Hydrogel based Silver Nanocomposites as Antimicrobials

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CERTIFICATE

This is to certify that the work which is being presented in the thesis entitled **"Hydrogel based silver nanocomposites as antimicrobials"**, in partial fulfillment of the requirements for the award of degree of Master of Technology in *Biotechnology* submitted in Biotechnology Department of Thapar University, Patiala, is an authentic record of candidate's (Ms. Navneet Kaur) own work carried out under the supervision of *Dr. Shekhar Agnihotri* and refers other researcher's work which are duly listed in the reference section.

The matter embodied in this thesis has not been submitted in part to any other university or institute for the award of any degree in India.

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This is to certify that the above statement made by the candidate is correct and true to the best of my knowledge.

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DECLARATION

I hereby declare that the dissertation work entitled **"Hydrogel based silver nanocomposites as antimicrobials"** is an authentic record of my own project work carried out at Biotechnology Department, Thapar University, Patiala under the guidance of ***Dr. Shekhar Agnihotri.*** The matter presented in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

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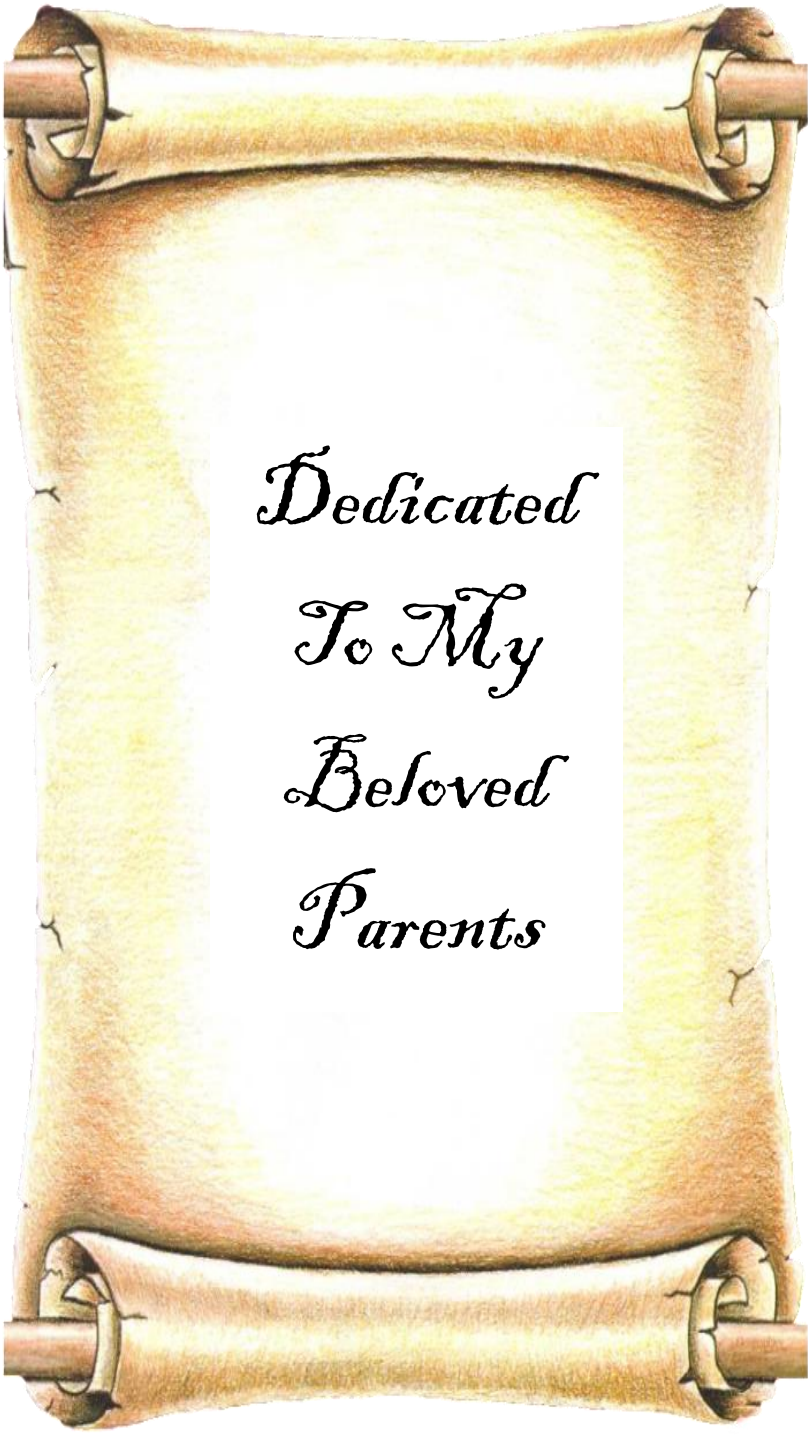
LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
&	And
°C	degree centigrade
µ	Micro
µl	microlitres
Ag	Silver
$AgNO_3$	Silver nitrate
AgNPs	Silver Nanoparticles
CFU	Colony forming units
CNTs	Carbon nanotubes
Cu	Copper
Da	Daltons
g	grams
hrs	hours
mg	milligrams
MIC	Minimum inhibitory concentration
ml	millilitres
mm	millimetre
mM	millimolar
MW	molecular weight
nm	nanometre
NPs	Nanoparticles
PVA	Poly (vinyl alcohol)
ROS	Reactive oxygen species
rpm	Revolutions per minute
TiO_2	Titanium dioxide
w/v	weight by volume
wt	weight
ZnO	Zinc Oxide
ZOI	Zone of inhibition
γ	gamma
µg	microgram


Dedicated
To My
Beloved
Parents

ABSTRACT

New generation nanomaterials provide an alternative approach for water disinfection systems. The development of such materials has an obligation to deal with some challenges to emerge out as the most efficient antimicrobial agents. Successful water disinfection systems should include the capability of destroying microorganisms in water without forming toxic byproducts and harming the environment. Currently few materials possess these characteristics but are practically limited by factors such as, mechanical instability, aggregation, leaching and toxicity etc. The present study describes the development of nano-silver based hydrogel nanocomposite consisting of porous networks of natural biopolymer chitosan and poly vinyl alcohol with well dispersed silver nanoparticles (CHT/PVA/Ag) synthesized by *in situ* chemical reduction. Field emission scanning electron microscopy (FE-SEM), Energy-dispersive X-ray spectroscopy (EDS/EDX), and Fourier Transform Infra-Red spectroscopy (FTIR) were employed to characterize the CHT/PVA/Ag hydrogel. The 3D structure of the hydrogel provided space for generation of AgNPs and acted as template matrix for their subsequent immobilization. Hydrogels exhibited excellent swelling capacities with improved structural rigidity. Disinfection experiments performed against three test microorganisms *i.e. E.coli, Bacillus licheniformis,* and *Lysinibacillus fusiformis* showed that the CHT/PVA/Ag hydrogels exhibited good antibacterial efficacy while the maximum time to achieve complete disinfection time was calculated to be 90 minutes. The Zone of inhibition (ZOI) studies indicated *Lysinibacillus fusiformis* as the most sensitive strain with clear zone diameter of 18mm. It is assumed the leaching level of Ag or AgNPs is considerably lower than drinking water standard due to its retention by hydrogel matrix. Therefore, this study sheds new light on developing nanomaterials for its application in water disinfection purposes.

CHAPTER 1

INTRODUCTION

In this modern world, health concerns are rising among growing population due to contamination of environment around them. There is dire need to make surroundings sustainable because it poses a direct threat in furtherance of public health. Pathogenic microorganisms that dwell in these contaminated areas are source of many infectious diseases and mortality among human beings. These diseases spread through food, air, and water is the most prominent of all transmission methods. Since, no other resource is as necessary for life as water its safety is inextricably linked to global health and worldwide 1.8 million children die every year from diarrhea mainly due to water contamination (WHO/UNICEF 2010). In India alone, 80% of the diseases such as gastrointestinal infections arise due to bacterial contamination of water. There is an imperative necessity to provide fundamental, reasonable water treatment in developing countries, where water and wastewater infrastructure are often nonexistent. Contemporary solutions (chemical and physical) to this problem include the use of chlorine and its derivatives, UV treatment, sediment filters, ozonization, activated carbon and water sediment filters (Droste *et al*., 1997). However some of the chemical/physical agents result in formation of disinfection by products (DBPs) in treated water which can prove to be harmful in one way or another (Boorman *et al*., 1999). For novel techniques research is being conducted to imply superior nanotechnology in water disinfection for safe drinking.

Nanotechnology to the rescue: Nanotechnology offers leapfrogging opportunities to develop next-generation treatment processes to eradicate microorganisms from water supply system. Nanomaterials with one external or internal dimension that measures 100 nm or less can be metals, ceramics, polymers, or composite materials which may be in the form of particles, tubes, rods or fibers demonstrating unique properties compared to conventional materials due to their novel features at nanoscale (Liu *et al*., 2007).The extraordinary properties of nanomaterials, such as high surface area (for adsorption), photosensitivity, catalytic and antimicrobial activity (for disinfection and biofouling control) provide useful features for many applications in various industries including waste water treatment. To provide effective disinfection without forming harmful disinfection byproducts (DBPs) various inorganic or organic

nanoparticles having strong antimicrobial activity are used such as nano-Ag, nano-ZnO, nano-TiO_2, graphene/graphene oxide and carbon nanotubes (CNTs) (Li *et al*., 2008; Klaine *et al*., 2008). These nanomaterials destruct microorganisms by releasing noxious metal ions undermining cell membrane integrity upon direct contact or generating reactive oxygen species (ROS) (Lee *et al*., 2011). Since silver and its compounds have been known for its antimicrobial activity dating back to ancient times when the Romans and Greeks used silver coins and vessels to make drinking water potable (Alexander, 2009). This historical use of silver as antimicrobial agent led to progression with development of silver nanoparticles owing to advent of nanotechnology. AgNPs due to their high surface areas have enhanced antimicrobial activity (Pettica *et al*., 2008; Rai *et al*., 2009).Therefore, Nano-Ag is a common choice for use in water treatment devices because of its strong wide-spectrum antimicrobial activity and low toxicity to humans. Silver nanoparticles are incorporated in apparels, footwear, paints, wound dressings, appliances, and cosmetics on account of their antibacterial properties and are also used in biosensors and various assays where the AgNPs embedded materials can be used as genetic tags for quantitative detection. The unique properties of silver nanoparticles make them ideal for abundant technologies, including biomedical, material sciences, optical as well as in nanotoxicological studies.

However, there are certain resistances to get utmost use out of these nanoparticles as antimicrobial agents. In the nonattendance of any substrate/support material, colloidal AgNPs tend to form aggregates in the aqueous phase, which gradually diminishes their efficacy in long term use (Li *et al*., 2012; De Gusseme *et al*., 2011; Gupta *et al*., 1998; Morones *et al*., 2005). Also, loosely held AgNPs cannot be reused keeping in mind the cost associated with it the system would become expensive and leaching of nanoparticles into the aquatic ecosystem can lead to adverse effects on life. 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.

These nanocomposites show at least one of the phase's dimensions in the nanometer range (1 nm = 10^{-9} m) (Roy *et al*., 1986) and can be classified according to matrix materials in 3 categories: ceramic, metal and polymer matrix nanocomposites (Camargo *et al*., 2009) each with distinct properties. However, polymer/metal

nanocomposites are gaining importance as a new generation broad spectrum antimicrobial material due to their ease of production, enhanced activity of inorganic fillers (natural or synthetic) present inside the network and also because of the biocompatibility, biodegradability, intrinsic antimicrobial activity of the polymer matrix. Natural/ bioinspired or synthetic polymers, dendrimers, latex particles, microgels, and hydrogels (Biffis *et al*., 2003; Dutta *et al*., 2004; Narayanan *et al*., 2004; Chen *et al*., 1998; Mohan *et al*., 2006) are preferred materials for immobilization of AgNPs for above mentioned reasons and have been studied broadly.

In compliance with recent researches, ideal candidates for matrix functionalization of AgNPs can be macroscopic gels like hydrogels. These hybrid 3D swollen composite materials can be used as "nanoreactor" for *in situ* synthesis of better stabilized and dispersed small size nanoparticles due to the presence of various functional groups in polymer matrix of gel. Advantages of using hydrogel as a template are that we can manage the size and shape of the nanoparticles by changing the quantity of monomer, varying cross linking density and/or modifying the functional groups of hydrogel polymer (Zhao *et al*., 2005; Lu *et al*., 2007; Aggor *et al*., 2010; Murthy *et al*., 2008) also, the swollen networks provide liberated space for nucleation and development of nanoparticles (Mohan *et al*., 2006). Previous works demonstrated the use of all synthetic polymers like poly (acrylamide), poly (N-isopropylacrylamide), 2-hydroxyethyl methacrylate (HEMA) (Henriquez *et al*., 2014) for immobilization of AgNPs. But in the past few years there has been growing interest in use of hydrogels synthesized from polysaccharides/ biopolymers like chitosan, cellulose, dextran and starch. Employing chitosan obtained from deacetylation of chitin (waste material of fisheries) 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). A study conducted by Jiao *et al*. (2015) demonstrated the potential of reduced graphene oxide/chitosan/AgNPs hydrogel for effective waste water treatment and dye removal. Rinehart *et al*. (2016), Agnihotri *et al*. (2012) demonstrated the antimicrobial activity of chitosan/PVA hydrogels decorated with AgNPs against *E.coli* and *S. aureus* with promising claim for microbial disinfection in water.

In the present study, we designed an immobilizing matrix of chitosan and PVA (Polyvinyl Alcohol) polymers for *in situ* synthesization of AgNPs in the hollow space of prepared hydrogel using chemical reduction. AgNPs containing chitosan/PVA and pristine hydrogels are tested for its swelling capacity; antimicrobial activity against some common water pathogens to demonstrate its potential for water disinfection and characterized by Fourier transform infrared spectroscopy (FTIR). AgNPs formed inside the network are characterized by Field emission Scanning electron microscopy (FESEM) and Energy-dispersive X-ray spectroscopy (EDS/EDX).

Objective of the Work

From the above introduction, we came to know about the wide applications of silver nanoparticles in present scenario and dire need to control the activity of these small particles to use and reuse them to the fullest. Basis of this study was provided by work done by Agnihotri *et al*., 2012.

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

- Synthesis and characterization of hydrogel.
- Hydrogel as a nanoreactor and an immobilizing matrix for silver nanoparticles.
- Evaluating antibacterial activity of these nanoparticles against various micro organisms.

CHAPTER-2

REVIEW OF LITERATURE

2.1 Nanotechnology Facets

In accordance with National science Foundation and NNI, Nanotechnology can be described as the discipline, art, and maneuvering inculcated in the regulation and deliberate molding of material at the atomic or molecular scale (Roco, 2007) to design new systems, devices and materials with new-fangled properties and utilities. Nanomaterials typically range between 1 -100 nm where, 1nm is equivalent to 10^{-9} m and this extremely small size give rise to their unique characteristics (Emerich *et al*., 2003; Sahoo *et al*., 2003). Nanotechnology holds the promise of providing momentous improvements in technologies for shielding the environment.

History and Development: Long before the start of this "nanoera" people in ancient times used nanosized objects and materials in daily life with the evidence of people using fabrics of cotton, silk, wool in BC with nanoporous structure (pore size 1-20 nm) which would quickly swell, dry and absorb sweat well. Another possible indication came from discovery of Roman Lycurgus cup (depicting mythical King Lycurgus), prepared of dichroic glass that holds different optical properties; shows green or red color depending upon direction of illumination. Development in techniques for visuals after 1959 showed contents of glass cup by electron microscope, detection silver and gold particles within size range 50-100 nm were held responsible for color change of cup (Lycurgus effect).

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 nanotechnological paradigm since, it suggested the creation of materials via direct manipulation of atoms. Ideas of nanotechnological strategy were put to action by Eric Drexler in 1986. However, the big word "nanotechnology" was used originally in scientific world by Norio Taniguchi in 1974 at a conference for describing processing of matter at nanometer accuracy and design of nanosized mechanisms. In 1980's two major breakthroughs provided a boost in nanotechnology and nanoscience: First, in 1981 the birth of the scanning tunneling microscope provided visuals of atomic and molecular particles followed by technique for their manipulation in 1989. Second, the discovery

of Fullerenes (C_{60} or Bucky Ball) in 1985 and subsequent structural assignment of carbon nanotubes in 1991 encouraged their use in electronics. USA initiated the first nanotechnological plan of National Scientific Fund in 1991 and permitted the National Nanotechnological Initiative (NNI) in 2001 (Tolochko, 2009). Since this point researches have been carried out in parts of the world especially in Japan, Germany, England, France, China and South Korea for nanomaterials synthesis and characterization. Nowadays with the progress in technology, a characterization method (non optical) for concurrent measurement of size, surface charge and concentration for a broad diversity of nanomaterials has been developed which is entitled as Tunable Resistive Pulse Sensing (TRPS) (Anderson *et al*., 2013).

2.2 Nanotechnology in Water Treatment

Nanotechnology has set to become an imminent discipline which will be able to safeguard long term feasibility and quality of water supplies through the exploitation of astonishing characteristics of nanomaterials such as photosensitivity, catalytic activity, high surface area, adjustable pore size and antimicrobial activity (Qu *et al*., 2012). Superior filtration systems incorporating nanomaterials that allow better water reuse and recycling have been developed. Novel nanosensors embedded with metal NPs, CNTs and quantum dots have been explored to identify low concentration levels of contaminants (biological/chemical) in water resources. New technologies in water decontamination and disinfection include use of natural & engineered nanomaterials like chitosan, carboxyfullerene, peptides, carbon nanotubes, ZnO and AgNPs (Li *et al*., 2008) which display strong antimicrobial properties and provide effective microbial control. A step ahead the antibacterial activities of AgNPs are implied in a nano filter systems which is achieved by immobilizing AgNPs onto a support system (gel or matrix).

2.2.1 Nanomaterials in microbial water disinfection

Chitosan: Chitosan is derivative of chitin (obtained from exoskeleton of crustaceans like crabs, shellfish etc) produced by deacetylation of the latter and accordingly it is a co polymer of N-acetyl glucosamine and glucosamine (Dutta *et al*., 2004). It has an intrinsic antimicrobial efficacy that amplifies with shift of dimension i.e. chitosan nanomaterials (ChNMs) have also been shown to have expansive antibacterial,

antiviral, and antifungal activity (Rabea *et al*., 2003) which is dependent upon several factors, including pH and solvent (Chung *et al*., 2003; Tavaria *et al*., 2013). ChNPs showed antifungal activity against *C. albicans* and *F. solani* with MIC ranging 0.5–1.2 mg/ml (Yien *et al*., 2012); Cu loaded ChNPs showed effective antibacterial activity against *S. typhimurium, E. coli, S. aureus* and *S. choleraesuis* with MIC ranging between 0.01 -0.13 µg/ml (Qi *et al*., 2004) due to the interaction of positively charged ChNPs with negatively charged cell membrane thereby, compromising the permeability of membrane. Chitosan at nanoscale has burgeoning applications in microbial disinfection of water sources where it can be applied as an antimicrobial means in surface coatings of water storage tanks, flocculants in waste water treatment membranes or sponges of filter systems.

Carbon nanomaterials: Nanomaterials with carbon as its foundation such as, Carbon nanotubes (CNTs), graphene, fullerenes as well as its derivatives are encouraged in water treatment because of their tremendous mechanical strength and biocidal properties (Cataldo *et al*., 2008; Sokolov *et al*., 1993). CNTs are hollow, nano-sized, cylindrical carbon structures which can be made by rolling multiple or single graphene sheets to form multi-walled (MWCNTs) and single-walled (SWCNTs) carbon nanotubes respectively (Wang *et al*., 2011). Kang *et al*. (2007) synthesized a filter membrane with its surface decorated with SWNTs and noticed that 87% of *E. coli* cells were killed in 2 hours. Srivastava *et al*. (2004) demonstrated antiviral & antibacterial activity of hollow fibers against poliovirus and *E. coli*. CNTs can be used to thwart biofilm formation on water filtration membranes and subsequent prevention in biofouling of these surfaces. Fullerenes (C_{60}) with its derivatives like fullerols have shown strong antibacterial activity (Sayes *et al*., 2004). Encapsulated C_{60} (due to the flexibility) and fullerols exhibit antimicrobial properties in water therefore, are considered for potential disinfection applications (Lyon *et al*., 2006).

Metal/metal oxide nanoparticles: Different nanoparticles such as nano silver, ZnO and TiO_2 are employed for water disinfection purposes.**TiO_2** is a semiconductor photo catalyst that can be irradiated by UV and visible light in order to eliminate contaminants from both air and water (Salthammer *et al*., 2007; Murray *et al*., 2007; Gelover *et al*., 2006); they show high sensitivity of antibacterial action against gram negative bacteria as compared to gram positive (Wei *et al*., 1994). Additionally, these metal NPs possess antiviral activity against hepatitis B virus, poliovirus 1, Herpes

simplex virus (Watts *et al*., 1995; Zan *et al*., 2007; Hajkova *et al*., 2007). MIC ranging between 100 and 1000ppm of TiO_2 is required for size dependent killing of bacteria (Wei *et al*., 1994) by ROS production, cell wall and membrane damage (Kikuchi *et al*., 1997). Doping of silver with TiO_2 greatly enhances photocatalytic bacterial inactivation with subsequent UV irradiation of TiO_2 surface (Page *et al*., 2007; Reddy *et al*., 2007). TiO_2 NPs are currently applied in wastewater treatment systems for degradation of organic contaminants and in air purification assemblies.

ZnO NPs show significant antibacterial activity against a wide variety of bacteria (Jones *et al*., 2008; Sawai, 2003; Adams *et al*., 2006) through hydrogen peroxide production (Sawai, 2003), release of Zn^{2+}ions (Atmaca *et al*., 1998), cell envelope and membrane disintegration (Huang *et al*., 2008; Brayner *et al*., 2006). ZnO NPs are commonly applied as antimicrobial agent in creams, sunscreens, ointments, paints and coatings (Franklin *et al*., 2007). Applications of ZnO NPs are restricted in drinking water treatment because ZnO disperses/dissolves easily in water and aquatic organisms are sensitive to Zn^{2+} ions release (Li *et al*., 2008).

2.3 Why Silver Nanoparticles?

A silver nanoparticle ranges from 1-100nm in size composed of several silver ions or atoms huddled together. Because of this diminutive size these AgNPs are capable of invading and killing bacteria and other microbes. However, nanosilver is not a new discovery silver and its compounds have been recognized for its antimicrobial efficacy dating back to ancient times when the Romans and Greeks employed silver coins and vessels for microbial disinfection of drinking water to make it potable. Colloidal silver had been used to cure lots of infections and diseases long before the discovery of penicillin in 1928; eye drops constituting of 1% silver nitrate solution were used in newborns to thwart post partum infections that lead to blindness (Alexander, 2009; Silvestry-Rodriguez, 2007).

Nowadays, the broad-spectrum antimicrobial properties of AgNPs encourage 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. Diverse range of consumer products use AgNPs including air sterilizer mists, textiles, pillows, moccasins, socks, ventilators, cosmetics, toothpastes, soaps &

shampoos, cleansers, water filters (Eurodia nanofilteration), washing machines, vacuum cleaners, refrigerator coatings, food preserving packaging (Top Screen DS13**),** dental fillers (Ketac Nano Light), contraceptives and mobile phones (Marambio-Jones *et al*., 2009).

But the real question is "Why AgNPs are so appealing"? The answer lies in their nature and unique properties. Because of their high surface to volume ratio AgNPs show enhanced bactericidal effects. Optical properties exhibit localized surface plasmon resonance which could be used to detect single molecules thus, can applied in biosensors. Catalytic activity help in reducing of dyes (Bhakya *et al.,* 2015) and anti inflammatory properties are functional in wound healing with improved cosmetic appearance (Tian *et al.,* 2007).

2.3.1 Antimicrobial aspect of silver nanoparticles

AgNPs show extensive antimicrobial activity in opposition to gram positive, gram negative and antibiotic resistant bacteria, protozoa, fungi, and few viruses (Slawson *et al*., 1992; Zhao *et al*., 1998). Numerous mechanisms have been projected to elucidate the inhibitory effect and mode of action of silver nanoparticles on microbes such as bacteria, viruses etc. Three assumptions made about the mechanism are: (1) interference in ATP production and DNA duplication after uptake of silver ions, (2) Induction of ROS species by AgNPs and silver ions, and (3) direct damage to cell membranes. The nanoparticles bind or append to the cell membrane and after that they break in inside the bacteria. 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 (Gupta *et al*., 1998; Matsumura *et al*., 2003). The nanoparticles deliberately assault the respiratory chain, hinder cell division as illustrated in *E. coli*, Gram-negative bacteria, AgNPs formed pits or holes in the cell wall by increasing the membrane permeability finally leading to cell death (Feng *et al*., 2000; Song *et al*., 2006; Sondi *et al*., 2004; Morones *et al*., 2005).

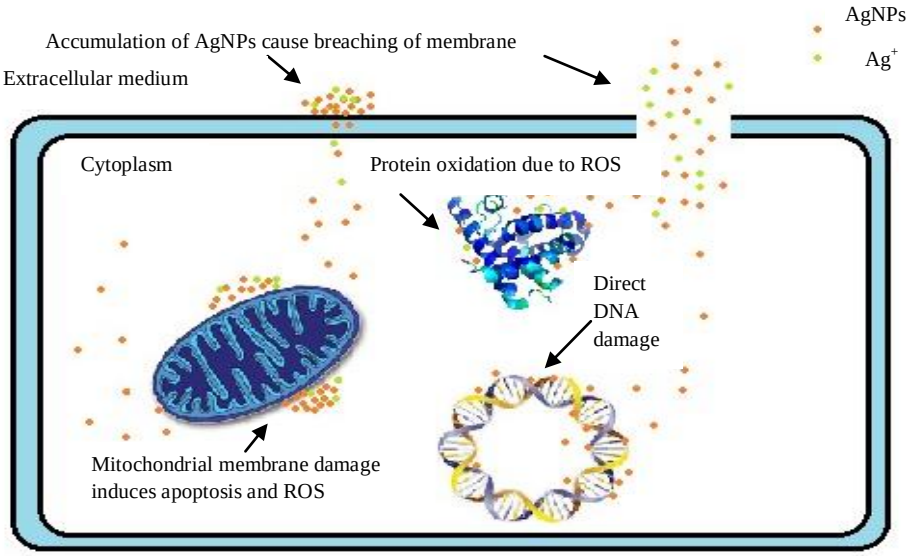


Fig 1: Representation of toxicity mechanisms exhibited by AgNPs.

Effect of shape and size: The antimicrobial activity is also particle size and shape dependent with smaller NPs (<10 nm) showing high antimicrobial activity than bigger NPs, these results might be possible due to high penetration rates when particle size is small because they have high surface to volume ratios (Cho *et al*., 2005; Baker *et al*., 2005; Martinez *et al*., 2008). AgNPs also showed interactions based on their shape such as Pal *et al*. (2007) reported enhanced bactericidal activity against *E.coli* in case of truncated triangular (1 µg needed) than spherical (12.5 µg) or rod shaped nanoparticles.

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):

- AgNPs go through brisk oxidation along with agglomeration in aqueous phase and as a result control of shape, size and stability could be at stake.
- Dispersed AgNPs cannot be reused, system becomes expensive and uneconomical.
- Release of AgNPs in uncontrolled fashion results in leaching of Ag^+ ions which leads to negative impact on aquatic environment.

One solution to this problem is to incorporate AgNPs in immobilization matrix of polymer material to form a nanocomposite which ensures stabilization of AgNPs. Hydrogels are being considered for this purpose with recent research efforts where nanoparticles and polymer material can retain their individual properties. Nanoparticles can either be applied onto the surface or embedded into the matrix. Two generic approaches are used for preparation of polymer/metal nanocomposites: (1) *in situ* in which nanoparticles are synthesized inside the polymer matrix; and (2) *ex situ*, in which nanoparticles are synthesized beforehand and then incorporated into the polymer matrix (Palza *et al*., 2015). Latter method is generally suitable for preparation of hydrogel based nanocomposites.

2.4 Synthesis of Silver Nanoparticles

There are wide varieties of methods applied for fabrication of nanoparticles, employing techniques inspired from physics, chemistry and biology. Silver nanoparticles can be manufactured by both 'top-down' and 'bottom-up' methods. The top-down process includes the mechanical kibbling of bulk metals followed by stabilization of the resultant metal nanoparticles with colloidal shielding agents (Gaffet *et al*., 1996; Amulyavichus *et al*., 1998). To the contrary, bottom-up process involves electrochemical methods and reduction of metals.

2.4.1 Physical methods (Qiaoxin *et al*., 2009)

These are usually swift, do not engage toxic chemicals and include procedures like laser ablation, laser pyrolysis, arc-discharge, physical vapor condensation and high-energy ball milling **(**Tien *et al*., 2008). Advantages of physical synthesis methods in contrast with chemical processes include the uniformity of nanoparticle distribution and lack of solvent contamination. Disadvantages include less thermal stability, expenditure of vast amount of energy, elevated neighboring environmental temperatures.

2.4.2 Chemical methods

Diverse procedures like chemical reduction, photochemical, electrochemical (Bakar *et al*., 2007) and pyrolysis (Qiaoxin *et al*., 2009) are applied for creation of AgNPs. **Chemical reduction** is the most frequently used method to synthesize stable,

Table 1: Antimicrobial properties of silver nanoparticles

Method	Size range	Antimicrobial	Organism tested	Reference
Biological reduction using whole plant extract (WPE) and callus extract (CE) of *Linum usitatissimum*	49-54 nm 19-24 nm	Antibacterial	*E. coli, K. pneumoniae, S. aureus*	Anjum *et al*.,2016
Biological reduction using probiotic *Bacillus licheniformis* cell free extract (BLCFE)	18.69-63.42 nm	Antibacterial Antibiofilm	*Vibrio parahaemolyticus Dav1*	Shanthi *et al*., 2016
Biological reduction using fungi	20-46 nm	Antiviral	Human parainfluenza virus type 3,Herpes simplex virus 1(HSV 1), Herpes simplex virus 2 (HSV 2)	Gaikwad *et al*., 2013
Chemical reduction using hydrazine hydrate	10-60 nm	Antibacterial	*B. cereus, E. coli, S. aureus, Proteus vulgaris*	Patil *et al*., 2012
Chemical reduction using grapheme oxide	5-25 nm	Antibacterial	*E. coli, P. aeruginosa*	Das *et al.*, 2011
Chemical reduction using ethylene glycol	8.5-11 nm	Antibacterial	*E. coli, S. aureus, S. epidermis, Micrococcus*	Dal Lago *et al*., 2011
Biological reduction using leaf extract *Nelumbo nucifera*	25-80 nm	Larvicidal	*Anopheles subpictus, Culex quinquefasciatus*	Santhoshkumar *et al*., 2010
Chemical reduction using Nitric acid	3 nm	Antifungal	*Candida albicans, Trichophyton, Mentagrophytes*	Kim *et al*., 2009
Physical reduction using γ ray irradiation	7-30 nm	Antibacterial	*E. coli, S. aureus*	Yoksan *et al*., 2009
Chemical reduction using HEPES buffer	10-50 nm	Antiviral	Hepatitis B virus	Lu *et al*., 2008
Chemical reduction using $NaBH_4$	13.5 nm	Antibacterial Antifungal	*E. coli, Yeast*	Kim *et al*., 2007
Chemical reduction using Ascorbic acid	15-21nm	Antibacterial	*E. coli*	Sondhi *et al*., 2004

colloidal dispersions in aqueous or organic solvents achieved by various reducing agents such as sodium borohydride ($NaBH_4$), sodium citrate and ascorbate. These agents reduce Ag^+ ions as a consequence, form metallic silver (Ag^0) followed by their agglomeration to form oligomeric bundles/clusters. These clusters ultimately assist to form and develop colloidal metallic silver particles (Wiley *et al*., 2005; Evanoff *et al*., 2004; Merga *et al*., 2007).With the establishment of innovative chemical or physical NP synthesis methods, the situation for environmental contaminations has become alarming therefore, more "greener" methods are being developed.

2.4.3 Biological methods

Existing organisms such as plants, fungi and bacteria have vast potential for the production of 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 approach. Additionally, green methods provide researchers with good control over size distribution of nanoparticles.

The above Table 1 compiles the broad spectrum antimicrobial properties of silver nanoparticles which are synthesized by different types of physical, chemical and biological methods.

2.5 Hydrogel as Immobilization Matrix

Hydrogels are 3D polymeric cross linked network structures made up of synthetic or natural polymers that can retain a large amount of water almost 10-20 times of its molecular weight and still remains insoluble in water (Kim *et al*., 1992).Upon hydration in aqueous environment the hydrophilic moieties present in polymer network form hydrogel structure.

2.5.1 Mechanism of network formation

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 this final form is known as the 'gel'/'network'. The

decisive point at which first appearance of gel occurs is called the 'gel point' (Rubinstein *et al*., 2003). This transition of a structure from finite to infinite branched polymer is known as 'sol-gel transition/sol-gel gelation') and gelation can be of 2 types: physical gelation (physical linking) or chemical gelation (chemical linking).

2.5.2 Methods of hydrogel production

Synthetic or artificial polymers such as polyvinyl pyrollidone (PVP), (polylactic acid (PLA) (Palumbo *et al*., 2006; Razzak *et al*., 2001), polymethacrylate (PMA), polyethylene glycol (PEG) and natural/organic biopolymers (Coviello *et al*., 2007) such as chitosan, carboxymethyl chitosan, carrageenan, alginate, hyaluronan, and cellulose to form cross-linked networks have been explored and reported. A variety of production techniques are adopted such as chemical, physical and radiation cross linking, grafting polymerization (Said *et al*., 2004; Fei *et al*., 2000; Hennink *et al*., 2002; Barbucci *et al*., 2004; Liu *et al*., 2002). **Chemical cross linking** method is quite popular method to obtain permanent hydrogels and it involves the connection/linking of two polymer chains via a cross linking agent such as, aldehyde (e.g. glutaraldehyde).The linking is achieved through the functional groups such as hydroxyl (OH), carboxyl (COOH), and amino (NH_2) present in polymers.

2.5.3 Characteristics

The most essential characteristic feature of a hydrogel is its water holding capacity. Upon hydration the polar hydrophilic groups come in proximity of water which induce the creation of 'primary bound water' as a result, exposure of the hydrophobic groups occurs which also merge with water molecules. As a consequence hydrophobically-bound water, as well called 'secondary bound water' is formed. 'Total bound water' is a term given for primary and secondary bound water combined together. Hydrogels have labile bonds that can be wrecked in physiological conditions either chemically or enzymatically, mostly by hydrolysis (Hoffman, 2002; Hennink *et al*., 2002) hence, these are **biodegradable** which makes them applicable for wound healing & tissue engineering etc.

The reason for why hydrogels have been selected as immobilization matrix for AgNPs is because these swollen cross linked structures provide hefty free space inside the network that can act as a nanoreactor and this space comes in handy for synthesis of

nanoparticles for its nucleation and growth (Wang *et al*., 2004; Mohan *et al*., 2006; Mohan *et al*., 2007). Also, the hydrogel can provide the targeted functional groups as active site/spot for metal nanoparticles.

2.6 Silver NP- Polymer Hydrogel Composites

Silver nanoparticles infused hydrogels combine the characteristics of both nanomaterials as well as polymeric hydrogels. The particle size distribution, agglomerations etc. of silver nanoparticles eventually impinge on the production and properties of AgNPs-polymer hydrogel composites. Current studies in this area are making use of the *in situ* production of metal nanoparticles inside the polymeric hydrogels.

Zeng *et al*. (2015) produced a reduced graphene oxide hydrogel doped with silver nanoparticles of size range 5-20 nm. Hydrogel displayed an exceptional antibacterial activity against *E. coli* with reduction rate of more than 95% and it can be applied as filter system for disinfecting water of microbes as demonstrated by this study.

Loo *et al*. (2013) synthesized poly (sodium acrylate) cryogels embedded with AgNPs (size <10nm) formed through intermatrix synthesis. These cryogels show high porosity, water absorption, greater mechanical strength than its pristine counter parts and significant antimicrobial activity against *B. subtilis* and *E. coli* with 5.4−7.0 log reduction of viable bacteria within few seconds of contact time. These cryogels can be reused without any decrease in disinfection efficacy and can be applied for disinfecting water in disaster relief situations.

Juby *et al*. (2012) created poly vinyl alcohol (PVA) /gum acacia hydrogel by γ radiation stimulated cross-linking with *in situ* synthesized AgNPs with size 10-40 nm dispersed in hydrogel matrix. The hydrogels with AgNPs were found to be more thermally stable and possessed significant antibacterial activity against *E. coli* and ZOI decreased with increasing concentration of gum acacia. These hydrogels can be applied for water disinfection purposes.

Table 2: Various antimicrobial silver hydrogel nanocomposites

AgNPs Hydrogel composites	NP Size	Antimicrobial activity	Organisms tested	Potential applications	MIC/Reduction rate/Inhibition zone diameter	Reference
AgNPs/ zwitterionic dopamine methacrylamide (ZWDO) Hydrogels	<20 nm	Antibacterial	*E. coli, S. aureus, P. aeruginosa*	Antimicrobial, Antifouling wound dressing	157, 148 and 129 % in ZOI diameters	Ghavami Nejad *et al*., 2016
AgNPs/ N-terminally 2(naphthalen-6-yl) acetic acid Phe–Phe–Cys peptide (Nap-FFC) hydrogel	15 nm	Antibacterial	*MRSA, Acinetobacter baumannii*	Antibacterial wound dressings.	MIC: 40 µg/ml	Simon *et al*., 2016
AgNPs/chitosan hydrogel	__	Antibacterial	*E. coli, B. subtilis*	Wound dressings, tissue engineering	ZOI: E.coli-10 mm *B. subtilis* -12mm	Kozicki *et al*., 2016
Ag/TiO_2 NPs/chitosan/PVA	292-443 nm	Antibacterial	*E. coli, S. aureus*	Wound management materials	ZOI: *E. coli*- 4mm *S.aureus*- 4.5 mm	Rinehart *et al*., 2016
AgNPs/Reduced graphene oxide hydrogel	5-20 nm	Antibacterial	*E. coli*	Bactericidal filter system in water disinfection	Reduction rate >94%	Zeng *et al*., 2015
Polyvinyl alcohol/ chitosan/AgNPs (PVA/CS/Ag)	27.5–12.8 nm	Antibacterial	__	Water disinfection, Anti-thrombogenic agent	__	Nguyen *et al*., 2015
AgNPs/methyl cellulose hydrogel	10 nm	Antibacterial Antifungal	*C. albicans. P. aeruginosa, S. epidermis*	Topical solution for treatment of wounds	MIC: 25mg/L	Panacek *et al*., 2014

AgNPs/ poly(sodium acrylate) (PSA) cryogels	<10nm	Antibacterial	*E. coli, B. subtilis*	Drinking water disinfection	5.4−7.0 log reduction	Loo *et al*., 2013
AgNPs/chitosan oligosaccharide/poly(vinyl alcohol) nanofibers	15–22 nm	Antibacterial	*E. coli, S. aureus*	Wound healing dressings	__	Li *et al*., 2013
Chitosan/PVA/AgNPs	20 nm	Antibacterial	*S. aureus, P. aeruginosa*	Wound dressing	ZOI:15mm	Hiep *et al*., 2013
AgNPs/PVA/gum acacia hydrogel	10-40 nm	Antibacterial	*E. coli*	Water disinfection	__	Juby *et al*., 2012
Chitosan/PVA/AgNPs	8-21 nm	Antibacterial	*E. coli*	Water disinfection	83.5% reduction rate	Agnihotri *et al*., 2012
Ag/P(HEMA/IA)/PVP hybrid hydrogels	_	Antibacterial Antifungal	*S. aureus, C. albicans, E. coli*	Regeneration of injured tissues.	Reduction rate : *S. aureus*- 70% *C. albicans*-95% *E. coli*-17%	Jovaševic *et al*., 2011
AgNPs/Poly (Acrylamide - co - Acrylic acid) Hydrogel	1-12 nm	Antibacterial Antifungal	*S. aureus, E. coli, , B. subtilis, C. albicans*	Antimicrobial swellable polymers	Maximum ZOI : 20 mm	Aggor *et al*., 2010
AgNPs/Dextran hydrogel	20–30 nm	Antibacterial	*Bacillus cereus*	Effective antimicrobial material	__	Ma *et al*., 2009
Poly(acrylamide)/poly(vinylpyrrolidone) semi-IPN hydrogel/AgNPs	3–5 nm	Antibacterial	*E. coli*	Antimicrobial material	100 % reduction rate	Murthy *et al*., 2007

2.7 Research in the Current Thesis

In the present work, we employed chitosan for fabrication of AgNPs which is a biopolymer and a promising applicant for production of antibacterial hydrogels on account of its innate bactericidal activity (Shi *et al.*, 2006), ability for gel formation, solubility in dilute acids, and low toxicity. But the main drawback with chitosan is its poor mechanical strength to uphold its structure for water disinfection purposes. In order to overcome this problem it is generally chemically cross linked with other polymers (Chandy *et al*., 1992) which offered the researchers a new window for changing the desire of interest. One such polymer is Poly (vinyl alcohol) PVA which is suitable for blending with chitosan due to its properties like high hydrophilicity, good film and gel forming ability, biocompatibility and fair mechanical strength. Thus, chitosan and PVA together form a hydrogel composite for AgNPs intended for antibacterial applications.

2.7.1 Preparation and characterization of Chitosan/PVA hydrogels

Abdeen, 2011 synthesized semi-synthetic hydrogel films by blending polyvinyl alcohol and chitosan employing solvent-casting technique and using glutaraldehyde as a cross linking agent. FTIR spectroscopy was applied to check the intermolecular interactions between chitosan and polyvinyl alcohol molecules.

Costa-Junior *et al*. (2009) produced intermingled films of chitosan and poly (vinyl alcohol) chemically cross linked with glutaraldehyde for utilization in repairing of skin tissue. Fourier Transform Infrared spectroscopy (FTIR), Energy-dispersive X-ray spectroscopy (EDX) and scanning electron microscopy (SEM) techniques were employed to study microstructure and morphology of the hydrogel films.

Tang *et al*. (2009) produced chitosan/polyvinyl alcohol hydrogels via 2 routes *in situ* and *ex situ*. The prepared thermo sensitive hydrogels were employed for protein delivery of hydroxyapatite. Characterization was done by rheological analysis, X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM).

He *et al*. (2008) prepared composite hydrogels comprising of poly (vinyl alcohol)/chitosan (CS) by physical cross linking and cyclic freezing/thawing techniques. Characterization of these hydrogels was done by scanning electron

microscope (SEM), infrared spectra (IR), and differential scanning calorimetry (DSC).

Yang *et al*. (2008) collectively used γ-Irradiation and freeze-thawing technique to yield hydrogels made up of water soluble chitosan and PVA for wound dressing applications. These gels were characterized by scanning electron microscope.

2.7.2 Antibacterial properties of Chitosan/PVA/AgNPs hydrogels

Rinehart *et al*. (2016) synthesized chitosan/PVA hydrogels doped with Ag/TiO_2 nanoparticles in size range 292-443 nm. These hydrogels displayed noteworthy antibacterial activity in opposition to *E. coli* and *S. aureus* with ZOI of 4 and 4.5 mm respectively. Ag functionalized hydrogels displayed improved structural rigidity without altering the swelling ability of the gel and provide controlled release of Ag+ ions for bacterial disinfection. Optimized hydrogels displayed great biocompatibility with dermal fibroblasts of human origin.

Li *et al*. (2013) produced chitosan oligosaccharide/poly (vinyl alcohol) nanofibers with AgNPs (15-22 nm) for use in bioactive wound healing dressings. These nanofibers displayed outstanding antibacterial efficacy against *S .aureus* and *E. coli* with no significant biocompatibility and cytotoxicity issues shown by human skin fibroblasts.

Hiep *et al*. (2013) synthesized chitosan/PVA hydrogels with AgNPs of average size 20 nm intended for wound dressing applications. These hydrogels confirmed first-rate antibacterial activity against *S. aureus* and *E. coli* with ZOI of 15mm compared to 12 mm of pristine chitosan/PVA hydrogels.

Vimala *et al*. (2013) produced chitosan/PVA hydrogel films embedded with AgNPs of size 16 nm and curcumin by chemical cross linking using glutaraldehyde. These hydrogel films showed excellent antibacterial and antifungal activities tested against *E. coli, P. pseudomonas, micrococcus, Staphylococcus, Candida albicus, and P. aeruginosa* showing ZOI ranging from 1.0 to 2.1 mm.

Agnihotri *et al*. (2012) synthesized chitosan/PVA hydrogel decorated with AgNPs (of average size 13 nm) using chemical cross linking and subsequent freeze thaw method.

The hydrogels showed remarkable antibacterial activity against *E.coli* (common water pathogen) by decreasing the growth rate by 83.5 %.

2.8 Characterization of Nanocomposites

Nanoparticles are generally characterized on the basis of their dimensions like size and surface properties like morphology by means of sophisticated microscopic techniques like scanning electron microscopy (SEM), differential scanning calorimetry (DSC), atomic force microscopy (AFM) and transmission electron microscopy (TEM) (Pal *et al*., 2011). Since most of the technical applications are directly or indirectly dependent upon surface properties therefore, quantitative and qualitative analysis of particles' surface is important and challenging at the time.

2.8.1 Scanning electron microscopy

The technique of SEM works by scanning the surface morphological features of sample via direct envision. To initialize characterization in SEM, a proper procedure is followed which requires the nanoparticles containing sample to be first air dried/freeze dried or pulverized into powder form. It is then mounted on a sample holder with the help of conductive tape accompanied by conductive metal (such as gold, platinum) coating with a sputter coater. Finally, by focusing a beam of electrons on the sample it is scanned (Jores *et al*., 2004) and the image of the surface morphology is acquired from the secondary (backscattered) electrons effused from the model surface hitting the detector. The mean size obtained by both SEM and dynamic light scattering are analogous to each other. Drawbacks include: the nanomaterials must be capable of enduring vacuum, damage to polymer via electron beam, high time consumption and cost (Molpeceres *et al*., 2000). Advanced version of SEM i.e. FESEM (Field Emission Scanning Electron Microscopy) is used nowadays to visualize very minute topographic details on the sample surface, in this technique electrons are launched by a field emission source thereby producing a more focused beam and secondary electrons are effused from every spot on the sample hence, producing a high resolution image.

2.8.2 Energy-dispersive X-ray spectroscopy (EDX)

EDS is a technique where one can be acquainted with elemental composition of the matter in question. It employs X-rays and its unique energy levels to detect the X-ray spectrum emanated from the sample after it is barraged by electron beams to obtain localized elemental chemical analysis. It works on the principle that when an atom is excited by incident beam of electrons like X-ray; inner shell electron is emitted out and as a result leaves a hole or spot behind. This is then filled by outer shell electrons and consequently the surplus energy is liberated through the emission of X-ray spectrum. Since each element in Periodic Table has a distinctive electronic configuration with different set of energy tiers therefore, X-ray spectral lines are an attribute of the element under examination (Ritchie *et al.*, 2013).

2.8.3 Transmission electron microscopy

TEM works in a different way in principle than SEM but the data obtained is relevant. The sample specimen is fixed on top of copper grids but to make the samples endure the apparatus vacuum either plastic embedding or staining of material is done with uranium, lead or uranyl acetate. Another way to assist handling is working with it as frozen material by exposing to liquid nitrogen temperatures. The surface morphology of the sample is attained when an electron beam is transmitted and diffracted through a very thin sample while electrons interact passing through the sample (Molpeceres *et al*., 2000) and image is obtained by analysis of the forward-scattered electrons. Disadvantage is that sample preparation is time consuming and complex because electron transmittance requires ultra thin sample (Pal *et al*., 2011).

2.8.4 Fourier Transform Infrared (FT-IR) spectroscopy

FTIR is a valuable practice for recognizing chemical constitution such as, specific functional groups of a material. Main principle behind its operation is that when IR radiation is passed through the sample it is either absorbed or transmitted. The resultant absorption and transmission spectra correspond to the vibration frequencies of atom bonds that form the framework of material. Since, each and every material is unique in its composition and has different arrangement of atoms the IR spectrum with absorption peaks represents the molecular fingerprint of the sample which cannot be shared by two compounds. Sample preparation is done by making KBr (Potassium

Bromide) pellets or discs for different types of material and solution cells are employed for liquid samples in transmission mode. FTIR can yield information regarding consistency and quality of sample. Fast pace, sensitivity, effortless calibration and mechanical simplicity are some of the other advantages of FTIR (Schmitt *et al*., 1998).

CHAPTER-3

MATERIALS & METHODS

3.1 Chemicals & 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. PVA (MW 85,000–124,000Da) was purchased from HiMedia ltd. Sodium hydroxide pellets were obtained from SD fine chemicals ltd. Glutaraldehyde was obtained from Loba Chemie ltd. For antibacterial experiments, various microbial strains *E.coli* DH5α, *Bacillus licheniformis* (NA11) and *Lysinibacillus fusiformis* (DGA) (isolated and characterized previously in research lab-3) were procured from research lab-3, department of biotechnology, Thapar University. For all experiments distilled or deionized water was used.

3.2 Sterilization procedure

Glasswares were first washed with detergent and then kept in oven at 40-50 ℃ till dried. After drying, glass wares were treated with Aqua regia for 15-30 minutes to dissolve metals and salts, followed by washing with water. Finally, they were kept in distilled water for 15 minutes and oven dried. All tips were autoclaved at 121°C for 15 minutes.

3.3 Reagent preparation

- **12 % NaOH**- 12g of NaOH dissolved in 100 ml of distilled water.
- **Aqua regia**- HCl : HNO_3 of 3:1
- **10mM $AgNO_3$-** 16.9 mg dissolved in 10 ml of distilled water.
- **Chitosan 2% w/v-** 0.3g of chitosan dissolved in 14.7 ml of distilled water with 300µL acetic acid.
- **PVA 4% w/v**-0.6g of PVA dissolved in 15 ml of distilled water.
- **$NaBH_4$ solution-** 4mg per 10 ml of distilled water.
- **NB media-** 2.6g of nutrient broth in 200ml of distilled water.
- **NA media-**10.4g of nutrient broth, 16g of agar in 800ml of distilled water.

3.4 Preparation of chitosan/ PVA hydrogel

For preparation of chitosan /PVA hydrogel procedure followed was given by Agnihotri *et al*., 2012 with few modifications.

1. Chitosan polymer was dissolved in dilute acetic acid solution at room temperature while stirring overnight at 700 rpm.
2. Poly vinyl alcohol (PVA) solution (4 % w/v- 0.6g in 15 ml of water) was prepared by dissolving PVA in distilled water at 80 °C with stirring for 4 hrs.
3. Chitosan and PVA solutions were mixed (total volume of 30 ml) and stirred at 60 °C for 2 hrs to obtain a homogenous phase and left overnight with continuous stirring.
4. For network formation, glutaraldehyde (200µL) was used and added slowly to the chitosan–PVA solution at 25 °C and was mixed well for 5 minutes before casting into test tubes.
5. Gel was cured in oven at 40- 50 °C until solid.
6. Hydrogel was removed from test tubes by precipitating in 12% wt. NaOH (sodium hydroxide) at room temperature overnight.
7. Removed hydrogel was washed with distilled water thrice to remove NaOH and then soaked in distilled water for 24 hrs.
8. Finally, porous semi-interpenetrating networks were developed by repeated freezing and thawing cycles at -20 °C for 6 hrs and 25 °C for 2 hrs, respectively, for 7 days.

In this way porous chitosan/ PVA hydrogel was formed which was designated as "CHT/PVA" and kept for further characterization.

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

Preparation of silver loaded hydrogel was performed as follows:

1. Previously prepared hydrogels were cut into circular discs (of diameter 10mm) of desired length with the help of sterilized blade.
2. These circular discs were introduced into 10mM silver nitrate (silver ion precursor) solution and kept in dark place.
3. Silver loaded gels were washed twice with distilled water to remove the unbound silver ions.

4. For chemical reduction of silver ions, these hydrogels were placed in 10mM sodium borohydride ($NaBH_4$) solution for few minutes until the color of gel changes.
5. Finally, the gel discs were washed with distilled water and kept for further characterization.

These hydrogel discs with silver (Ag) were designated as "CHT/PVA/Ag".

3.6 Swelling studies

In order to study swelling ratios, chitosan/PVA (CHT/PVA) and Ag loaded chitosan/PVA (CHT/PVA/Ag) circular discs (diameter 10mm) were dried under vacuum for 72 hrs to absorb all the moisture content and their dry weights were determined (W_2). Water content was measured by dunking the hydrogels in distilled water at room temperature for 2 hrs. After wiping the surface of gels with a filter paper or blotting sheet wet weight (W_1) was determined. The absorbed water content was then calculated by determining in term of the swelling ratio (S %) using Equation :

$$S(\%) = \frac{W1-W2}{W2} \times 100 \ldots\ldots\ldots\ldots \text{equation (1)}$$

3.7 Antibacterial activity of hydrogels

Potential for biocidal activity of these hydrogel nanocomposites against *E.coli* DH5α, *Bacillus licheniformis* (NA11) and *Lysinibacillus fusiformis* (DGA) was checked by two methods i.e. by standard colony forming and disc diffusion assay. Procedures for conducting these experiments are given as follows:

3.7.1 Colony forming assay

1. Nutrient broth (NB), nutrient agar (NA) media were prepared and autoclaved at 121°C for 15 minutes. NA Media was cooled then poured in Petri plates and kept for 30 minutes for solidification.
2. Sterilized NB media flasks (50 ml) were inoculated with 10µL of preferred microbial culture and kept in rotary shaker at 37°C at 120 rpm for 16-18 hrs.

3. During log phase of the microbial growth, culture was centrifuged to obtain pellet which was washed thrice with phosphate buffer solution (PBS) and finally the pellet was resuspended in PBS.
4. Adjustment for final concentration of 1×10^9 CFU/ml was done to unit absorbance at 600 nm using UV spectrophotometer.
5. Final working concentration of 10^3 CFU/ml was achieved with serial dilution in distilled water.
6. Flasks containing microbial solution with 2 hydrogel (CHT/PVA and CHT/PVA/Ag) discs (diameter 10mm) of each were incubated at 37°C at 120 rpm in rotary shaker.
7. Test solution samples (100µL) from flask were withdrawn at different time intervals (0, 15, 30, 60, 90, 120, 240 minutes) for plating over agar plates in duplicates.
8. Colonies of micro organisms were checked and counted after incubation period of 24 hrs.

3.7.2 Disc diffusion assay

1. Steps from 1-4 were repeated from previous assay.
2. Final working concentration of 10^6-10^7 CFU/ml was achieved with serial dilution in distilled water.
3. Microbial solution (100 µL) was spread onto NA plates and single hydrogel disc (CHT/PVA/Ag) was placed in center of the plate along with control hydrogel (CHT/PVA) in separate plate. Both discs were 10mm in diameter and experiment was carried out in duplicates
4. Plates were incubated at aforementioned conditions and zone of inhibition was recorded in each case after 24 hrs.

3.8 Characterization of hydrogel nanocomposites

3.8.1 Field emission scanning electron microscopy (FE-SEM)

The surface morphology of the pristine and Ag loaded hydrogels was determined by FE-SEM (Hitachi SU-8010) at Punjab university, Chandigarh. The hydrogel samples

were broken into small thin pieces and completely air dried to remove every bit of moisture from them. Gold coating was done using sputter system onto hydrogels supported on stub with the help of double sided carbon tape to make them conductive for analysis and image generation. The machine was operated at high vacuum and finally surface analysis was done by generating images at different resolutions.

3.8.2 Energy-dispersive X-ray spectroscopy (EDX)

It is a method useful for elemental breakdown and chemical characterization of a sample. EDX analysis can be done on SEM side by side with no extra preparation for test samples. Results are displayed as per the detector's recognition of X ray signals in terms of peaks for corresponding elements present in the sample. The EDX normally reveals the presence of phases.

3.8.4 Fourier Transform Infra-Red spectroscopy (FTIR)

FTIR analysis of both pristine and Ag loaded hydrogels was carried out through using Nicklet 380 Thermo, US Fourier Transform Infrared Spectrometer. Sample preparation was done by drying the hydrogels in hot air oven at 50 °C for 3 hrs and grinding them with pestle & mortar to obtain coarse powder type consistency. These samples were read between 500 and 4000 cm^{-1} and peaks were obtained for individual test materials.

4.1 Formation of CHT/PVA hydrogel

Hydrogel was formed according to the protocol discussed in the 'Materials & Methods' (Section 3.4). With subsequent freeze thaw treatment of precipitated hydrogel, porous network structures were developed and the color of the hydrogel was changed from transparent golden yellow to opaque yellow (Fig 2).

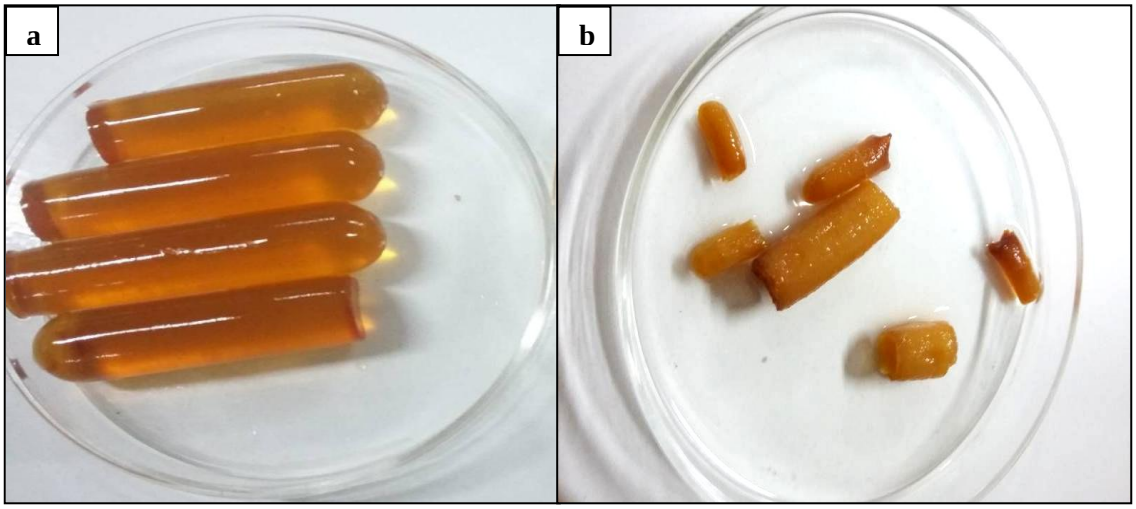


Fig 2: Synthesized CHT/PVA hydrogel (a) Before freeze thaw (b) After freeze thaw cycles.

4.2 *In situ* AgNPs synthesis in hydrogel

When silver loaded hydrogels (previously soaked in silver nitrate solution) were kept in contact with sodium borohydride solution (strong reducing agent), the color of the hydrogel was changed within 20-30s from golden yellow to brownish yellow and eventually to brownish black over time. This color change is an indicative of the *in situ* formation of silver nanoparticles (as shown in Fig 3) within the porous network of hydrogel.

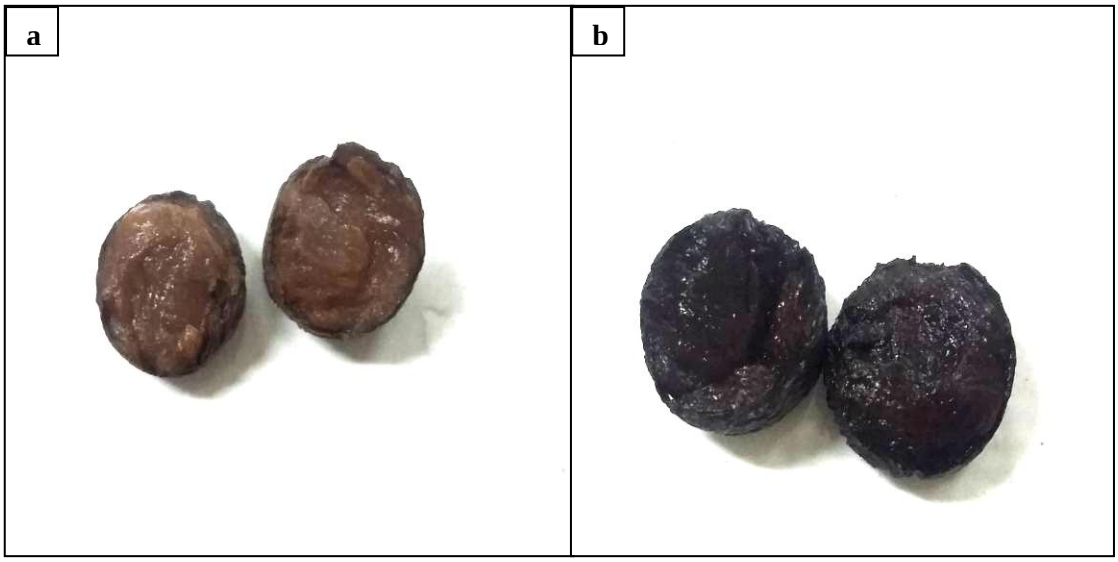


Fig 3: Synthesized silver loaded CHT/PVA hydrogels (a) Before reduction (b) After reduction

The possible explanation for synthesis of silver nanoparticles is provided by the fact that completely inflated hydrogel provide sites for adsorption of Ag^{+} ions from silver nitrate solution (ion exchange method) by bestowing them with its functional groups such as hydroxyl (–OH), amino ($–NH_2$), carbonyl (–C=O) (Tarnavchyk *et al*., 2009; Bozanic *et al*., 2010). Silver ions may interact with functional moieties through non covalent mechanism and/or get trapped in the liberated spaces between the cross linked polymer structures (Mohan *et al*., 2010) and subsequently their reduction with appropriate reducing agent ($NaBH_4$ in this case) results in the formation of silver nanoparticles distributed uniformly all over the surface and inside of the hydrogel. Hence, hydrogel acted as a 'nanoreactor' as well as an immobilization matrix silver nanoparticles (Agnihotri *et al*., 2012).

4.3 Swelling studies

Swelling capacity of both CHT/PVA (pristine) and CHT/PVA/Ag hydrogels was determined and consequence of inclusion of silver nanoparticles into pristine gel networks was also evaluated. Swelling ratios are provided in Table 3 calculated by equation 1 given in section 3.6.

Table 3: Swelling ratios of blended hydrogels

Hydrogel	Swelling ratio (%)
CHT/PVA	67.2 ± 5.9
CHT/PVA/Ag	52.5 ± 3.6

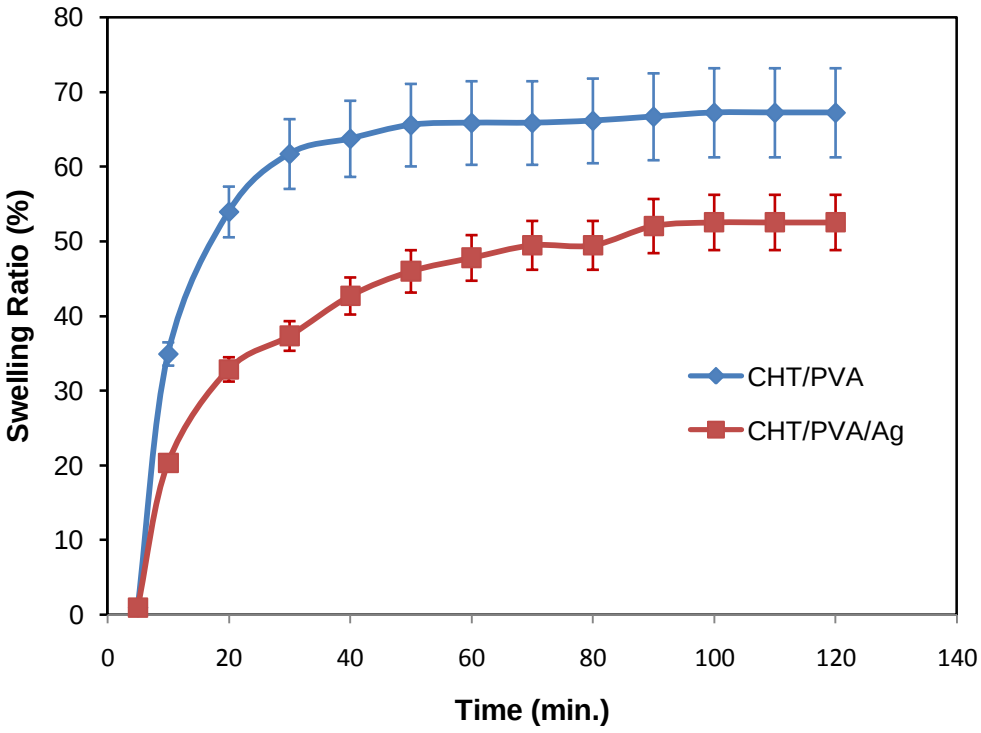


Fig 4: Swelling kinetics of CHT/PVA and CHT/PVA/Ag hydrogels at room temperature

The results for swelling studies/kinetics are concluded in Fig 4. It is evident from the figure that the nano-silver hydrogel nanocomposites (CHT/PVA/Ag) have lower swelling capacities than their pristine counterparts (CHT/PVA). Swelling ratios were found to be 67.2 ± 5.9 % and 52.5 ± 3.6 % for pristine and silver NPs loaded hydrogels respectively. The swelling ratios increased gradually up to 90 min after which no noticeable change was observed for both hydrogels. The decrease in swelling ratio is attributed to the fact that AgNPs formed inside the gel network were electrostatically stabilized with the functional moieties of chitosan and PVA polymers. Also, AgNPs have the tendency to form further additional cross-links with the electrons of O and N atoms of amine ($-NH_2$) and carboxyl (-COOH) groups present in the chains of both chitosan and PVA polymers (Bal *et al.*, 2015).The

resultant cross linked network severely affected the diffusion of water inside the hydrogel (Vimala *et al*., 2011; Ostrowska-Czubenko *et al.*, 2009) thereby the entire swelling behavior was lowered.

In a different study by Ma *et al*. (2009), the possibility of enhancement in elasticity of the hydrogel complex after incorporating AgNPs within the polymer network was proposed. Owing to the stable interactions between Ag and the hydrophilic groups of polymer, a decline in hydrophilicity of the nanocomposite hydrogel was observed which in turn reduced the swelling behavior.

4.4 Characterization studies of hydrogels

4.4.1 FTIR analysis

FTIR spectra for both CHT/PVA and CHT/PVA/Ag hydrogels are given in Fig 5. Examination of the spectra reveals various absorption peaks corresponding to chemical structure of bonds. In the CHT/PVA spectra, an absorption peak was observed at 3445 cm^{-1} which corresponds to O-H/N-H stretching due to the hydrogen bonding. However in CHT/PVA/Ag hydrogel, this peak was shifted to 3287 cm^{-1} which indicates that AgNPs might be well bonded to the chitosan/PVA polymer during their synthesis procedure (Li *et al*., 2013). Moreover, the peaks at 2925 cm^{-1} and 2910 cm^{-1} in CHT/PVA and CHT/PVA/Ag respectively are representative of C-H stretching (Vimala *et al*., 2011). Absorption peak at 1674 cm^{-1} in pristine hydrogel spectra indicated carbonyl (-C=O) bond stretching (Yang *et al*., 2004) and the emergence of these functional groups in CHT/PVA hydrogel subsequent to cross linking by glutaraldehyde is a sign of the presence of liberated hydroxyl (–OH) groups in PVA (Mansur *et al*., 2008). These functional moieties acted as nucleation sites for the attachment of Ag^{+} ions and helped in their subsequent immobilization as AgNPs. A peak was observed at 1453 cm^{-1} in CHT/PVA due to C-H bending (Vimala *et al*., 2011) and at 1323 cm^{-1} in CHT/PVA/Ag representing C-N stretching.

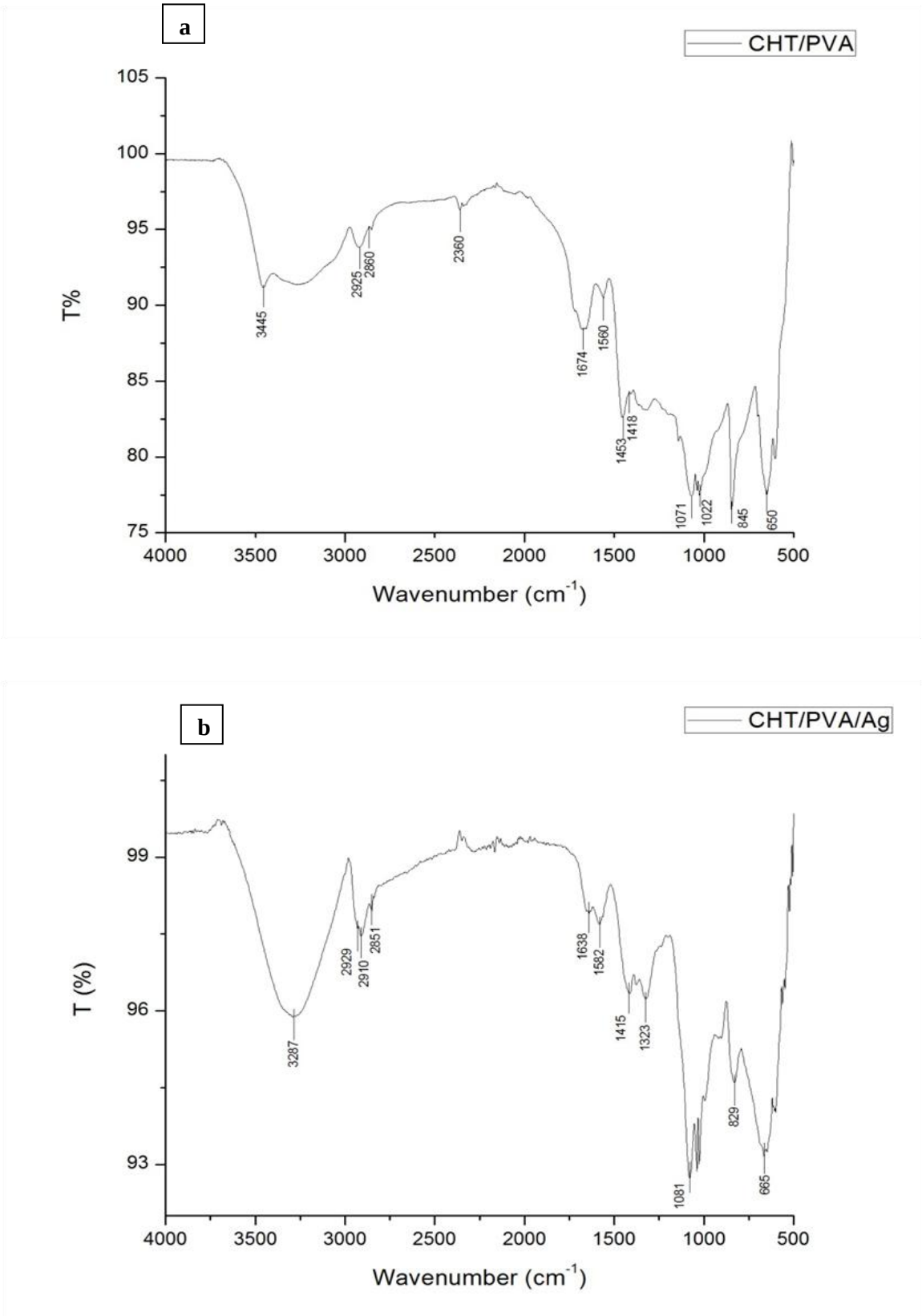


Fig 5: FTIR spectra of (a) CHT/PVA and (b) CHT/PVA/Ag hydrogels.

4.4.2 FE-SEM Analysis

The FE-SEM analysis of pristine and AgNPs loaded hydrogels are compiled in Fig 6.

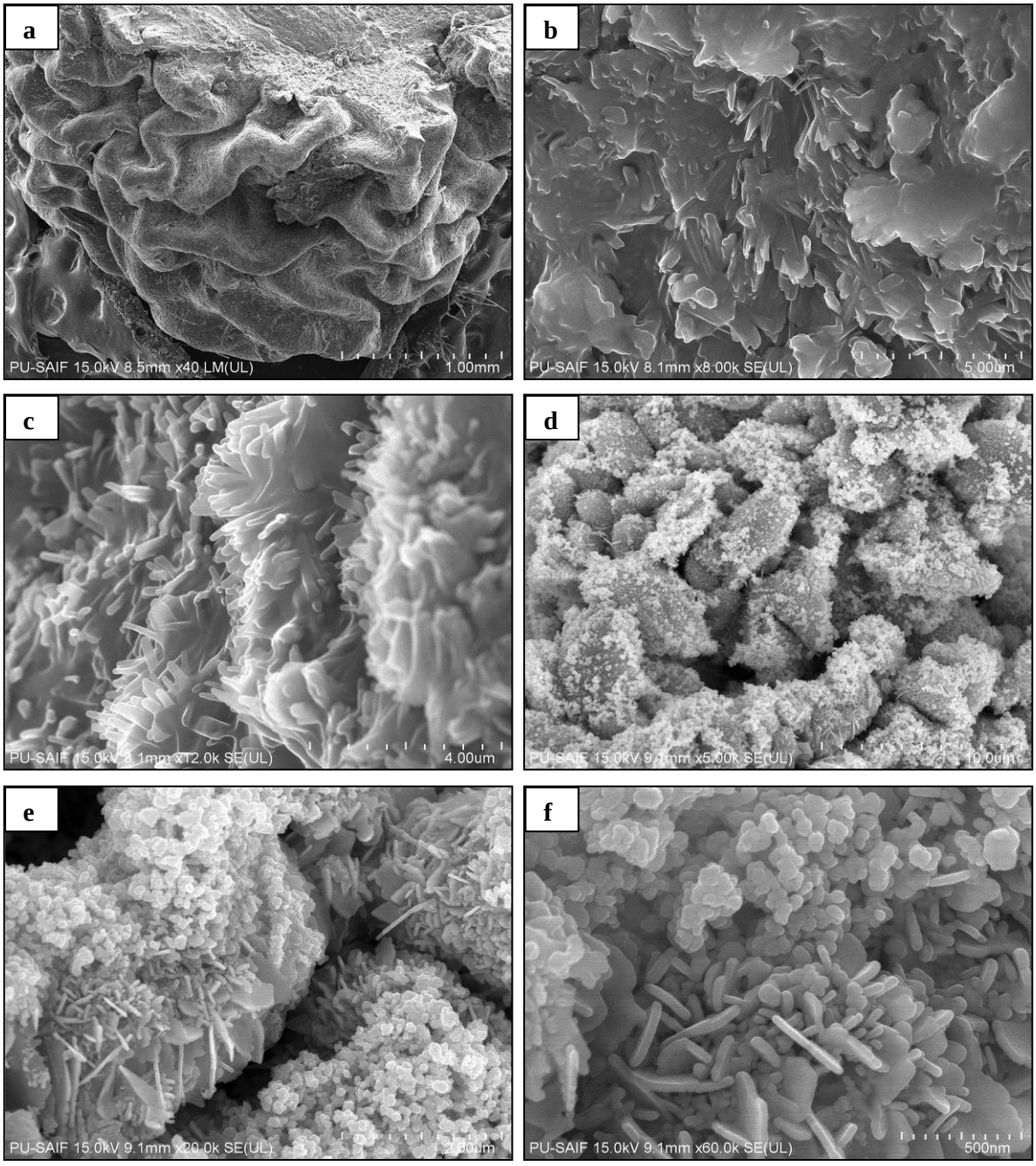


Fig 6: FE-SEM images of CHT/PVA hydrogel at (a) x 40 (b) x 8.00k (c) x12.0k and CHT/PVA/Ag hydrogel at different resolutions (d) x5.00k (e) x20.0k (f) x60.0k

The pristine CHT/PVA hydrogels displayed absence of nanoparticles and its surface had almost leaf like morphology at 8.00 k in Fig 6 (b) (Govindan *et al*., 2012) while CHT/PVA/Ag hydrogels showed the attendance of silver nanoparticles which were

spherical in shape (Govindan *et al*., 2012) in Fig 6 (f) . These synthesized AgNPs were in aggregated form, uniformly dispersed over the surface of blended polymer material and were embedded in the polymer matrix of chitosan/PVA. No significant variation in the morphology of chitosan/PVA hydrogel was observed after immobilizing AgNPs. This indicates a stable intercalation of AgNPs within the polymeric matrices without distorting their inherent topographical identifications.

4.4.3 EDX Analysis

Table 4 illustrates different constitutional elements present in CHT/PVA and CHT/PVA/Ag hydrogels on the basis of atomic and weight percentage. CHT/PVA/Ag hydrogels showed silver content of 15.78 % according to weight.

Table 4: Elemental composition of hydrogels

Element	Pure CHT/PVA hydrogel		CHT/PVA/Ag hydrogel	
	Weight %	**Atomic %**	**Weight %**	**Atomic %**
C K	48.66	56.63	41.42	55.26
O K	45.77	39.99	41.27	41.33
Na K	5.57	3.39	1.53	1.07
Ag L	0.00	0.00	15.78	2.34

Fig 7 shows the EDX plot of SEM image taken of both these hydrogels. It verifies the absence of any silver or silver nanoparticles content in the pristine hydrogel. The plot also shows the presence of carbon (C), sodium (Na) and oxygen (O) elements which were higher in weight percentage than in CHT/PVA/Ag hydrogel. The EDX evaluation also proved that the required phase of silver (i.e. AgNPs) is present in the test sample (CHT/PVA/Ag) of hydrogel. The plot of silver loaded hydrogel also demonstrates the presence of carbon (C), sodium (Na) and oxygen (O) elements just like in its pristine counterparts. These elements might have been present due to polymer backbone consisting of chitosan and PVA.

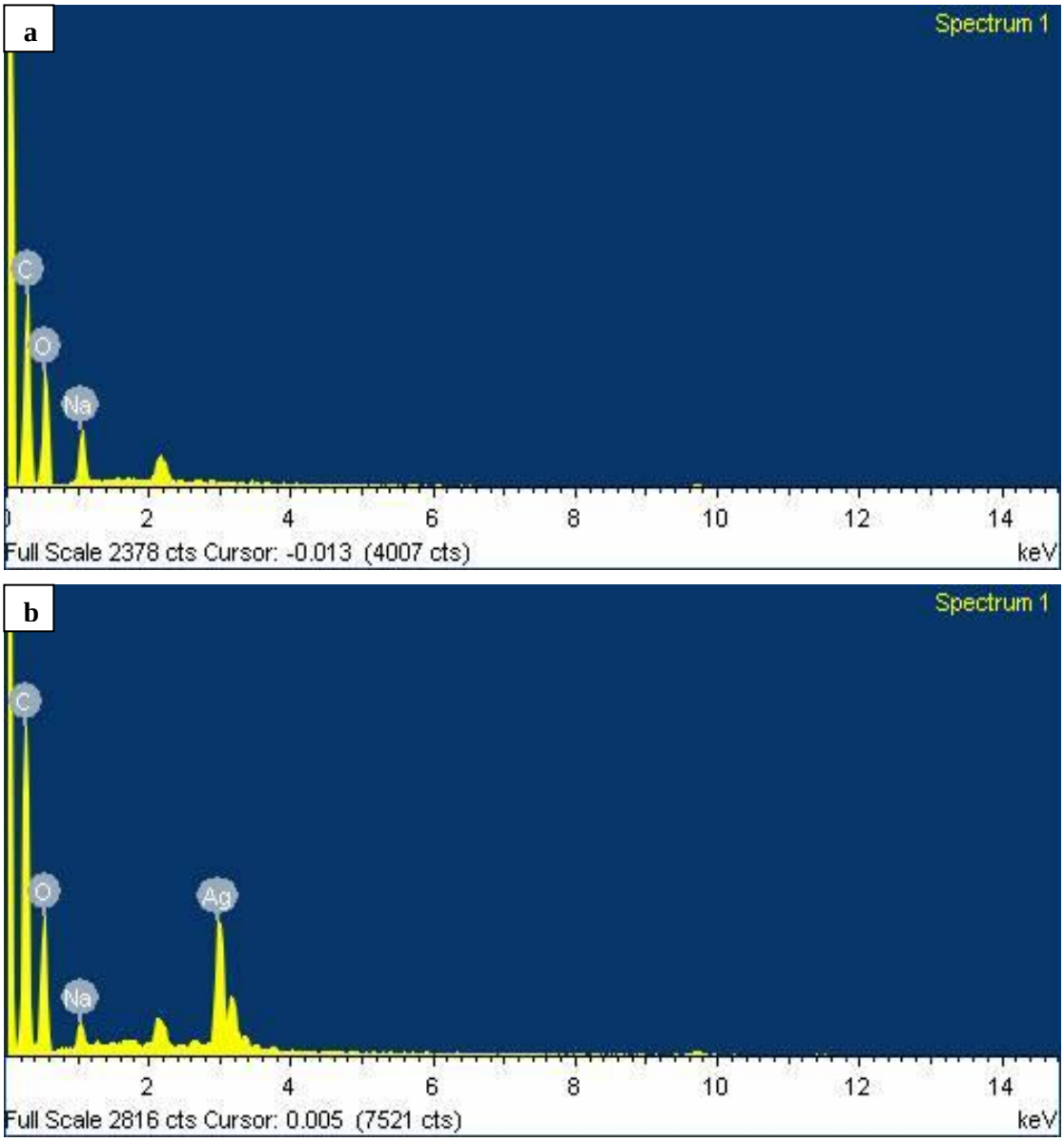


Fig 7: EDX plot of (a) CHT/PVA (b) CHT/PVA/Ag hydrogels

4.5 Antibacterial Assays

The bactericidal effect of pristine (CHT/PVA) and AgNPs loaded (CHT/PVA/Ag) hydrogels was tested in opposition to *E.coli*, *Bacillus licheniformis* and *Lysinibacillus fusiformis* with the help of colony forming assay in which reduction in number of colonies and growth kinetics of bacterial cells was studied, disc diffusion test where zone of inhibition was studied.

4.5.1 Colony forming assay

Fig 8 and Fig 9 shows the number of microbial colonies grown on nutrient agar plates as a function of incubation time which is required to disinfect the microorganisms.

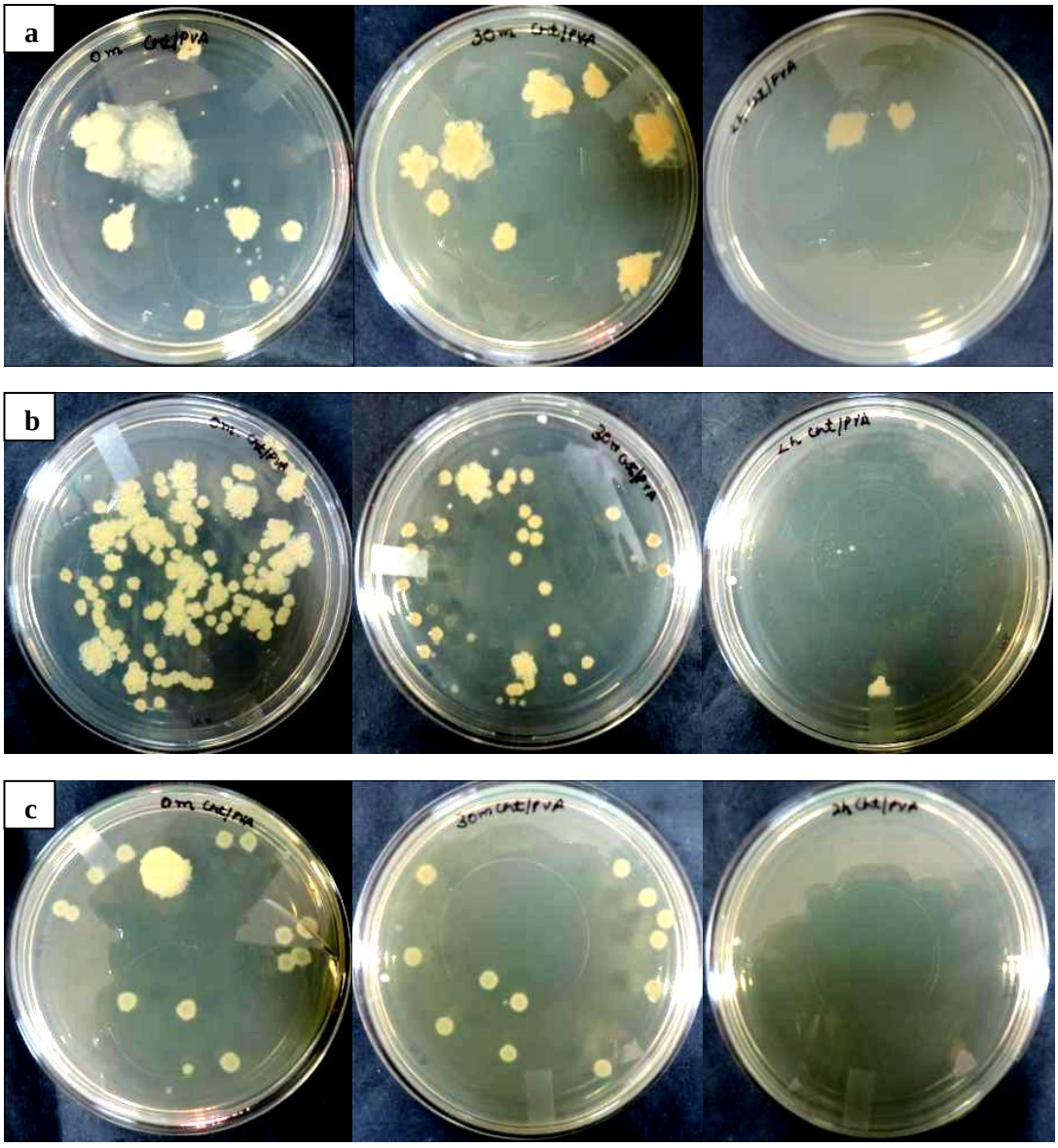


Fig 8: CHT/PVA treated cultures of (a) *E.coli* (b) *Bacillus licheniformis* (c) *Lysinibacillus fusiformis* plated at different time 0 min, 30 min, 120 min (left to right)

The number of colonies significantly reduced in both the hydrogel (pristine as well as AgNPs loaded) samples with increase in incubation time from 15 minutes to 4 hours. After 18-24 hours complete eradication of colonies was observed in the case of

CHT/PVA/Ag hydrogel. However, reduction in growth of micro organisms was significantly higher in case of silver loaded hydrogels (CHT/PVA/Ag).

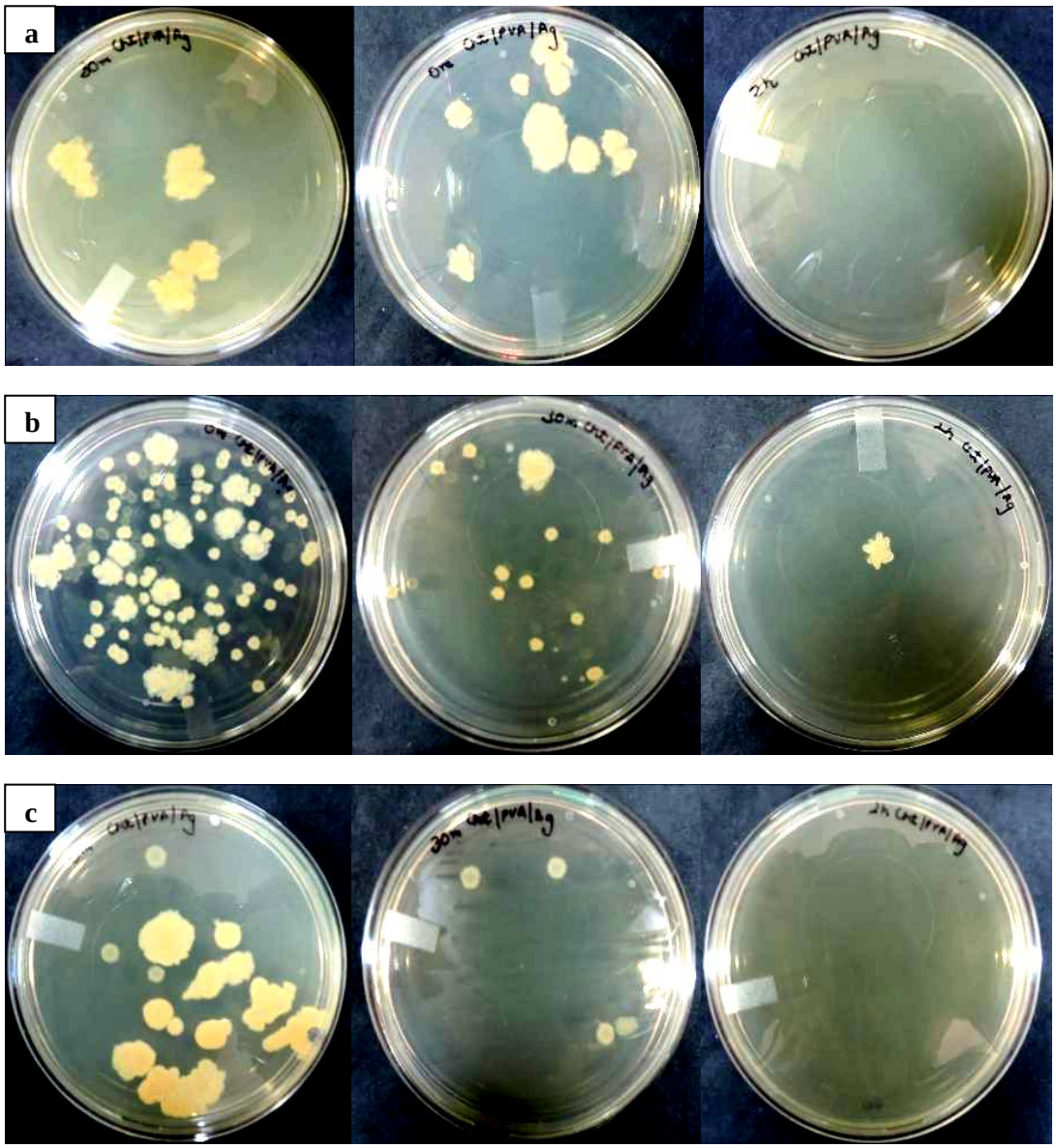


Fig 9: CHT/PVA/Ag treated cultures of (a) *E.coli* (b) *Bacillus licheniformis* (c) *Lysinibacillus fusiformis* plated at different time 0min, 30min, 120min (left to right)

Possible explanation for this phenomenon is that the AgNPs shielded within hydrogel matrix might come out or release gradually in medium (Mohan *et al*., 2007) where they interact with microorganisms and compromise their viability via mechanisms cited in literature review. Justification for antibacterial action in pristine CHT/PVA

hydrogel is provided by the innate antimicrobial character of cationic chitosan polymer. It can bind electrostatically to negatively charged cell membranes of microbes in order to destroy them while compromising cell permeability (Rabea *et al*., 2003).

4.5.2 Growth Inhibition kinetics

The synthesized CHT/PVA and CHT/PVA/Ag hydrogel discs were introduced into microbial solutions to examine their growth behavior. To draw any inference, graphs were drawn between -Ln N/N_o and time to depict the decrease in growth pattern. Fig 10 demonstrates the strain specific antibacterial activity of CHT/PVA and CHT/PVA/Ag hydrogel present in 100 ml of working solution with initial bacterial concentration of 10^3 CFU/ml.

It is evident from the graphs that extent of inhibition i.e. time take to achieve complete disinfection was same (90 minutes) for all and irrespective of the microbial strains tested. This shows that CHT/PVA/Ag hydrogel can demonstrate an efficient performance against a variety of microbial strains under similar test conditions.

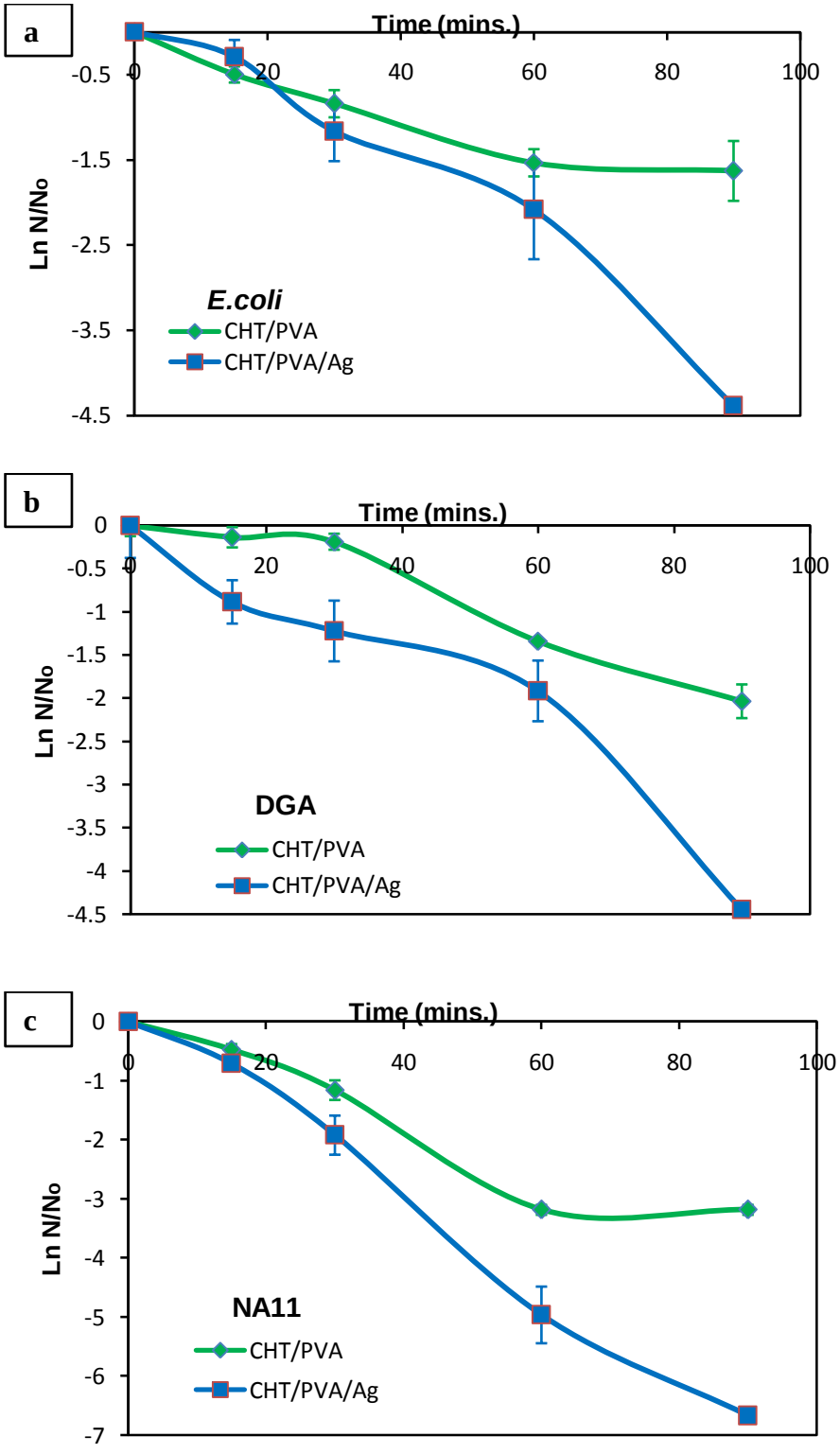


Fig 10: The effect of CHT/PVA/Ag gels on growth of (a) *E.coli* (b) *Bacillus licheniformis* (c) *Lysinibacillus fusiformis*

4.5.3 Disc diffusion test

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

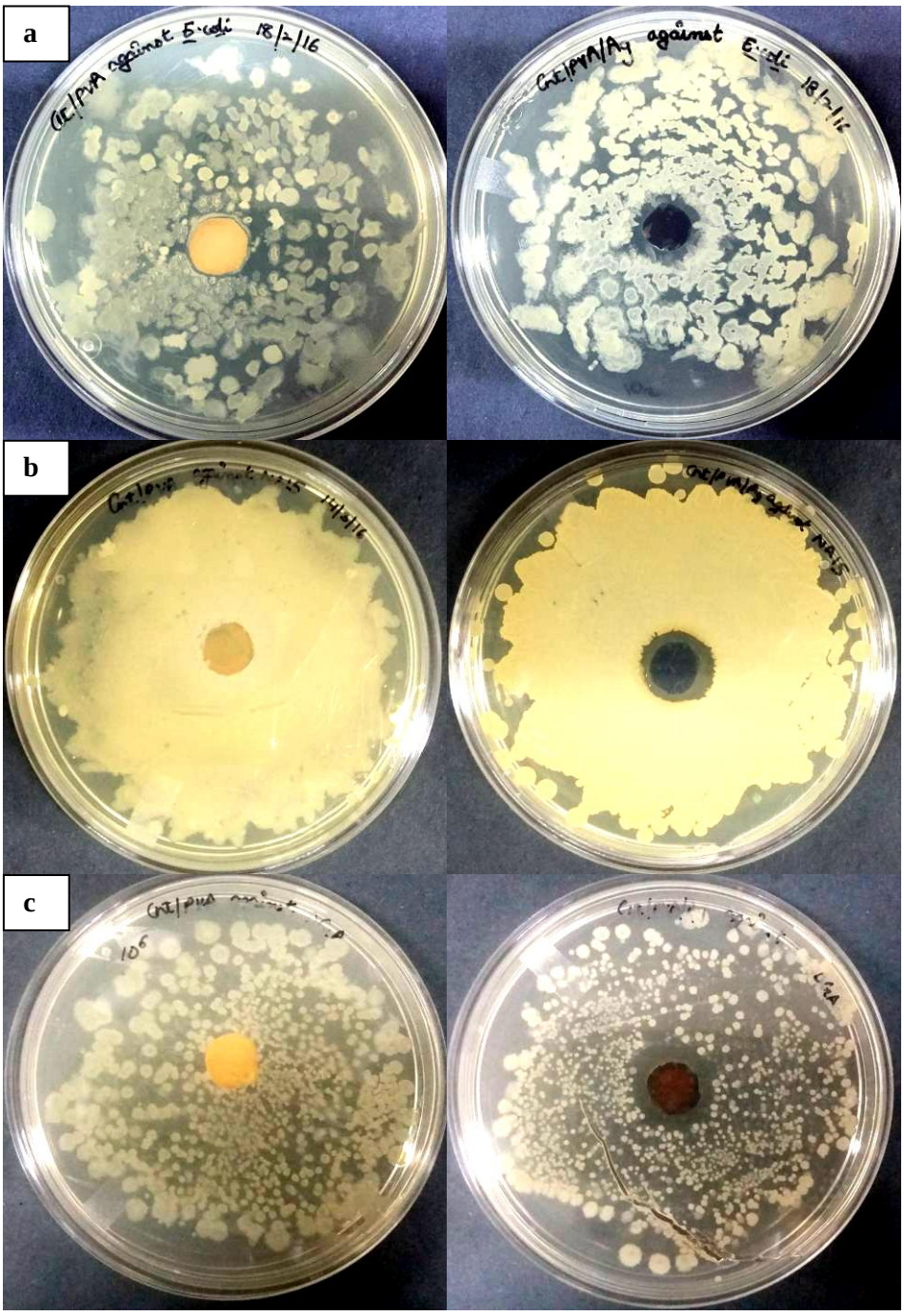


Fig 11: Antibacterial activity of CHT/PVA (left column) and CHT/PVA/Ag discs (right column) against (a) *E.coli* (b) *Bacillus licheniformis* (c) *Lysinibacillus fusiformis*

Table 5: Zone of inhibition measured with different bacteria

Test Organisms	ZOI (mm)	
	CHT/PVA	**CHT/PVA/Ag**
E.coli	NIL	14
Bacillus licheniformis	NIL	16
Lysinibacillus fusiformis	NIL	18

It is clearly evident from Table 5 that pristine hydrogel without silver does not participate in inhibition of microbes. On the contrary CHT/PVA/Ag hydrogel showed noteworthy antibacterial activity against all three test microorganisms namely *E.coli, Bacillus licheniformis,* and *Lysinibacillus fusiformis* with zone of inhibition ranging from 14 to 18 mm. Maximum ZOI with 18mm was revealed in case of *Lysinibacillus fusiformis.* The clear zones in agar plates indicate the release of high amount of silver and silver nanoparticles from hydrogel which then inhibit the growth of surrounding microbes. Results also showed strain specific sensitivity towards released silver nanoparticles and is justified by maximum inhibition zone diameter in *Lysinibacillus fusiformis.*

All of the antibacterial assays concluded the superior capability of silver nanoparticles modified CHT/PVA hydrogel to inhibit microbes (gram positive as well as gram negative) and can be compared with reported literature. CHT/PVA composite does show some inhibition action because of antibacterial nature of chitosan but in present work CHT/PVA/Ag hydrogel nanocomposites were more efficient in killing bacteria which is probably attributable to synergistic effect of AgNPs and chitosan in the composite.

The conclusions drawn from the present work are as follows:

1. Chitosan/PVA hydrogels of good elasticity were produced by freeze thaw cycles and subsequently *in situ* synthesis of AgNPs was done by chemical reduction using $NaBH_4$.
2. CHT/PVA/Ag hydrogel nanocomposites were characterized in detail by Field emission scanning electron microscopy (FE-SEM), Energy-dispersive X-ray spectroscopy (EDS/EDX), and Fourier Transform Infra-Red spectroscopy (FTIR).
3. Prepared hydrogels showed satisfying properties like swelling capacity. Swelling percentages were 67.2 ± 5.92 and 52.5 ± 3.6 for CHT/PVA, CHT/PVA/Ag hydrogels respectively.
4. Antibacterial potential of silver hydrogel nanocomposites and pristine hydrogels as a function of incubation time was tested against three different bacteria like *Escherichia coli*, *Bacillus licheniformis,* and *Lysinibacillus fusiformis*. The test was carried out by both Disc diffusion and colony forming assay. From the study, both types of composites were observed to have antimicrobial efficacy but silver loaded hydrogels displayed superior properties.
5. Growth inhibition kinetics was also studied for all three strains. As the incubation period increased CHT/PVA/Ag hydrogels gradually eradicated microorganisms from the solution.
6. Zone of inhibition studies revealed a maximum halo of 18mm in case of *Lysinibacillus fusiformis* inhibited by CHT/PVA/Ag hydrogels. On the contrary, pristine CHT/PVA hydrogels did not show ZOI in any of the cases.

Future scope of the present work is concluded as follows:

- Methods for immobilizing silver nanoparticles in matrix can be investigated further to counteract destabilization, non reusability and leaching of nanoparticles.
- Various “green” matrix materials can be searched as immobilizing template for silver nanoparticles.

- Greener and more economical methods without the use of toxic chemicals for synthesis of polymer nanocomposites can be considered.
- Wide array of properties like antifungal, antiviral and anti-cancerous activity of AgNPs and chitosan can be studied in detail.
- Detailed study of mechanism of antibacterial action of silver nanoparticles may be elucidated.
- Antimicrobial aspect of pristine and silver loaded hydrogels can be taken further for inspection in water disinfection and various biomedical applications like wound dressing materials etc.

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