

**Carboxymethyl Chitosan Electrospun Nanofibers:
Preparation and its Antibacterial Activity**

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ABSTRACT

Electrospinning of carboxymethyl chitosan (CMCS) solutions in water was studied by adding poly (vinyl alcohol) (PVA) as “guest” polymer. The properties of the CMCS/PVA solutions measured, and effects of the polymer concentration, CMCS/PVA mass ratio and processing parameters on the electrospinnability of CMCS/PVA were investigated. Morphology of the electrospun CMCS/PVA observed by using scanning electron micrographs. Results showed that the ultra-fine fibers could be generated after addition of PVA in different mass ratios of CMCS to PVA from 4–8 wt. % CMCS/PVA solutions. Antibacterial activity of electrospun fibers prepared against Gram (-ve) and Gram (+ve) bacteria is measured via Disk Diffusion Method. Electrospun CMCS/PVA nanofiberwebs show good antibacterial effects towards Gram (+ve) and Gram (-ve) bacteria. Antibacterial activities of electrospun nanofiberwebs increased by increasing the CMCS percent in the electrospun membrane. Results of the higher antibacterial activity of electrospun CMCS/PVA nanofiberwebs oriented its potential applications in wound dressings.

Keywords: Antibacterial fibers, electrospun nanofiberwebs, antibacterial activities

1. Introduction:

Electrospinning is a fiber-forming process by which either polymer solutions or melts are charged by high voltage to form fine jets. Fiber formation by electrospinning of polymer solutions has been extensively studied in terms of voltage, tip-to-collector distance, extrusion rate of polymer solution, and polymer solution properties¹⁻⁵. It is a very effective, simple and expensive method of making nanofibers from polymer solution compared to other methods such as self-assembly and phase separation and has the potential for large-scale

manufacturing^{6, 7}. The nanofibers provide a very large surface area due to their extreme small size which makes them compatible with human cells' size and suitable to form products for filtration of sub-particles, adsorption of biological and chemical warfare gases, medical industry as artificial blood vessels and organs, sutures, surgical facemask, wound dressing, and drug delivery systems^{8,9}.

Chitosan is partially deacetylated chitin, the second most abundant polysaccharide next to cellulose. It is also one the few nitrogen containing natural

polysaccharides. Its inherent biological and physicochemical characteristics are attributed to the unique amine functionality, and it has attracted extensive attention in research because of its potential in biomedical and technological applications¹⁰. Like other polysaccharides, chitosan is non-thermoplastic and insoluble in organic solvents. Chitosan is only soluble in acids, and it has been wet spun from acetic acid and formic acid into alkaline coagulating media¹¹⁻¹³. Smaller electrospun chitosan fibers have also been produced by electrospinning, either alone or with another polymer, from trifluoroacetic acid, formic acid and acetic acid^{8, 14-19}. Since the traces of the residual acids or organic solvents in the produced electrospun fibers is harmful, reduced its biocompatibility, and increase its toxicity. To overcome these problems, carboxymethyl chitosan was produced as water-soluble chitosan to allow fiber generation from non-acidic aqueous solution affords obvious advantages for biological and biomedical applications²⁰⁻²².

PVA is a water-soluble polymer with semi-crystalline molecular structure. It shows good properties, such as nontoxicity and chemical stability. Preparation of PVA nano fibers by electrospinning has been reported^{7, 23, 24}, and electrospun inorganic nanofibers were often obtained with the help of PVA^{7, 25}.

In the present study, electrospun fibers were obtained by electrospinning of the blended carboxymethyl chitosan/PVA solutions in water. The properties including surface tension and viscosity of the carboxymethyl chitosan and PVA solutions were measured. Effects of system parameters on the morphology of nanofibers were studied and the antibacterial activity of the electrospun CMCS/PVA fibers were measured and its effect on processing parameters via disk diffusion method.

2. Experimental:

2.1. Materials

Chitosan (CS) (Aldrich, viscosity 1860cps, degree of deacetylation 79.0%). PVA (MW 88,000, degree of deacetylation 89% and viscosity 35-50 cps for 4% solution

at 20 °C) was obtained from Fine Chemicals Pvt. Ltd, India. All other chemicals and reagents were of analytical grade, and were used without further purification. Four bacterial strains from the Faculty of women for Art, Science & Education, Ain Shams University, Cairo, Egypt were employed. They include gram-positive, gram (+) bacteria: *Staphylococcus aureus* (*S. aureus*) and *Bacillus subtilis* (*B. subtilis*) and the gram-negative, gram (-) bacteria: *Escherichia coli* (*E. coli*), and Proteus. These bacterial strains were selected as test cells because they are the most frequent bacteria in the wound infection and represent gram-positive and gram-negative bacteria, respectively. Fresh inoculants for antibacterial assessment were prepared on nutrient broth at 37°C for 24 hours.

2.2. Methods

2.2.1. Carboxymethylation of chitosan:

The carboxymethylation of chitosan (CMCS) was prepared as reported in our previous work²⁰ as follow; chitosan (5g), sodium hydroxide (50%), isopropanol (80ml), and water (20ml) were added into a three necked flask (250ml) to swell and alkaline at room temperature for one hour. The monochloroacetic acid (2.5M) was dissolved in isopropanol (20ml), and added into the reaction mixture drop wise for 30 minutes and reacted for proper time (3 hrs) at temperature (60°C), then stopped by adding 80% ethyl alcohol. The solid was filtered and rinsed in 70-90% ethyl alcohol to desalt and dewater and dried at room temperature.

2.2.2. Preparation electrospinning solutions:

The CMCS solutions with concentrations ranging from 3 to 9 wt% were prepared by dissolving 0.3, 0.5, 0.7 and 0.9 g of CMCS in 10 ml distilled water for 1 h. PVA solution of 8 wt% was prepared by dissolving 0.8 g of PVA in 10 ml distilled water with moderate stirring at 60 °C for 2 h. To determine the most favorable spinnable concentration of CMCS with PVA, each CMCS solution was mixed with 8 wt% of PVA solution (in 1:3 ratio) with the help of a

magnetic stirrer. The selected concentration of CMCS, 7 wt% CMCS solution, was mixed with 8 wt% PVA solution in five different weight compositions such as 0/100, 25/75, 50/50, 75/25, and 100/0. All the solutions were mixed well in a beaker for 2 h to get a homogenous mixture.

2.3. Characterization of electrospinning solutions:

Surface tension and viscosity play important role in extrusion of the solution through the needle and decides fiber diameter and morphology. Surface tension was measured using Fisher Tensiomat Model 21. Viscosity of the solution at different concentration was measured with a Brookfield viscometer at room temperature of 25° C. The surface morphology of the electrospun fibers were studied by scanning electron microscope (SEM) examination by mounting the samples on stub with double stick adhesive tape and coated with gold in a S150A sputter coater unit (Edwards, UK). The gold film thickness was 150Å. The samples were then viewed in a JEOL JXA-840 electron probe microanalyser, Japan. The nanofiberwebs for SEM were frozen in liquid nitrogen, immediately snapped, then freeze-dried under vacuum at – 60°C and cover by gold vapors.

2.4. Electrospinning of carboxymethylchitosan solutions:

The electrospinning equipment used consists mainly of extrusion system (syringe pump), fiber collection system, and high voltage supply (Figure 1). The extrusion system (New Era Pump Systems Inc., Model number: NE-300) is used to provide controlled feed rate of the spinning solution. The extrusion system has a barrel filled with polymer solution. The solution is filled to required level of the barrel, so that the solution gives the specific amount of yield. The polymer solution is given a positive field with the help of a high voltage power supply (25 kV, Leybold Didactic GMBH). The terminal wire (metal electrode) from the high voltage power supply is fixed to the extrusion needle (1 mm inner diameter) with the help of a rubber holder. The spinneret and plunger are fixed onto the pumping block of the syringe pump, whereas the barrel is fixed into the stationary holder. The extrusion system allows the user to control the flow rate of solution and the volume through a user-friendly interface. These parameters control the fiber yield and hence the basis weight of the electrospun fiberweb.

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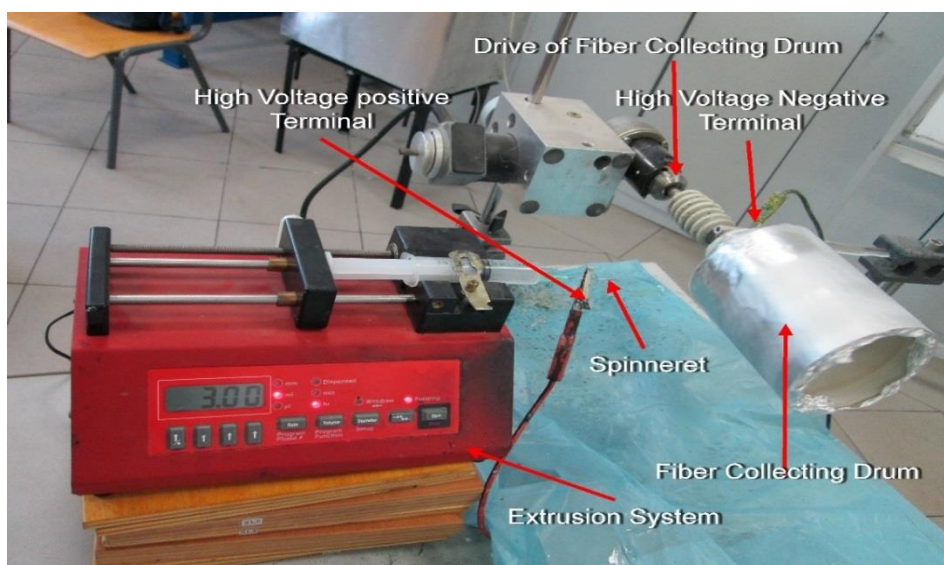


Figure 1. Electrospinning equipment,

2.5 Evaluation of antibacterial activity via disk diffusion method:

In all electrospinning trials, a strip of 10-cm wide aluminum foil was mounted securely around the fiber collecting drum (Figure 1). Gauze of the same width was mounted over the aluminum foil. Part of the aluminum foil was not covered by gauze. By rotating the drum with low speed (1-3 m/min), carboxymethyl chitosan nanofibers layer was spun over the gauze and the aluminum foil without any difficulty since the gauze structure is very open and allowed the electrical field to form nanofibers. Samples from nanofiber web with aluminum foil were taken for SEM imaging and samples from nanofiberwebs/gauze were taken for antimicrobial evaluation.

The disc diffusion method^{26, 27} was used for assessing the carboxymethyl chitosan nanofibers webs for antimicrobial activity. Discs of 7 mm diameter were cut from the composite structure of gauze and carboxymethyl chitosan layer. Nutrient agar plates were incubated with microbial culture. The cut discs of gauze/carboxymethyl chitosan structures were placed onto the surface of inoculated plates. The plates were incubated at 37°C for 48 hours. The inhibition zone (distance from disc circumference in mm) was determined for each disc

3. Results and Discussion

3.1. Electrospinning

As carboxymethyl chitosan (CMCS) could not be easily electrospun in water solutions, blending with another polymer was supposed to interfere with the strong interaction between the carboxymethyl chitosan macromolecules to improve its electrospinnability.

In the present work, polyvinyl alcohol (PVA) introduced as a “guest” polymer because PVA was show interactions with CMCS through hydrogen bonding at a molecular level via amino, carboxyl or hydroxyl groups. Another reason was that PVA could be conveniently electrospun from its aqueous solutions. Electrospinnability of CMCS/PVA was studied by the analysis of the solution properties including surface tension and viscosity, which would have effects on the morphology of the electrospun fibers. These solution properties were mainly related to the system parameters such as the solution concentration and the mass ratio of chitosan to PVA. So, the polymer concentration and the CMCS/PVA mass ratio in the blend solutions were particularly examined. The processing parameters including the applied field, the extrusion rate, and the needle tip-to-collector distance were also studied.

Our initial studies for determining the optimum CMCS concentration required for electrospinning was determined at four different concentrations of CMCS (3%, 5%, 7% and 9%) with PVA. Electrospinning generally produces non-woven matrices with randomly arranged, ultrafine fibers with nanometer diameters. Apart from other concentrations, 7% CMCS showed comparatively more uniform morphology and was found to be the distinctiveness of the chitin & chitosan based polymer systems as reported earlier²⁸A massive difference in the fiber morphology and viscosity was found when the optimized CMCS concentration (7%) was mixed with PVA in different weight compositions. Table 1 shows the details of the parameters used for the Electrospinning of CMCS solution in the presence of PVA.

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Table 1. Parameters of electrospinning trials

<i>PVA/CMCS, W/W</i>	Potential, kv	Distance, cm	Field, kv/cm	Extrusion Rate, ml/hr
100/00	30	6 , 10	3, 5	1, 3
75/25	30	6 , 10	3, 5	1, 3
50/50	30	6 , 10	3, 5	1, 3
25/75	30	6 , 10	3, 5	1, 3
00/100	30	6 , 10	3, 5	1, 3

SEM micrographs of the nanofibers obtained at different weight ratios are shown in Figure 2. It could be found that, when CMCS/PVA (100/00) instead of fibrous structure, pure CMCS gave globular drop like deposition in solution phase on the collecting target (Fig. 1a).when CMCS/PVA is 75/25, the jet was not stable, and a bead-on-string morphology with several big beads was obtained, as shown in (Figure 2b). With an increase of PVA content, the number of spindles among the fibers decreased and the fiber formation ability improved (CMCS/PVA) 50/50, (Figure 2c). As the CMCS/PVA ratio reached up to 25/75, the bead-on-string morphology disappeared and smooth, homogeneous fibers were produced (Figure 2d). When the content of PVA increased above 50%, the Electrospinning process became increasing fluent and the

fiber diameter became larger (Figure 2d,e).When electrospun, it was hard to get fibers for 100% of CMCS, Fiber formation efficiency was found to be increasing when PVA was blended with CMCS. Remarkable differences in the morphology of fibers were observed when the CMCS concentration decreased to 25% (Figure 2d). In this structure, all the fibers showed exactly the same structural similarity of fine nanofibers of 100% PVA as shown in (Fig. 1e) [29]. Other processes parameters effects on morphology e.g. Field expressed in voltage per needle tip – collector distance and the extrusion rates were also studied at different values but they are had a little effect on the electrospinnability of carboxymethyl chitosan/PVA. Similar results were ever obtained during Electrospinning in chitosan and polysulfone in the literatures^{7, 30}.

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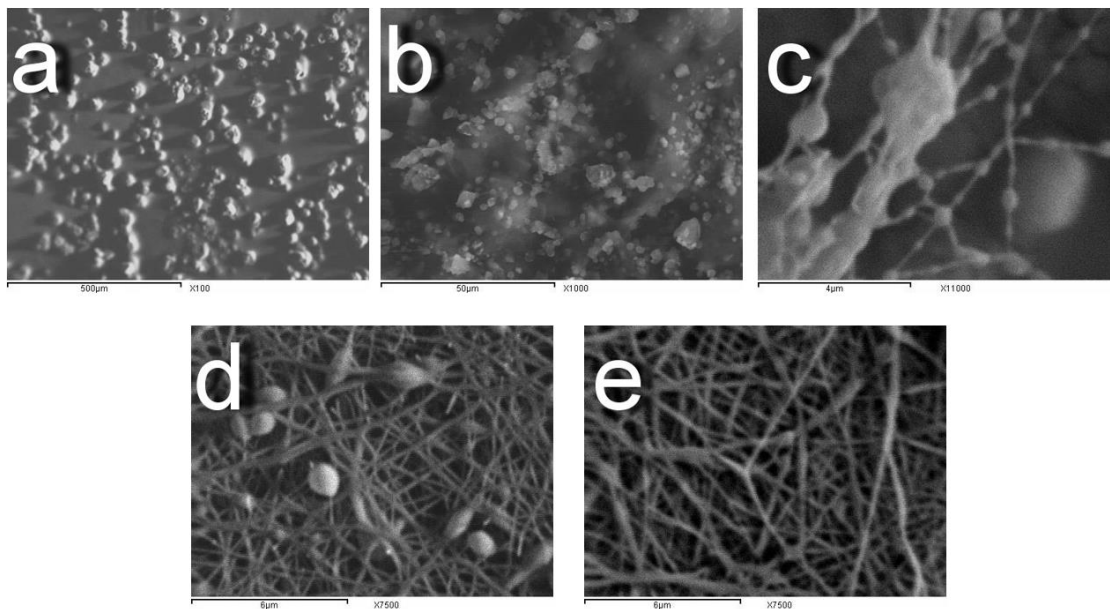


Figure 2. Effect of the composition of spinning solution on the morphology of CECS/PVA nanofiber. CECS/PVA weight ratio: (a) 80/20; (b) 60/40; (c) 50/50; (d) 40/60; (e) 30/70; (f) 20/80; (g) 10/90. At 1 ml/hr extrusion rate and (25 Kv / 10 cm) 2.5 field,

3.2. Surface tension and viscosity measurements

At low electrospinning solution concentrations, the solutions do not have sufficient viscosity to produce solid continuous fibers. With increasing polymer concentration (7% and 9%), the viscosity increases along with the increase in the number of interchain associations of the polymer molecules in the solution that leads to continuous fiber formation. At these levels of concentrations (7% and 9%) the surface tension is reduced (Table 2). The combination effect of increase in viscosity and decrease in surface tension provides excellent Electrospinning solution conditions for fiber formation with little beads. Actually, CMCS not form fiber alone due to Because CMCS is a polyelectrolyte polymer, and lead to an increase in the conductivity of the solution²⁹. Previous studies have shown that, during the smooth fiber process, the relatively higher conductivity and viscosity are both favorable factors for improving electrospinnability^{31, 32}. Higher viscosity

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polymer solutions usually exhibit longer stress relaxation times, which could prevent the fracturing of ejected jets during Electrospinning³³. Meanwhile, the high conductivity could enhance the electric force, which helps to strengthen the whipping instability and to improve the formation of smooth fibers³³. However, conventional CMCS solution formulations displayed electrical conductivities that were unacceptably large for electrospinning, where the liquids underwent deep atomization and broke into polydisperse electrospays. So that we PVA mixed with CMCS as fiber, forming material because of it is nonionogenic polymer. In this work, when CMCS/PVA) 75/25, the blend solution could not be electrospun because of too low viscosity and too high conductivity. With an increase in the PVA content, the conductivities of the solutions gradually fell, whereas the viscosities of the solutions increased high enough to enhance the molecular entanglement necessary for fiber formation, which made the blend solutions electrospun (Table 2).

Table 2. show the surface tension and viscosity of carboxymethylchitosan solutions at different concentrations

A			B		
Concentration, %	Surface Tension, dynes/cm	Viscosity, cP	CMCS/PVA, V/V	Surface Tension, dynes/cm	Viscosity, cP
3	55.0	78	100/00	79.2	880
5	54.6	540	75/25	64.5	1102
7	48.9	880	50/50	61.5	14521
9	50.6	1150	25/75	58.0	650
			00/100	55.0	1850

3.3. Effect of the electrospinning process parameters on antibacterial activity:

Table 3 shows the antibacterial activity expressed in inhibition zone of the electrospun carboxymethylchitosan, according to the disk diffusion method, on gram-negative bacteria and gram-positive bacteria as described in the experimental section. Table 3 shows inhabitation zone results for different microorganism cultured on discs of gauze layer without carboxymethyl chitosan layer (control sample) and gauze/carboxymethyl chitosan composite structures with carboxymethyl chitosan nanofibers webs (or beads/films). It can be seen from Table 3 that the control sample provided no antimicrobial protection (expressed in zero inhibition zone), while the samples containing carboxymethyl chitosan layer were very effective in contact kill of the

microorganisms as indicated by the large inhabitation zone.

Chitosan, a cationic antibacterial agent, has been widely used, particularly for wound dressing, and the target site of the cationic biocides is the cell envelope of bacteria. The mechanism of antibacterial activities of chitosan that the amino group of chitosan is bound to surface components of the bacteria and then inhibits their growth was developed^{34, 35}. They thought that at lower concentration chitosan may have bound to the negatively charged bacterial surface to disturb the cell membrane and cause cell death due to leakage of intracellular components³⁶, at high concentration, chitosan may have additionally coated the bacterial surface to prevent leakage of intracellular components as well as to impede mass transfer across the cell barrier³⁷.

Table 3. Effect of process parameter on antimicrobial activity of carboxymethyl chitosan nanofibers

Sample No.	PVA/CMCS, W/W	Extrusion Rate; ml/hr.	Field Kv/cm	Inhibition zone, mm			
				gram (+)		gram (-)	
				<i>S. aureus</i>	<i>B. subtilis</i>	<i>E. coli</i>	<i>Proteus</i>
I.1	100/00	1	3	0	0	0	0
I.2	75/25	1	3	5	3	6	7
I.3	50/50	1	3	9	9	14	10
I.4	25/75	1	3	15	14	19	13
I.5	00/100	1	3	24	21	20	17
I.6	100/00	1	2	0	0	0	0
I.7	75/25	1	2	3	3	5	1
I.8	50/50	1	2	10	9	10	7

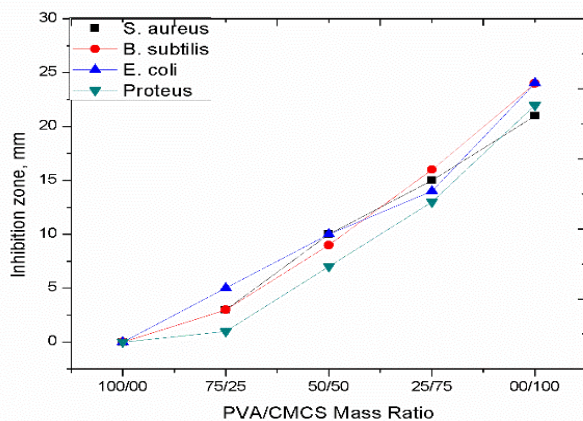
I.9	25/75	1	2	15	16	14	13
I.10	00/100	1	2	21	24	24	22
I.11	100/00	3	3	0	0	0	0
I.12	75/25	3	3	9	8	8	7
I.13	50/50	3	3	14	13.5	10	10
I.14	25/75	3	3	17	19	19	19
I.15	00/100	3	3	20	24	22	26
I.16	100/00	3	3	0	0	0	0
I.17	75/25	3	2	8	6.5	8	7
I.18	50/50	3	2	12	12	10	10
I.19	25/75	3	2	17	16	17	14
I.20	00/100	3	2	21	21	24	20

The antimicrobial effectiveness of the material increased with the CMCS/PVA ratio. During electrospinning same amount of solution was used to form the nanofibers webs that led to formation of more fibers (or polymer in beads and films form) as concentration gets higher as shown in Figure 3 (from A to D) at different extrusion rate and applied field. In other words, the area covered by the nanofibers webs from carboxymethyl chitosan gets higher with concentration a matter that led to an increase of the inhabitation zone with concentration.

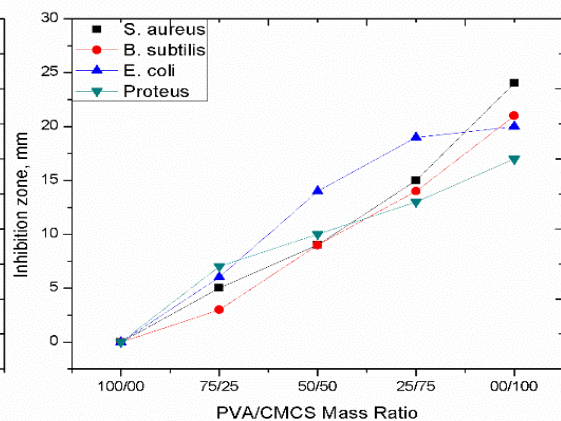
As mention in previous work that chitosan and its derivatives had more effective inhibition on gram (+) bacteria than gram (-) bacteria^{26, 38-40}. This may be attributed to their different cell walls of gram-positive bacteria; its cell wall is fully

composed of peptide polyglycogen. The peptidoglycan layer is composed of networks with plenty of pores, which allow foreign molecules to come into the cell without difficulty. On the other hand, the cell wall of gram-negative bacteria is made up of a thin membrane of peptide polyglycogen and an outer membrane constituted of lipopolysaccharide, lipoprotein and phospholipids. Because of the bilayer structure, the outer membrane is a potential barrier against foreign molecules^{39, 41}. But our results shows that electrospun carboxymethyl chitosan nanofibers shows the same effective inhibition on both gram (+) and gram (-) bacteria due to its transform to nano structure that made it able to penetrate the bacterial cell wall even it had potential barrier.²⁷

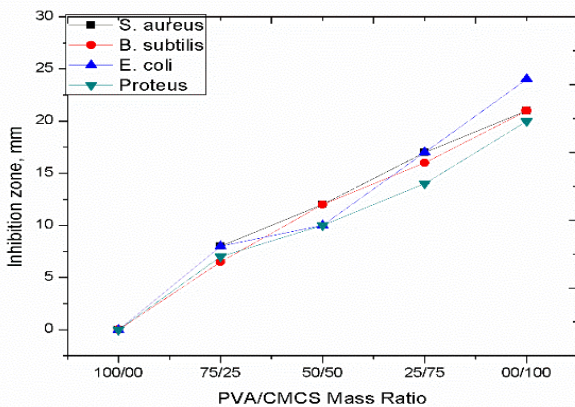
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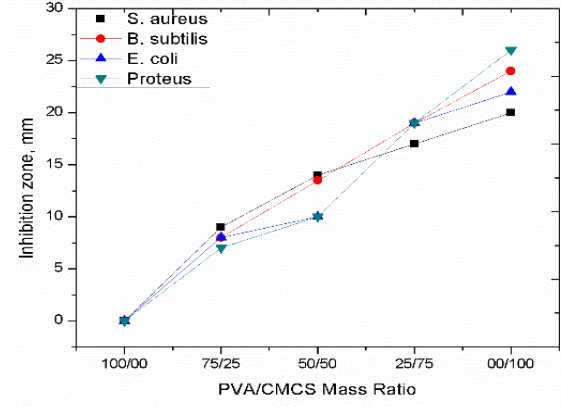
A. Effect of PVA and CMCS Ratio on Inhibition zone at rate 1 and field 2



B. Effect of PVA and CMCS Ratio on Inhibition zone at rate 1 and field 3



C. Effect of PVA and CMCS Ratio on Inhibition zone at rate 3 and field 2



D. Effect of PVA and CMCS Ratio on Inhibition zone at rate 3 and field 3

Figure 3. Effect of PVA and CMCS Ratio on antibacterial activity,

As shown in Table 3, when the solution extrusion rate increased from 1 to 3 ml/h, the morphology and the antibacterial activity of the electrospun carboxymethyl chitosan/PVA fibers did not show obvious changes. The antibacterial activity of the

electrospun carboxymethyl chitosan/PVA fibers were almost the same at the same CMCS/PVA mass ratio due to the amount of CMCS nanofiberweb deposit at the collectors are the same for all CMCS/PVA mass ratios from 100/00 to 00/100.

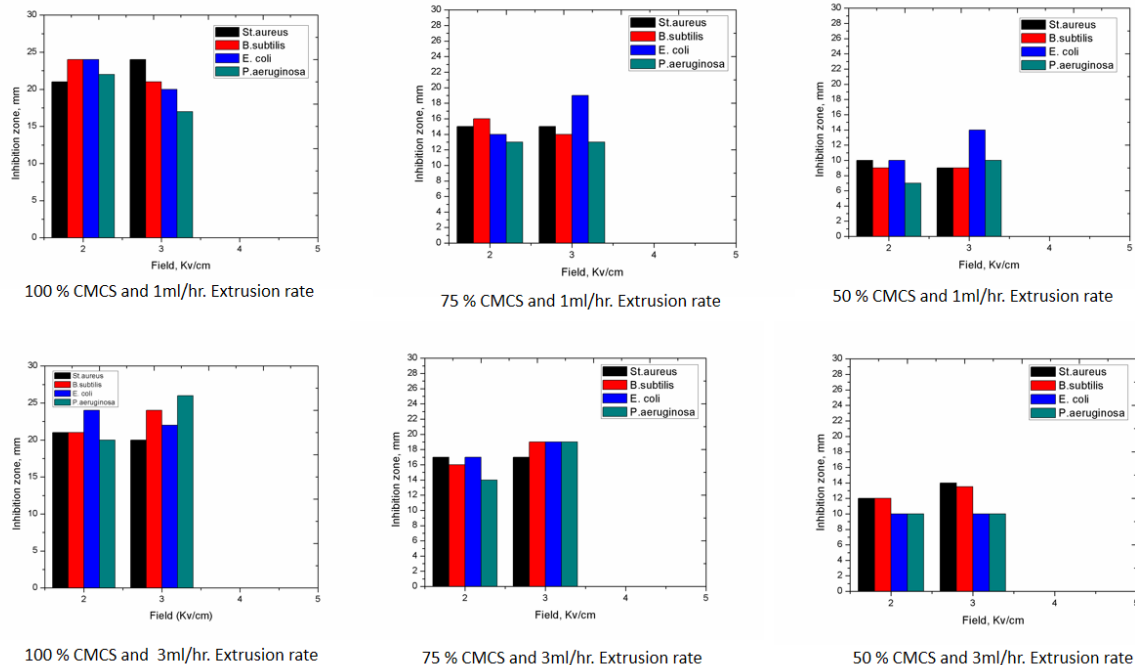


Figure 4. Effect of extrusion rate on antibacterial activity,

Figure 5 shows the effect of antibacterial activity on the needle tip-to-collector distance of the electrospun carboxymethyl chitosan/PVA fibers. When the distance was 10 cm, inhibition zone of the

electrospun carboxymethyl chitosan/PVA fibers was in the same range when the distance were 6 cm. and the antibacterial activity related only with fiber formation not beads or films.

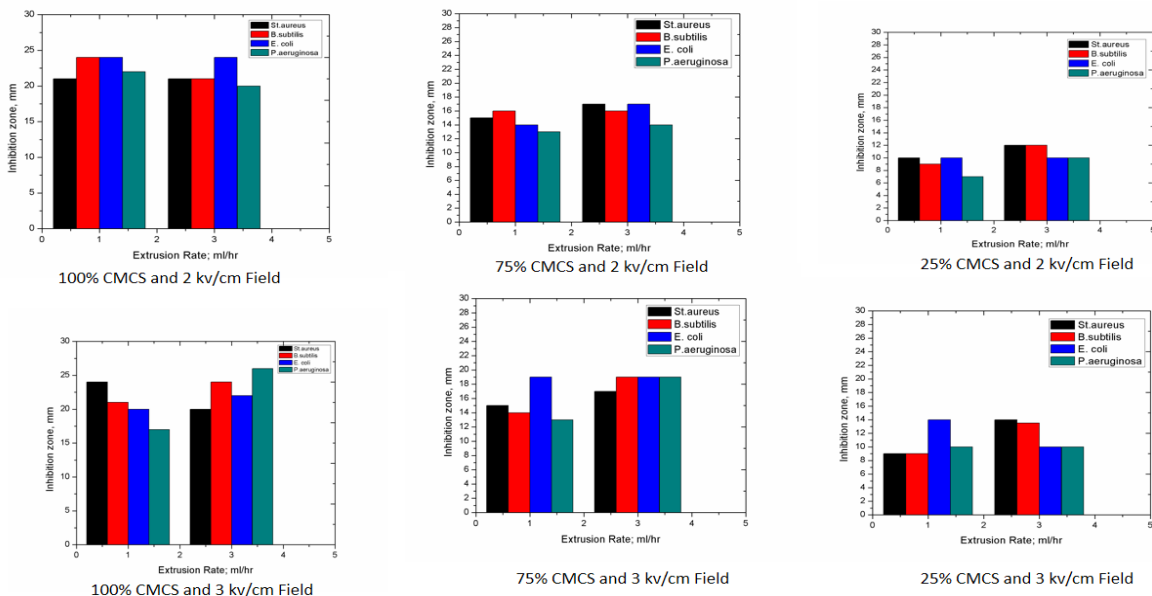


Figure 5. Effect of applied field on antibacterial activity,

4. Conclusion

- Electrospinning of carboxymethyl chitosan (CMCS) solutions in water was studied by adding poly (vinyl alcohol) (PVA) as “guest” polymer.
- The Nano fibers could be generated after addition of PVA in different mass ratios of CMCS to PVA from 4–8 wt. % CMCS/PVA solutions.
- The optimum concentration of CMCS forming electrospun fiber with PVA as 7% based on combined viscosity and surface tension measurements.
- During electrospinning of the CMCS/PVA solutions, ultrafine fibers were often obtained along with beads
- Electrospun CMCS/PVA nanofiberwebs show good antibacterial effects towards both gram (+) bacteria and gram (-). Antibacterial activities of electrospun nanofiberwebs increased by increasing the CMCS percent in the electrospun membrane.
- The higher antibacterial activity of electrospun CMCS/PVA nanofiberwebs oriented its potential applications in medical textiles as wound dressing and wound healing.

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