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Synthesis of Graphene oxide + Silver Nanocomposite blended with Cellulose Acetate Membranes - Evaluating Antimicrobial and Filtration Efficiency of developed CA+GO-AgNC membranes

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ABSTRACT

The present study was aimed to investigate the effect of developed nanocomposite (GO-Ag$_{NC}$ - graphene oxide+silver nanoparticles) on cellulose acetate (CA) membrane performance in terms of its antibacterial activity, anti-fouling properties and microbial filtration efficiency. Silver nanoparticles and graphene oxide were chemically synthesized. UV-Vis spectrophotometer of Silver nanoparticles (AgNPs) showed a plasmon peak shifted to longer wavelength at 390nm. FTIR absorption spectra of AgNPs significant band at 1610cm^{-1} indicating the presence of amide group arises due to carbonyl stretch in proteins; attributed to the stretching vibration of (NH) C=O group. SEM analysis of synthesized silver nano-particles were found evident in different shapes like pentagons and hexagons and its size were measured in the range from 65nm to 85nm in size. Antibacterial activity of synthesized graphene oxide (GO), Silver nanoparticles (AgNps) and GO-Ag$_{NC}$ were tested separately and GO-Ag$_{NC}$ showed comparatively more inhibitory zones (21mm to 23mm) than the synthesized individual graphene oxide (14mm to 16mm) and silver nanoparticles (19mm to 21mm). Minimum inhibitory concentration of GO-Ag Nanocomposite showed 40µg/ml for Escherichia coli and Klebsiella pneumoniae; and 60µg/ml for Staphylococcus aureus, Staphylococcus epidermidis and Pseudomonas aeruginosa. Morphological characteristics of the CA+GO-AgNC membrane using Field Emission Scanning Electron Microscopy revealed the presence of pores of uniform size throughout the sample. Developed CA+GO-AgNC membrane showed good antibacterial activity of 23.3±1.25mm, and 23.6±0.57mm against Staphylococcus aureus and Pseudomonas aeruginosa. Microbial filtration efficiency study revealed that, CA+GO-Ag$_{NC}$ membrane filtered bacterial cells to significant level proving that graphene oxide+silver nanoparticles were more lethal to microorganisms.

Keywords: Graphene oxide, Silver nanoparticles, Cellulose acetate, Nanocomposite, Anti-fouling

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INTRODUCTION

The most widely used membrane in industry during the period ranging from 1964 to the mid of 1970s is the reverse osmosis membrane which mainly made from cellulose diacetate (CA), cellulose triacetate or blend of them with different ratios [1]. CA membranes are obtained from most available natural polymer (cellulose) by acetylation process. CA membranes are characterized with several advantages as moderately low cost, neutral surface, good resistance to free chlorine (at low level), highly hydrophilic surface and highly potential water flux [2]. Growth of the bacteria on the surfaces of the membrane forming biofilms is a major problem in most of desalination plants and it is very difficult to be removed, either through disinfection or chemical cleaning [3].

To overcome this problem, a number of approaches have been established, such as pretreatment procedures and membranes modification [4, 5]. The blending of different polymers give some advantages as a better hydrophilicity of membranes, enhancing physical-chemistry stability, improving the film-forming characteristics of the polymers and improving anti-fouling properties. Wu et al.[6] enhanced the anti- fouling properties and hydrophilicity of polyvinyl chloride ultrafiltration membrane via blending it with synthesized amphiphilic copolymer PVC-g-poly (ethylene glycol) methyl ether methacrylate to create a new membrane with outstanding properties.

Zhang et al.[7] coated the polyamide membrane surface with chitosan derivative, which was then reacted with aqueous solutions of glutaraldehyde (as cross-linking agent) and Cu2+ solution that in-situ reduced to Cu nanoparticles and fixed in the covering layer. The modified membranes showed antibacterial efficiency of above 99% for more than 90 days' immersion in water.

To enhance bio-fouling resistance, cellulose acetate (RO) membranes were modified by incorporation of Graphene oxide + Silver Nanocomposite in the casting solution. No study had been found in literature for using Graphene oxide + Silver Nanocomposite for modification of reverse osmosis membrane. So; we aimed in this study to investigate the effect of developed nanocomposite on cellulose acetate membrane performance in terms of its antibacterial activity, anti-fouling properties and microbial filtration efficiency.

MATERIAL AND METHODS

Synthesis of Graphene Oxide (GO)

Graphene oxide was synthesized using the modified Hummers method. About 1.2g of graphite powder and 2g of sodium nitrate (Merck, India) were taken in a beaker containing 50mL of concentrated sulfuric acid (97%, v/v; Fisher Scientific) to increase the interlayer spacing of the carbon source. The reaction mixture was stirred for 2h in ice-cold water, maintaining its temperature (0 to 6°C). About 6g of $KMnO_4$ was slowly added to the reaction mixture and stirred for 2h in an ice bath; and further stirred at 30°C until it turns into a brownish paste. The solution mixture was finally treated with 8mL of hydrogen per oxide to stop the reaction. The change in color from dark brown to golden yellow was found evident indicating the formation of graphene oxide. Finally, the mixture was centrifuged and rinsed with 8% hydrochloric acid; followed by adding distilled water to obtain the precipitates. The precipitates were filtered, dried, and ground in fine powder form; stored under room temperature in dark amber bottle.

Synthesis of Silver Nanoparticles (AgNPs)

Silver nitrate solution (Solution-A) was prepared by adding 3.4g of $AgNO_3$ in 20mL of distilled water. Polyvinylpyrrolidone (PVP) solution (Solution-B) was prepared by dissolving 1g of PVP, glucose (1g) and NaOH (1g) in 60 mL of distilled water. Solution-B was heated to 60°C with continuous stirring, and solution-A was drop wise added into it. The obtained mixture was stirred for 10min and centrifuged, washed with distilled water and dried to obtain silver nanoparticles (AgNPs).

Purification of synthesized silver nanoparticles was done according to the procedure suggested by Chaudhary et al. The synthesized AgNPs were kept in 12N hydrochloric acid solution for 24h. The Ag NPs were isolated by the process of centrifugation from the mixture. The mixture was washed with distilled water till hydrochloric acid was completely removed. Purified silver nanoparticles were further stored in room temperature for characterization studies.

Characterization of the silver nanoparticles

UV-Visible spectroscopy (UV-Vis) analysis

Synthesized silver nanoparticles was confirmed by sampling the aqueous component of different time intervals and the absorption maxima was scanned by UV-Vis spectrophotometer at the wavelength of 300 – 800 nm on Perkin-Elmer Lambda 25 spectrophotometer. The UV-visible reading was recorded and then analyzed using the Origin Pro or Microsoft Excel analysis tool.

Fourier transform infrared spectroscopy (FTIR) analysis

The purified silver nanoparticles was subjected to FTIR analysis (Shimadzu IR). AgNPs were mixed with KBr and subjected to IR source 500–4000 cm−1.

Scanning Electron Microscopy (SEM)

The morphology of the developed silver nanoparticles using field emission scanning-electron microscopy (JSM-7500F, JEOL) was analyzed. A minute drop of nanoparticle powder was cast on to a carbon-coated copper grid and subsequently transferred to the microscope. The high-resolution images of silver nanoparticles were recorded and the morphology was further studied.

Synthesis of Graphene oxide – Silver Nanocomposite (GO-Ag_{NC})

The coupled nano-system of Graphene oxide – Silver Nanocomposite (GO-Ag_{NC}) was synthesized by mixing graphene oxide and silver nanoparticles in a 2:1 ratio within 5mL of methanol. The mixture was sonicated for 2 days followed by thermal annealing at 100°C and subsequent cold freezing in a deep freezer. Final obtained powder was dried and homogenized for further testing.

Antibacterial activity of GO, AgNPs and GO-Ag Nanocomposite

The antibacterial activity of Graphene oxide, silver nanoparticles and GO-Ag Nanocomposite was evaluated against the test organisms by well diffusion method. Sterile Nutrient Agar (Composition g/L: Peptone: 5g; Yeast extract: 5g, Beef extract: 3g, Sodium chloride: 5g, Agar 15 g; Final pH (7.0 ± 0.2) plates were prepared and allowed to solidify. About 0.1% inoculum suspensions of the each test organism (*Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Staphylococcus epidermidis* and *Pseudomonas aeruginosa*) were swabbed uniformly over the agar surface separately. Under sterile conditions, 6mm wells were cut on the agar surface of each MHA plates. About 20µl of each synthesized particles (Graphene oxide - 100µg/ml, silver nanoparticles - 100µg/ml and GO-Ag Nanocomposite - 100µg/ml) were loaded into the well and the plates were incubated at 37ºC for 24h. The antibacterial activity was evaluated in terms of

zone of inhibition around the wells in all the inoculated MHA plates. The inhibition clear zones were measured and recorded in millimeter.

Determining the MIC of Synthesized GO-Ag Nanocomposite

To determine the MIC of GO-Ag Nanocomposite, a set of Mueller-Hinton Agar (MHA) plates (Composition g/L: Acid hydrolysate of Casein: 17.5g; Starch: 1.5g, Sodium chloride: 5.0g, Agar 17.0 g; Final pH - 7.0 ± 0.2) were prepared under sterile conditions. GO-Ag Nanocomposite at different concentrations (20µg/ml, 40µg/ml and 60µg/ml) was added to their respective wells on each culture pre-seeded (*Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Staphylococcus epidermidis* and *Pseudomonas aeruginosa, Candida albicans* and *Candida tropicalis*) MHA plates. All the inoculated plates were incubated at 37°C ± 0.2°C for 24 to 48hours. Plates were observed for inhibitory zones around the wells after incubation period. The zone around the lowest GO-Ag Nanocomposite concentration was selected as MIC.

Preparation of cellulose acetate filtration membranes by phase inversion method

Cellulose acetate filtration membranes were prepared from a homogeneous solution composed of Cellulose acetate (14 wt%), acetone (17.5 wt%), dioxane (45.9 wt%), methanol (13.9 wt%) and acetic acid (8.6 wt%). The solution was stirred using a magnetic stirrer and left overnight then cooled to 4°C. The solution was casted using a micrometer applicator (with 250µm clearance gap) on a clean glass plate at room temperature. The solvents were allowed to evaporate within different durations (120s) and the membranes were given the names as M120. The cast films obtained were immersed into a water bath (4°C for 2h). The coagulated membranes were washed using deionized water, then the prepared membranes were thermally annealed by immersion in a hot water bath of 80°C–85°C and allowed to hold for 10min for consolidating the dense layer which provides the high solute rejection of such membranes.

Developing Cellulose Acetate + GO-Ag Nanocomposite (CA+GO-Ag_{NC}) blended membranes

The developed cellulose acetate filtration membranes were sterilized under Ultra-Violet light for 15min kept at distance of 30cm. The filtration membrane was coated with GO-Ag nanocomposite using a modified two dip-coating procedure. In this method, the UV sterilized membrane was placed in a sterile glass beakers on a shaker (120 rpm) for 3 hours, with a drying period of about 15 minutes between the two coating procedures. The coated materials were rinsed in phosphate buffered saline (PBS) to remove surface accumulation of particles, followed by drying at room temperature. All coating steps were carried out under aseptic conditions in a laminar airflow hood. Each nanocomposite coated membrane samples were further tested for its filtration and permeation efficiency.

Field Emission Scanning Electron Microscopy (FESEM)

CA+GO-Ag_{NC} membrane developed was observed using Field-Emission Scanning Electron Microscopy (FESEM). The test materials were prepared for SEM using a suitable accelerating voltage (10 KV), vacuum (below 5 Pa) and magnification (X 3500). Metal coating was used as the conducting material to analyze the sample.

Antibacterial activity of CA+GO-Ag_{NC} membranes

The antibacterial activity of CA+GO-Ag_{NC} membrane was evaluated against the five test organisms (*Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Staphylococcus epidermidis* and *Pseudomonas aeruginosa* by modified Kirby-Bauer method or disc diffusion method. Sterile Nutrient Agar plates were prepared and allowed to solidify. About 0.1% inoculum suspensions of each of the bacterial cultures were swabbed with the sterile cotton swab three times by turning the plate at 60^0 angle between each streaking. Under sterile conditions, membrane size of 20mm in diameter was cut and placed on the agar surface of each Nutrient Agar (NA) plates. Filter paper disc impregnated with standard antibiotic solution (Gentamicin – 10µg/ml) was also placed on the same plate to compare the efficacy of PHB films. All the plates were incubated at 37ºC for 24 - 48h. The antibacterial activity was evaluated in terms of zone of inhibition around the wells of each extract in all the inoculated NA plates. The inhibition clear zones were measured and recorded in millimetre.

Anti-biofouling properties of CA+GO-Ag_{NC} membranes

Static adhesion test for cellulose acetates membranes was employed; an immersion test was used for examination the anti-adhesion performance of the modified CA membranes to bacterial cells. RO membranes used in this method were cut into 1 × 1cm^2 and immersed in the bacterial suspension for about 4 days. *Escherichia coli* was the selected strains used in the biofouling tests (supplied from the American type culture collection (ATCC; Rockville, MD, USA)). This bacterium was selected to determine how the nanocomposite modified cellulose acetate membrane affected model Gram negative bacteria [8]. The bacterial strain was inoculated into nutrient broth medium at 37°C for 24h.

Microbial filtration efficiency CA+GO-Ag_{NC} membranes

Escherichia coli was inoculated into Nutrient broth medium at 37°C for 24h. The samples were filtered over a 47mm diameter CA+GO-Ag_{NC} membrane filter (F1) with a pore size of 0.45µm. After filtration, the

membrane was placed on Plate Count Agar media and incubated for 12 to 24h to obtain bacterial colonies. Similar experiment was carried out for plain CA filter membrane (F2). Difference in the number of colonies from both control and nanocomposite membranes were calculated and presented as CFU/ml. The difference in number of colonies between F1 and F2 exposed samples were calculated using the following bacterial reduction percentage formula.

$$\text{Bacterial reduction percentage} = A - B/A \times 100$$

Where,
A – No of colonies from F1,
B – No of colonies from F2.

RESULTS AND DISCUSSION

Characterization of the silver nanoparticles

UV-Visible spectroscopy

The effect of filtration on the AgNPs, produced using UV–vis spectrophotometer was illustrated in Fig. 1. After filtration of the sample, the plasmon peak shifted to longer wavelength of 390nm. The formation of these can be confirmed by means of a spectrum for the colloids that used silver nitrate since a strong plasmon band was observed near 390nm, which confirms that the Silver ions were reduced to Ag^0 in phase watery. There is no change in peak position, suggesting that nucleation of silver nanoparticles starts with initiation of reaction time only, and the size remains unchanged throughout the course of reaction.

Fourier transform infrared spectroscopy (FTIR) analysis

FTIR measurements were carried out to identify the potential functional groups which are responsible for the reduction of silver ions into silver nanoparticles. FTIR absorption spectra of AgNPs are shown in Fig. 2. The band at 1610cm^{-1} indicates the presence of amide group arises due to carbonyl stretch in proteins; attributed to the stretching vibration of (NH) C=O group. That amide groups present in carbohydrates, proteins are dominant reducing agents and play an important role in the reduction of Ag+ ions to Ag^0 leads to nanoparticles synthesis. The observed peaks at 1,610cm^{-1} indicating C=C groups, 1,308cm^{-1} occurring to the germinal methyls and 1,008cm^{-1} shows ether linkages, suggesting the presence of silver nanoparticles.

Scanning Electron microscopic (SEM) analysis of synthesized silver nanoparticles

SEM analysis of synthesized silver nano-particles were found evident in different shapes like pentagons and hexagons. The obtained nanoparticles were clearly distinguishable and its size were measured in the range from 65nm to 85nm in size. In some of the focused area, the particles were also found in the form of agglomerates (Fig. 3).

Antibacterial activity of GO, AgNPs and GO-Ag Nanocomposite

Antibacterial activity of synthesized GO, AgNps and GO-Ag_{NC} against all the test bacteria was presented in Table-1. All three samples showed good antibacterial inhibitory zones against the organisms. The zone size was comparatively more for GO-Ag_{NC} compared to the synthesized individual graphene oxide and silver nanoparticles against all the test bacteria. The inhibitory zones for GO ranged from 14mm to 16mm; for AgNPs ranged from 19mm to 21mm and for GO-Ag_{NC} ranged from 21mm to 23mm respectively (Fig. 4).

MIC of Synthesized GO-Ag Nanocomposite

Minimum inhibitory concentration of GO-Ag Nanocomposite against five test bacteria using three different concentrations were presented in Table-2. Among the three concentrations, 20µg/ml did not exhibited inhibitory zones against any organisms tested. *Escherichia coli* and *Klebsiella pneumoniae* showed MIC at 40µg/ml. And other test organisms (*Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas aeruginosa*) exhibited MIC at 60µg/ml. Standard antibiotic (gentamicin) exerted inhibitory zones ranging from 17mm to 19mm against the test organisms (Fig. 5).

FESEM analysis of CA+GO-AgNC membranes

Morphological characteristics of the CA+GO-AgNC membrane were examined using Field Emission Scanning Electron Microscopy (FE-SEM). Images obtained from scanning electron micrographs reveal the presence of pores of uniform size throughout the sample. The topographical images of the developed membrane were presented in Fig. 6.

Antimicrobial activity of CA+GO-AgNC membranes

Developed CA+GO-AgNC membrane showed good antibacterial activity against all test organisms. Inhibitory zones of 22.6±0.75mm, 23.3±1.25mm, 23.6±0.57mm, 22.9±0.75mm and 23.6±0.57mm exhibited for respective organisms *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. The obtained results when compared to standard ampicillin drug loaded paper disc, significant antibacterial activity in terms of zone of inhibition was observed. In Table-3, the inhibitory zones obtained for the developed PHB films showed significant values against all the test bacteria compared to standard ampicillin.

Anti-biofouling properties of CA+GO-AgNC membranes

Anti-biofouling properties of the developed membrane was presented through SEM image analysis in Fig. 7. Control CA membrane adhered more bacterial cells was evident in Fig. 7A indicating the absence of nanocomposite. In Fig. 7B, only few bacterial cells were found adhered on the CA+GO-AgNC membrane, which proved that the nanocomposite significantly prevented the adherence of bacterial cells on the membrane surface. The images indicated that the CA membrane containing graphene oxide+silver nanoparticles have enhanced antibacterial properties; the obtained results were thus in accordance with the earlier anti-fouling results described by Weng et al.[9], Zidan et al.[10], Pan et al.[11] and Ardila et al.[12].

Microbial filtration efficiency of CA+GO-Ag_{NC} membranes

Microbial filtration efficiency study revealed that, number of colonies are significantly higher in the bacterial plates exposed to plain CA filter membrane (F2) compared to plates exposed to CA+GO-Ag_{NC} membrane filter (F1) for all three sampling volumes (100ml, 200ml and 300ml) respectively. In Table-4, the difference in number of colonies and its statistical values were presented. The obtained results indicated that the CA+GO-Ag_{NC} membrane filter (F1) filtered the bacterial cells and its growth in their respective tested volumes. As graphene oxide+silver nanoparticles has more lethal potential on microorganisms, the obtained results were found significant and satisfactory to develop more products related to water filtration in near future by replacing with conventional antibacterial agents. Similar results were obtained by Liu et al [13].

Table-1: Antibacterial efficacy of synthesized particles

S. No.	Test Bacteria	Zone of Inhibition (mm)		
		Graphene oxide	Silver Nanoparticles	GO-Ag Nano composite
1	*Escherichia coli*	16	19	22
2	*Staphylococcus aureus*	14	20	21
3	*Klebsiella pneumoniae*	15	21	23
4	*Staphylococcus epidermidis*	16	19	23
5	*Pseudomonas aeruginosa*	15	20	21

Table – 2: MIC of Synthesized GO-Ag Nanocomposite

S. No.	Test Bacteria	Zone of Inhibition (mm)			
		20µg/ml	40µg/ml	60µg/ml	S
1	*Escherichia coli*	0	11	15	18
2	*Staphylococcus aureus*	0	0	14	17
3	*Klebsiella pneumoniae*	0	11	14	18
4	*Staphylococcus epidermidis*	0	0	15	17
5	*Pseudomonas aeruginosa*	0	0	16	19

Table-3: Qualitative Antibacterial activity of developed films

Sample	Zone of inhibition (mm)				
	Escherichia coli	*Staphylococcus aureus*	*Klebsiella pneumoniae*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
CA+GO-AgNC membrane	22.6±0.75	23.3±1.25	23.6±0.57	22.9±0.75	23.6±0.57
Standard (Gentamicin)	24.6±0.57	25.3±0.75	24.9±1.05	24.6±1.25	24.9±0.57

All values calculated using mean ± standard deviation (SD)

Table-4: Microbial filtration efficiency of CA+GO-Ag_{NC} membranes

Sampling volume (ml)	No. of colonies		Reduction percentage (%)	Statistical analysis Between Plain CA and CA+GO-Ag_{NC} membrane	
	Plain CA filter membrane (F2)	CA+GO-Ag_{NC} membrane filter (F1)		F-Value	Significance
100	164	27	83.5	F = 89.3	P = 0.000
200	128	09	92.9		
300	71	06	91.5		

Fig. 1: UV-Visible spectroscopic analysis of synthesized silver nanoparticles

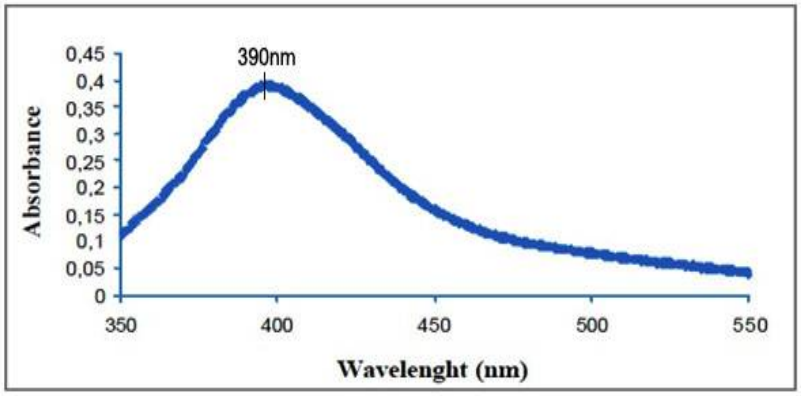


Fig. 2: FTIR analysis of synthesized silver nanoparticles

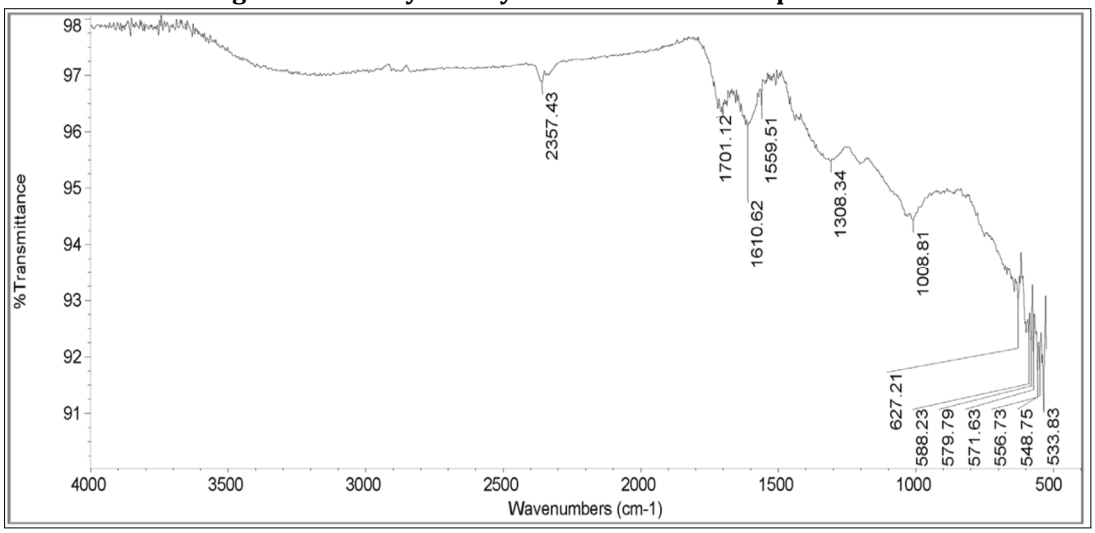


Fig. 3: SEM analysis of synthesized silver nanoparticles

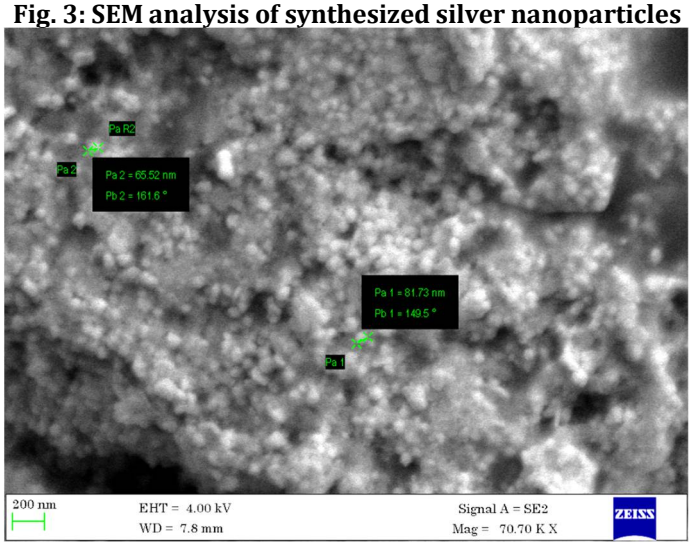
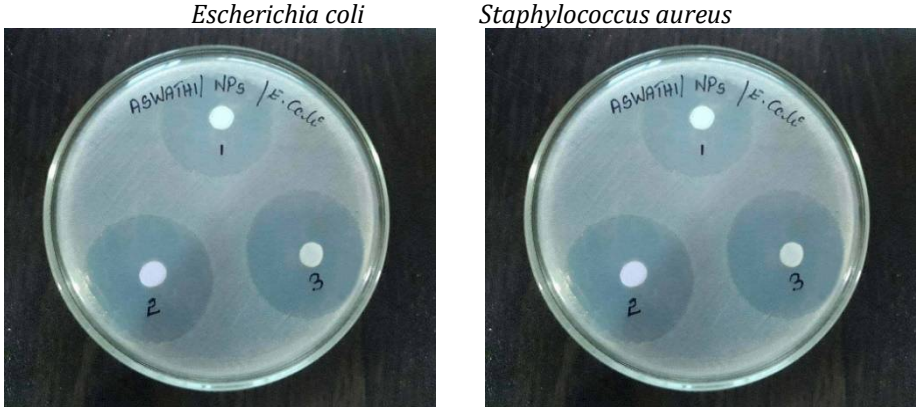
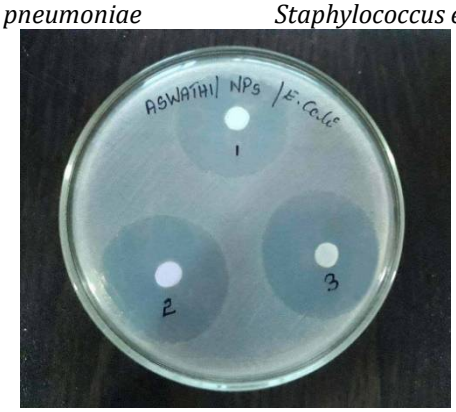


Fig. 4: Antibacterial efficacy of synthesized particles

Escherichia coli *Staphylococcus aureus*



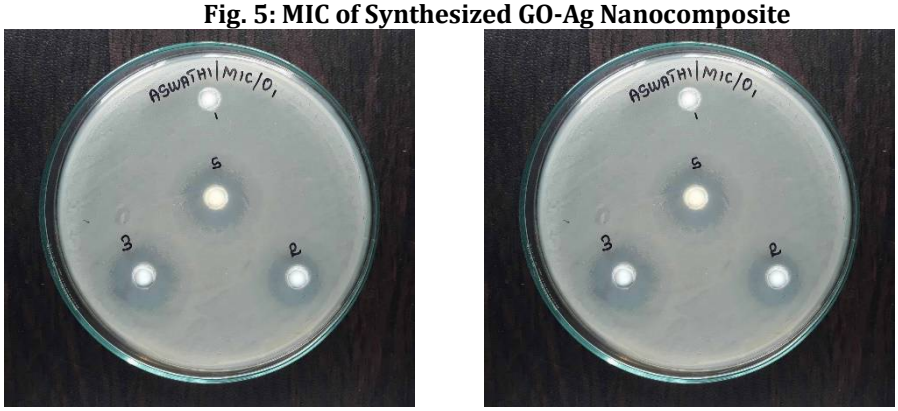
Klebsiella pneumoniae *Staphylococcus epidermidis*



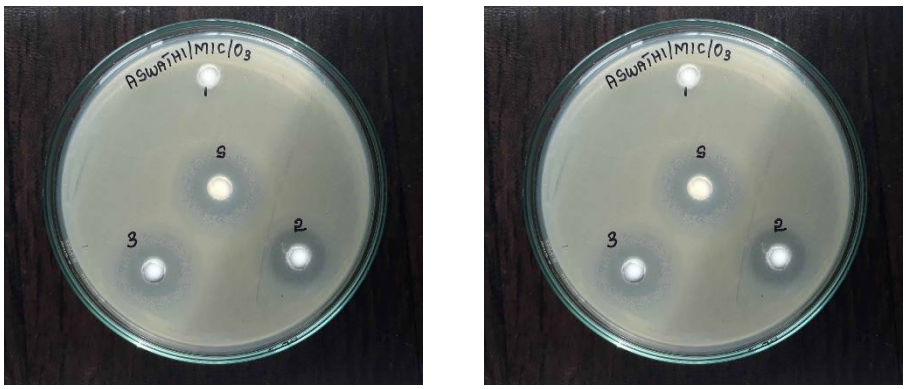
Pseudomonas aeruginosa

(1- Graphene oxide, 2 – Silver NPs, 3 – GO-Ag composite)

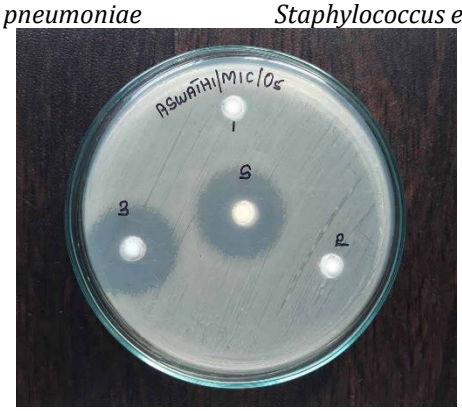
Fig. 5: MIC of Synthesized GO-Ag Nanocomposite



Escherichia coli *Staphylococcus aureus*



Klebsiella pneumoniae *Staphylococcus epidermidis*



Pseudomonas aeruginosa

(1, 2, 3 Different concentrations of GO-Ag composite, S- Streptomycin)

Fig. 6: Developed CA+GO-AgNC membrane

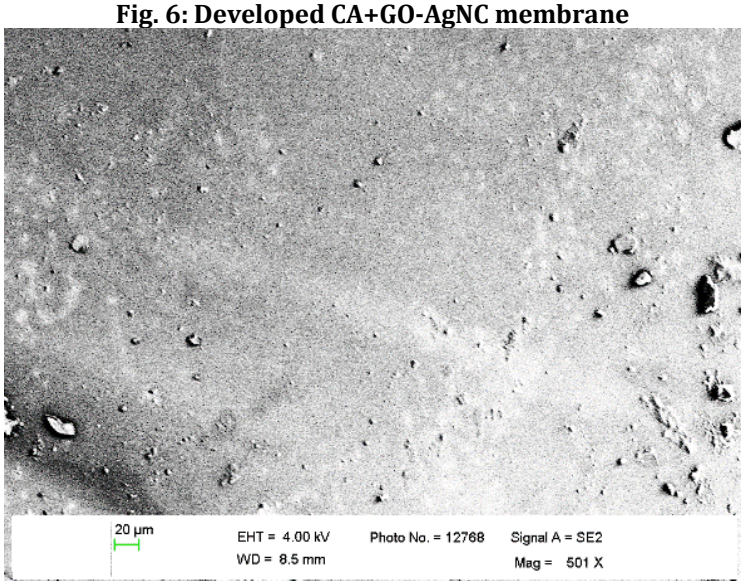
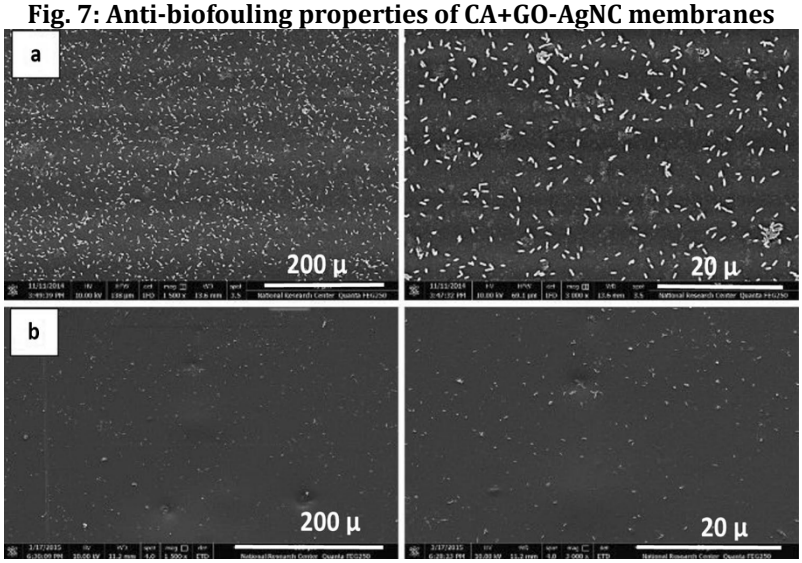


Fig. 7: Anti-biofouling properties of CA+GO-AgNC membranes



CONCLUSION

Silver nanoparticles and graphene oxide were chemically synthesized. UV-Vis spectrophotometer of AgNPs showed a plasmon peak shifted to longer wavelength at 390nm. FTIR absorption spectra of AgNPs significant band at 1610cm^{-1} indicating the presence of amide group arises due to carbonyl stretch in proteins; attributed to the stretching vibration of (NH) C=O group. SEM analysis of synthesized silver nanoparticles were found evident in different shapes like pentagons and hexagons and its size were measured in the range from 65nm to 85nm in size. Antibacterial activity of synthesized GO, AgNps and GO-Ag$_{NC}$ were tested separately and GO-Ag$_{NC}$ showed comparatively more inhibitory zones (21mm to 23mm) than the synthesized individual graphene oxide (14mm to 16mm) and silver nanoparticles (19mm to 21mm). Minimum inhibitory concentration of GO-Ag Nanocomposite showed 40µg/ml for *Escherichia coli* and *Klebsiella pneumoniae*; and 60µg/ml for *Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. Morphological characteristics of the CA+GO-AgNC membrane using Field Emission Scanning Electron Microscopy revealed the presence of pores of uniform size throughout the sample. Developed CA+GO-AgNC membrane showed good antibacterial activity against all the test bacteria. Microbial filtration efficiency study revealed that, CA+GO-Ag$_{NC}$ membrane filtered the bacterial cells to a significant level proving that graphene oxide+silver nanoparticles were more lethal to microorganisms. The obtained results in the present study were thus found significant and satisfactory to develop more products related to water filtration in near future by replacing with conventional antibacterial agents.

Conflict of Interest

Authors declare no conflict of interest in the current research

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