the removal of water molecules. Water molecules can interact with two different polar groups in chitosan; amine and hydroxyl groups. Initially, the water molecules selectively binds to the amine groups, which are easily removed as compared to those bound to hydroxyl groups. The second stage weight loss of 39% from 284 °C to 314 °C corresponds to the thermal and oxidative decomposition of chitosan. On the other hand, the **CST** undergo a single stage weight loss between 60 and 80 °C corresponding to concomitant loss of water molecules and decomposition of the hydrogels. Due to the lesser content of amine groups present in the **CST** hydrogels, the water molecules are bound to the free hydroxyl groups, which are difficult to remove [29,30]. The formation of intra-cross linkages between polysaccharides chains, which interferes with previously existing stronger hydrogen bonds, is found at the site of cross-linking. Also, it is reported that the cross linking increases the water content in the chitosan hydrogels.It is also evident from the XRD data and the crystallinity index that cross-linked chitosan has undergone structural changes compared to the pure chitosan. Further the presence of double bond or oxygen containing structures (TAL) make the chitosan backbone less resistant to high temperatures [31]. All these factors contribute to the low temperature of decomposition and maximum weight loss of 80–90%.

9. pH dependent swelling study

Hydrogels are usually characterized by the degree of swelling; its capacity can be determined by the amount of space inside the hydrogels network available to accommodate water molecules and the nature of functional group. There will be stronger polymer-water interaction, if the polymers are more hydrophilic in nature. The pH dependent

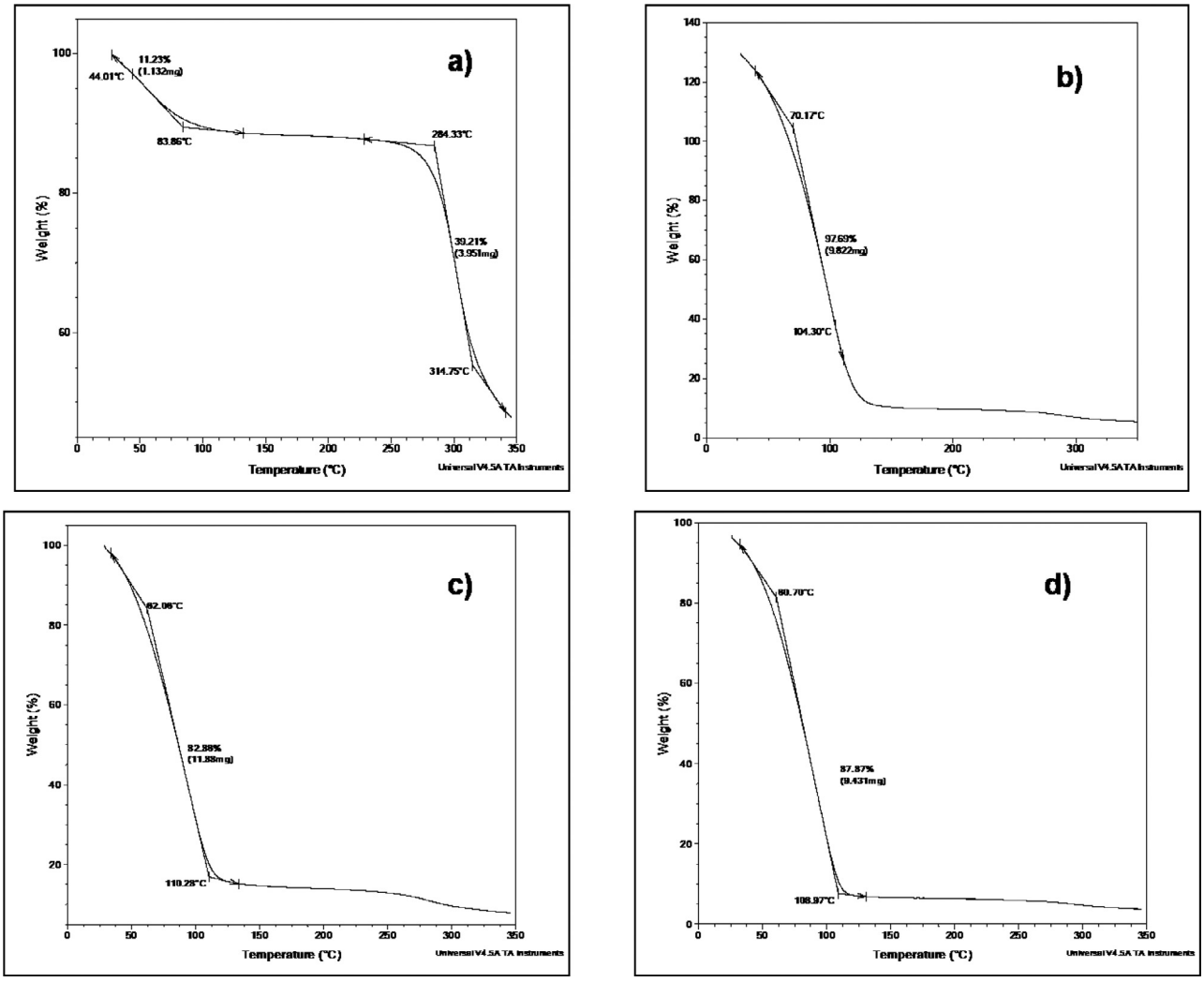


Fig. 4. TGA patterns of a) **CS**, b) **CST-I** c) **CST-II** and d) **CST-III**.

swelling behavior was studied for chitosan and the prepared **CST** hydrogels. The plot of pH vs swelling percentage shown in Fig. 5, suggest that pure chitosan swells at pH 1 and the swelling percentage increases till neutral pH (pH 7) and falls down in alkaline pH range, Chitosan being a hydrophilic polyelectrolyte polysaccharide it swells up in aqueous media, the pH dependent swelling is controlled by the -NH_2 groups present in chitosan backbone, the protonation of -NH_2 group at acidic pH leads to hydrophobic repulsion [32]. On the other hand, for the cross-linked hydrogels the swelling is not observed until pH 3. When cross-linked, the chitosan molecules stay rigid, hence the pH dependent swelling or de-swelling, arising from the ionization of the -NH_2 groups, is withheld. Thus, it requires a higher pH and corresponding ionization of -NH_2 groups to overcome the rigidity of the cross-linked hydrogels. Accordingly, the swelling percentage of the hydrogels increases up to pH 7 and decreases in alkaline pH range [33]. From the Fig. 5, it is observed that **CST-II** has shown the least water absorption compared to other cross-linker **CSTs** and **CS** at all pH and lowest swelling at pH 7, mainly attributed to the lower degree of crystallinity witnessed from the XRD studies of **CST-II**. The amount of cross linker in **CST-II** serves as the optimum concentration between the semi crystalline nature of pure **CS** and highly rigid and defined structure of **CST** hydrogels with higher concentration of terephthaldehyde. The cross-linking of chitosan with TAL demonstrate a pH dependent volume change of chitosan, attaining a maximum swelling at pH 7 for **CST-I** making it an ideal choice for pH dependent drug delivery applications at physiological pH. The least swelling of **CST-II,** makes it suitable for biomedical applications requiring lower water retention like in burn wound dressing which requires maintaining a moist environment in the wound site for tissue generation [34,35].

Table 3
Zeta potential measurement of **CST-(I-IV)**.

Sample	Zeta potential in mV
CST-I	46.5
CST-II	52.4
CST-III	51.9
CST-IV	53.1

10. Antimicrobial activity

There are several reports on the antimicrobial activity of polycationic chitosan [6,36]. The modification of chitosan into antimicrobial hydrogels makes them suitable for various applications. The polycationic chitosan hydrogels, as inferred from the zeta potential values are given in Table 3, have shown an increase in anti-microbial activity towards the anionic gram negative bacteria. Of the two gram negative bacteria,

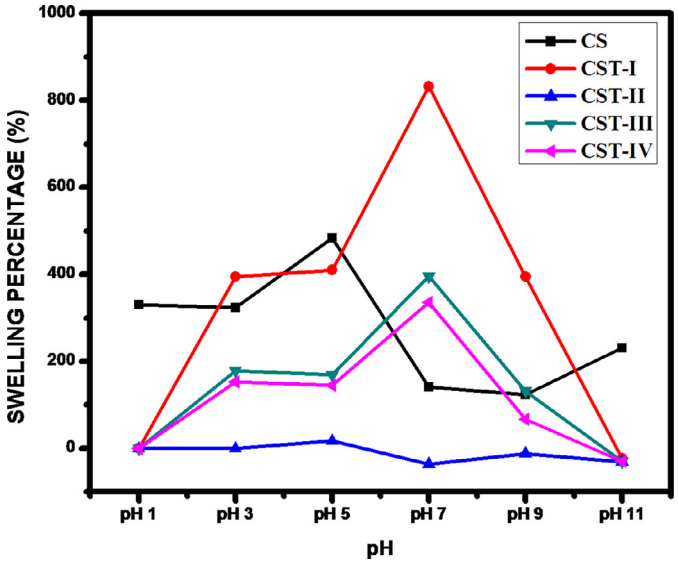


Fig. 5. pH dependent swelling of **CS** and **CST** hydrogels.

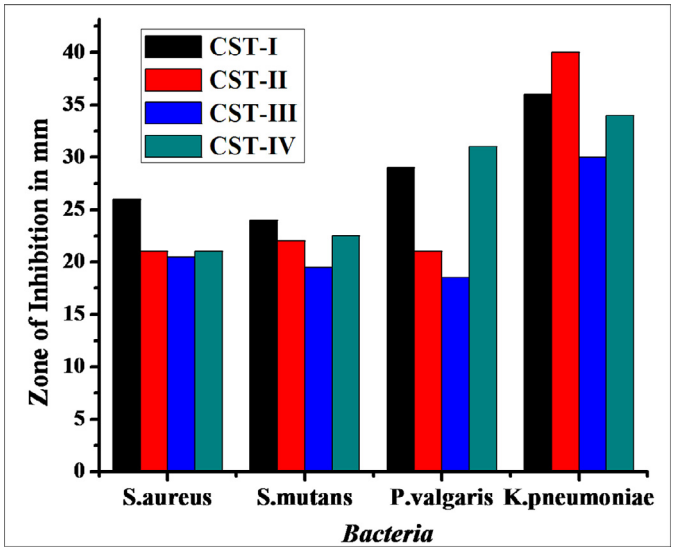


Fig. 7. The antimicrobial activity of **CST-I-IV** with *S.aureus,S.mutans, P.vulgaris and K.pneumonia*.

maximum inhibition was observed towards *Klebsiella pneumonia.* While comparing the antimicrobial activity with respect to the concentration of cross-linking **CST-I** and **CST-II** has shown the maximum inhibition with *Klebsiella pneumonia* (Figs. 6 and 7). Antimicrobial activities of the prepared **CST** hydrogels were exhibited increased activity with increase in concentration of the hydrogels (Fig. 8**)**. It has been proposed that the interaction between the anionic cell surface of bacteria and the cationic amino group of chitosan weakens the cell membrane of the bacteria. Another mechanism proposed for antimicrobial activity is the binding of chitosan with microbial RNA by penetration into the nuclei of the microorganism which leads to inhibition on mRNA and protein synthesis. The hydrophilic terephthaldehyde cross-linker separates the chitosan chains apart and decreases their intermolecular hydrogen bonds and increases their solubility, allowing easy penetration of the hydrogels into the cells of the bacteria and prevents their proliferation [20]. This explains the potential antimicrobial activity of chitosan cross-linked with

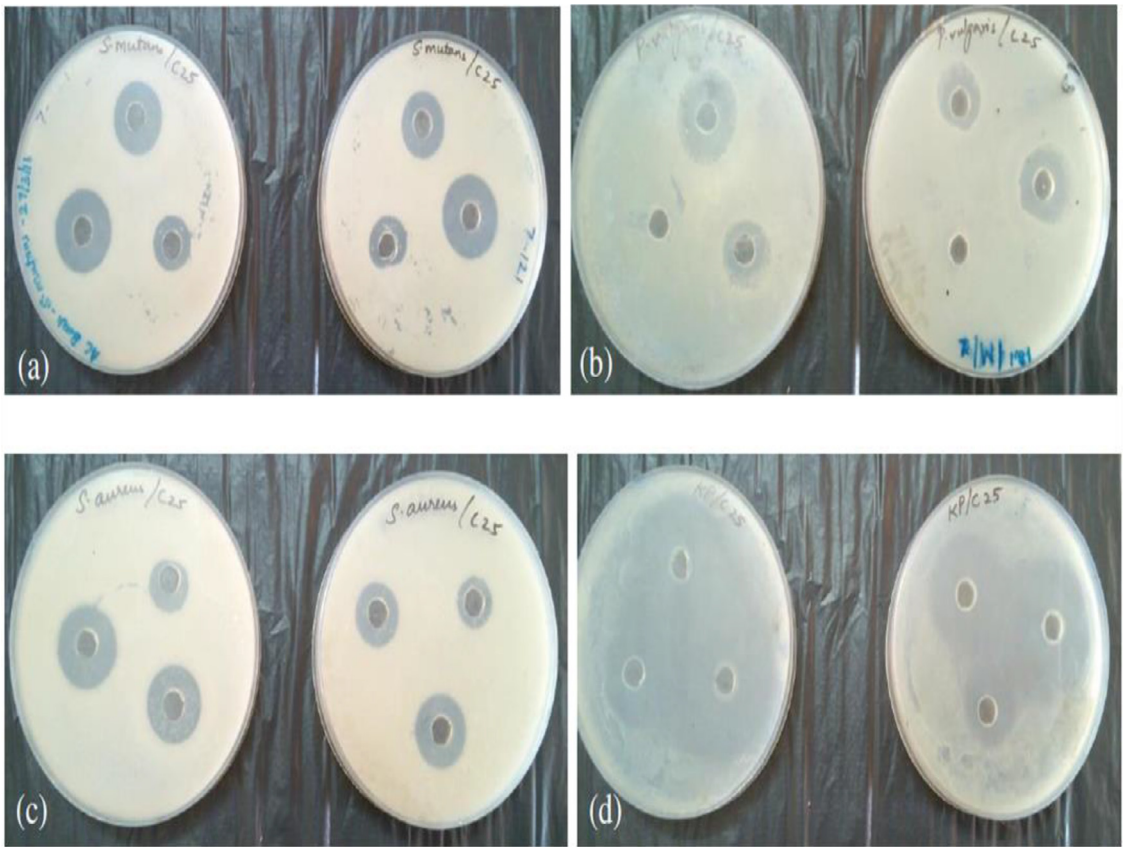


Fig. 6. Antibacterial activity of **CST-II** with (a)*K.pneumonia* (b) *S.mutans,* (c) *P.vulgaris,* (d)*S.aureus*.

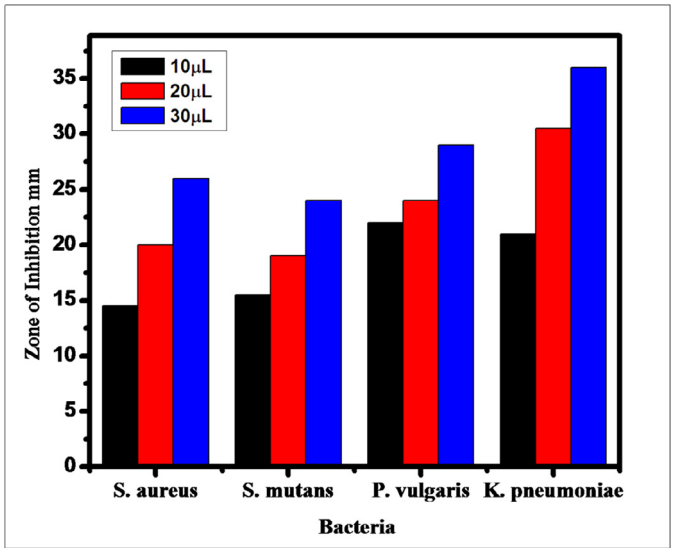


Fig. 8. The antimicrobial activity of different concentration of **CST-I** with *S.aureus,S.mutans,P.vulgaris and K.pneumonia.*

hydrophilic terephthaldehyde. **CST-II** exhibit least swelling in water at pH 7 and with higher antimicrobial activity, this is essential requirement for the application in wound dressing material, in view of the fact that the wound dressing material must ensure an optimum moisture condition for faster healing [37].

11. Conclusion

The effect of concentration of the aromatic cross-linker, terephthaldehyde with chitosan on structure, thermal stability, pH dependent swelling characteristics and antimicrobial studies were studied. The formation of imine bonds between chitosan and TAL were confirmed using FT-IR technique. SEM analysis indicates that hierarchical morphological changes observed at high degree of cross-linking. The **CST** hydrogels showed single stage thermal decomposition at a temperature lower than that of pure chitosan. The swelling behavior was found to be pH dependent and the positively charged **CST** hydrogels exhibited good antimicrobial response towards gram negative bacteria. This study suggests that chitosan-terephthaldehyde hydrogels could be suitable for various biomedical applications at physiological pH by tuning the cross-linker concentrations.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

We are greatly thankful to Science and Engineering Research Board, Department of Science and Technology (DST-SERB), Government of India for the grant in aid. We thank Sophisticated Analytical Instrumentation Facility-SAIF IIT Madras for the characterization of the samples.

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