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Enhanced Antibiofilm Activity of Chitosan Stabilized Chemogenic Silver Nanoparticles Against Escherichia Coli

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***Abstract*-** Microbial biofilms, which are formed when bacterial and/or yeast cells adhere to abiotic and biotic surfaces represent the most prevalent type of virulent factor involved in the crucialdevelopment of clinical infection exhibiting resistance to antimicrobial agents. Metalnanotechnology chemistry has the potential to prevent the formation of these life-threatening biofilms on life supporting devices. The present study is undertaken to evaluate the antibiofilm effect of metallic silver nanoparticles (AgNP) stabilized with biocompatible chitosan polymer against biofilm of clinical isolate of Escherichia coli and their effect on biochemical composition of biofilm matrix in terms of total carbohydrate and total protein under in vitro condition. Free and chitosan stabilized AgNPs were characterized by Fourier Transform Infrared Spectroscopy(FTIR), Transmission Electeron Microscopy (TEM), Scanning Electron Microscopy (SEM) and EDS (Energy Dispersive Spectroscopy). Both free as well as the stabilized nanoparticles were found to be effective in bringing about the inhibition of biofilm formation. But enhanced biofilm inhibitory effect inhibition was observed in case of polymer stabilized nanoparticles. Biochemical composition of biofilm matrix in terms of total carbohydrates and total proteins was appreciably reduced in all tested concentration of polymer stabilized nanoparticles compared to free AgNPs.

***Index Terms*-** Silver Nanoparticles; Chitosan; *Escherichia coli*; Biofilm; Matrix,Inhibition

I. INTRODUCTION

Nanobiotechnology, the convergence of nanotechnology and biotechnology and in particular its applications in the medical sector are considered as one of the most promising and most advanced areas of nanotechnology [1] The application of nanotechnology in the field of healthcare has come under great attention in recent times. There are many treatments today that take a lot of time and are also very expensive. Using nanotechnology, quicker and much cheaper treatments can be developed. By performing further research on this technology, cures can be found for diseases that have no cure today. The application of such a technology can be used for the inhibition of biofilm formation on the surgical and medical devices which are of higher threat in the process of treatments. Microbial biofilms develop when microorganisms irreversibly adhere to a submerged surface and produce extracellular polymers that facilitate adhesion and provide a structural matrix [2, 3]. This surface may be inert, nonliving material or living tissue.Biofilm-associated microorganisms behave differently from freely suspended organisms with respect to growth rates and ability to resist antimicrobial treatments and therefore pose a public health problem [4, 5]. Due to increasing tolerance of the biofilm community to antibiotics,biocides and mechanical stress, it has become difficult to completely eradicate mature biofilms.Common treatments to prevent or remove biofilm include disinfection, minimizing nutrients in the feed or altering surface materials to prevent bacterial attachment or clean-in-place (CIP) to remove mature biofilm by chemical or mechanical shear.Nevertheless, nanoscale materials have recently appeared as one of the most promising strategies to control biofilm infections related to indwelling medical devices, especially due to their high surface area to volume ratio and unique chemical and physical properties [6]. A nanomaterial has a diameter ranging from1 and 100 nm, and they can be made from different materials like copper, zinc, titanium, magnesium, gold, alginate and silver. The use of silver nanoparticles (NPs) is now considered as one of the most promising strategies to combat biofilm infections related to indwelling medical devices [7].Another important factor is the protection provided by the encapsulation of the drug in the biological milieu, decreasing toxicity and allowing the drug to reach the specific site [8].Chitosan a natural polymer has been reported as a polymer-based protective agent to stabilize the metal nanoparticles [9]. Because of the biocompatibility, biodegradability, nontoxicity and adsorption properties of chitosan, it was used as a stabilizing agent to prepare Ag, Au and Pt nanoparticles. These chitosan-protected nanoparticles can be easily integrated into systems relevant for pharmaceutical, biomedical, and biosensor applications. Therefore, it has attracted considerable interest due to its medicinal properties, such as antifungal, antibacterial, antiprotozoan, anticancer, antiplaque, antitartar, hemostatic, wound healing and potentiates anti-inflammatory response, immunopotentiation, antihypertensive, serum cholesterol lowering,immune enhancer, increases salivary secretion (anti-xerostomial) and helps in the formation of bone substitute materials [10]. Hence an attempt has been made in this study to evaluate anti biofilm activity of biocompatible chitosan stabilized silver nanoparticles against clinical isolate of *Escherichia coli* under *in vitro* conditions.

II. MATERIALS AND METHODS

Synthesis of silver nanoparticles

Silver nanoparticles were synthesized by chemical reduction of 0.1 M silver nitrate with 0.1 M sodium borohydride as reducing agent[11].Synthesis of silver nanoparticles (AgNPs) was confirmed by the conversion of the reaction mixture into brown colour.Further characterization of the synthesized AgNPs was carried out with the determination of plasmon absorption maxima using UV-Visible spectroscopy (Thermoscientific Spectrascan UV 2700 spectrophotometer) operating in the absorbance mode. The particle morphology was recorded by Scanning Electron Microscopy (SEM) by using Carl Zeiss subra Germany equipped with an energy dispersive spectroscopy (EDS) capability. Synthesized nanoparticles were purified by successive centrifugation at 10,000 rpm and the collected pellets were washed thrice with deionized water, the washed suspension thus obtained was freeze dried.

Synthesis and characterization of chitosan coated AgNPs nanoparticles

For the synthesis of chitosan stabilized silver nanoparticles (CS-AgNPs), 5ml of 0.1 M silver nitrate, 1ml of 0.1 M sodium borohydride and 10 ml of a solution containing chitosan (6.92 mg mL-1) were mixed and stirred for 3 hours to obtain a homogeneous solution. The homogenous solution thus obtained was transferred to the screw cap vial and incubated for 12 h at 95°C [12].The colour of the solution changed from colourless toil ght yellow and finally to yellowish brown which primarily confirmed the coating of chitosan onto silver nanoparticles.

Bacterial strain and growth condition

Clinical isolate of *Escherichia coli* was obtained from Madurai Medical college hospital,Madurai, Tamil Nadu, India. The strain was isolated from patient with severe urinary tract infection and maintained on the slope of tryptic soy agar slant. *E. coli* from the slant was inoculated in to tryptic soy broth (Hi media) and incubated in an orbital shaker at 150 rpm at 37□C for 24 hours. Cells were collected by centrifugation and the collected cell pellets were washed twice in Phosphate buffered saline (PBS) and resuspended in PBS to give a suspension with an OD value of 0.8 (Standard MC value) at 520 nm.

Biofilm inhibition assay in microtitre plates

Freeze dried free and the polymer stabilized AgNPs were dissolved in deionised water at different concentrations (10, 25, 50, 75 and 100 µg /ml) in sterile screw cap vials, shaken well to obtain complete homogenous mixture and were used for biofilm inhibition assay. Biofilm inhibition was carried out in 96 well plate and on nitrocellulose membrane adopting modified spectrophotometric assay described below [12]. 100µl of *E coli* cell suspension was prepared and added into 96 well titre plate and different concentrations of nanoparticles (25, 50, 75 and 100 µg/ml) were added and the plates were at incubated 37º C for 3 days. After the incubation, the liquid suspension was removed and 100 µl of 1% w/v aqueous solution of crystal violet was added. Following staining at room temperature for 30 minutes, the dye was removed and the wells were washed thoroughly and 95% ethanol was added and incubated for 15 minutes. The reaction mixture was read spectrophotometrically at 570 nm. Inhibition of biofilm formation was calculated by using the following formula.

% inhibition = (OD Control –OD Treated) / OD Control × 100

Biofilm inhibition assay on nitrocellulose membrane

Sterile nitrocellulose membrane filter (Rankem, New Delhi, India) with 47 µm and 0.45 µm dia was placed in the 6 well tissue culture plate to which 100 µl of cell suspension of *E. coli*,free and coated nanoparticles were added separately in the above mentioned concentrations and incubated for 3 days at 37□C. Five replicates including a control were maintained. After the incubation period, the inoculated filter was taken and the biofilm inhibition assay was carried out by the modified spectrophotometric inhibition assay described earlier. The filter was stained with 1.0% crystal violet and incubated for 1 hour. After staining, the filter was washed thoroughly with 1 % ethanol and the washed solution was collected in sterile screw cap vial and the reaction mixture was read at 570 nm. Biofilms were examined by SEM after processing of samples by a freeze-drying technique [13] .Biofilms formed on membrane were fixed with glutaraldehyde (2.5%, v/v, in 0.1 M cacodylate buffer, pH 7.0), washed thrice in distilled water and then plunged into a liquid propane/isopentane mixture (2: 1, v/v) at 2196 µC before freeze-drying under vacuum (1026 torr, 1.361024 Pa). Samples were finally coated with gold and palladium and viewed under Carl zeiss subra (Germany) scanning electron microscope.

Isolation and biochemical analysis of biofilm matrix

Biofilm matrix material was isolated from the microtitre plate and nitrocellulose membrane as previously described [14] .Adherent biofilms were transferred to screw cap bottles containing 10 ml distilled water. The bottles were sonicated for 5 min in an ultrasonic water bath and vortexed vigorously for 1 min to disrupt the biofilms. Cell suspensions were then pooled and centrifuged. The collected supernatant was used as source for studying biochemical composition in terms of total protein determined by Lowry et al. and total carbohydrate by Dubois et al [15]

III. RESULTS AND DISCUSSION

The harsh and potentially fatal consequences of microbial biofilm infections generated efforts to prevent their formation, particularly on indwelling medical devices using chemical and mechanical approaches [16.] Biofilms, the predominant mode of device-related microbial infection, exhibit resistance to antimicrobial agents. They can serve as hides for disease and are often associated with high level antimicrobial resistance of the associated organisms. The metallic nanoparticles are thoroughly being explored and extensively investigated as potential antimicrobials [16]..Biofilm inhibitory effect of metallic nanoparticles against pathogenic bacteria specifically against the biofilms formed by them has been gaining importance recently as the indwelling device-related infections constitute a major cause of morbidity and mortality in hospitalized patients [17]. While studies on the biofilm inhibitory effect of silver nanoparticles are going at an accelerated rate [18, 19, 20] their stabilization using biocompatible polymer for increased antibiofilm activity still needs more attention.

Synthesis and characterization of free AgNPs

Silver nanoparticles synthesis adopting chemical reduction was primarily confirmed by colour change of the reaction mixture from pale yellow to brown which clearly indicates the formation of nanoparticles (Fig. 1a Inset). The characteristic brown colour due to the excitation of plasmon vibrations in the nanoparticles provides a convenient signature of their formation.Synthesized AgNPs were characterized by UV-Vis spectroscopy which reveals a strong broad surface plasmon peak located at 420 nm (Fig. 1). Particle morphology and size recorded by SEM analysis reveals smooth, spherical particles with the size in the range of 19-44 nm (Fig. 2a). The elemental composition of the sample was disclosed by EDS analysis in which strong signals of silver were observed (42.44% in mass) at 3 keV, while weaker signals from C, O, Al and S were also recorded confirming the presence of silver nanoparticles (Fig. 2b).

Synthesis and characterization of chitosan stabilized AgNPs

The AgNPs synthesized were coated with the biopolymer chitosan which acts as a stabilizing agent. The rationale behind selecting chitosan as stabilizing agent is that it shows unique polycationic, chelating and film forming properties as it is an oxygen rich linear polysaccharide having active amino and hydroxyl groups [21]. Therefore, chitosan exhibits a number of interesting biological activities such as biocompatibility, biodegradability, non-toxicity,non-antigenicity and adsorption properties.[22.] These chitosan stabilized nanoparticles were characterised by FTIR, TEM and EDS.The IR spectra of the free as well as chitosan capped silver nanoparticles (Fig. 3) show two prominent peaks, one at 3438 cm-1 corresponding to O – H stretching and a second peak at 1642 cm-1 corresponding to C=C stretching vibrations respectively. Another peak at 794 cm-1 corresponding to -C-H bending vibrations was found only in stabilized silver nanoparticles indicating that chitosan is involved in the process of stabilisation as this peak was also found in the IR spectrum of chitosan.The TEM and EDS analyses were carried out for quantitative deduction of size, morphology and localization of elements in the nano specimens. The TEM micrograph of CSAgNPs reveals the uniform spherical smooth morphology within the size range of 101.78 nanometers and electron dense thin chitosan coated shell of the diameter in the range of 3-5 nanometers (Fig. 4a). Such size distribution analysis primarily confirms that the particles are well dispersed and less aggregated. The EDS graph recorded to unearth the presence of various elements is given in Fig. 4b. Peaks of silver were observed along with significant peaks of C, O and N reflecting the presence of elements constituting chitosan.

Biofilm inhibition

The biofilm inhibition studies carried out using both the free and chitosan stabilized AgNPs at all the tested concentration have successfully inhibited the biofilm formation of *E. coli*.The results were represented as inhibition percentage of biofilm development (Table 1). Both the free and stabilized nanoparticles have been found to show distinct effect on biofilm formation in dose dependent manner. But significant effect (P>0.05) was recorded in case of chitosan stabilized nanoparticles. The results in Table 1 clearly indicate the enhanced antibiofilm effect of coated nanoparticles in microtitre plate assay. While 100 µg/ml of free AgNPs resulted in 80 % inhibition, the same concentration of CS-AgNPs brought about complete inhibition of the biofilm. Namasivayam et al [23] have reported the synergistic effect of biogenic silver nanoparticles and plant products and also with antibiotics on the biofilm of clinical isolates of *Staphylococcus aureus* and *Candida tropicalis*. His group has further studied the biofilm inhibitory effect of chemogenic silver nanoparticles and antibacterial antibiotics coated catheters against *Staphylococcus aureus* biofilm [24]. nAg immobilized on glass was found to inhibit the biofilm formation of *Streptococcus oralis* which is of great importance for materials that require durable antibacterial effect on their surfaces, as it is the case of dental implants [17]. The increased antibiofilm effect of chitosan stabilized metallic nanoparticles may be due to the inhibition of exopolysaccharide synthesis limiting the formation of biofilm [25] or due to diffusion of CS-AgNPs through the channels present in the biofilms followed by the sustained release of metal nanoparticles which may then impart antimicrobial functions [26, 27]. A study carried out by Mohanty et al. [28] revealed that starch stabilised silver nanoparticles was showing considerable antimicrobial and antibiofilm activity. Further he has reported that these AgNPs were found to disrupt biofilm formation and exhibit better antibacterial activity compared tohuman cationic antimicrobial peptide LL-37.Nitrocellulose membrane consists of a series of repeating structural units called nitrocellulose polymer. Nitrocellulose membrane is suitable to catch bacteria in a filtration processbecause its tiny pores can capture microorganisms like bacteria [29]. So these nitrocellulose membranes were selected for further studies on the biofilm inhibitory effect of chitosan coated metallic nanoparticles. The results of biofilm inhibition studies on nitrocellulose membrane using free and coated AgNPs were given in Table 1. It has been identified that 81 % biofilminhibition was brought about by 100 µg/ml of free AgNPs while complete inhibition (100%) was successfully achieved with 75 µg/ml of CS-AgNPs. Scanning electron microscopy of the biofilm derived from nitrocellulose membranes treated with CS-AgNPs reveals degeneration of biofilm with weakened cell masses (Fig. 5b) while the control exposed compact tightly packed cell aggregates (Fig. 5a). ***3.4.***

Effect on biochemical composition of biofilm matrix

The matrix is one of the most distinctive features of a microbial biofilm. It forms a three dimensional, gel- like, highly hydrated and locally charged environment in which the microorganisms are largely immobilized. Matrix-enclosed micro colonies, sometimes described as stacks or towers, are separated by water channels which provide a mechanism for nutrient circulation within the biofilm. The composition of the matrix varies according to the nature of the organism and reduction in the biochemical composition of the biofilm matrix leads to weakening of the biofilm thus facilitating the entry of the drugs [13].Biochemical composition of biofilm matrix in terms of total carbohydrate and total protein were used as an index of inhibition. Table 2 shows a gradual reduction in the concentrations of carbohydrates and proteins with increasing concentrations of free and stabilize dnanoparticles. Another notable feature is the significant reduction in the biochemical compositionbrought about by the stabilized nanoparticles

compared to the free counterparts. Similar findings were reported earlier by Namasivayam et al.[24] and Ganesh and Namasivayam [30] in which drastic reduction in carbohydrates and proteins of biofilm matrix derived from *Staphylococcus aureus* treated with silver nanoparticles and nano zero valent iron respectively. These results clearly indicate that these nanoparticles brought about antibiofilm effect mainly by inhibiting the formation of biofilm rather than disrupting the preformed biofilms.

IV. CONCLUSION

Particular attention is oriented nowadays towards the need for antimicrobial textiles and polymers that are able to reduce or eliminate infections completely especially those caused by antibiotic-resistant bacterial strains. Therefore, the development of nanoparticles possessing antimicrobial properties has recently received growing interest from both academic and industrial sectors. The present study demonstrated the synthesis of metallic silver nanoparticles by chemical reduction method followed by stabilization using biocompatible polymer chitosan which was found to exhibit enhanced antibiofilm activity. This study can further be used to prevent or minimize bacterial infections leading to the development of new generation of antimicrobial agents.

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Table 1. Effect of free and CS-AgNPs on biofilm inhibition of *E.coli*

S.No	Concentration (µg/ml)	Biofilm inhibition (%)			
		Microtitre plate		Nitrocellulose membrane	
		Free AgNps	CS-AgNps	Free AgNps	CS-AgNps
1	10	32.1	71.6	40.4	80.1
2	25	54.4	80.0 a	54.3	89.0
3	50	65.0	89.7 a	62.1	94.3
4	75	70.5	95.0 a	73.5	100.0
5	100	80.0	100.0 a	81.0	100.0

Table 2. Total carbohydrates and total protein of biofilm matrix of *E.coli* grown in microtitre plate and nitrocellulose membrane treated with free and stabilized nanoparticles

S.No	Concentration **(µg/ml)**	Assay	Total carbohydrate **(mg)**		Total protein **(mg)**	
			Free AgNp	**CS-AgNp**	**Free AgNp**	**CS-AgNp**
1	10	MP	92.4	76.1	91.0	75.4
		NA	87.4	71.1	87.3	70.2
2	25	MP	81.3	68.1	84.1	64.1
		NA	76.2	64.2	76.5	60.2
3	50	MP	74.3	55.2	72.0	58.2
		NA	70.2	51.2	68.4	52.0
4	75	MP	63.2	34.1	67.4	49.2
		NA	59.2	31.2	62.1	42.1
5	100	MP	55.1	21.2	55.1	35.2
		NA	50.2	18.5	50.1	28.5
	Control	125.0			102.4	

Figure 1. UV-Vis Spectra of synthesized AgNPs and inset shows the colour change during synthesis,

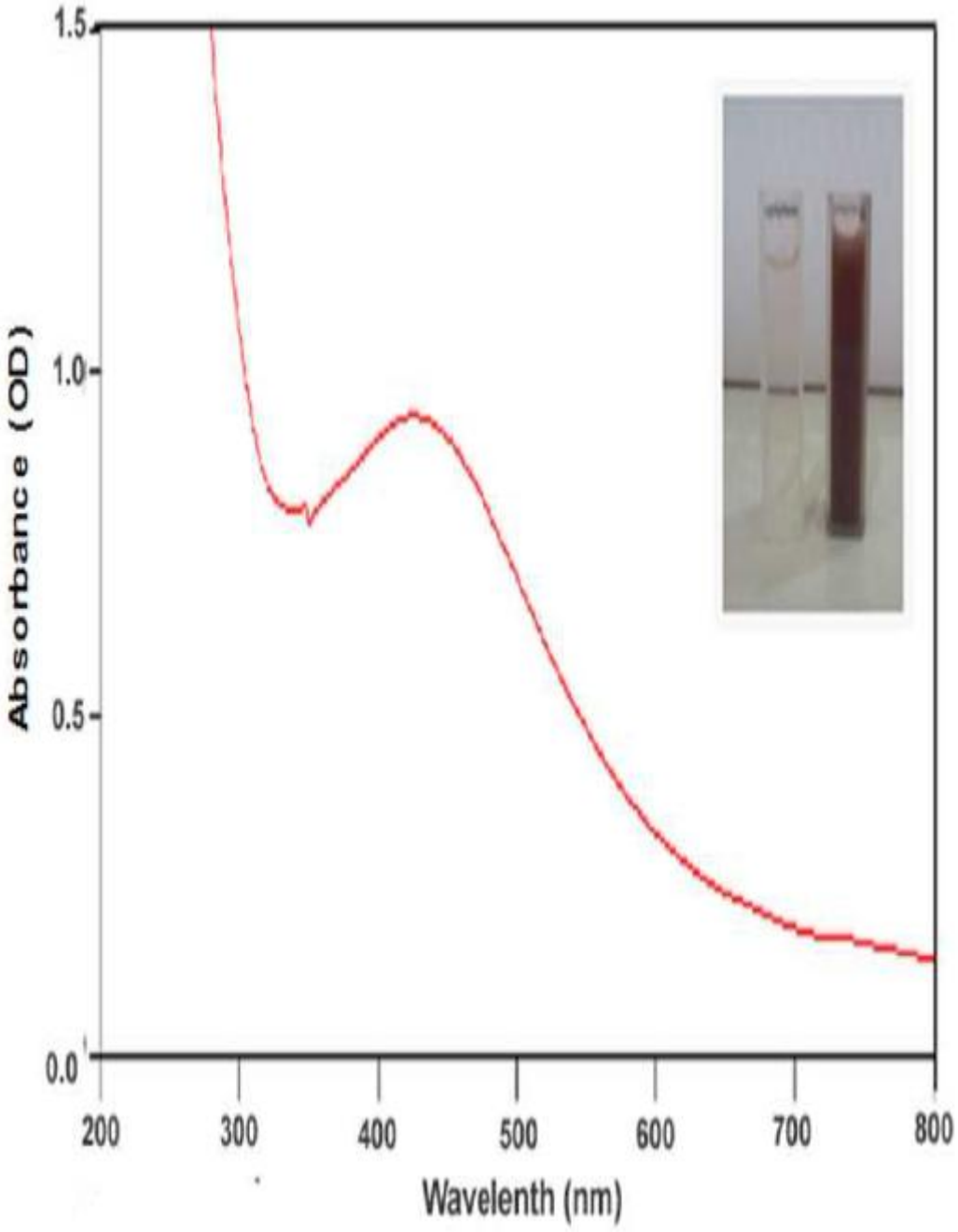

Figure 2 a) SEM and b) EDS images showing the presence of AgNPs

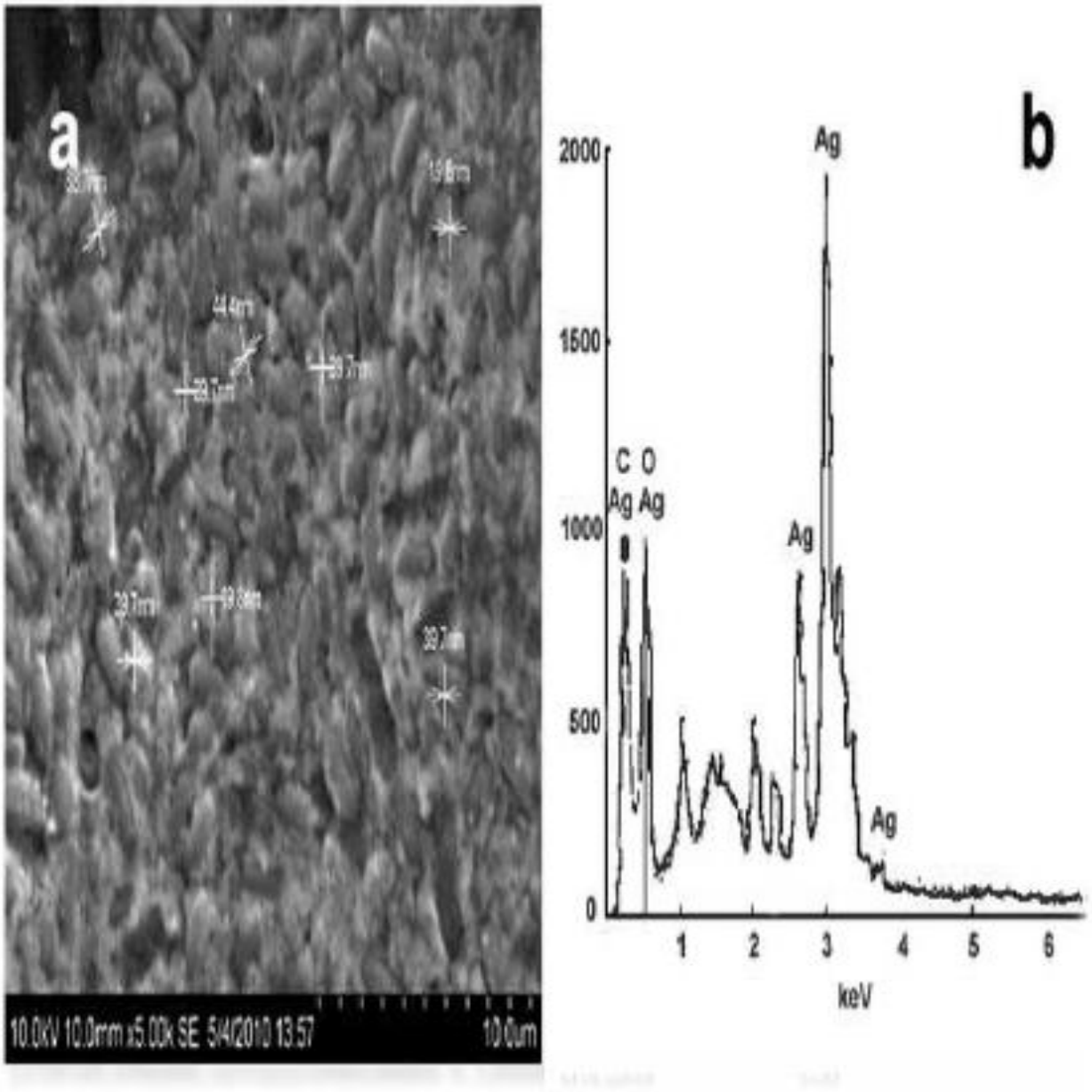

Figure 3. a) FTIR Spectra of CS-AgNPs

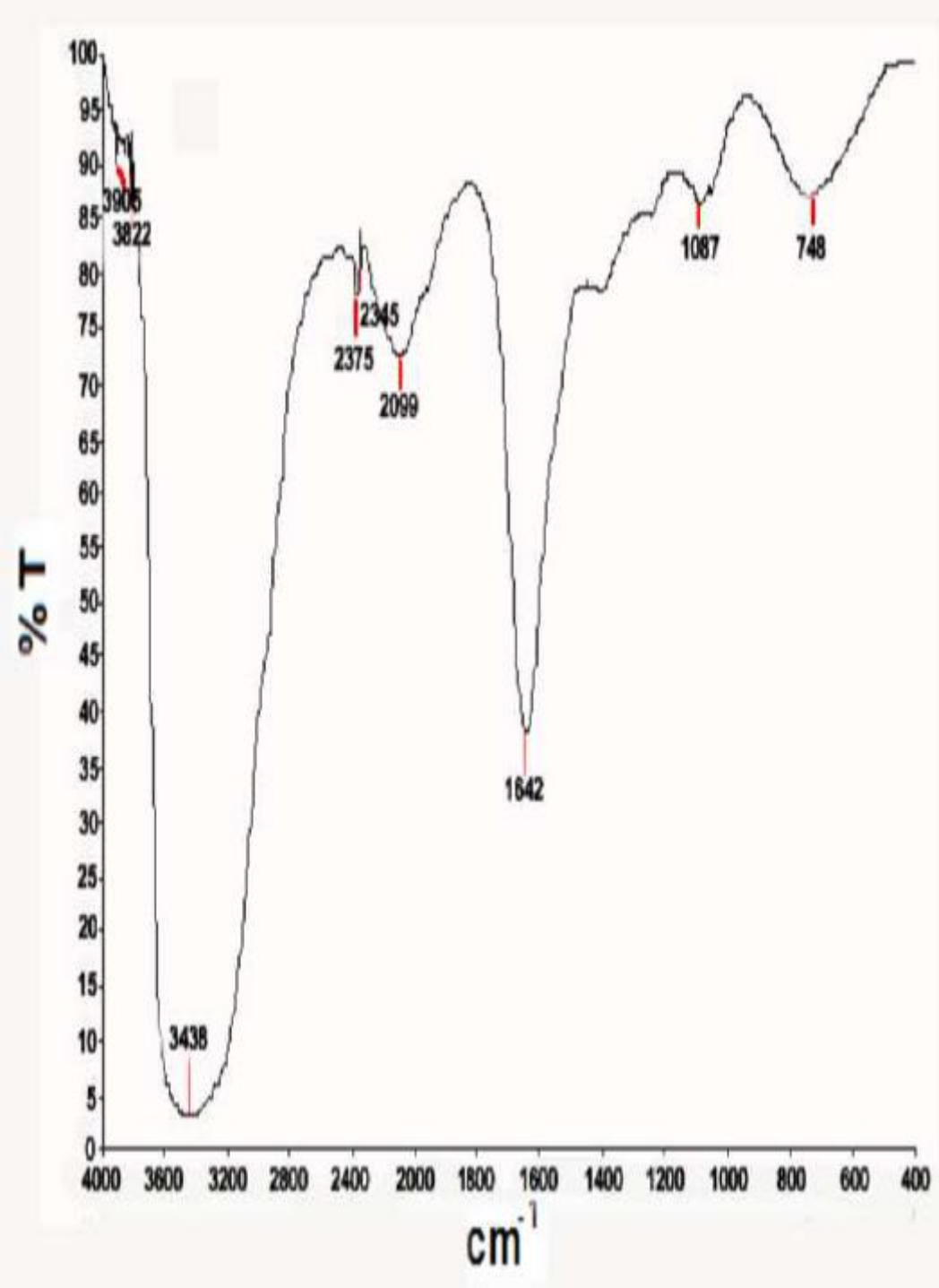

Figure 4. a) TEM and b) EDS images showing the presence of CS-AgNPs.

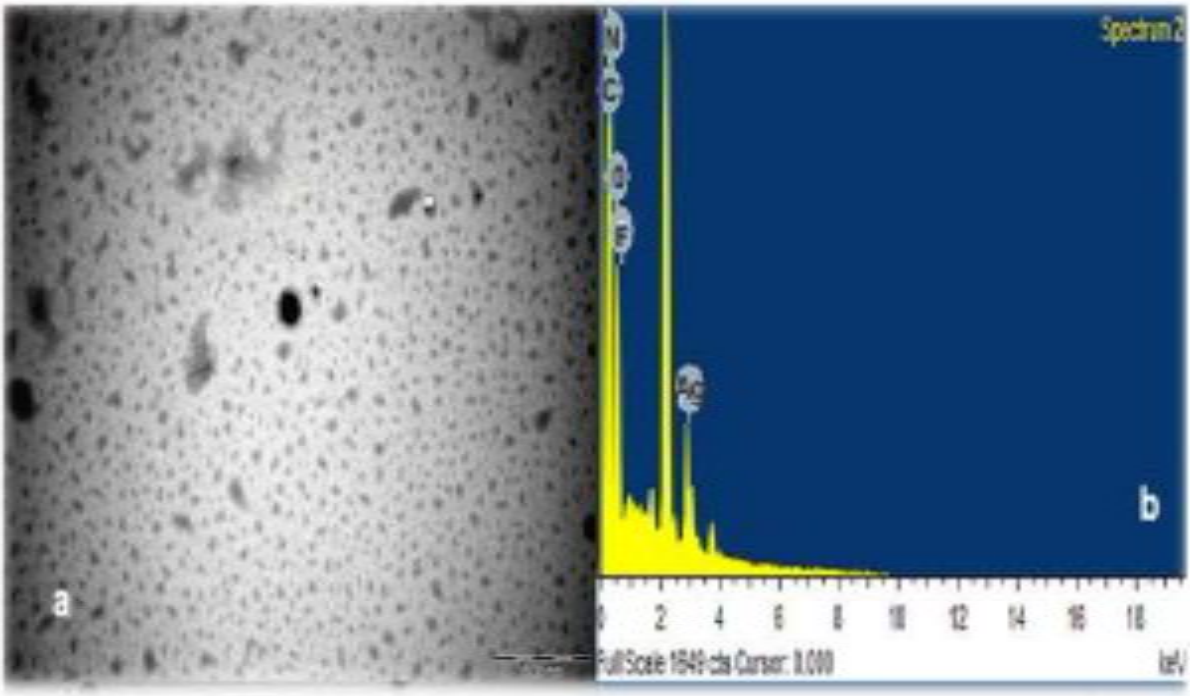

Figure 5. SEM images showing (b) the effect of CS-AgNPs on *Escherichia coli* biofilm derived from nitrocellulose membrane along with (a) being the control