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Antimicrobial activity of carboxymethyl chitosan/polyethylene oxide nanofibers embedded silver nanoparticles

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ABSTRACT

A facile method to synthesize silver nanoparticles (AgNPs) using carboxymethyl chitosan (CMCTS), which act as reducing agent for silver ions as well as protecting agent for the formed AgNPs, is reported. CMCTS embedded AgNPs are mixed with polyethylene oxide (PEO). The blend polymers containing AgNPs are electrospun resulting in blend nano-fiber mats. The formation of AgNPs has been confirmed using UV–vis and TEM. The diameter range of 12–18 nm of well-dispersed AgNPs with a concentration of 100 ppm was obtained. The electrospun mats are characterized using SEM, EDX as well as TGA. Antimicrobial activity against different species of pathogenic/nonpathogenic; *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 in addition to the fungus *Candida albicans* ATCC 10231 was studied. The results show excellent antimicrobial activity compared with nanofibers without AgNPs and AgNPs alone.

1. Introduction

In electrospinning, the simplest and most cost-effective method of fabricating polymer nanofibres, various polymers have been electrospun into ultrafine fibers with diameters range of 20–400 nm (Huang, Zhang, & Kotaki, 2003). In electrospinning, the polymer solution is placed into syringe with millimeter size nozzle. Strong electric field is applied on a droplet of polymer solution held by its surface tension at the tip of a syringe's needle. As a result, the pendent drop becomes highly electrified and the induced charges are distributed over its surface. Increasing the intensity of electric field, the surface of the liquid drop will be distorted to a conical shape known as the Taylor cone (Taylor, 1969). Once the electric field strength exceeds a threshold value, the repulsive electric force dominates the surface tension of the liquid and a stable jet emerges from the cone tip. The charged jet is then accelerated toward the target and rapidly thins and dries as a result of elongation and solvent evaporation. As the jet diameter decreases, the surface charge density increases and the resulting high repulsive forces split the jet to smaller jets. Ultimately, solidification occurs and fibers are deposited on the surface of the collector as randomly oriented nonwoven mats (Derch, Greiner, & Wendorff, 2004). Besides charge density and applied voltage other parameters also influence the final nanofibrous structure and its properties, for example: polymer types and concentration, type of solvent, presence of electrolyte, type and concentration of electrolyte, viscosity, surface tension, tip-to-collector distance, flow rate of the polymer solution, inner diameter of the tip, material of the tip, etc.

The field of nanoparticle research has witnessed tremendous growth due to the unique chemical and physical properties from the bulk. Silver nanoparticles have gained considerable attention today due to their potential applications in medical field, since it has been widely used in the production of biodegradable surgical sutures. Recently, electrospun nanofibers embedded AgNPs have a great antimicrobial potential. Different methods have been used to prepare AgNPs, where one of these is chemical reduction method in which the polymer can be used as both reducing and stabilizing agents for the formed AgNPs (Abou-Okeil, 2012; Abou-Okeil, Amr, & Abdel-Mohdy, 2012; El-Rafie et al., 2011; Hebeish, El-Naggar, et al., 2011; Textor, Fouda, & Mahltig, 2010).

Chitosan is one of the most important biopolymers, obtained from chitin, the second most abundant natural polysaccharide present on the earth next to cellulose (El-Shafei, Fouda, Knittel, & Schollmeyer, 2008; Hebeish, Abdel-Mohdy, et al., 2011). The poor solubility of chitosan in water, due to its rigid crystalline structure, limits its effective utilization in electrospinning process. To overcome this drawback, it is necessary to convert chitosan to water-soluble derivative (El-Shafei et al., 2008). Chemical

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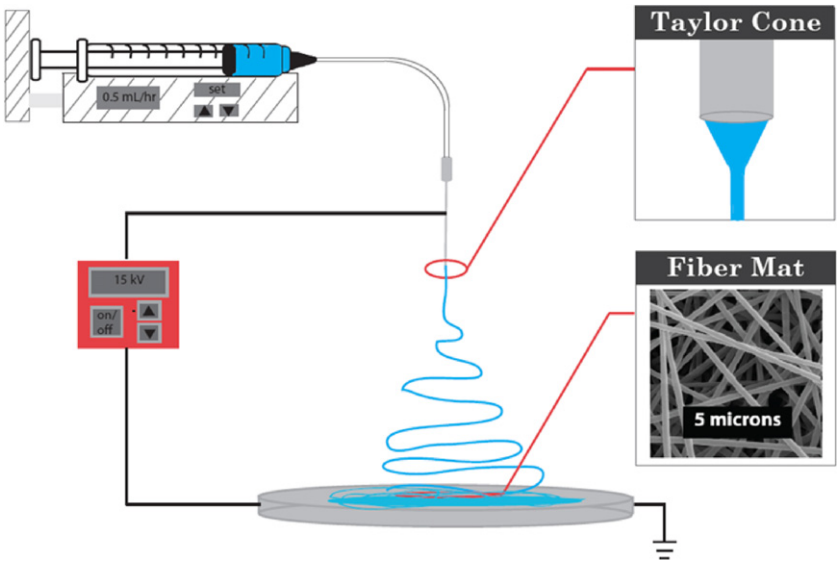

Fig. 1. Schematic diagram of the typical electrospinning setup.

modification is anticipated to be quite promising. Carboxymethylation is one of the chemical methods to prepare water-soluble chitosan. This reaction takes place preferentially either at C-6 hydroxyl groups or at the NH_2-group resulting in N/O–carboxymethyl chitosan (CMCTS). Both products are water-soluble and contain an amino group either as the primary ($-NH_2$) or as secondary amine ($-NH-CH_2COOH$).

Polyethylene oxide (PEO) is one of the few biodegradable synthetic polymers approved for internal use in food, cosmetics, personal care products, and pharmaceutical. PEO is an effective ion conductive polymer (Morgado et al., 1999). Therefore, it is added in order to enhance the spinnability of the modified natural polymer.

The objective of this research work is to synthesize well-stabilized AgNPs using CMCTS followed by electrospinning of CMCTS–AgNPs/PEO solution. The structure, morphology and the antimicrobial activity of the resulted nanofiber mats are characterized.

2. Experimental

2.1. Materials

Polyethylene oxide (PEO) (≥95%, average Mw 124–186 kg/mol) was obtained from Scientific Polymer Products, Inc. Silver nitrate ($AgNO_3$) (99.998%) was purchased from Aldrich, Germany. Chitosan, DDA 95% was obtained from Aldrich Chemical Co., Germany. All other solvents and reagents were used as received without any further purification.

2.2. Synthesis of carboxymethyl chitosan (CMCTS)

The experimental technique adopted for carboxymethylation of chitosan was as follows: certain volume of sodium hydroxide solution (30%, w/v) was added to 16 g chitosan suspended in isopropyl alcohol. The mixture was left under stirring for 30 min at room temperature. To this mixture 34 g of monochloroacetic acid was added and the content of the flask was subjected to continuous stirring for 3 h. At the end, the excess alkali was neutralized using glacial acetic acid and chitosan was precipitated by adding acetone. Finally, modified chitosan was filtered and washed with isopropyl alcohol/water (70:30) several times and dried at 60 °C (El-Shafei et al., 2008). The final product was soluble in water.

2.3. Synthesis of silver nanoparticles (AgNPs)

Silver nanoparticles (AgNPs) were prepared according to the procedure described by El-Rafie et al. (2011) and can be summarized as follows: 0.5 g of CMCTS is dissolved in 100 ml of distilled water, the temperature of the reaction is raised to 60 °C and the pH is adjusted to 11.5 by using 10 M NaOH. 1 ml of AgNO3 (1.7 M) is added dropwise to the previous solution under continuous stirring for almost 1 h. The formation of silver nanoparticles solution was observed by monitoring the color change (visually, when the color of the solution started to change from its original color to the different degrees of the yellow color, then the reduction reaction started to work and silver nanoparticles started to seed). The AgNPs formed are characterized by (UV–vis), transmission electron microscope (TEM).

2.4. Electrospinning of CMCTS–AgNPs/PEO solution

2 g of CMCTS is added to CMCTS solution containing silver nanoparticles (0.5 wt%) while stirring. To this solution, PEO (5 wt%) is added slowly under continuous stirring till homogeneity occurs. Electrospinning of the prepared blend polymers solution containing AgNPs was carried out using two different methods; typical electrospinning technique and Nanospider technology as a modified electrospinning technique. A schematic diagram of the complete electrospinning apparatus is shown in Fig. 1. It consisted of a syringe and stainless needle, a grounded electrode, an iron plate covered by aluminum foil as a collector, and an adjustable high voltage supply.

2.5. Testing and analysis

UV–vis spectrum was used to prove the formation of AgNPs (Hebeish, El-Naggar, et al., 2011). Particle shapes and sizes of AgNPs were obtained by transmission electron microscope (TEM); JEOL-JEM-1230. Scanning electron microscope (SEM) (JEOL GSM-6610LV) and (JEOL GSM-7600F) field emission scanning electron microscope were used to study the surface characteristics of electrospun nanofibers. Specimen in the form of films was mounted on the specimen stabs and coated with thin film of gold by the sputtering method. The micrograph was taken at magnification of 1000× using (KV) accelerating voltage. FT-IR spectra were obtained using FT-IR spectrometer, Bruker, TENSOR Series FT-IR

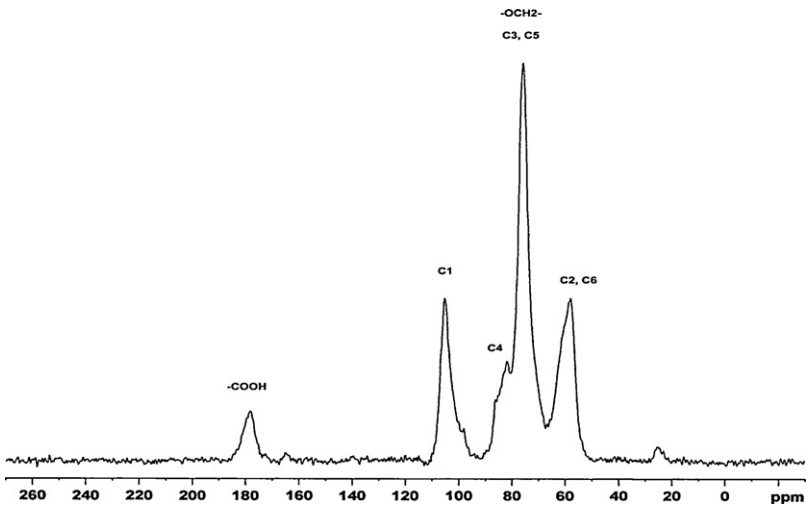

Fig. 2. Solid state ^{13}C NMR spectrum typical for *O*-carboxymethyl chitosan.

Spectrometer, Germany, connected to a PC, and the data were analyzed by IR Solution software, where analytical methods are standard in OPUS^{TM} software.

2.6. Antimicrobial evaluation of electrospun nanofibers

In order to evaluate the antimicrobial properties of electrospun nanofiber mats with/without AgNPs against microbial pathogens and to compare this effect with the commonly used antibiotics in addition to AgNPs alone as +ve control, the zone of inhibition test was performed against the gram positive bacterium *Staphylococcus aureus* ATCC 25923, the gram negative bacteria *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 in addition to the fungus *Candida albicans* ATCC 10231. To perform the test, several colonies of each strain, obtained from a fresh culture in blood agar plate, were suspended in 5 ml of Mueller-Hinton broth to achieve turbidity equal to the 0.5 Mac-Farland standards. The suspensions were inoculated with sterile swabs onto 150 mm diameter Mueller-Hinton agar plates and after the agar surfaces were allowed to dry, tested disks were applied on each plate. Plates were incubated at 37 °C for 24 h and the zones of inhibition (IZ) were measured. Same was performed for the *Candida* except that it was inoculated in Sabouraud dextrose agar medium and incubated for 2–3 days at 37 °C. The antimicrobial agents tested in this study were CMCTS–PEO nano-fiber incorporated with silver nano-particles (CMCTS–AgNPs/PEO), the antibiotic Amikacin (AK), ampicillin/clavulinic acid (AMC), 100 ppm AgNPs solution (10 μL per disk) as a positive control, in addition to negative controls as empty disks of CMCTS–PEO nano-fibers.

3. Results and discussion

3.1. Characterization of (CMCTS) by solid state ^{13}C NMR

Carboxymethylation of chitosan (CTS) is achieved with monochloroacetic acid and sodium hydroxide. According to El-Shafei et al. (2008) this reaction takes place preferentially either at C-6 hydroxyl groups or at the NH_2-group resulting in N/O–carboxymethyl chitosan (CMCTS). The solid state ^{13}C NMR spectrum for a typical N-carboxymethyl chitosan shows signals attributed to the N-carboxymethyl substituent, at 47.7 and 168.7 ppm, for N—CH_2 and COOH, respectively (El-Shafei et al., 2008). But in case of our results, the solid state ^{13}C NMR described in Fig. 2 shows signals at 73 and 175 ppm which attributed to —O—CH_2— and COOH respectively. This downfield shift of the carbon indicates the formation of *O*-carboxymethyl chitosan. The formation of this product agrees with the higher reactivity of hydroxyl group of C_6 in this heterogeneous reaction. The *N*-carboxymethyl substituent is not present because of the absence of peaks at 47 and 168 ppm for N—CH_2 and COOH respectively.

3.2. Characterization of the synthesized silver nanoparticles (AgNPs)

In this research work, CMCTS was used as reducing and as stabilizing agent too. The formation of AgNPs could be visualized from changes in the color of the solutions from colorless to light yellow. The reduction of Ag^+ could occur via the reduction effect of CMCTS at 60 °C and pH 11.5 for 30 min. Fig. 3 shows the UV–visible absorption spectra of AgNPs. The surface plasmon absorption bands are centered around 409 nm (El-Rafie et al., 2011). The absorption band at 405 nm becomes stronger and narrower which means higher conversion of Ag^+ to Ag^0 with smaller nanoparticles size. Fig. 4a and b shows the TEM image and the histogram of the size and size distribution of the AgNPs. Results revealed that the size range of prepared AgNPs was between 12 and 18 nm.

3.3. Morphology of the CMCTS–AgNPs/PEO-electrospun nanofibers

The performance and morphology of the electrospun fiber were affected by the electrospinning process parameters. In the present study, two different electrospinning setups were used. In the first setup, a typical electrospinning setup was used (Fig. 1) along with

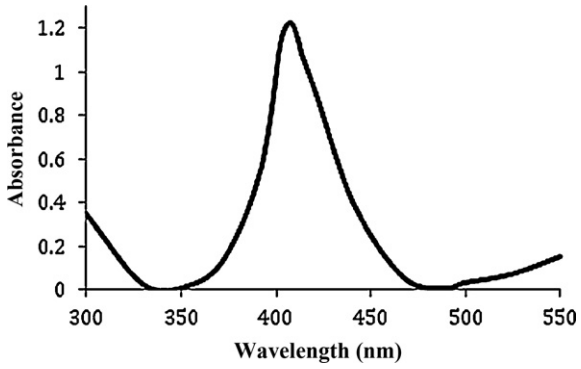

Fig. 3. UV–vis spectra of silver nanoparticles embedded in CMCTS.

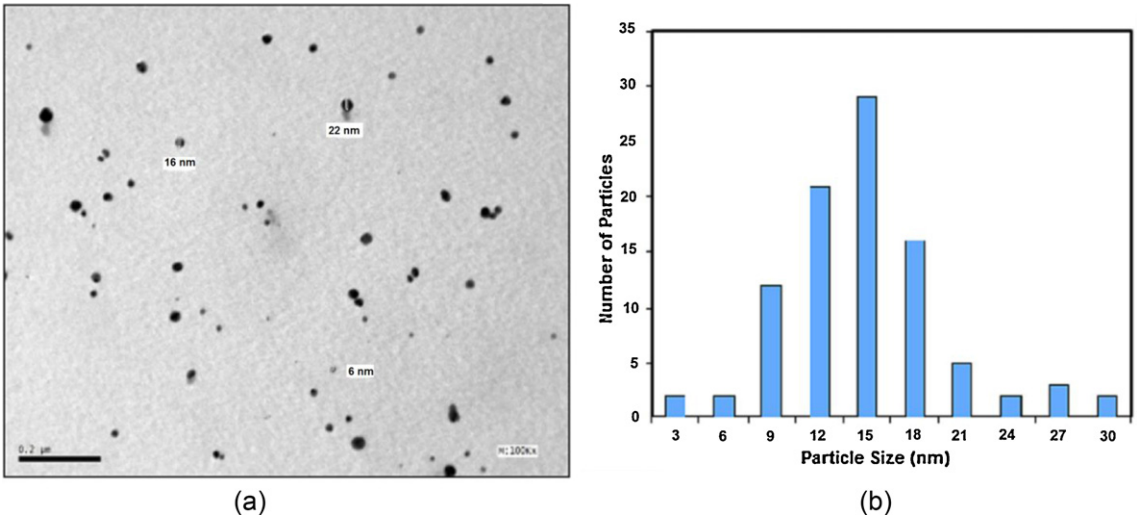

Fig. 4. (a and b) TEM image and the histogram of the size and size distribution of the AgNPs.

Nanospider technology. Both electrospinning setup and Nanospider are used in order to prepare nanofiber mats, but, Nanospider is used in large scale/sample production of the selected and best nanofiber mats from the results obtained, in addition, no difference in morphology of the resulted mats for both techniques is observed. Generally, the electrospun mat is opaque due to light scattering from the fibrous structure. The obtained fibers (Fig. 5a and b) had cylindrical morphology and no fiber bundles, indicating that applied parameters were adequate for the formation of fibers and proper evaporation of the solvent. On the other hand, the presence of AgNPs in CMCTS has little effect on the electrospun fiber morphology. The fiber diameter ranged from 50 to 300 nm. In addition, after the encapsulation of AgNPs into CMCTS–PEO nanofiber, the fiber diameter decreases compared to fibers consisting of CMCTS–PEO without AgNPs, due to the high conductivity, which plays a key role in decreasing of the fiber diameter during electrospinning (Sheikh et al., 2010). The presence of AgNPs results in high electric charge and subsequently high conductivity of the polymer solution which leads to high charge values during electrospinning process and possibly forming thinner fiber diameter (Nirmala et al., 2010; Nirmala, Navamathavan, Kang, El-Newehy, & Kim, 2011; Nirmala, Park, et al., 2011). At the same time the

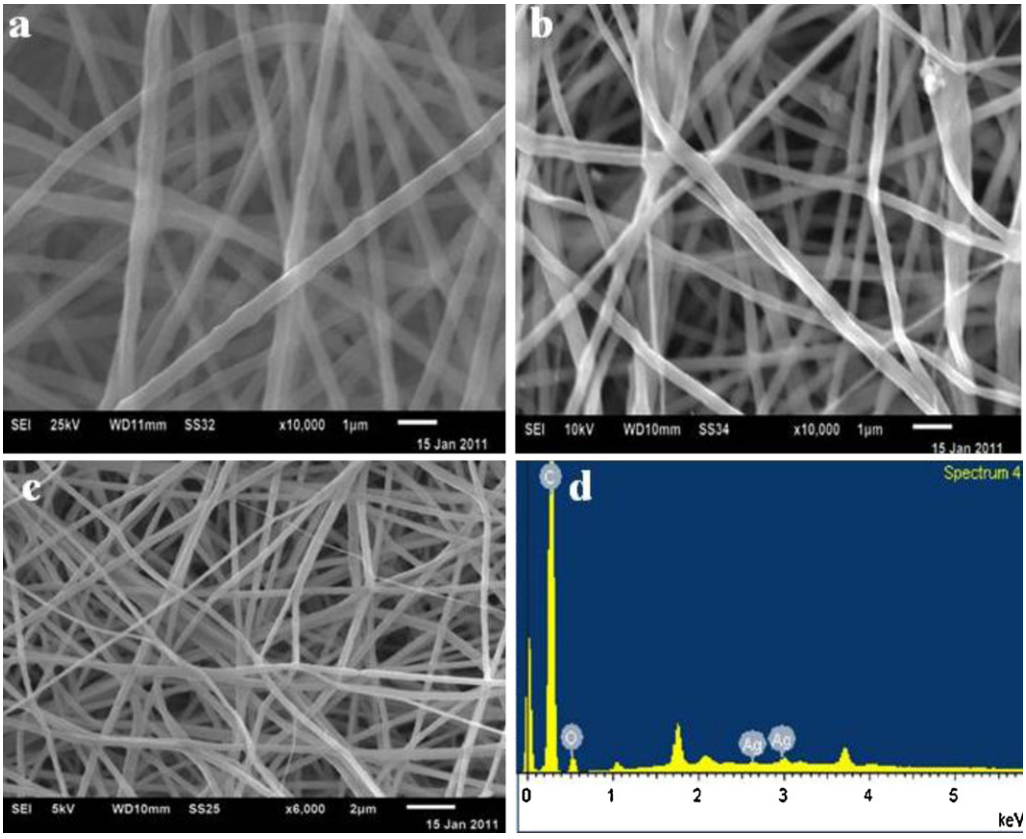

Fig. 5. SEM images of electrospun nanofibers containing AgNPs; (a) CMCTS; (b) CMCTS–Ag, (c) CMCTS/PEO–Ag and (d) CMCTS/PEO–Ag (EDX).

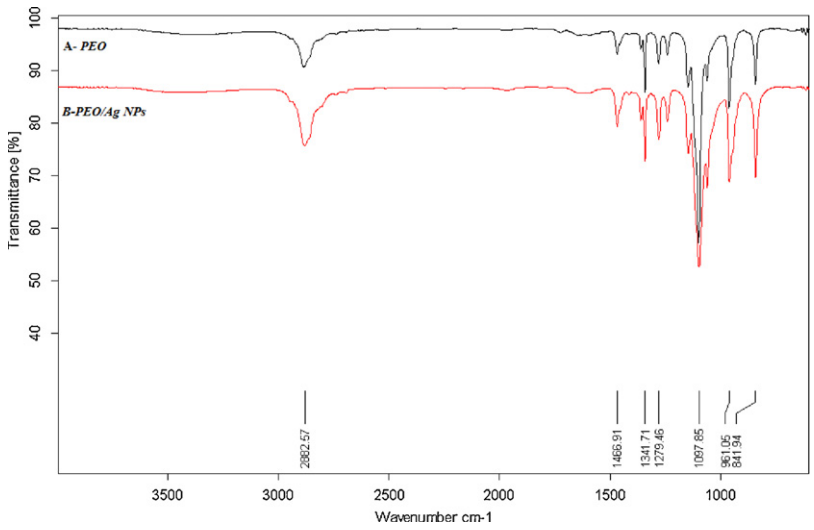

Fig. 6. FT-IR spectra of electrospun nanofibers; (a) PEO and (b) PEO–AgNPs.

fibrous structure assures much more loading of AgNPs into the fibers. EDX was used to analyze the elemental constitution of solid samples. Elementary analysis of CMCTS–PEO/silver nanocomposite was carried out by using SEM–EDX. Fig. 5c displays a spectrum of CMCTS–PEO/silver nanocomposite obtained by elemental microprobe analysis of EDX. The results show that carbon, oxygen, and Ag were the principal element of CMCTS–PEO/silver nanocomposite. EDX analysis, as a result, provides direct evidence that AgNPs are embedded in the CMCTS–PEO composite. The quantitative analysis of CMCTS–PEO/silver nanocomposite is presented in Table 1. At the same time, Fig. 5a and b shows SEM images of CMCTS–PEO/AgNPs nanofibers, which revealed that the AgNPs were evenly distributed in the CMCTS–PEO ultrafine fibers with an average size less than 12–18 nm. This suggested that the AgNPs were well stabilized by CMCTS during the preparation of AgNPs.

Table 1
Stoichiometric ratio of CMCTS–AgNPs.

Element	Weight%	Atomic%
(PEO)/Ag		
C K	83.90	88.17
O K	14.81	11.68
Ag L	1.29	0.15
Total	100.00	

3.4. FT-IR spectra of electrospun nanofibers

FT-IR spectra of electrospun CMCTS–PEO and CMCTS–AgNPs/PEO are shown in Fig. 6. The frequencies and assignments for the pristine PEO are indicated as follows:

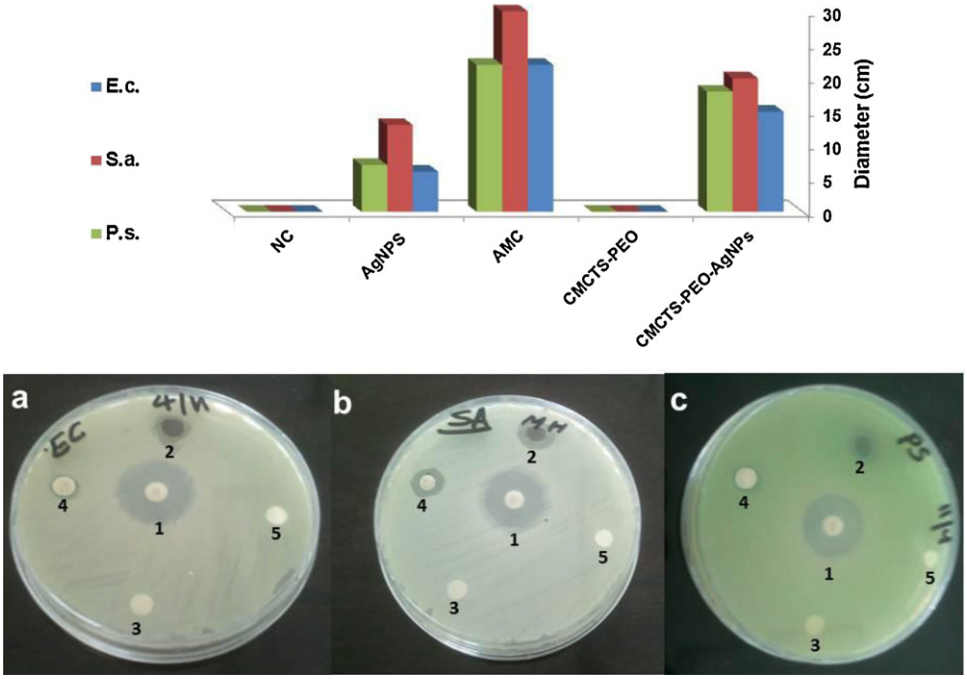

Fig. 7. Diameter inhibition zone (cm) of electrospun nanofibers against *Staphylococcus aureus* (Sa), *Pseudomonas aeruginosa* (ps) and *Escherichia coli* (Ec).

2882 cm^{-1} due to the CH_2 group stretching vibration, 1097 cm^{-1} and 841 cm^{-1} due to the C—O—C asymmetric stretch and bending vibrations. On the other hand, for the electrospun CMCTS–AgNPs nanofiber shows the same characteristic bands, in which the intensity of the bands at 2882 cm^{-1} and at 841 cm^{-1} was increased due to the CH_2 and C—O—C stretching vibration upon the presence of AgNPs.

3.5. Antimicrobial of electrospun nanofibers

Fig. 7 shows chart of inhibition zone of the tested antimicrobial samples and the corresponding plates (a, b, c). Results illustrated that *S. aureus* was the most sensitive microbe against antimicrobial disk (AMC), CMCTS–PEO–AgNPs nanofiber, and AgNPs solution with inhibition zone 30, 22 and 15 millimeters (mm) respectively. *C. albicans* was the least sensitive against all tested antimicrobial agents with IZ of 0 mm, except for CMCTS–PEO–AgNPs that showed IZ of 12 mm. It was found that the CMCTS–PEO–AgNPs nanofibers were the most effective silver containing material with IZs of 20, 18, 15 and 12 against *S. aureus*, *P. aeruginosa*, *E. coli* and *C. albicans*, respectively. In contrast, the AgNPs showed the least antimicrobial activity among silver containing nanofibers with IZ of 13, 7, 6 and 0 mm against *S. aureus*, *P. aeruginosa*, *E. coli* and *C. albicans*, respectively. It was observed that CMCTS–PEO–AgNPs nanofibers are the most effective silver containing material against all tested microbes. Also, it was found that CMCTS–PEO–AgNPs nanofiber was more than twofold strength of the positive control (AgNPs). However, its efficacy was less than any of the tested antibiotics, but this can be compensated with the less hazardous effect of antibiotics and the less chance of resistance development compared with silver nanoparticles.

4. Conclusion

Silver nanoparticles (AgNPs) have been successfully prepared using carboxymethyl chitosan (CMCTS) which acts as both reducing and stabilizing agent for the formed AgNPs. CMCTS–AgNPs with polyethylene oxide (PEO) are well mixed together and subjected to electrospinning process. The resulted nanofiber mats' embedded AgNPs are characterized using different analytical tools. The presence of silver ions in the polymer structure was found to be strongly affecting the electrospun nanofibers diameters due to enhancement of electrical conductivity of the nanofibers. The obtained results indicated that the number of Ag^+ ions that were converted into Ag^0 increased with increasing the aging time. Antimicrobial activity of the prepared sample was evaluated against different types of microorganisms. It was observed that CMCTS–PEO–AgNPs nanofibers are the most effective silver containing material against all tested microbes. Also, it was found that CMCTS–PEO–AgNPs nanofiber was more than twofold strength of the positive control (AgNPs). Finally, the prepared CMCTS–AgNPs/PEO nanofibers matrix could be properly employed as recommended candidate for many biological applications such as prolonged antimicrobial wound dressing materials.

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