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Preparation of nylon-6/chitosan composites by nanospider technology and their use as candidate for antibacterial agents

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Abstract−Electrospun nylon-6/chitosan (nylon-6/Ch) nanofibers were prepared by nanospider technology. Quaternary ammonium salts as antibacterial agent were immobilized onto electrospun nylon-6/Ch nanofibers via surface modification by soaking the mat in aqueous solution of glycidyltrimethylammonium chloride (GTMAC) at room temperature overnight to give nylon-6/*N*-[(2-hydroxy-3-trimethylammonium)propyl] chitosan chloride (nylon-6/HTCC). The morphological, structural and thermal properties of the nylon-6/ch nanofibers were studied by field-emission scanning electron microscopy (FE-SEM), X-ray diffraction (XRD), Fourier transform-infrared (FT-IR) spectroscopy, and thermogravimetric analysis (TGA). Biological screening has demonstrated the antibacterial activity of the electrospun nanofibers against Gram negative bacteria, *Escherichia coli* 35218, and *Pseudomonas aeruginosa* and Gram positive bacteria, *Staphylococcus aureus* 24213 among the tested microbes. Thus, the study ascertains the value of the use of electrospun nanofibers, which could be of considerable interest to the development of new antibacterial materials for biomedical applications.

Key words: Chitosan, Nylon-6, Onium Salts, Antibacterial Polymers

INTRODUCTION

In the last few years, polymer nanofibers have received much attention due to their small diameter, which mimics the topology of extra cellular matrix present in the human body, and hence are used as scaffolds in tissue engineering [1]. Among the processes used to prepare nanofibers, electrospinning is a simple and versatile method to generate nano- to submicrometer fibrous structures [2]. Electrospun nanofibers have become promising materials for many biomedical applications such as wound dressing [3] drug delivery, and scaffold for tissue engineering [4-10].

Nanospider is a modified electrospinning method which requires the use of a high voltage electrostatic field (up to 80 kV) to create an electrically charged stream of polymer solution or melt. The innovative idea of the Nanospider is based on the possibility of producing nanofiber from a thin layer of liquid polymer. In this case Taylor cones (the source of nanofiber) are created on the surface of a rotating roller, immersed in a polymer solution. This commercial method for the production of polymeric nanofibers is used in industrial range. It is a simple and versatile method for the production of ultra-thin fibers from a variety of materials that include polymers. In addition, Nanospider has the ability to process a wide range of polymers in diameters of 50-300 nm into nonwoven webs [11,12].

Chitosan is a polysaccharide composed of D-glucosamine residues that are structurally linked similarly to various glycosaminoglycans (GAG) present in the extracellular matrix (ECM) [13-15]. As a natural polymer, chitosan is biodegradable and biocompatible in addition to its unique properties which include antimicrobial activity, non-toxicity and versatile chemical and physical properties [16, 17]. On the other hand, nylon-6 is a biodegradable, biocompatible and synthetic polymeric material which has good mechanical and physical properties [18-20]. Unfortunately, poor solubility of chitosan under normal physiological pH has limited its further applications. So far, many techniques have been applied to overcome this disadvantage, including quaternization or guanidination of NH_2 groups or conjugating dextran and/or polyethylene glycol to the chitosan backbone [21,22]. Therefore, to improve the characteristic features of chitosan, we modify the nylon-6/Ch nanofibers to nylon-6/*N*-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (nylon-6/HTCC). *N*-(2-hydroxy)propyl-3-trimethyl ammonium chitosan chloride (HTCC) can be easily prepared by reacting chitosan with glycidyltrimethylammonium chloride (GTMAC), which shows good water solubility, antibacterial activity, mucoadhesivity and enhanced permeability due to hydration and strong steric hindrance of positively charged quaternary amino groups [23]. Although there have been several reports based on the antimicrobial activity of HTCC with other composites [24-26], the modification of nylon-6/Ch nanofibers with GTMAC to produce nylon-6/HTCC has not previously been reported.

In our work, we take the advantages from both chitosan and nylon-6 by blending them into composite nanofibrous scaffolds for anti-

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bacterial applications by introduction of quaternary ammonium salts via surface modification of electrospun nylon-6/Ch nanofibers. Nylon-6/chitosan composite nanofibers having 8% w/w chitosan were fabricated using Nanospider.

EXPERIMENT

1. Materials and Characterization

Nylon-6 (Ultramid® B24 N 03) was purchased from BASF. Chitosan, low molecular weight, was purchased from Aldrich. Glycidyltrimethylammonium chloride (GTMAC) (technical, ≥90%) was purchased from Fluka. All materials and solvents were used as received without further purification.

1-1. Electrospinning Setup

Electrospinning was carried out using the Nanospider laboratory machine NS LAB 500S from Elmarco s.r.o.

1-2. Field-emission Scanning Electron Microscope (FE-SEM)

The morphology of electrospun nanofiber was observed using field-emission scanning electron microscopy (FE-SEM, Hitachi S-7400, Japan). The morphology of the species cells was examined by SEM (S-4100, Hitachi, Japan).

1-3. X-ray Diffraction (XRD)

Structural characterization was carried out by X-ray diffraction (XRD, Rigaku, Japan) operated with Cu-Kα radiation (λ=1.540 Å).

1-4. FT-IR Spectroscopy

Fourier-transform infrared (FT-IR) spectra were recorded to study the surface modification of the electrospun nanofiber using TENSOR 27, Bruker.

1-5. Thermogravimetric Analysis (TGA)

Thermal properties of electrospun nanofibers were examined through using thermogravimetric analysis (TGA), which was carried on TA-Q500 System of TA. Samples of 5-10 mg were heated in the temperature range 30-800 °C at a scanning rate of 10 °Cmin^{-1} under nitrogen atmosphere.

2. Preparation of the Spinning Solutions

Nylon-6/chitosan nanofibrous mat was fabricated using electrospinning process in which nylon-6 was dissolved in formic acid at 60-70 °C with gentle stirring in order to prepare 12% (w/v) homogeneous solution. Chitosan with concentration of 8% (w/w) was mixed with the nylon-6 solution with gentle heating and stirring till complete dissolution. The prepared solution was then used for electrospinning at room temperature.

3. Electrospinning of Nylon-6/Chitosan Nanofibers (Nylon-6/Ch)

Nylon-6/chitosan nanofibers were electrospun using nanospider technology, the main advantage of which is to synthesize composite nanofibers by blending higher weight percentage chitosan (8%) which is difficult to spin with the conventional electrospinning process. The electrospinning was accomplished with the following conditions: active electrode to collecting electrode distance is 14 cm at a driving voltage of 75 kV, active electrode speed is 2.2 rpm and humidity of 37%. The electrospun nanofiber was collected on aluminum sheet and was dried in a hood at room temperature till constant weight.

4. Surface Modification of Electrospun Nylon-6/Chitosan Nanofibers (Nylon-6/HTCC)

Electrospun nylon-6/Ch nanofibers mat was treated with excess GTMAC in water with stirring for 24 h, and then the mat was washed with excess water and finally dried in hood at room temperature [21].

5. Antimicrobial Assessment

5-1. Bacterial Strains and Culturing Conditions

Pure strains of Gram negative *Escherichia coli* 35218, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Proteus* spp., *Salmonella* spp. and Gram positive *Bacillus cereus*, *Streptococcus pneumonia* and *Staphylococcus aureus* 24213 were used for biological assessment. The strains were obtained from the Central Laboratory Research, Department of Botany and Microbiology, College of Science, King Saud University. Bacterial strains were pre-cultured and enriched in nutrient broth overnight in a rotary shaker at 37 °C, centrifuged at 10,000 rpm for 5 minutes. The pellet was suspended in distilled water and the cell density was standardized by spectrophotometer (A_{570} nm).

5-2. Antibacterial Activity

The inhibitory effect of nylon-6/HTCC mat was tested by the disc diffusion method [27]. The tested microbes were seeded into respective medium by spread plate method. The control plates nylon-6/Ch were assessed in parallel to monitor the changes. The 5 mm diameter discs of nylon-6/HTCC were placed on organism-seeded plates. The antibacterial assay plates were incubated at 37 °C for 12 hrs. The diameters of the inhibition zones were measured in mm. These assays were performed in triplicate and repeated at least three times.

5-3. Scanning Electron Microscopy (SEM)

The cells were prepared according to the initial fixation and dehydration steps previously described [28]. The cells were fixed at 24 °C for 60 min with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (20.15 g sodium cacodylate trihydrate, 0.1 mL HCl in 250 ml distilled water, pH=7.4) and then dried on a critical point dryer (HCP2; Hitachi Company, Japan). The dried cell samples were coated with gold, and examined using a scanning electron microscope (S-4100, Hitachi, Japan).

RESULTS AND DISCUSSION

We successfully obtained electrospun nylon-6/chitosan nanofibers with chitosan concentration 8% (w/w) of nylon-6 using Nanospider technology as a needless electrospinning technique in which we used up to 75 kV of electrostatic force. The experimental parameters were optimized to obtain nanofibers with continuous and without any beads in their morphology. The main idea for increasing the chitosan concentration is to use its functional amino group as a carrier for antimicrobial agents. The antimicrobial agent was introduced via surface modification of the electrospun nylon-6/Ch nanofibers by treatment in aqueous solution of GTMAC at room temperature. The surface modification was confirmed by different methods including FE-SEM, XRD, FTIR spectroscopy as well as TGA.

1. Scanning Electron Microscopy

Fig. 1 shows the FE-SEM images of electrospun nylon-6/chitosan nanofibers before and after surface modification (nylon-6/Ch & nylon-6/HTCC), respectively. The images showed nanofibers with a smooth surface and uniform diameters along their lengths. As shown in Fig. 1, a very clear arrangement of ultrafine mesh-like nanofibers strongly bound with the main fibers was observed. These

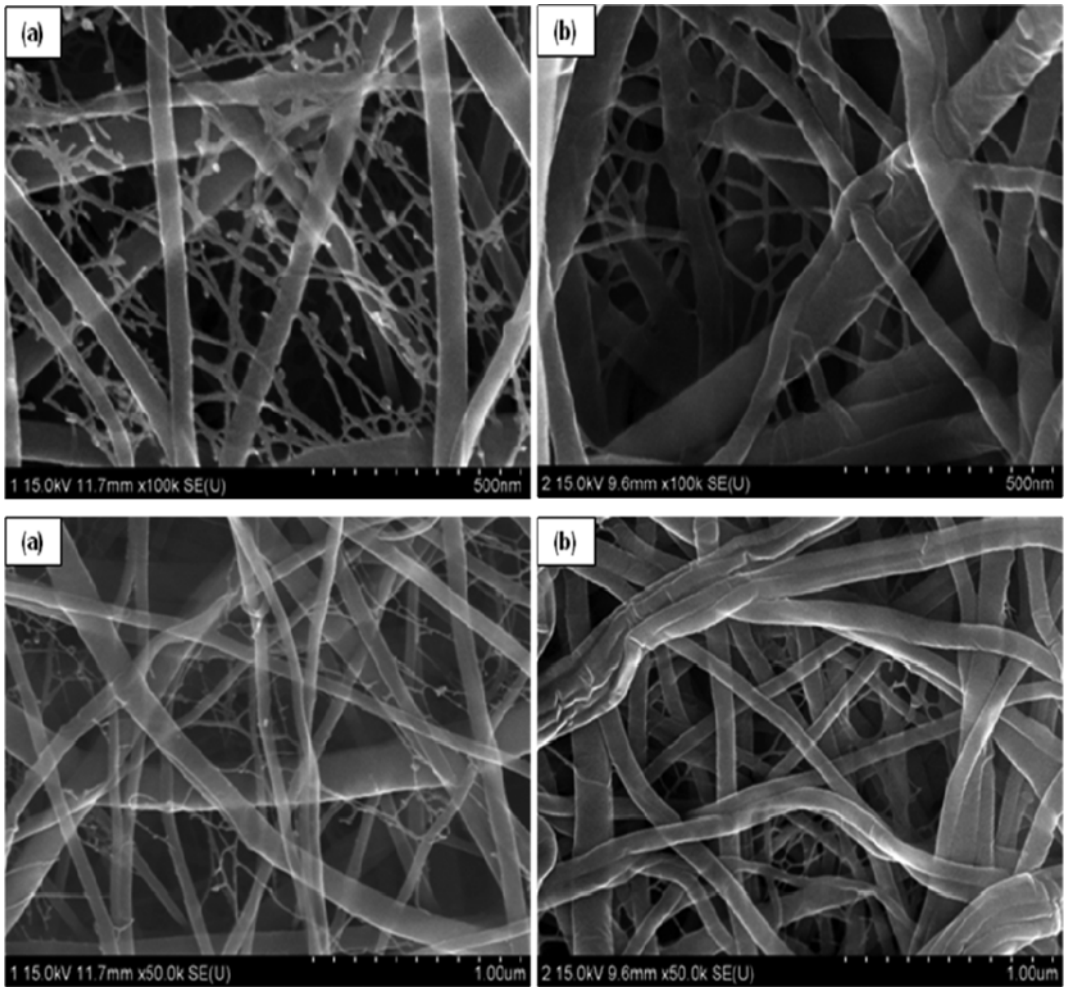


Fig. 1. FE-SEM images of electrospun nylon-6/Ch nanofibers before and after surface modification; (a) Nylon-6/Ch; (b) Nylon-6/HTCC.

ultrafine nanofiber structures resulted in a large surface area-to-volume ratio. The size of the ultrafine nanofibers is one order less than those of main fibers. The diameter of the nylon-6 nanofibers was observed to be in the range of 44-114 nm, whereas the mesh-like nanofibers structure consisted of regularly distributed very fine nanofibers with diameters of about 10-14 nm. It is believed that the formation of large surface area to volume ratio nanofibers was due to the strong applied voltage created between the electrodes.

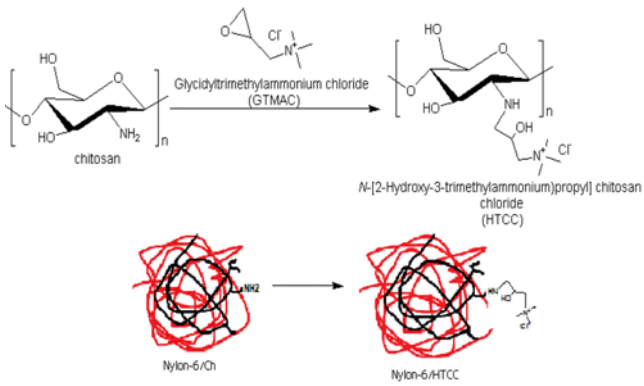
Also, the fibers had cylindrical morphology and no fiber bundles, indicating that the distance between the active electrode and collecting electrode 14 cm was adequate for proper evaporation of the solvent.

2. Proposed Reaction

Here, we present an appropriated chemical reaction for the possible surface modification which is illustrated in Scheme 1. As shown in the scheme, the quaternary ammonium salt group (-NH-CH_2-CH(OH)-$N^+(CH_3)_3Cl^-$) was introduced onto chitosan backbone during surface modification process. This proposed mechanism is in a good agreement with the XRD and FTIR data as shown in Figs. 2 and 3, respectively.

3. XRD

The crystalline structures of electrospun nylon-6/Ch and nylon-6/HTCC nanofibers were characterized by XRD, and the result was



Scheme 1. Surface modification of nylon-6/Ch with GTMAC.

compared with that acquired from the pristine. The XRD patterns of the electrospun nylon-6/Ch nanofibers before and after surface modification are shown in (Fig. 2). The diffraction pattern of nylon-6/Ch nanofibers exhibited a narrow peak appeared at 2θ=20°. As can be seen from the XRD data, the diffraction peak corresponding to the nylon-6 modified with HTCC appeared to be broader compared to that of unmodified nylon-6/Ch nanofibers. Moreover, a shoulder appeared at 22° for nylon-6/HTCC that corresponds to the

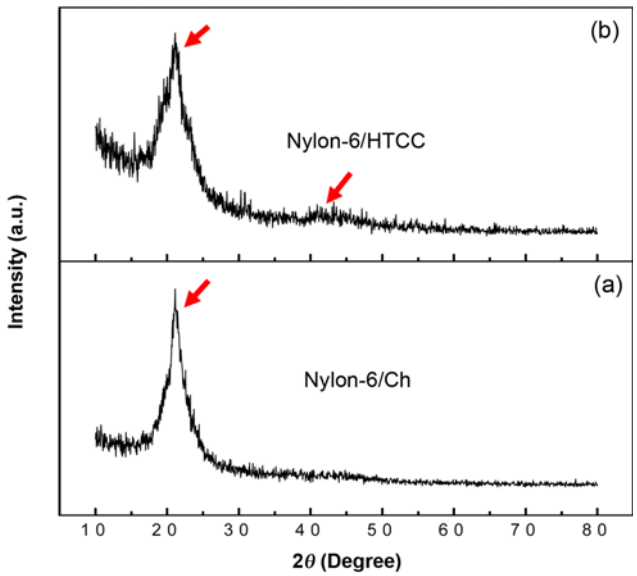


Fig. 2. XRD patterns of electrospun nylon-6/Ch nanofibers before and after surface modification; (a) Nylon-6/Ch; (b) Nylon-6/HTCC.

modified structure, which is in a good agreement with that of previous report [29]. On the other hand, too feeble peak at 2θ=42° corresponding to the characteristic of the γ phase also appeared [30] for modified nylon-6/HTCC nanofibers. This result clearly confirms that surface treatment efficiently occurred via immobilization of quaternary ammonium salt group onto the electrospun nylon-6/HTCC nanofibers.

4. FT-IR Spectra

The FTIR spectra of the electrospun nanofibers as shown in Fig. 3 showed a resonance band at 1,168 cm^{-1} characteristic of its saccharide structure (unsymmetrical stretching of C-O-C bridges. The band at 1,645 cm^{-1} is assigned to C=O stretching of the secondary amide band (amide I) and the -NH_2 bending of the primary amino groups as well as the stretching vibrations C=O of polyamide. Peaks at 1,120 and 1,074 cm^{-1} were due to the skeletal vibrations involving the C=O stretching. The absorption band in the 2,856 cm^{-1} region is characteristic of the stretching vibrations -CH_2- in polyamide backbone. The FTIR spectrum also shows an evidence for the introduction of the quaternary ammonium salt group onto chitosan backbone; a new peak at 1,462 cm^{-1} confirms the C-H bending of trimethylammonium group. The peak at 3,298 cm^{-1} is assigned to the hydroxyl group stretching. It should be also noted that the N-H bending at 1,541 cm^{-1} is assigned to the stretching of the secondary amine due to the change of the primary amine to the secondary amine. Also, the intensity N-H bending (1,541 cm^{-1}) of the primary amine was reduced and broadened due to the change of the primary amine to the secondary amine (aliphatic) for the nylon-6/HTCC nanofibers [31]. According to the FTIR spectrum, the epoxide group of GTMAC has been reacted with the NH_2 groups rather than the OH groups of chitosan. This confirmed the occurrence of the *N*-alkylation reaction in chitosan.

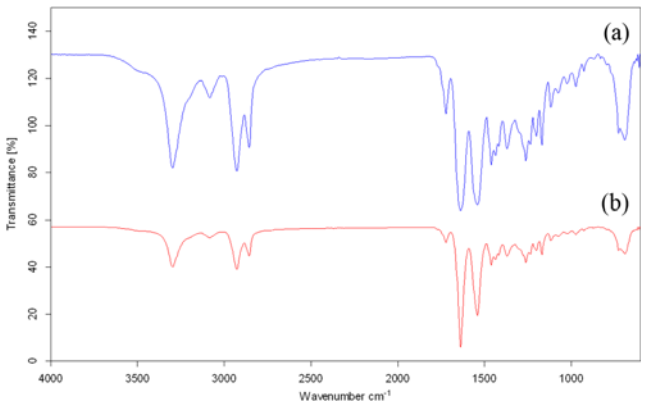


Fig. 3. FT-IR spectra of electrospun nylon-6/Ch nanofibers before and after surface modification; (a) Nylon-6/Ch and (b) Nylon-6/HTCC.

5. Thermogravimetric Analysis (TGA)

The TGA thermogram of the electrospun nanofibers before and after modification (nylon-6/Ch & nylon-6/HTCC) is shown in Fig. 4. The thermogram of nylon-6/Ch showed a weight loss of 1.1% in the range starting from 25 to 100 °C due to the evaporation of the residual absorbed solvents, followed by slow weight loss of 7.6% in the range starting from 100 to 335 °C due to the decomposition of polymer with low molecular weight chitosan, dehydration of the saccharide rings, depolymerization and decomposition of the acetylated and deacetylated units of the chitosan. In addition, a maximum weight loss was observed from 335-500 °C, which may be due to the degradation nylon-6. On the other hand, the thermogram of nylon-6/HTCC showed similar results to that of nylon-6/Ch in addition to the slow weight loss of 4.6% in the range starting from 100 to 223 °C due to the degradation of the immobilized active group. Moreover, after the nylon-6/Ch modification the nanofibers mat showed a residue weight of 7.0-14.2% at 500 °C. On the other hand, the significant weight loss at 422 and 366 °C indicates the onset (T_{on}) of electrospun nylon-6/Ch and nylon-6/HTCC, respectively, due to HTCC modification. Table 1 summarized the TGA results of nylon-6/Ch and nylon-6/HTCC electrospun nanofibers.

The results demonstrated the confirmation of the surface modification of amino group of chitosan with biological active group which leads to the loss of thermal stability of nylon-6/HTCC compared to

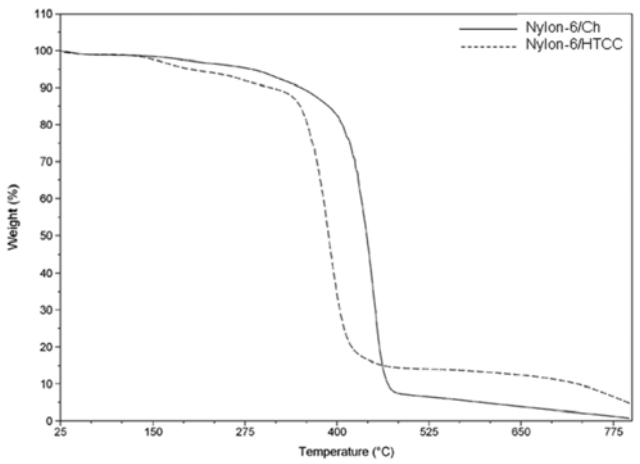


Fig. 4. TGA thermogram of electrospun nylon-6/Ch nanofibers before and after surface modification; nylon-6/Ch and nylon-6/HTCC, respectively. The heating speed was 10 °C/min.

Table 1. Proximate analysis (wt%) of electrospun nanofibers based on thermogravimetric analysis (TGA)

Sample code	Distribution of volatile ranges (temperature range) (°C)				Residue at 500 °C	50% Loss at (°C)
	Solvents evaporation	Active group degradation	Chitosan degradation	Nylon degradation		
	Zone I 30-100	Zone II 100-223	Zone III 223-328	Zone IV 328-500		
Nylon-6/Ch	1.1	7.6*		84.3	7.0	440
Nylon-6/HTCC	0.7	4.7	5.5	74.6	14.2	388

*Represent degradation of chitosan in the range of 100-335 °C

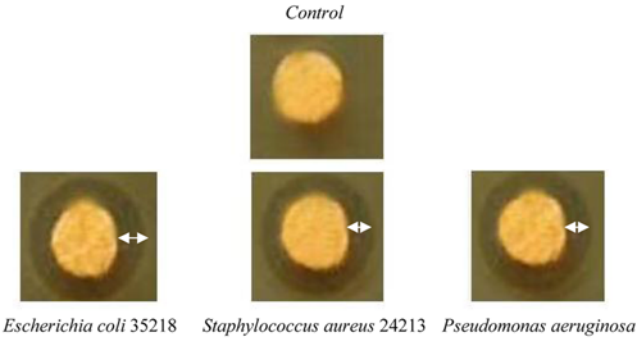


Fig. 5. Inhibition Zones of nylon-6/HTCC mat against *Escherichia coli* 35218, *Staphylococcus aureus* 24213 and *Pseudomonas aeruginosa*.

the original nylon-6/Ch.

6. Antimicrobial Assessment

The biological assessments explored by the disc diffusion method in the present work revealed that nylon-6/HTCC mat exhibited potential antibacterial activity against the pathogenic Gram negative *Escherichia coli* 35218, *Pseudomonas aeruginosa* and Gram positive *Staphylococcus aureus* 24213. A reduction of the colony formation was observed after 12 hrs post treatment. The zone diameter was 10 mm as shown in Fig. 5. The control sample (nylon-6/Ch) exhibits no activity. There was no pronounced activity against Gram negative *Klebsiella pneumonia*, *Proteus* spp., *Salmonella* spp. and Gram positive *Bacillus cereus*, *Streptococcus pneumonia.*

In general observation, Gram negative *Escherichia coli* 35218, *Pseudomonas aeruginosa* and Gram positive *Staphylococcus aureus* 24213 examined using SEM were totally deformed and exhibited severe destruction as shown in (Fig. 6). In case of *Pseudomonas aeruginosa*, the surfaces of the bacterial cells were totally damaged. It was found also that the intact cells had a smooth surface, while most of the exposed bacterial cells exhibited severe destruction. Furthermore, the damage to the surface structure of *Pseudomonas aeruginosa* cells may, therefore, be the main reason for exposure by nylon-6/HTCC.

In contrast, for *Escherichia coli* 35218 they became rough and swollen, the structure of the cell wall surface layer was wrinkled, and round pores were partially deformed, indicating that the cytoplasmic structures were flushed out of the cells but they were unlysed. The results suggestd that the nylon-6/HTCC exposed cells remained unlysed in suspension, in some cases of the cells. Intact cells had a smooth surface with overall intact morphology. For *Staphylococcus aureus* 24213, many cells were enlarged, elongated and highly irregular. However, there was a pronounced deformation and visible shrinkage and this was mainly due to the binding of antimicrobial agents to the certain receptors of the bacterial membrane that lead to the disruption of the cytoplasmic membrane and thus inhibits the growth.

The lethal action of polycationic biocides can be interpreted by which the biocide target the cytoplasmic membranes of bacterial cells and the following elementary processes (6 steps) were identified as modes of action: (1) adsorption onto the bacterial cell surface; (2) diffusion through the cell wall; (3) binding to the cytoplasmic membrane; (4) disruption of the cytoplasmic membrane; (5) release of the cytoplasmic constituents such as K^+ ions, DNA, RNA; and (6) death of the cell [32,33].

In fact, it is well known that bacterial cell surfaces are negatively charged. Therefore, the adsorption onto the negatively charged cell surface (process 1) is expected to be enhanced with increasing the charge density of the cationic biocides. It is reasonable to assume that process 2 is much more enhanced for polymers than for model compounds [34,35]. A similar situation can also be expected in process 3 because there are many negatively charged species present in the cytoplasmic membrane, such as acidic phospholipids and some membrane proteins [35-38]. The disruption of the membrane (process 4) is a result of the interaction of the bound polymers with the membrane disruption and, therefore, is expected to be facilitated with increasing amounts of the bound polymers.

In fact, polymers containing antibiotics are one of our most important weapons in fighting bacterial infections and have greatly benefited the health-related quality of human life since their introduction. However, over the past few decades these health benefits are under threat as many commonly used antibiotics have become less and less effective against certain illnesses, not only because many of them produce toxic reactions but also due to emergence of drug resistant bacteria (processes 5 and 6). It is essential to investigate newer polymers containing drug with lesser resistance. Systematic studies among various pharmacological compounds have revealed that any drug may have the possibility of possessing diverse functions, and thus may have useful activity in completely different spheres of medicine. Therefore, the results clearly indicated the antibacterial activity of the used polymer and ascertained its value in the development of new antimicrobial materials.

CONCLUSIONS

Electrospun nylon-6/Ch nanofibers were prepared using nano-

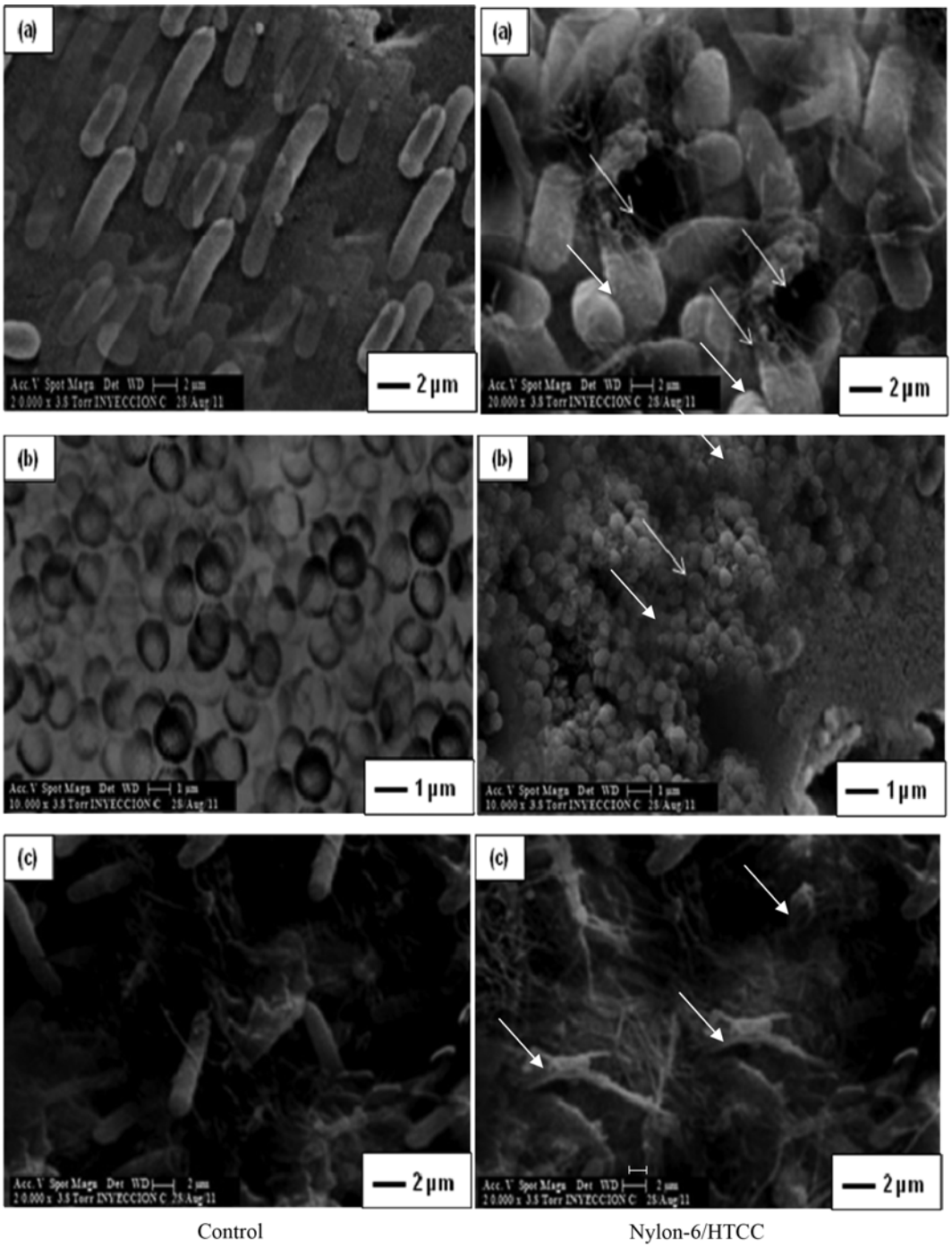


Fig. 6. Scanning Electron Micrograph (SEM) demonstrating effect of nylon-6/HTCC mat on Gram negative (a) *Escherichia coli* 35218, (b) *Staphylococcus aureus* 24213, and (c) *Pseudomonas aeruginosa*.

spider technology as a carrier for antimicrobial drugs. The introduction of glycidyltrimethylammonium chloride (GTMAC) as antimicrobial drug was confirmed by FTIR spectra, XRD, TGA and FE-SEM. The electrospun nanofibers exhibited high inhibitory effects against the microbes. The results clearly indicated that the antimicrobial activity of the electrospun nanofibers varies with the species of the organisms used. Thus, the study ascertains the value of the use of electrospun nanofibers which could be of considerable interest in the development of new antimicrobial materials for biomedical applications. SEM image of the affected microbes was totally deformed and exhibited severe destruction. Abnormal cell division was observed at high frequencies among cells that tried to divide in the presence of the polymer. Many cells were enlarged, elongated, empty ghosts, or fragmented, consistent with the extremely low viability modified technique of electrospinning. Therefore, this finding puts the spotlight on the nanotechnology applications in the fields of medical and life sciences. Moreover, the adverse affects of nylon-6/Ch could be also used in general toxicology of nanoscale

materials.

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