Nanocomposite Hydrogel based on Silver Nanoparticles/Graphene-Oxide Self Assembly for Potable Water Disinfection

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CERTIFICATE

This is to certify that the dissertation work entitled **"Nanocomposite hydrogel based on silver nanoparticles/ graphene oxide self-assembly for potable water disinfection"** submitted by Anjana Mittal (Roll No. 301701003) in the partial fulfillment for the award of degree of Master of Science in Biotechnology from Thapar Institute of Engineering and Technology, Patiala, Punjab, is the record of the candidate's own independent and original research work carried out under my supervision and guidance. The matter embodied in this dissertation has not been submitted in part to any other University/Institute for the award of any degree or diploma in India.

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DECLARATION

I hereby declare that the work which is being presented in dissertation entitled "**Nanocomposite hydrogel based on silver nanoparticles/graphene oxide self-assembly for potable water disinfection**" is an authentic record of my own project work carried out at Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala under the supervision of Dr. Shekhar Agnihotri. This matter presented in this dissertation has not been submitted to any other University/Institute for the award of any degree in India or abroad.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

µg	microgram
Γ	Gamma
ZOI	Zone of inhibition
ZnO	Zinc oxide
Wt	Weight
TiO_2	Titanium dioxide
Rpm	revolutions per minute
NPs	Nanoparticles
nm	nanometer
MW	molecular weight
mM	millimolar
mm	milimetre
ml	milliliter
MIC	Minimum inhibitory concentration
mg	milligrams
hrs	hours
gm	grams
Cu	Copper
◦C	degree centigrade
µ	micro
µl	microliter
Ag	Silver
AgNPs	Silver nanoparticles
$AgNO_3$	Silver nitrate
CFUs	Colony forming units
GO	Graphene oxide
DI	Deionized water

ABSTRACT

New generation nanomaterials provide many opportunities for the treatment of waste water. The development of these kinds of materials has a commitment to deal with some challenges to become most efficient antibacterial agent. Successful water disinfection must have the capability to destroy microorganisms without forming any disinfection by products and disturbing the environment. At present very few materials possess this activity but they are practically limited because of aggregation in solvent, mechanical instability and toxicity etc.The present work describes the synthesis of nano-silver based hydrogel nanocomposite consisting of porous networks of natural biopolymer chitosan and graphene oxide with uniformly dispersed AgNPs (CH/GO/Ag) synthesized by *in situ* chemical reduction. Scanning Electron microscopy (SEM), Fourier Transform Infrared (FT-IR) Spectroscopy, Energy-dispersive X-ray spectroscopy (EDX) and X-ray diffraction (XRD) were used to characterize CH/GO/Ag hydrogels. 3D structure of hydrogel provided space for silver nanoparticles and act as matrix for the immobilization of AgNPs. Hydrogels show excellent swelling capacity and rigidity. Disinfection tests were performed against *Escherichia coli*, *Staphylococcus aureus, Staphylococcus epidermidis* and *Enterobacter aerogenes* showed that CH/GO/Ag hydrogels exhibited good antibacterial activity and time required to achieve complete disinfection was 120 min.

Keywords: Hydrogel, Immobilization, Antibacterial, Nanomaterials, Nano-silver

CHAPTER-1

INTRODUCTION

Water contamination is one of the insidious problems affecting people throughout the world. It has become a matter of concern as it directly affects human health. Pathogenic microorganisms that reside in water cause many infectious diseases and impermeance among human beings. Mostly these diseases spread through consumption of water, food and inhalation of air. Among these, water is the leading source for transmission of diseases. Since, water is an essential resource for life its safety or disinfection is indispensably important for global health. Influential presence of microorganisms and their associated diseases in water bodies and healthcare areas have taken a huge toll on humanity.

Research data for water contamination suggests when water contains bacteria or other microbes it can cause water related diseases like typhoid fever, cholera, *E.coli* infection, botulism, malaria, dysentery and amoebiasis which are responsible for millions of death every year (Cabral *et al*., 2010). Every year, water pollution causes more than 1.7 million deaths, most of which are children under the age of five (WHO, 2017). These health hazards are more prominent in rural areas where access to water disinfection infrastructure is insufficient. Therefore, in order to halt the outbreaks caused by water pollution, there is dire need to clean, treat or disinfect the water resources. For this purpose various conventional treatment methods are currently employed which include coagulation, flocculation, sedimentation, and filtration (Wei *et al*., 2018) to remove suspended pollution particles but in order to inactivate or kill pathogenic microorganisms, disinfection treatments are applied. Standard disinfection methods that were used for water disinfection are chlorination, UV, heat sterilization and filtration but these methods are unfavorable for long term use as they affect human well-being. As in one of the methods like chlorination, chlorine tends to react with already present natural organic matter (fulvic and humic acid) in water which in turn forms disinfection by products (DBPs) like trihalomethanes. These DBPs has been proven to be carcinogenic in nature and have serious health hazards to humans and water bodies (Rahman *et al*., 2010). Therefore, we require some other alternative methods which are not associated with production of any by products. As the use of

fresh water increases drastically due to increase in population, there is dire need to find some unconventional methods that are safer, cheap and ecofriendly. Nanotechnology solves all the problems that are caused by previous unconventional methods.

In the last few years, nanotechnology has provided very large number of opportunities for the manipulation of substances at nanoscale by changing their physiochemical properties and transforming them into potential microbicidal agents. Many bulk metals do not show any antimicrobial activity, but their nanoforms can so by altering them they can be converted into antimicrobial agents. With very small size and high surface area to volume ratios, nanomaterials also have high mechanical flexibility, fast dissolution and high reactivity, physical and chemical stability. Due to these properties, nanomaterials are found to be very effective even at very low concentrations than their bulk form (Zeng *et al*., 2018; Sharma *et al*., 2009; Rai *et al*., 2009; Mauter *et al*., 2008). The multiple mechanisms were used by nanomaterials to kill the microorganisms due to which it is not possible for the microorganisms to develop resistance against them. As a result, nanomaterials are predominantly effective to kill the several drug resistant microbial strains. Due to these reasons nanomaterials are being applied universally in various applications such as water disinfection/purification, biomedical devices/coatings, and food packaging materials etc. Nanomaterials with carbon as its foundation like carbon nanotubes, graphene and fullerenes are also employed in water treatment because of their high mechanical strength and biocidal properties. Different type of metal and metal oxide NPs were also employed in water treatment such as carbon nanotubes, ZnO, TiO_2 and AgNPs (Sharma *et al.,* 2009). However, nanomaterials in colloidal form have limited practical applications owing to its lesser stability due to high reactivity, no reusability and high expense when used in colloidal conditions without any solid support or matrix. All such problems can be limited by immobilizing or stabilizing the nanomaterials onto a support matrix to enhance their antimicrobial efficacy and reusability. In the last few years, interest has been increased in use of hydrogels synthesized from polysaccharides/ biopolymers like chitosan, cellulose, dextran and starch. Employing chitosan obtained from deacetylation of chitin have been encouraged to form a hydrogel due to its biocompatibility, biodegradability, nontoxicity , intrinsic antimicrobial activity, blending ability with other polymers and presence of various functional groups (Dutta *et al*., 2004). Therefore, there is need to

develop an immobilization matrix system for nanoparticles to form a nanocomposite material and acquire the most out of their efficacy in addition to reduction of environmental and health risks. Hydrogels mainly remove contaminants by the adsorption of bacteria on its surface.

In the present work, we designed an immobilization matrix of chitosan and graphene oxide for *in situ* synthesis of AgNPs in the synthesized hydrogels using chemical reduction. AgNPs containing Chitosan/GO and pristine loaded hydrogels were tested for its swelling capacity, antimicrobial activity against common water pathogens to check its potential for water disinfection and characterized by Field emission scanning electron microscopy (FTIR), Energy-dispersive X-ray spectroscopy (EDX/EDS), Scanning electron microscopy (SEM) and X-ray diffraction (XRD).

Objectives of the Work

Considering the importance of the present study following work objectives have been designed:

- Synthesis and characterization of Chitosan/GO hydrogel.
- Chitosan/GO hydrogel as an immobilization matrix for AgNPs.
- Evaluation of antibacterial activity of hydrogel on clinically relevant strains.

CHAPTER-2

REVIEW OF LITERATURE

2.1 History

From the ancient times Greeks, Romans and Egyptians used silver in the different forms for the preservation of food and water, this was used through World War II. North American people during early days when there was no refrigeration, silver coins were dropped into the vessel for the preservation of water.In 1890s silver nitratewas used to cure burn injuries. Richard Feynman‘s (known as ―Father of Nanotechnology) oration on ―There's Plenty of Room at the Bottom‖ in 1959 is known as the origin of Nano technological paradigm since, it suggested the creation of materials via direct manipulation of atoms. One of the American surgeon in 1990s used silver foil for wound dressing and silver sutures in surgical cuts to prevent infection. In 20th century, silver has been used for water purification; wound dressing (Alexander, 2009).

2.2 Nanotechnology in Water Treatment

Nanotechnology offers many opportunities for next generation water supply. Nanotechnology is widely used because of their extraordinary properties like high surface area, photosensitivity, catalytic and microbicidal activity, magnetic properties and small pore size etc. (Xio *et al.*, 2012; Qu *et al.*, 2013).

Superior filtration systems incorporating nanomaterials that allow better water reusage and recycling have been developed. Novel nanosensors embedded with metal NPs, CNTs and quantum dots have been explored to identify low concentration levelsof contaminants (biological/chemical) in water resources. New technologies in water decontamination and disinfection include use of natural & engineered Nanomaterials like chitosan, carboxy fullerene, peptides, carbon nanotubes, ZnO and AgNPs (Emelita *et al.*, 2018) which display strong antimicrobial properties and provide good microbial control. Steps ahead the antibacterial activities of AgNPs are implied in anano filter systems which are achieved by immobilizing AgNPs onto a support system (gel or matrix).

2.2.1 Carbon nanomaterials in microbial water disinfection

Chitosan

It is a polysaccharide having high molecular weight that is composed of β– (1, 4) -1-acetomido-2-deoxy –D glucose and it was formed by deacetylation of chitin. It has wide range of applications ranging from pharmaceuticals to material sciences and it also shows excellent biological properties such as biocompatibility, biodegradability and coagulation activity (Achaby *et al*., 2017). CH is a good reducing agent for the reduction of silver nitrate and it also has ability to provide protection to AgNPs (Jing *et al*., 2018). It has also been proved that incorporation of chitosan increases its biocompatibility (Xiong *et al.,* 2015).Chitosan hasfilm forming capacity and its insoluble in organic solvents and water. Chitosan alone has low mechanical properties so to improve its physical and mechanical properties several fillers can be used such as nanoclays, cellulose, whiskers and carbon nanotubes (Fernandez *et al.,* 2017). Chitosan and its derivatives show antimicrobial activity against viruses, bacteria and fungus (Qi *et al.,* 2004; No *et al.,* 2002; Badawy *et al*., 2005). Antibacterial activity of chitosan is greater for gram positive than gram negative bacteria. Main mechanism followed by chitosan is that positively charged chitosan interacts with cell membrane that is negatively charged and this leads to increase in the permeability of cell membrane which ultimately leads to cell death (Qi *et al.,* 2004).

Graphene Oxide

GO is two dimensional sheet of sp^2 hybridized carbon atoms and it show excellent properties (Raccichini *et al.,* 2015; Bonaccori *et al.,* 2015). Hydroxyl and epoxide groups on the side and carboxylic functional groups at edges of GO (Cap *et al*., 2010; Deng *et al.,* 2017). Presence of these groups helps in its dispersion in water and they also provide surface for the growth of various NPs (Kamal *et al*., 2010; Deng *et al.,* 2017). GO is incorporated to increase the mechanical properties of chitosan (Xie *et al.,* 2013; Cobas *et al.,* 2017). GO contains epoxy groups and chitosan contains amino groups on its surface, due to presence of these groups crosslinking reactions takes place between chitosan and GO (Zhang *et al.,* 2013).

2.2.2Metal/Metal oxide nanomaterials

Many different types of metal and metal oxide nanomaterials are used such as TiO_2, ZnO etc.

TiO_2: It is a photosensitive nanomaterial and photocatalyst semiconductor (Han *et al.*, 2016). It is a good opacifier so can be used in paper, inks and plastic (Laurak; Adamas *et al.*, 2006). With doping TiO_2 shows high degradation rate than simple TiO_2. Doping is of TiO_2 is mainly done with carbon into titanium lattice (Znu *et al.*, 2012). A conc. of 100-1000 ppm completely disinfects water within 30 min (Wei *et al.*,1994; Maness *et al.*, 1999). Doping of TiO_2 with silver has greatly increased the photo catalytic inactivation of viruses (Kim *et al.*, 2006) and bacteria (Paget *et al.*,2007; Reddy *et al.*, 2007).

ZnO: ZnO nanoparticles have the ability to inhibit bacterial growth and it is also stable under tough conditions (Lu *et al.*, 2018). ZnO nanoparticles are more effective against gram negative than gram positive bacteria (Sawai *et al.*, 2003). Primary mechanism followed by them to kill microorganisms is generation of hydrogen peroxide. It shows good durability, selectivity and heat resistance (Chun *et al.*, 2018).

2.3 Why Silver Nanoparticles?

AgNPs show unique physical and chemical properties like optical, electric and biological. Due to these unique properties AgNPs can be applied in different applications which include antimicrobial activity, in food industries, pharmaceutical industries, in health care products, cosmetics, textiles etc. (Chernousova *et al.*, 2013). Main reason for showing these properties is their high surface area to volume ratio (Zhang *et al.*, 2017; Sharma *et al.*, 2009). AgNPs have important applications in antimicrobial and catalysis field because of its high surface area to volume ratio and high fractions of surface atoms (Jing *et al.*, 2018). Nowadays, the broad-spectrum antimicrobial properties of AgNPs stimulate its use in biomedical applications such as, in wound dressings replacing the need of silver sulfadiazine based agents, in bone cements, surgical implements like gloves and masks.

2.3.1 Antimicrobial activity of AgNPs

Ag ions stop DNA replication and also affect the permeability as well as structure of cell membrane (Matsumma *et al*., 2003), due to change in the permeability of cell membrane microbes do not survive, thus leads to cell death. Mechanisms of antimicrobial activity of silvers nanoparticles are postulated: - (a) Adhesion of silver nanoparticles on the surface of cell surface affect the cell membrane (Tripathi *et al.,* 2017; Sondi *et al.,* 2004), (b) Penetration of AgNPs inside the bacterial cells leads to DNA damage and c) Dissolution of AgNPs leads to the release of antimicrobial Ag^{+} ions (Monores *et al.,* 2005). AgNPs show antimicrobial activity by forming reactive oxygen species (Tripathi *et al.,* 2017). The cell membrane of bacteria has copious amounts of sulfur-containing proteins inside or outside the surface so, the AgNPs interacting with these sulfur containing amino acids consequently compromise the cell viability through the inhibition of enzyme functions. AgNPs release Ag+ ions which have high affinity towards the phosphorus containing moieties like DNA, their interaction results in inactivation of DNA replication process (Tripathi *et al.,* 2017; Gupta *et al*., 1998; Matsumura *et al*., 2003). The nanoparticles deliberately assault the respiratory chain; hinder cell division as illustrated in figure 1. AgNPs formed pits or holesin the cell wall by increasing the membrane permeability finally leading to cell death (Tripathi *et al*., 2017; Duran *et al*., 2015; Song *et al*., 2006; Sondi *et al*., 2004; Morones *et al*., 2005).

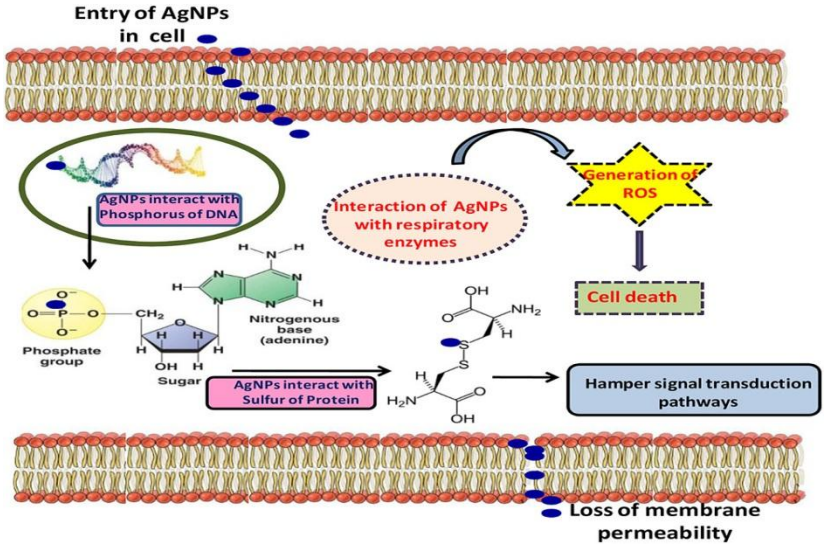

Figure1: Representation of toxicity mechanism of AgNPs (Tripathi *et al.,* 2017**)**

Effect of shape and size of AgNPs

AgNPs having size smaller than 13nm show good antimicrobial activity (Agnihotri *et al*., 2012). Antibacterial activity of AgNPs is greatly affected by its shape and size (Raza *et al.,* 2016). Many researchers reported that an isotropic shapes of Ag particle such as nano plates or triangular nano prism play a significant role in biocidal activity of silver nanoparticles (Raza*et al.,* 2016). Truncated triangular AgNPs show better antibacterial activity than spherical and rod shaped AgNPs (Pal *et al.,* 2007; Raza *et al.,* 2016).

The above mentioned antimicrobial properties of AgNPs can be exploited in water disinfection purposes but there are some restrictions associated with it (Agnihotri *et al*., 2012):

- Silver nanoparticles form aggregates in the aqueous solution, which leads to decrease in their efficacy.
- Loosely held AgNPs cannot be reused keeping in mind the cost associated with it the system becomes expensive.
- Leaching of nanoparticles into the aquatic ecosystem can lead to adverse effects on aquatic life.

2.4 Synthesis of silver nanoparticles

AgNPswere mainly synthesized using three different approaches that includephysical, chemical and biological methods. AgNPs can be produced by both top – down and bottom – up method. Top down method involves mechanical grinding of bulk metals and their stabilization (Korbekandi *et al.,* 2009; Kruis *et al.,* 2000; Iravani *et al.,* 2014). Bottom down methods involves reduction of metals, sonodecomposition.

2.4.1 Physical methods

This method mainly involves evaporation, condensation and laser ablation (Iravani *et al.,*2014). Advantages of this method are that they do not use chemicals, fast process and AgNPs produced were uniformly distributed (Kruis *et al.,*2000; Iravani *et al.,* 2014). But low yield and high energy consumption are some of the disadvantages.

2.4.2 Chemical methods

This method involves the use of organic solvents and water for the production of AgNPs. Three main components which were used in the chemical synthesis of AgNPs are metal precursors, reducing agents and stabilizing agents (Zhang *et al*., 2007; Iravani *et al*., 2014). Advantage of these methods is high yield and major disadvantage is use of chemicals like citrate, borohydride that show toxic effects (Wiley *et al.*,2005; Merga *et al*., 2007; Iravani *et al*., 2014).

2.4.3 Biological methods

To overcome the disadvantages of physical and chemical methods, we shift to biological methods. Advantages of biological methods are simple, rapid, non-toxic .plants, bacteria, fungi and algae are used for the synthesis of silver nanoparticles.Organisms such as plants, fungi and bacteria have the ability to produce metal nanoparticles. Microorganisms have recently been distinguished as potential biofactories for the synthesis of metallic nanoparticles such as gold (Armendariz *et al*., 2004; Ankamwar *et al*., 2005) and silver (Sastry *et al*., 2003; Gericke *et al*., 2006) and emerged as an eco-friendly and exhilarating method. Additionally, green methods provide researchers with good control over size distribution of nanoparticles.

2.5 Hydrogels as an immobilization matrix

Hydrogels are 3D structures of polymer chains cross linked together which are highly hydrophilic and have the ability to hold large amount of water without changing their structure. The ability of hydrogels to absorb water arises from hydrophilic functional groups present on the polymer backbone while their resistance to suspension arises due to cross linkage between network chains. The features that make hydrogel so useful include its durability & stability in the swelling atmosphere and at the time of storage, water absorption capacity, non-toxicity and biological activities.

Hydrogels' hydrophilic nature leads to its swelling in water but don't dissolve in water. Hydrogels have the ability to absorb more water than its weight is considered to have great potential for biomedical applications (Daniele *et al*., 2014; Yu *et al*., 2016; Billiet *et al*., 2012; Hoffman *et al.*, 2012; Wang *et al*., 2015).

2.5.1 Mechanism of network formation

Mixture that contains soluble branched polymer and water is known as “sol”. Solubility of these polymers decreases with increase the dimensions of structure and this infinite polymer is known as gel or network and this is formed by several finite branched polymers (Li *et al*., 2012). According to gelatin mechanism, hydrogels can be divided into two types, namely, physically and chemically cross-linked. Chemical crosslinking is common method to synthesize polymer hydrogels (Biao *et al*., 2014)

2.5.2 Synthesis methods of polymer nanocomposites

There are diverse methods for synthesis of polymer nanocomposites. Most important factors to consider in the synthesis of nanoparticles are size and shape of NPs in order to obtain predefined properties of nanomaterials. Nanomaterials that are used to impart antimicrobial properties in the polymer matrix can either be applied onto the surface or embedded into the matrix. Two methods are used for preparation of polymer/metal nanocomposites: *in situ* in which nanoparticles are generated by decomposition of metal precursor inside polymer matrix. Firstly the monomers get polymerized in solution containing metal ions chemical species, then metal nanoparticles were obtained by reduction of metal ions through chemically, thermally or photolysis methods; and *ex situ*, in which nanoparticles are synthesized before hand chemically, physically or biologically and are then incorporated into the polymer matrix (Plaza *et al*., 2015).

Under chemical methods, *in situ* reduction procedure is quite popular for generation of nanoparticles where reduction of metal ion precursors from their salt solution occurs at the polymer-solution interface. Also, this method is generally suitable for preparation of antimicrobial bio-nanocomposites in which nanoparticles are suspended throughout the matrix.

Base or the polymer matrix comprising of two or more components have been combined through a variety of production techniques such as chemical, physical and radiation cross linking, grafting polymerization (Said *et al*., 2004; Fei *et al*., 2000; Hennink *et al*., 2012; Barbucci *et al*., 2004; Liu *et al*., 2002). Chemical cross linking method is quite popular method to obtain permanent & mechanically strong composites and it involves the connection/linking of two polymer chains via a cross linking agent such as aldehydes which involves glutaraldehyde, silanes.The linking is

achieved through the functional groups such as hydroxyl (OH), carboxyl (COOH), and amino (NH_2) present in polymer. Mechanism of network formation in hydrogels occurs at first linking of macromolecular chains together present in the preliminary material leads to formation of large branched but soluble polymer which is called 'sol'. When linking process is continued it results in increase in the size, decrease in solubility of the branched polymer and thus obtained final form is known as the 'gel/network'.

2.5.3 Antibacterial activity of nanocomposite hydrogel

Dubey *et al.* (2016) synthesized chitosan beads that contain AgNPs or AgGO and 100% reduction of *Staphylococcus aureus* and *Escherichia coli* was achieved.

Ghavami *et al.* (2016) produced AgNPs/ zwitter ionic dopamine methacrylamide hydrogels having size < 20 nm and they show antibacterial activity against *Escherichia coli, Staphylococcus aureus, and Pseudomonas aerogenosa* showing reduction by 157, 148 and 129% respectively.

Dubey *et al.* (2016) synthesized GO based nanocomposite grafted CS/ PVA that showed the antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* with zone of inhibition 7 nm and 11 nm respectively.

Zeng *et al*. (2015) produced a reduced graphene oxide hydrogel containing silver nanoparticles of size range 5-20nm. Hydrogel displayed an excellent antibacterial activity against *E.coli* with reduction rate of more than 95%.

Loo *et al.* (2013) synthesized poly (sodium acrylate) cryogels with AgNPs (size <10nm) formed through inter matrix synthesis. These cryogels show high porosity, water absorption, greater mechanical strength than its pristine counter parts and significant antimicrobial activity against *Bacillus subtilis* and *Escherichia coli* with 5.4−7.0 log reduction of viable bacteria within few seconds of contact time.

Rest of the studies are listed in table 1.

Table 1: Antimicrobial activity of various silver hydrogel nanocomposites

AgNPs hydrogel composite	NP size	Antimicrobial activity	Organism tested	Potential application	MIC/Reduction rate / Inhibition zone diameter	References
CS/PVA/Gr/Ag hydrogel	_	Antibacterial	*E.coli,& S.aureus*	Antibacterial	ZOI- *E.coli* -13.5nm, *S.aureus*- 19.5nm	Nesovic *et al*.,2019
CS/RGO/Ag colloids	30nm	Antibacterial	*E.coli, S.aureus*	Antibacterial	95% reduction	An *et al.,* 2018
Chitosan beads contains AgNPs or AgGO	_	Antibacterial	*E.coli & S.aureus*	Antibacterial	100% reduction rate	Chook *et al*., 2017
Graphene based antimicrobial nanoparticle	10-40 nm	Antibacterial	*E.coli, B.subtilis*	Antibacterial	*E.coli*–4log reduction *B.subtilis*-3log reduction	Zeng *et al*., 2017
AgNPs/zwitter ionic dopamine methacrylamide hydrogels	<20nm	Antibacterial	*E.coli, S.aureus, P.aerogenosa*	Antimicrobial, Antifouling, wound dressing	157, 148 and 129% in ZOI diameters	Ghavami *et al*., 2016
GO based nanocomposite grafted CS/ PVA	140 & 133 nm	Antibacterial	*E.coli, S.aureus*	Wound dressing	Zone of inhibition 11nm for *S.aureus* , 7 nm for *E.coli*	Dubey *et al*., 2016
Silver/ RGO hydrogel	5-20 nm	Antibacterial	*E.coli, Coliforms*	Antimicrobial	97% for *E.coli*, 97% for Coliforms	Zeng *et al.*, 2015

2.6 Research in current thesis

In the present work, we used chitosan for the fabrication of AgNPs which is a biopolymer and a favorable applicant for synthesis of antibacterial hydrogels on account of its bactericidal activity (Achaby *et al.*, 2014), ability to form gel, solubility in dilute acids and low toxicity. But there is one major disadvantage of chitosan that it has poor mechanical strength, so it does not maintain its integrity in the solution. To overcome this problem, other polymers are used to crosslink with chitosan (Dubey *et al.*, 2016). One such polymer is graphene oxide (GO) which is suitable for combination with chitosan due to its properties like hydrophilicity, gel forming ability, biocompatibility and mechanical strength.

2.6.1 Preparation and characterization of CH/GO hydrogels

Nesovic *et al.* (2019) synthesized silver/poly (vinyl alcohol)/chitosan/graphene (Ag/PVA/CS/Gr) nanocomposite hydrogels by *in situ* electrochemical reduction of silver ions in hydrogel and then characterized them using field emission scanning electron microscopy (FE-SEM), X-ray photo electron spectroscopy (XPS), Raman and UV-visible spectroscopy.

Jing *et al.* (2018) synthesized chitosan and reduced graphene oxide colloids containing AgNPs and they characterized the colloids using Transmission electron microscopy (TEM), Scanning electron microscopy (SEM) and X-ray diffraction (XRD).

Yang *et al*. (2017) synthesized CH/PVA/GO composite nanofibrous membranes via electro spinning and then characterized them with FTIR, XRD, DSC and TCA.

Zheo *et al*. (2015) synthesized GO-chitosan composite hydrogel via self-assembly of chitosan and graphene oxide and these composites were characterized by XRD, TEM, SEM and TG-DSC.

2.7 Characterization methods

2.7.1 Fourier Transform Infrared (FT-IR) spectroscopy

FTIR is a diagnostic technique that is constantly developing, which allows a non-destructive, rapid, high-throughput, reagent less analysis of various ranges of samples. FTIR is adaptable technique as sample requirement is much lower. Typically few milligrams of sample can be spotted on plate for analysis and data can be collected within seconds (Trenerry *et al.*, 2012). The basic principle behind this technique is that most of the molecules absorb radiations of IR- region in electromagnetic spectrum. A molecule bond specifically corresponds to this absorption. The range of FT-IR is measured as wave number with the range of 4000-6000 cm. In this technique, IR spectrum of emission or absorption of gas, liquid, or solid is obtained, and it collects high resolution data over wide range of spectra.

2.7.2 Scanning Electron Microscopy (SEM)

SEM is type of electron microscopy that is used for the production of images of samples by scanning the surface with focused electron beams. This method works on the principle that when electron beam interact with sample at various depths they produce the image of the sample at different magnifications. To initiate the characterization, firstly nanoparticles containing samples are air dried. Then sample is mounted on sample holder with the help of conductive tape. Then by focusing a beam of electrons, a sample is scanned (Jores *et al.*, 2004).

2.7.3 Energy dispersive X-ray Spectroscopy (EDX)

EDS is an analytical technique that is used for the elemental analysis of sample. This method relies on the interaction of some source of X-ray excitation and sample. EDS works on the principle EDX detector contains crystal that absorbs energy of incoming X-rays, yields free electrons in crystal & then they become conductive and produce electrical charge bias. Then X-ray absorption convert the energy of individual X-rays into electrical voltage of proportional size. These electrical pulses correspond to X-ray of the element.

2.7.4 X-ray Diffraction (XRD)

XRD is a fast systematic tool that is commonly used to identify the phase of crystalline substance and also provides the information about cell dimensions. The sample is finely grounded, mixed well in solvent (homogenized), and bulk composition is also determined. It is basically, depends upon the interference of X-rays and the sample; the source of X-rays is cathode ray tube, which generates the monochromatic light wave that directly concentrated on the sample. When the circumstances fulfill the Bragg's law, then the constructive interference produced after the interaction between sample and incident rays. Bragg's law co-relates the parameters that are diffraction angle, wavelength of EM radiations, and the lattice spacing (Pandian *et al.*, 2014). Unique "fingerprint" of the crystalline sample was obtained after analysis with XRD, and the data from XRD can be used to depict the crystalline form after some comparisons with standard measurements and patterns.

CHAPTER-3

MATERIALS & METHODS

3.1 Chemicals and Reagents

Chitosan (purity 99%; deacetylation degree 85%; pH 5-6; MW 100,000-300,000 Da) was procured from Nano Wings Pvt. Ltd., Telangana. Silver nitrate and sodium borohydride were obtained from Sigma Aldrich Ltd. Graphene oxide (produced in Biotechnology lab-2) was procured from Biotechnology lab-2 TIFAC-CORE, TIET, Patiala. Glutaraldehyde was obtained from Loba Chemie Ltd. For various antibacterial experiments, various strains, *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis* and *Enterobacter aerogenes* were procured from NCL, Pune. For all experiments distilled or deionized water was used.

3.2 Sterilization procedure

All glasswares were first washed with detergent and then kept in oven at 40-50 °C until they dried and after that glasswares were treated with Aqua regia for 15-20 minutes to dissolve metals, ions and salts. After 15 minutes glasswares were kept in DI water and then they rinsed with DI water and oven dried. All tips were autoclaved at 121℃ for 15minutes at 15 psi.

3.3 Synthesis of graphene oxide

Graphene oxide is synthesized by modified hummers method followed by Zaaba *et al*., 2017:

Sulphuric acid (H_2SO_4) and phosphoric acid (H_3PO_4) were taken and mixed in 9:1 and then stirred for few minutes. After that 0.225 gm of graphite powder was added into the prepared solution under stirring conditions. Then 1.32 gm of potassium permanganate ($KMnO_4$) was added into the solution and the solution was stirred for 6 hrs until it becomes dark green. Then 0.675 ml of H_2O_2 was added dropwise and then again stirred for 10 min. After that 10 ml of HCl and 30ml of DI water was added and solution was centrifuged for 10 min at 5000 rpm. After centrifugation supernatant was discarded and pellet was washed with HCl and DI water thrice. At the end slurry obtained in the pellet was kept in oven at 90°C for 24 hrs.

3.4 Preparation of Chitosan /GO hydrogel

Chitosan polymer (3%) was dissolved in 2.5 % AA solution while stirring overnight at 600rpm.GO solution (0.05% in 2.5 % AA solution) was prepared by dissolving GO powder in dilute AA solution andthen sonicating for 3 hours.Then GO was mixed with chitosan solution under stirring conditions and then kept on shaker for 24 hours. For network formation glutaraldehyde (280µL) was added dropwise to Chitosan/GO solution under stirring conditions at 25° C. After mixing gel solution was poured into test tubes and then kept in oven at 45-55˚C until it solidifies. Hydrogels were then removed from test tubes by keeping in 12% NaOH solution overnight. Hydrogels then water with DI water thrice and then soaked in DI water for 24 hours.After that gels was continuously freezed and thawed (-20 ˚C for 6 hours and 25 ˚C for 2 hours) for 7 days to form pores inside the hydrogel.

3.5 *In situ* synthesis of silver nanoparticles in chitosan/GO hydrogel

Preparation of silver loaded hydrogel was performed as follows:
Previously prepared hydrogels were cut into circular discs having diameter 10 mm and desired length with the help of sterilized surgical blade and then these circular discs were introduced into 10mM silver nitrate (silver ion precursor) solution and placed in dark .Silver loaded gels were then washed with DI water thrice to remove unbound silver ions. For chemical reduction of Ag ions, these hydrogels were placed in 10mM sodium borohydride ($NaBH_4$) solution for some time until the color of hydrogel changes. Finally, the hydrogel discs were washed with DI water.

3.6 Swelling studies

In order to know the swelling ratios, chitosan/Graphene oxide (CH/GO) and Ag loaded Chitosan/Graphene oxide hydrogels (CH/GO/Ag) were cut into circular discs (diameter 10mm) and then dried in lyophilizer for 72 hrs to remove all the moisture content and then their dry weight of hydrogels were determined (W2). Water content was measured by soaking the hydrogels in DI water at room temperature for 120-180 mins. After dabbing the surface of gels with tissue paper, wet weight (W1) of hydrogels was determined. The absorbed water content was then calculated in term of the swelling ratio (S %) using Equation:

$S\% = \frac{w1-w2}{w1} * 100$…….Equation (1)

3.7 Antibacterial activity of hydrogels

Antimicrobial activity of these hydrogel nanocomposites against *Escherichia coli*, *Staphylococcus aureus, Staphylococcus epidermidis* and *Enterobacter aerogenes* was tested by two methods i.e. by colony forming method and disc diffusion assay. Procedures followed for conducting these experiments are given as follows:

3.7.1 Colony forming assay

Nutrient broth (NB), nutrient agar (NA) media were prepared and autoclaved at 121°C for 15 minutes at 15 psi.NA media was then poured in petri plates and then allowed to solidify for 30 minutes. Then 50ml autoclaved NB was taken and inoculated with microbial culture and kept in rotary shaker at 37°C at 120 rpm for 14-16 hrs.During log phase, microbial culture was centrifuged. After centrifugation supernatant was decanted out andpellet was washed with phosphate buffer saline (PBS) thrice. Finally the pellet was dissolved in PBS. After that final concentration of 1×10^9 CFU/ml was prepared to unit absorbanceat 600 nm using UV spectrophotometer and then dilutions were made up to 10^3 CFU/ml in 0.85% saline by serial dilution. Flasks containing microbial solution with 2 hydrogels (CH/GO and CH/GO/Ag) discs having diameter 10 mm were incubated at 37°C at 120rpm in rotary incubator shaker. 100µl culture was withdrawn from flasks at definite intervals of time (0, 15, 30, 60, 90, 120, 240 minutes) for plating on nutrient agar plates. Colonies of microorganisms were counted after 24 hrs of incubation period.

3.7.2 Disc diffusion assay

First four steps were repeated from previous assay. Final working concentration of 10^6 CFU/ml was achieved with serial dilutions in 0.85% saline.Then 100 µl microbial solution was spread on nutrient agar plates and then one hydrogel disc (CH/GO/Ag) was placed in center of plate and one with control hydrogel (CH/GO) in separate plate. Both discs were 10mm in diameter and test was carried out in duplicates and those plates were incubated at mentioned conditions and zone of inhibition was measured in each case after 24 hrs of incubation period.

3.8 Characterization of hydrogel nanocomposite

3.8.1 Fourier Transform Infra-Red spectroscopy (FTIR)

FTIR analysis of both CH/GO and CH/GO/Ag hydrogels was carried out using Nicklet 380 Thermo, US Fourier Transform Infrared Spectrometer. Sample preparation was done by drying the hydrogels in lyophilizer for 3 days and grinding them in pestle & mortar to obtain coarse powder. These samples were read between 500 and 4000cm^{-1} and peaks were obtained for individual test materials.

3.8.2 Scanning Electron Microscopy (SEM)

The surface morphology of pristine and Ag loaded hydrogels was determined by SEM at SAI labs, TIET, Patiala. The hydrogel sample was broken into small thin pieces and completely dried under vacuum to remove moisture from them. Coating was done onto hydrogels supported on end with the help of double sided carbon tape to make them conductive for analysis & image generation. SEM machine was operated at high vacuum & finally surface analysis was done and images were generated at different resolutions.

3.8.3 Energy-dispersive X-ray spectroscopy (EDS/EDX)

EDS is method used for used for elemental breakdown and chemical characterization. EDS was performed along with SEM therefore, no separate preparation of sample was done for this. Results were displayed in terms of peaks for corresponding elements present in the sample.

CHAPTER-4

RESULTS & DISCUSSION

4.1 Formation of CH/GO Hydrogel

Hydrogel was formed according to the protocol given in 'Materials and Methods' (Section 3.4) with subsequent freeze thaw treatment porous network structure was developed in the hydrogels and color of hydrogel was changed (Fig. 2).

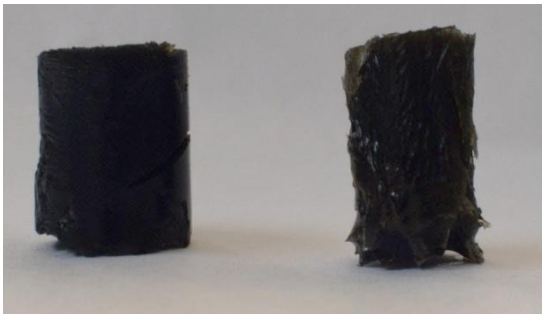

Figure 2: Synthesized CH/GO hydrogels before freeze thaw (left) and after freeze thaw (right) respectively.

4.2 *In situ* AgNPs synthesis in hydrogel

When silver loaded hydrogels (previously soaked in silver nitrate solution), came in contact with sodium borohydride solution (strong reducing agent), the color of hydrogel was changed within 50s from greenish brown to black over time. For the complete reduction of silver ions hydrogels were incubated for 30 min. From color change it become clear that AgNPs were synthesized *in situ* within the porous network of hydrogel.

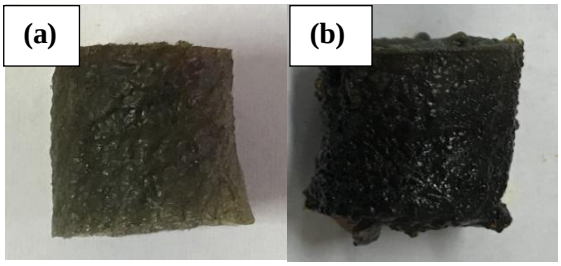

Figure 3: Synthesized silver loaded CH/GO hydrogels (a) Before reduction (b) After reduction.

Synthesis of AgNPs inside the hydrogel is provided by the fact that hydrogels provide site for the adsorption of silver ions from silver nitrate solution by giving its functional groups like hydroxyl (-OH), amino ($-NH_2$), carbonyl (-C=O). Ag^+ interacts with functional moieties and gets trapped in the porous area of hydrogel. After that reduction with $NaBH_4$ leads to the synthesis of AgNPs that were uniformly distributed on the surface as well inside the hydrogel (Agnihotri *et al.*, 2012)

4.3 Swelling studies

Swelling capacity of both CH/GO and CH/GO/Ag hydrogels was determined. Swelling ratios are provided in Table 2, calculated by equation-1(Section 3.6)

Table 2: Swelling ratios of CH/GO and CH/GO/Ag hydrogels.

Hydrogel	Swelling ratio (%)
CH/GO/Ag	76.86
CH/GO	80.92

The results for swelling studies were concluded in Figure 4. It is clear from the figure that swelling ratio was more for pristine hydrogels than CH/GO/Ag hydrogels. Swelling ratio was 80.82% for pristine and 76.33% for silver loaded hydrogel respectively. Swelling ratio increases for approximately 80min, after that no change in the swelling ratio of hydrogel observed. Ag loaded hydrogels show low swelling ratio because AgNPs form network inside the gel. It also form cross linkage with O & N atoms of amine and carboxyl group present in the backbone of CH and GO.

4.4 Characterization studies

4.4.1 UV Visible spectroscopy of GO

UV visible spectra of aqueous GO is shown in figure 5. Two kinds of characteristic features were observed in the spectra. First characteristic feature was observed at 232nm and this peak was corresponding to the occurrence π-π^* transition of aromatic C-C bond. Second was the shoulder peak which was attributable to 304 nm and this peak corresponds to n-π^* transition of atomic C-C bond (Lai *et al.*, 2012; Shahriari *et al.*, 2014).

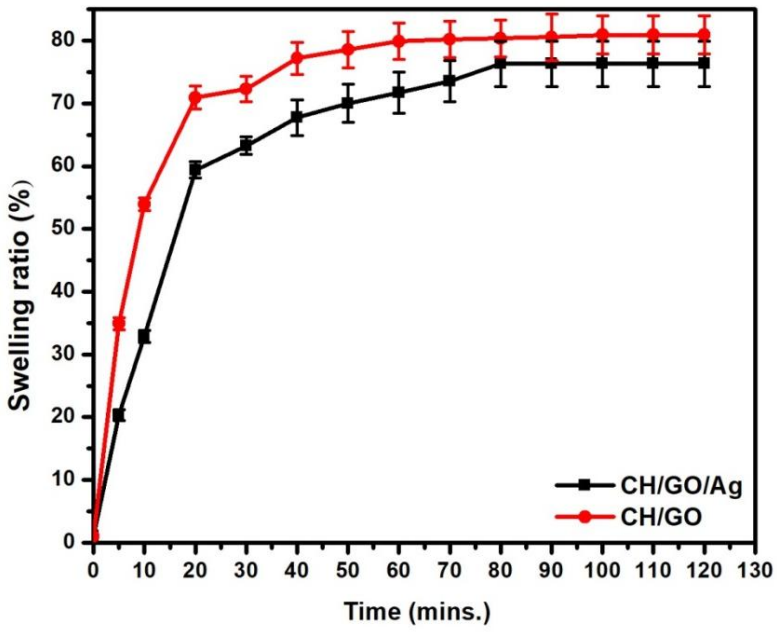

Figure 4: Swelling kinetics of CH/GO and CH/GO/Ag hydrogels at room temperature

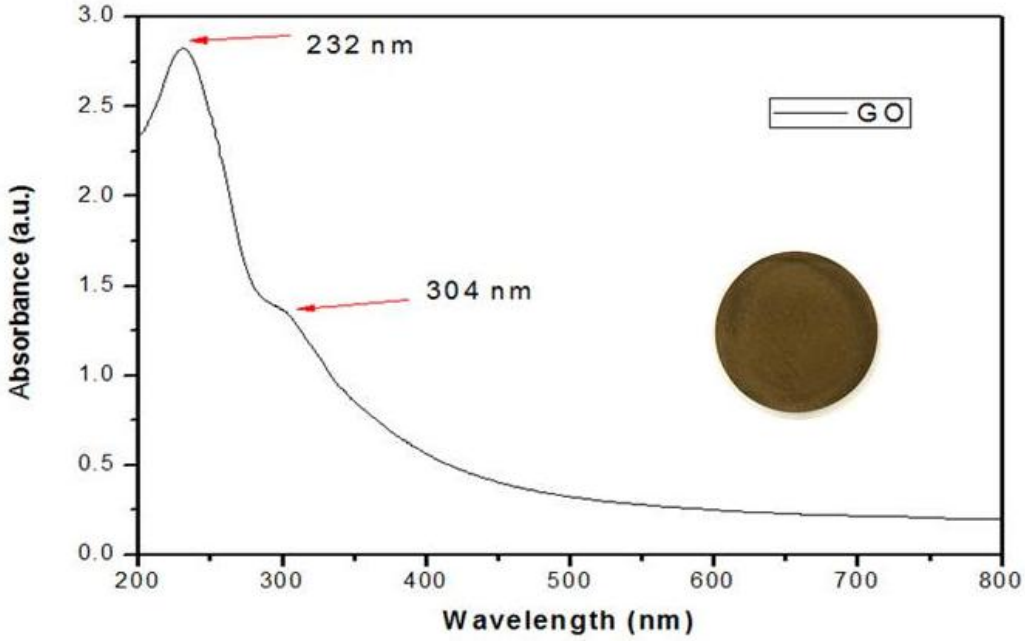

Figure 5: UV visible spectroscopy of graphene oxide.

4.4.2 Fourier Transform Infrared (FT-IR) spectroscopy

The FTIR spectrum of CH/GO and CH/GO/Ag hydrogels is given in figure 6. The absorption peaks obtained are corresponding to the chemical structure of bonds. In CH/GO spectra, peak observed at 3431cm^{-1} which is corresponding to O-H/N-H stretching because of hydrogen bonding. But in case of CH/GO/Ag hydrogels, this absorption peak was moved to 3430 cm^{-1}which reveals that AgNPs might forms the bond with CH/GO during its synthesis process (Zhang *et al.*, 2010). However the peaks observed at 1632 cm^{-1} and 1618 cm^{-1} are corresponding to C-H stretching in CH/GO and CH/GO/Ag respectively. The absorption peak at 1568 cm^{-1}in CH/GO hydrogel indicates the (–C=O) carbonyl bond stretching. Also the appearance of these functional groups in pristine hydrogel subsequent to cross linking by glutaraldehyde which indicates the presence of hydroxyl (-OH) groups in GO (Achaby *et al.*, 2014). These functional groups act as sites for the attachment of Ag+ ions and helps in their immobilization within the gel. Another peaks observed at 1018 cm^{-1}in CH/GO and at 1017 cm^{-1}in CH/GO/Ag is directly corresponds to C-H and C-N stretching respectively.

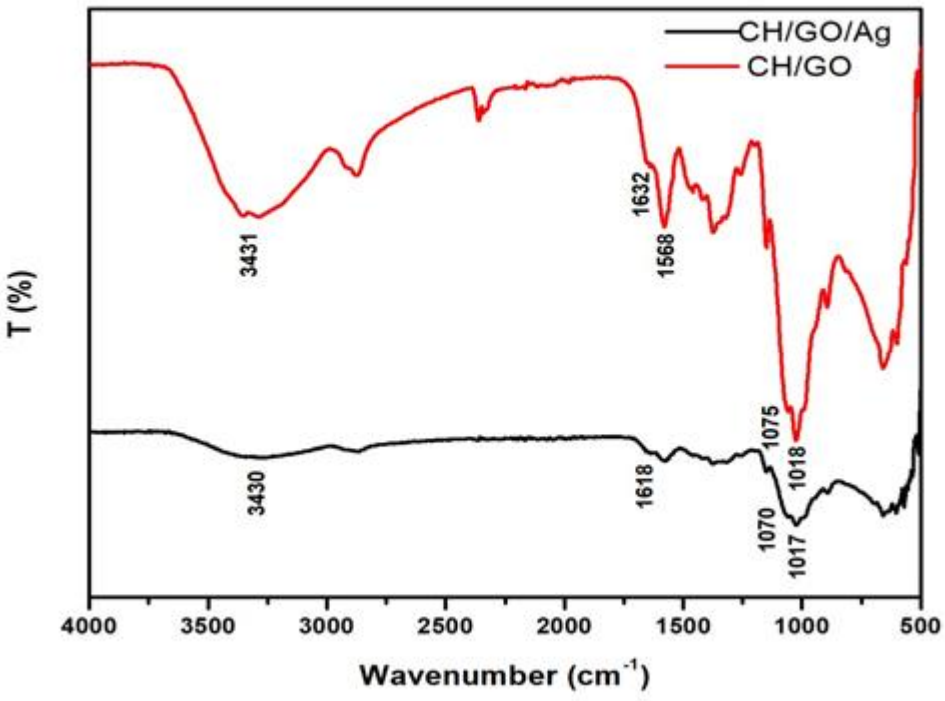

Figure 6: FTIR spectra of CH/GO and CH/GO/Ag hydrogels

4.4.3 Scanning Electron microscopy (SEM) analysis

SEM analysis of pristine and AgNPs loaded hydrogels are compiled in figure 7.

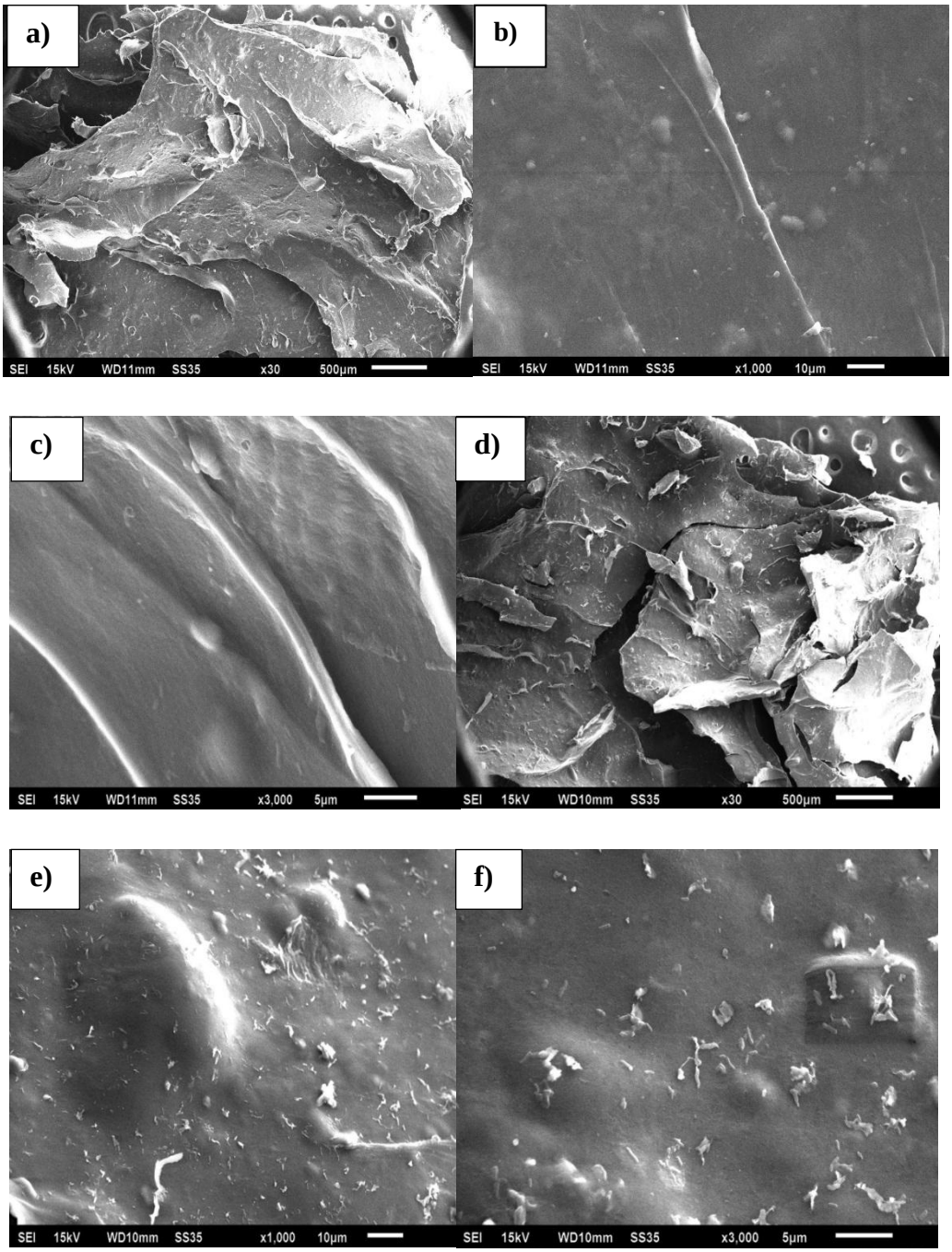

Figure 7: SEM images of CH/GO at 30x, 1000x and 3000x (a-c) respectively and CH/GO/Ag hydrogels at 30x, 1000x, 3000x (d-f) respectively.

SEM images of pristine CH/GO hydrogel show rough surface morphology with porous texture (Nath *et al.*, 2018) while CH/GO/Ag showed the presence of silver

nanoparticles. However, synthesized AgNPs were in aggregated form but they were uniformly distributedon the surface of CH/GO/Ag hydrogel and were embedded in polymer matrix of CH/GO. There was no change in the morphology of CH/GO hydrogel after immobilization of AgNPs within the polymeric matrices.

4.4.4 Energy dispersive X-ray spectroscopy (EDX) analysis

Table 3 illustrates different elements present in CH/GO and CH/GO/Ag hydrogels on the basis of weight and atomic percentage. CH/GO/Ag hydrogels showed silver content of 9.31% according to weight percentage.

Table 3: Elemental composition of hydrogels

Element	Pure CH/GO hydrogel		CH/GO/Ag hydrogel	
	Weight %	Atomic %	Weight %	Atomic %
C K	48.66	56.63	52.92	64.64
O K	45.77	39.99	35.83	32.85
Na K	5.57	3.39	1.94	1.24
Ag L	0.00	0.00	9.31	1.27

Fig 7 shows the EDX plot of SEM image taken of both the hydrogels. It confirms the absence of any silver or silver nanoparticles in CH/GO hydrogel. The plot also shows the existence of sodium (Na) and oxygen (O) elements which were higher in weight percentage than in CH/GO/Ag hydrogel because the lattice space in occupied by AgNPs. But carbon content is more in weight in CH/GO/Ag hydrogel than pristine hydrogel according to weight percentage.The EDX evaluation also proved that silver (AgNPs) was present in the test sample (CH/GO/Ag) of hydrogel. The EDX plot of silver loaded hydrogel also shows the presence of carbon (C), sodium (Na) and oxygen (O) elements just like in CH/GO hydrogel.

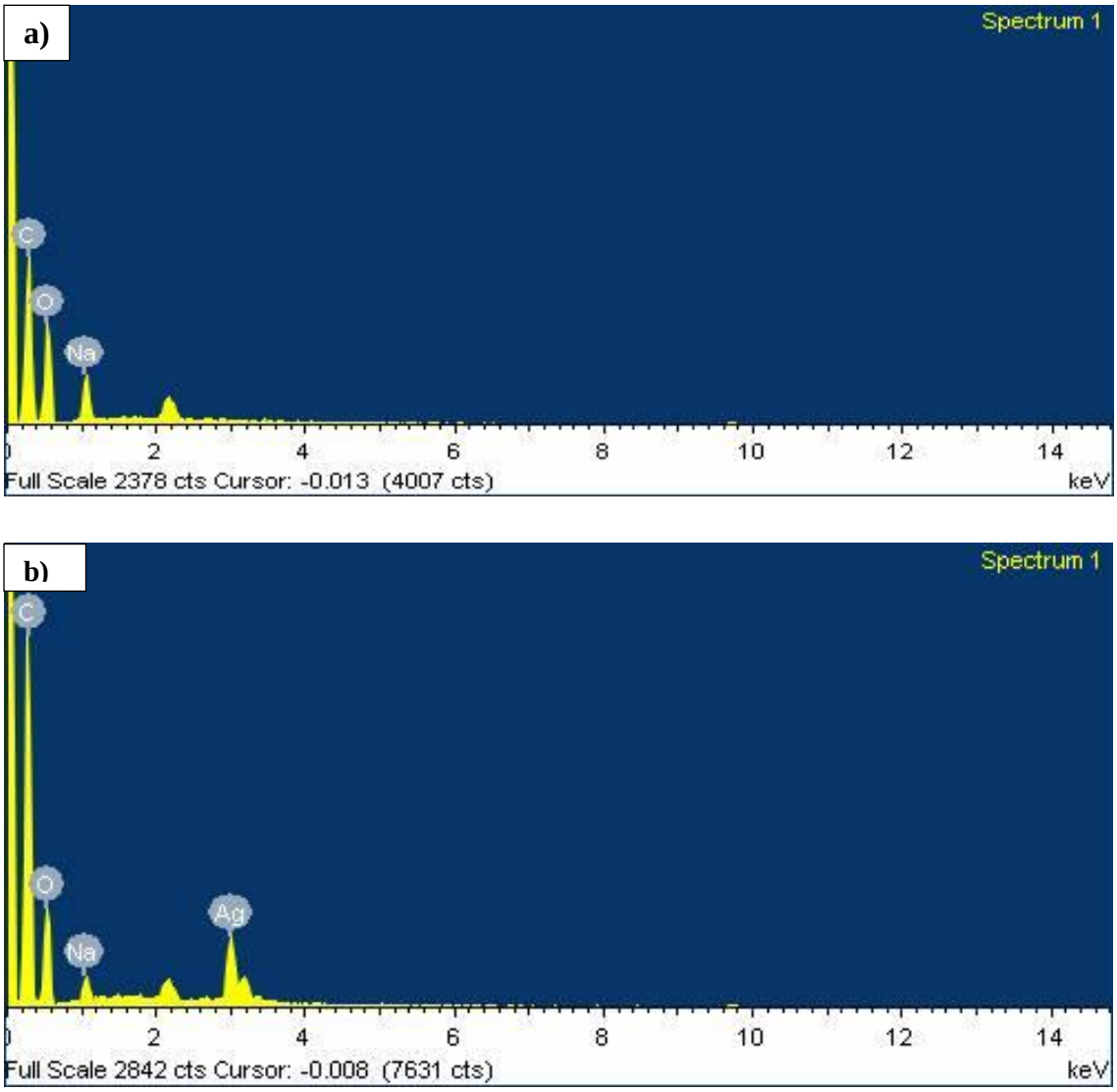

Figure 8: EDX plot of a) CH/GO and b) CH/GO/Ag hydrogel

4.4.5 X-ray diffraction (XRD)

The XRD pattern of graphene oxide shows sharp peak at 2θ=10.5˚, but the XRD pattern of CH/GO nanocomposite was different because of the synchronization of GO with chitosan sharp diffraction peak which first appears 2θ = 10.5˚ now shifted to 2θ=16.1˚ because of strong interaction between chitosan and GO (Ashiri *et al.*, 2018). In case of CH/GO/Ag hydrogel Ag does not cause any change in the crystallinity so all the peaks remain same and peak of Ag was observed at 2θ= 38.7˚.

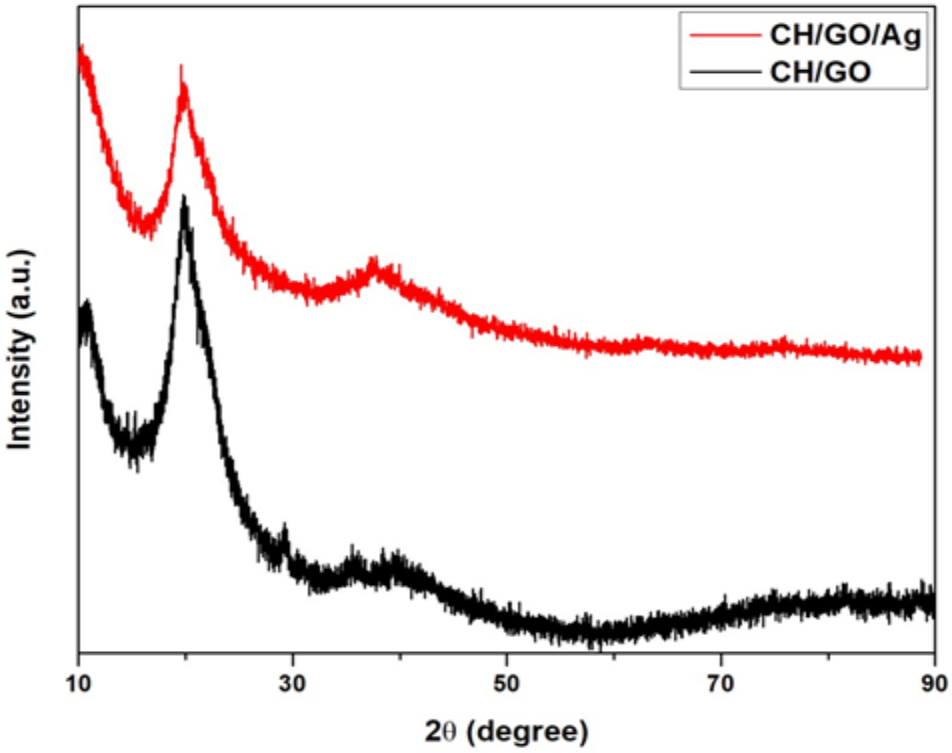

Figure 9: XRD plot of CH/GO and CH/GO/Ag hydrogels

4.5 Antibacterial assay

The antibacterial effect of pristine (CH/GO) and AgNPs loaded (CH/GO/Ag) hydrogels was verified against *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis* and *Enterobacter aerogenes* with the help of colony forming assay in which reduction in number of colonies and growth inhibition kinetics of bacterial cells was studied and in disc diffusion test, zone of inhibition was studied.

4.5.1 Colony forming assay

Figure 8 and Figure 9 shows the number of microbial colonies grown on nutrient agar plates after appropriate incubation period which is required to disinfect the microorganisms.

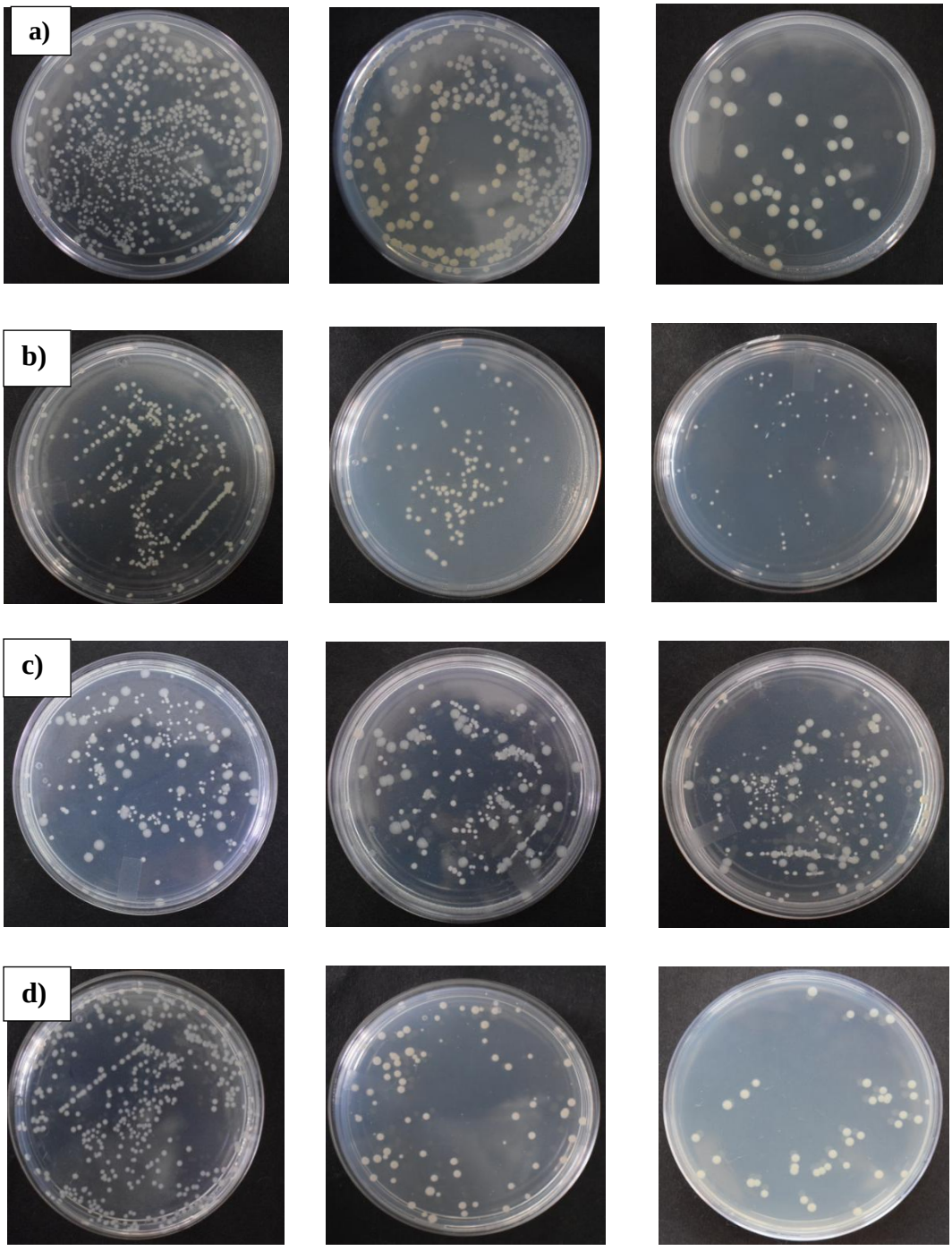

Figure 10: CH/GO treated cultures of (a) *E. coli* (b) *S. aureus* (c) *S. epidermidis* (d) *E. aerogenes* plated at different time intervals 0, 30 and 120min (left to right).

The number of colonies significantly reduced in both the hydrogels (pristine as well as AgNPs loaded) samples with increase in incubation time from 15 minutes to 2 hrs. After 2 hrs complete eradication of colonies was observed in case of CH/GO/Ag hydrogels.

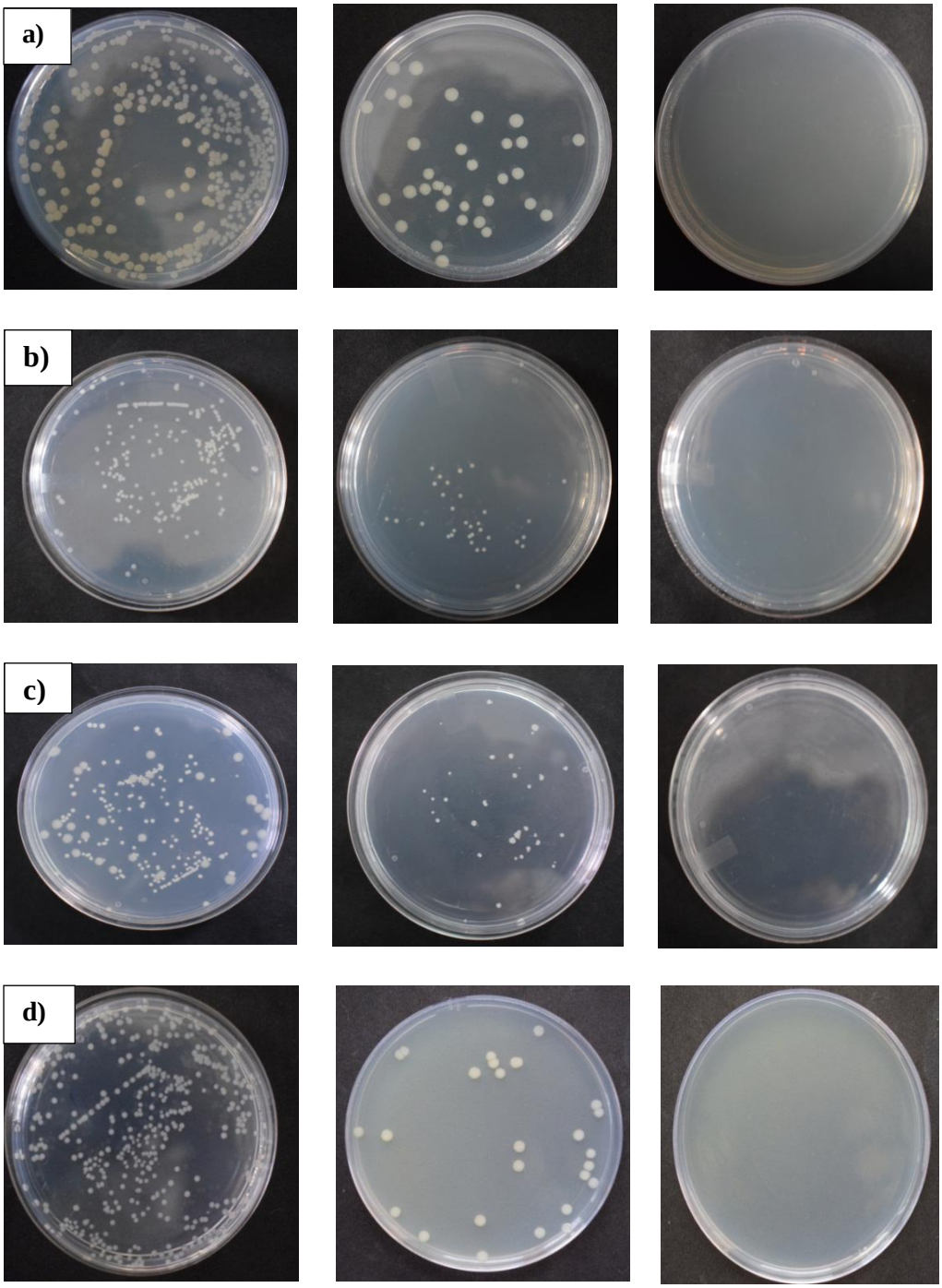

Figure 11: CH/GO/Ag treated cultures of (a) *E. coli* (b) *S. aureus* (c) *S. epidermidis* (d) *E.aerogenes* plated at different time intervals 0, 30 and 120min (left to right).

4.5.2 Growth inhibition kinetics

CH/GO and CH/GO/Ag hydrogels were introduced into microbial solution (in separate flasks) to check the antibacterial activity of the synthesized hydrogels. Then graph was plotted between $\ln N/N_o$ and time to illustrate the decrease in growth of

bacteria. Fig 11 shows the antibacterial activity of CH/GO and CH/GO/Ag hydrogels introduced in 100 ml of the microbial culture having initial working concentration of 10^3CFU/ml.It is clear from the graphs that CH/GO/Ag show superior antibacterial activity than pristine hydrogel. With CH/GO/Ag hydrogel complete disinfection was obtained within the time period of 120 min.

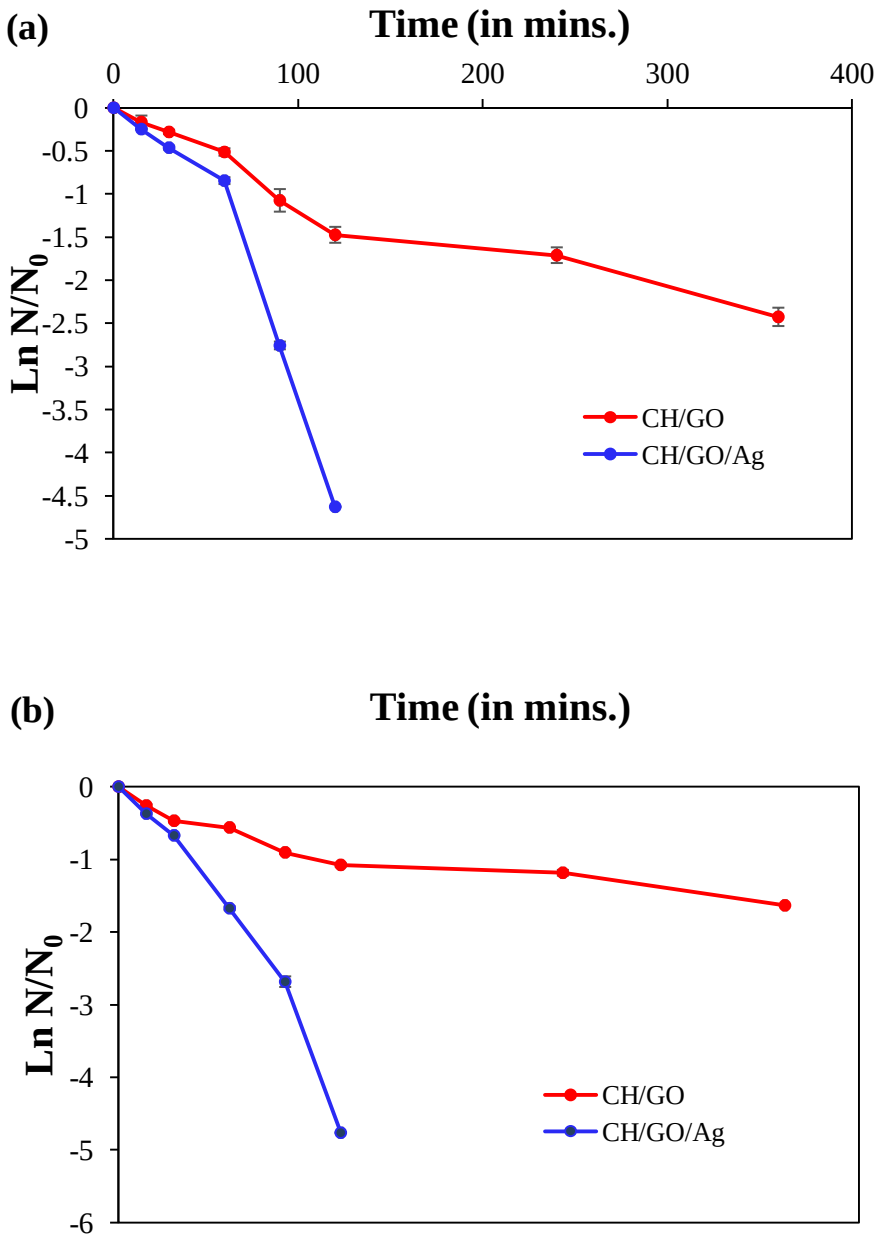

Figure 12: The disinfection potential CH/GO/Ag hydrogels against (a) *Escherichia coli* and (b) *Staphylococcus aureus* microbial strains

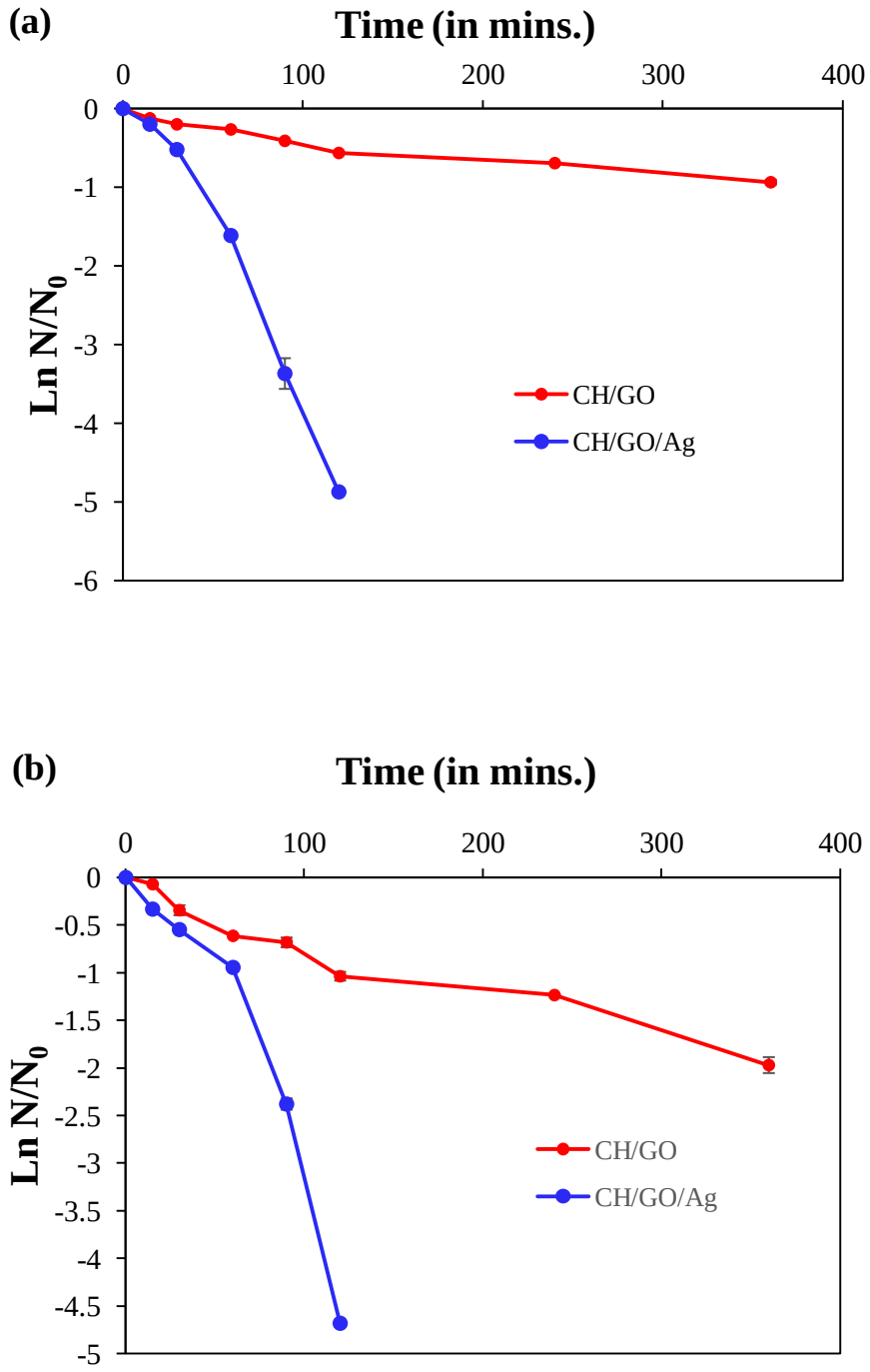

Figure 13: The disinfection potential CH/GO/Ag hydrogels against (a) *Staphylococcus epidermidis* and (b) *Enterobacter aerogenes* microbial strains

4.5.3 Disc diffusion test

The effect of AgNPs loaded gel discs on microorganisms at an initial bacterial concentration of 10^6CFU/ml are concluded below in figure 11.

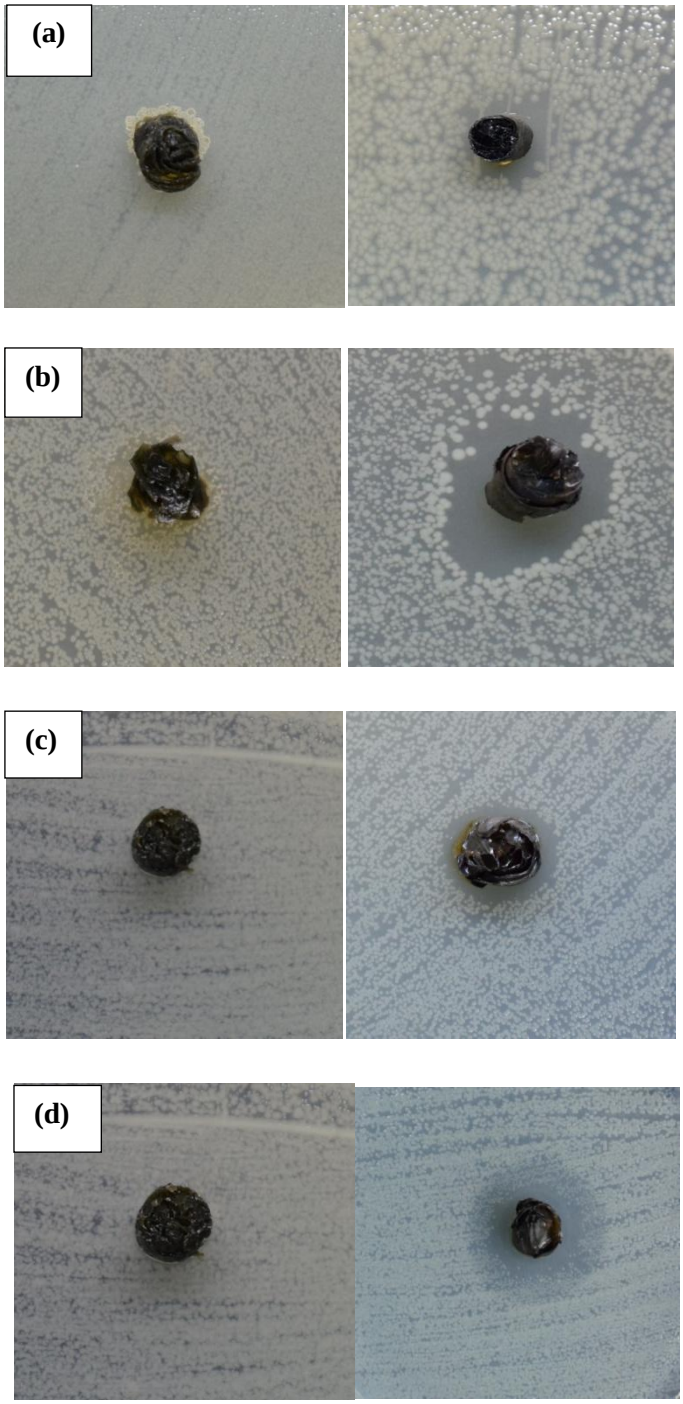

Figure 14: Antibacterial activity of CH/GO (left column) and CH/GO/Ag discs (right column) against (a) *Escherichia coli* (b) *Staphylococcus aureus* (c) *Staphylococcus epidermidis* (d) *Enterobacter aerogenes*

Table 4: Zone of inhibition measured with different bacteria

Test Organism	ZOI (mm)	
	CH/GO	**CH/GO/Ag**
E. coli	NIL	17
S.aureus	NIL	18
S. Epidermidis	NIL	15
E.aerogenes	NIL	13

It is clear from the table 6 that CH/GO hydrogels do not show any antimicrobial activity. On the other hand CH/GO/Ag displayed good antibacterial activity against all four test microorganisms that are *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis* and *Enterobacter aerogenes* with ZOI ranging from 13 to 18 mm. From all the antibacterial assays it was concluded that CH/GO/Ag has greater capability to inhibit the growth of microorganisms which is due to the synergistic effect of AgNPs in the polymer matrix.

CONCLUSIONS

- Chitosan/GO hydrogels were successfully synthesized and pores were created by freeze thaw cycles. After the synthesis of hydrogel silver nanoparticles were synthesized in situ in the porous matrix of hydrogel.

- CH/GO/Ag hydrogel nanocomposites were characterized in detail by Field emission scanning electron microscopy (FT-IR), Scanning electron microscopy (SEM), Energy-dispersive X-ray spectroscopy (EDS/EDX) and X-ray diffraction (XRD).

- Prepared hydrogels showed good properties like swelling capacity. Swelling percentage was 76.8% and 80.9% forCH/GO and CH/GO/Ag respectively.

- Antibacterial activity of pristine as well as Ag loaded hydrogel was tested against four clinically relevant strains like *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis* and *Enterobacter aerogenes.* The test was carried out by both disc diffusion and colony forming assay. For further study, both types of composites were observed to have antibacterial activity butAg loaded hydrogels show superior activity than pristine hydrogels.

- Growth inhibition kinetics was also studied for all the four bacterial strains. With the increase of incubation period CH/GO/Ag hydrogel gradually eradicated the microorganisms from solution.

- By diffusion method maximum ZOI was obtained against *S.aureus* i.e., 18mm and by colony forming assay complete disinfection was obtained within the time period of 120 min.

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