Indian Journal of Science and Technology Vol. 5 No. 2 (Feb 2012) ISSN: 0974- 6846

Silver nanomembrane and ceramic silver nanofilter for effective removal of water borne diarrhoegenic *Escherichia coli*

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Abstract

The provision of safe drinking water to rural and urban areas is one of the challenges of present century in the realm of public health. The present study focuses on an *in vitro* testing of the effectiveness of nanoscale silver based filtration units for the removal of bacterial pathogen contaminated water. Clinical isolates of diarrhoegenic *Escherichia coli* (*E.coli)* in measured volume of water was tested for most probable number (MPN) of *E.coli* by multiple tube test. The water samples were filtered through the ceramic silver nanofilter, plain polysulfone membrane and polysulfone membrane coated with silver nanoparticles with a pore size small enough to retain the pathogenic *E.coli.* The water sample was filtered through plain polysulfone nanomembrane without silver nanoparticles and ceramic silver nanofilters and the results were compared. Silver nanomembrane and ceramic silver nanofilter effectively retained and destroyed the pathogenic organism and thus exhibiting better protection over the plain polysulfone membrane. Research on metal nanoparticles having significant antimicrobial effect would be utilized for making cost effective water filter systems which could protect us from the water borne diseases.

Keywords: *Polysulfone silver nanomembrane, Ceramic silver nanofilter, diarrhoegenic E.coli, membrane filtration method, water contamination.*

Introduction

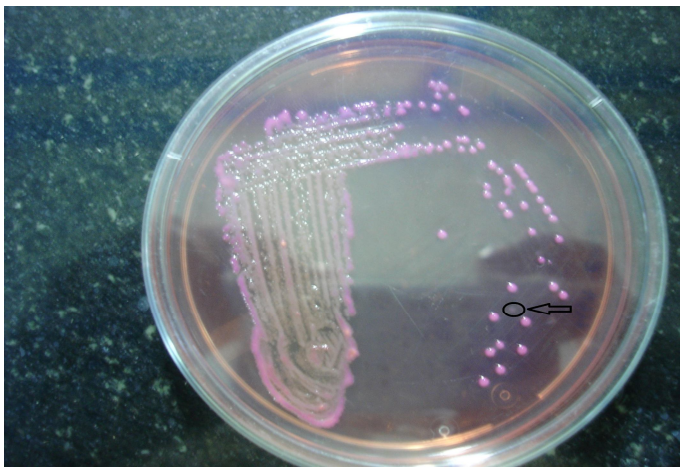
Nanofilters prepared by using various nanoparticles immobilised on polysulfone membrane are ideal matrices or templates used by researchers as an effective biocide against various bacteria and reduce the chance of formation of biofilm on the filter surface (Jun Sung Kim *et al.*, 2006; Mostafavi *et al*., 2009). Advances in nanoscale science and engineering are providing unprecedented opportunities to develop cost effective and environmentally acceptable water purification process (Savage & Diallo, 2005). Recently, membrane filtration in water treatment has been used worldwide for reduction of particle concentration and natural organic material in water (Tahaikta, 2007).

Varying levels of virus removal by membranes have been reported in the literature like complete removal of polio virus by using nanofiltration with membranes of 20 nm nominal pore size (Bohonak & Zydney 2005; Song, 2007). Nowadays polymer membranes are finding widespread applications. Polysulfone describes a family of thermoplastic polymers. These polymers are known for their toughness and stability at high temperatures. They contain the subunit aryl-SO_2-aryl, the defining feature of which is the sulfone group. Due to the high cost of raw materials and processing, polysulfones are used in specialty applications and often are superior replacement for polycarbonates (Munari *et al.*, 1988). Nanoscale silver, silver oxides and silver alloys can be prepared by wet chemical methods and immobilized on suitable polymeric or ceramic media (Zhang, 2003).

The *E.coli* count is the most useful test for detecting faecal contamination of water supplies in water quality analysis. Two principal techniques are available for counting faecal coliforms (Guidelines for drinking water quality, WHO, 1998) namely multiple tube/ most probable number (MPN) and membrane filtration.

The objective of our study is to develop a cost effective nanofilter for drinking water purification after testing the contaminated water. The expected outcome of this study is the development of the cost effective nanofilter that would be able to filter out bacteria, organic and inorganic impurities and undesirable dissolved metal

Fig.1.Non sorbitol fermenting bacterial colony in MSA agar plate



Indian Journal of Science and Technology Vol. 5 No. 2 (Feb 2012) ISSN: 0974- 6846

ions while keeping the water potable. This should prove to be of great utility in rural and urban areas.

Fig. 2. Hemolysis observed on washed sheep's RBC with $CaCl_2$ with controls

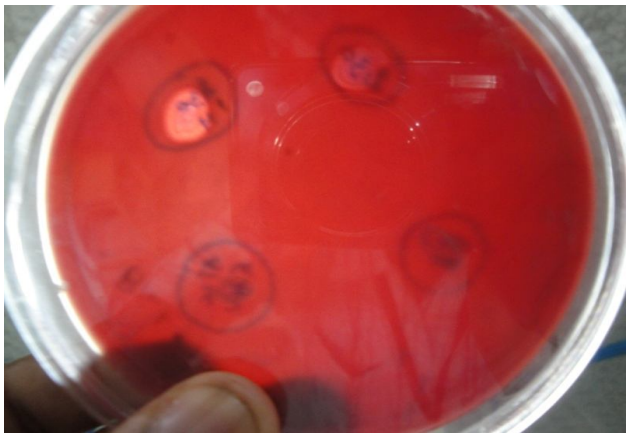
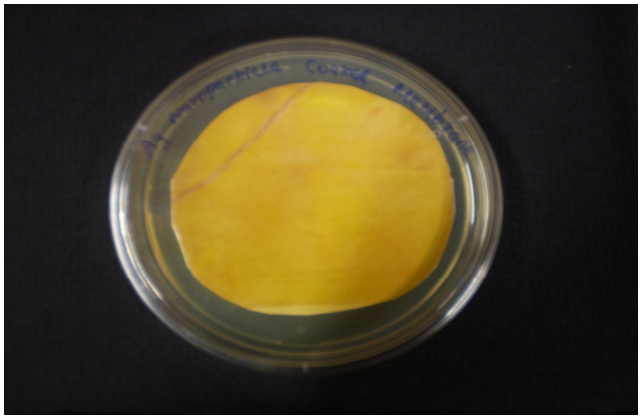


Fig.3. Plain Polysulfone membrane showing confluent growth of E.coli

Fig.4. No colonies on Silver nanomembrane.



Materials and methods

Preparation of water sample using diarrhoegenic E.coli

Stool sample was collected from a child admitted in SRM Hospital for acute gastroenteritis. The sample was cultured and colourless colonies of non sorbital fermenting *E.coli* on the selective medium MacConkey Sorbitol agar containing cefexime and potassium tellurite (CT-SMAC) were isolated (Fig.1). The strain was inoculated into a blood agar plate, made by washed sheep red blood cells with Calcium Chloride ($CaCl_2$). When the hemolytic zone was observed after 24 hours of incubation at 37^0C, it was confirmed as enterohemolysin producing *E.coli,* which is diarrhaegenic (Fig.2). The Enterohemolysin is active only on washed red blood cells in the presence of calcium (Ca 10 mMol/L in 5% defibrinated sheep blood washed three times) (Beutin *et al*., 1989).

Water quality analysis by Membrane Filtration Method and Multiple Tube Test to standardise the sample:

The stock culture was prepared using the above non sorbital fermenting, enterohemolysin producing *E.coli* colony by sub culturing in to nutrient agar from the selective medium (CT-SMAC) and the plates were incubated at 37^0C for 24 hours. The isolated single colony from nutrient agar plate was transferred to 5 ml of sterile nutrient broth and incubated at 37^0 C for 2 hours and the solution was made to match with 0.5 Mcfarlands standard ($1.5 \times 1O^8$ *E.coli* / ml). This was considered as a standard culture.

Different dilutions of water samples were prepared by mixing 5 µl, 10 µl, 20 µl and 30 µl of standard culture broth with 100 ml of sterile distilled water in different conical flasks. The samples were filtered through the sterile membrane procured from a commercial source (Sartorious) filter membrane of high quality 47 mm in diameter and 0.45 µm pore size (Marmion *et al*., 1996). Among the dilutions, the dilution of 10µl of standard culture in 100ml water sample showed the number of colonies in the acceptable range of about 74 colonies, after keeping the membrane on nutrient agar at 37^0C for 24 hours of incubation. According to the general formula, count per 100ml of water sample = (No. of colonies counted / Volume of sample filtered in ml) X 100, the usual acceptable range of count is between 20-80 colonies per membrane (Bordner *et al*., 1978). In the present study, we selected 10µl of standard culture for testing most probable number of *E. coli* in 100ml water by multiple tube method and membrane filtration.

Multiple tube / most probable number (MPN)

In this technique, a 100 ml water sample is distributed in tubes of sterile selective culture broth of different dilutions containing lactose and an indicator. After incubation of 24 hrs and 48 hrs, the media is to be inspected and the number of cultures of each volume of water that show the production of acid (colour change) and gas (a bubble large enough to fill the cavity at the top of the Durham tube) are to be noted. These acid and gas producing cultures are considered to be presumptive positive growth of coliform bacilli, cultures not showing production of both acid and gas at 48 hrs are considered to be negative. By referring to the tables of most probable

Indian Journal of Science and Technology Vol. 5 No. 2 (Feb 2012) ISSN: 0974- 6846

numbers in respect of the combination of positive and negative results observed, the most probable number of presumptive colifom bacilli present in 100 ml of sampled water can be ascertained (Swaroop, 1951).

Following this technique, five 10 ml volumes of water sample were pipetted aseptically into tubes containing 10 ml double-strength MacConkey medium with bromocresol purple as indicator and five 1 ml volumes into tubes containing 5 ml single strength medium. Then, a 1-in-10 dilution of the water in quarter strength Ringer's solution was prepared. Five 1 ml volume of the 1-in-10 dilution were pipetted into tubes containing 5 ml of single strength medium. The seeded media was incubated at 37^0C and observed for 24 hours and 48 hours. The colour change to yellow, the formation of acid from the lactose in the broth and the gas collection in an inverted Durhams tube placed in each tube of the medium confirmed the positive reaction. Cultures not showing production of both acid and gas after 48 hours were considered as negative.

We referred the revised tables of Tillet *et al.* (1988) to get the most probable number value with 95 % confidence limit. As per the results of our test, the following showed positive reaction matched with MPN value of 1600 *E.coli* per 100ml and most probable range (MPR) of 1350-1990 organisms (Tillet *et al.,* 1988).

- All the five tubes of 10 ml volume of sample
- Five tubes of 1 ml volume of sample
- Four tubes of 1-in-10 dilution of sample

After the standardization, three sets of 100 ml water sample contaminated with a clinical isolate of diarrhoegenic *E. coli* was filtered through the plain polysulfone nano sized pour membrane, silver coated polysulfone membrane and ceramic silver nanofilter.

Membrane filtration through nano membranes

According to the principle of this technique, a 100 ml water sample or a diluted sample is filtered through a membrane filter. The membrane, with the coliform organisms on it, is then cultured on a pad of sterile selective broth containing lactose and an indicator. After incubation, the number of coliform colonies can be counted. This gives the presumptive number of *E.coli* in the 100 ml water sample.

The polysulfone nanomembranes were prepared by the Dept of Nanotechnology, SRM University. All the chemicals used in the preparation of the membrane were obtained from commercial sources as guaranteed analytical grade and used without further purification. Silver nitrate was procured from Rankem, New Delhi. Sodium borohydride and Polyvinylpyrrolidone procured from SRL, Mumbai and Loba Chemi, Mumbai respectively. Polysulfone Resin pellets 75000 (GPC) and 1-methyl-2-pyrrolidone were procured from Acros Organics, Belgium. By phase inversion polymerization technique, the poly sulfone membranes were prepared with a diameter of 100nm pore sizes, small enough to retain the test organism to be counted.

The nano membrane was prepared by 18% of (w/v) polysulfone (PSF) dissolved in the solvent N-methyl pyrrolidone and heated at 65°C- 70 °C with continuous stirring to dissolve it completely until it formed a homogenous mixture, then 15% of (w/v) of polyvinyl pyrrolidone (PVP) was added and mixed. Sufficient time was given (8 hrs) for the polymers to dissolve, 0.1% silver nitrate was mixed, then this mixture was dragged on a glass plate and immersed in water containing reducing agent sodium borohydride to reduce silver nitrate to silver. This was kept in Elcometer for 24 hrs to form a film. Then the dry film was removed and kept in a vacuum. The thickness of the dry film was 150 µm, when measured using a screw gauge. Then the film was immersed in Milli - Q deionized water. The characteristic morphology of the membrane, their pore size and the number was studied using scanning electron microscopy (SEM) and X-ray diffraction (XRD).

The filtration apparatus was cleaned and sterilized by autoclaving at 120^0 C for 15 minutes. The polysulfone membrane was highly heat stable. The plain polysulfone membrane and silver coated nano membrane were sterilized by autoclaving for 10 minutes at 115^0 C. The plain polysulfone membrane was fitted facing upwards in the filtration apparatus. Then it was connected to a vacuum source. The first sample was filtered slowly through the nano membrane by applying a vacuum of about 500 mm of mercury. The membrane was transferred aseptically from the funnel, keeping its upper side upwards, on to a nutrient agar medium avoiding air bubbles from being trapped between the membrane and the medium. The same membrane filtration procedure was repeated for polysulfone membrane coated with silver nanoparticle with the second sample of same quantity. The third sample was reserved for the test with ceramic silver nanofilter.

The plates holding the membranes were incubated at 37^0 C for 24 hours. After 24 hours of incubation, confluent colonies were noticed on the surface of plain polysulfone membrane (Fig.3) and no colonies were observed on the surface of silver coated nano membrane (Fig.4). The colonies observed on plain polysulfone membrane were identified as *E. coli* since they formed acid and gas from lactose at 44^0C and indole from tryptophan at 44^0 C by routine confirmatory fecal coli form tests. The filtrate of both plain polysulfone membrane and silver coated polysulfone membrane were tested for most probable number *E. coli* per 100 ml water sample by multiple tube test method and it was observed that, MPN / 100 ml of filtrates was zero.

Preparation of ceramic silver nanofilter

The ceramic silver nano filter unit was prepared in nano laboratory. Aluminium oxide powder was added with sufficient amount of silica and the mixture was mixed well and the mixture made to bind each other by using sodium silicate as binder. This mixture was made as a pellet by using a molding apparatus and passing CO_2, and the

Indian Journal of Science and Technology Vol. 5 No. 2 (Feb 2012) ISSN: 0974- 6846

Table 1. Observation after the filtration of sample through different membranes and ceramic nanofilter

Sample No	Test samples	Plain polysulfone membrane		Polysulfone membrane with silver nanoparticles		Ceramic silver nano filter	
		Countable colonies on membrane surface	Filtrate (MPN/100ml)	Countable colonies on membrane surface	Filtrate (MPN/100ml)	Organisms on candle surface	Filtrate (MPN/100ml)
1	100ml	Confluent colonies	0	-	-	-	-
2	100ml	-	-	0	0	-	-
3	100ml	-	-	-	-	0	0

MPN= Most Probable Number

pellet was sintered at 1000^0 C for 1 hour. The silver nanoparticles were characterized by UV-visible spectroscopy and immobilized in suitable porous ceramic supports. The candle filtration apparatus was setup and used for the water filtration. The pore size of the ceramic filter was measured as roughly 1 µm using porosimeter.

Filtration in ceramic silver nanofilter

The 100 ml of the third water sample was filtered through ceramic silver nanofilter and the filtrate was collected aseptically. A sterile swab was taken and all the sides of candle surface of the ceramic filter were scraped and cultured on nutrient agar plate and incubated at 37^0 C for 24 hours. No colonies were observed. The filtrate was tested for MPN method and the MPN / 100 ml of filtrate was found to be zero.

Results

The observation after the filtration of the sample through different membranes was recorded (Table 1). The first sample filtered through plain polysulfone membrane showed confluent colonies on its surface, whereas the MPN per 100 ml of filtrate was zero. The second sample filtered through polysulfone membrane with silver nanoparticle showed no colonies on its surface and the MPN per 100 ml of filtrate was zero. The third sample filtered through ceramic silver nano filter showed no colonies on nutrient agar streaked with the swab taken from the candle surface after filtration. The filtrate of ceramic candle filter was completely sterile which was proved by MPN test. The final interpretation of these tests is the plain polysulfone membrane retained the organism on its surface as confluent colonies instead of countable colonies. The countable colonies can be obtained by improving the quality of polysulfone membrane. The polysulfone membrane with silver nanopartilces and silver coated ceramic nano filters destroyed the organisms completely. The filtrate of all the membranes were sterile.

Discussion

E. coli is regarded as the essential indicator organism of faecal pollution of human or animal origin in water and their presence indicates serious contamination of other microorganisms including viruses. Many research workers utilized *E.coli* ATCC strain as control for testing the water quality by different methods. We utilized clinical isolates of pathogenic *E. coli* as an indicator organism for testing the effectiveness of nano filters. The viability of the organisms on nano membranes or on the nano filters after filtration can be proved by routine sub culturing on nutrient agar and confirmed with routine biochemical tests.

In order to get countable colonies on nanomembrane after membrane filtration instead of confluent colonies, the exact fitting of the membrane on filtration unit, the surface integrity of the membrane, distribution of pores evenly on the surface of the membrane, quality of the membrane and the duration of filtration are to be improved during preparation of the membrane and during filtration. Further study is necessary to know the exact contact time of silver nanoparticles to inactivate or kill the microorganisms.

Silver ions and silver based compounds are highly toxic to micro organisms and showing strong biocidal effects on many bacteria including *E. coli*. Silver ions, silver based compounds and silver coated matrices or templates are being used by researchers as an effective biocide against various bacteria, also reduce the formation of biofilm on its surface and showing strong biocidal effects on as many as species of bacteria (Zhao & Stevens, 1998).

The growth inhibition effect of silver nanoparticles was observed by researchers in a concentration dependent manner and its minimum inhibitory concentration (MIC) against various organisms like yeast, *Staphylococus aureus, E. coli* were proved, but the mechanism of the growth-inhibitory effects of silver nanoparticles on micro organisms has not been well understood (Jun Sung Kim *et al.,* 2006). The silver nanoparticles show efficient antimicrobial property compared to other salts due to their extremely large surface area which provides better contact with microorganisms. Experiments showed the nanoparticles damaged the Escherichia coli cell wall by forming pits on it (Sondi & Salopek-Sondi, 2004). Also silver acts by inhibiting the uptake of phosphate. The silver ions interact with disulfide or sulfhydryl groups of enzymes, causing structural changes in bacteria that lead to disruption of metabolic processes followed by cell death (Feng *et al.,* 2000).

It has been widely reported that the antimicrobial activity of silver nanoparticles on microorganisms do not affect the quality of the water. It results in less negative

Indian Journal of Science and Technology Vol. 5 No. 2 (Feb 2012) ISSN: 0974- 6846

effects like minor alterations in taste, odour and colour of water and there is no toxic effect. It is safe to animal cells while toxic on bacterial cells. Silver has the most effective antibacterial action out of all the metals and the least toxins to animal cell (Guggenbichler *et al.,* 1999).

The nonporous polysulfone membranes formed by a phase inversion process are known to possess a uniform pore structure. The use of different polymers, which are the pore formers, and reduction in size of the polymers before the phase inversion allows one to adjunct the pore diameter quite reliably. The surface morphology of the nanoporous polysulfone membrane fabricated using phase inversion polymerization can be studied under SEM. As the nanoporous polysulfone membranes are ideal matrices or templates for the synthesis of nano structured materials and the membrane pore dimension can be reduced in the range of 1-100 nm by phase inversion polymerization technique (Rajashri Bhattacharya *et al.*, 2003). These type of nano sized pore membranes can be utilized for filtering water contaminated with several microorganisms having nano meter size. The pore size can be controlled to a significant extent by reducing the particle size of the pore former. The approximate pore dimension was observed to be in the 100 nm range. Further work on the effect of the thickness, surface, number and size of pores on the membrane is under way.

The commercially available water filter membranes made by cellulose ester have 47 mm in diameter and pore size of 0.45 µm. Organisms, especially viruses, causing water borne diseases vary widely in size, in the range of 20 nm to 300 nm. For example, rotaviruses with 75 nm in diameter size will easily pass through the membrane with mean pore diameter of 0.45 µm. The retaining capacity of nano membranes and the antimicrobial effect of nano membrane with metal particles have great application in drinking water purification. To get good quality membranes, the membranes of the pure polymers (PVP&PSF) should be homogenous, smooth and transparent. But due to the sensitivity of PVP to moisture and also because of the immiscible nature of the two polymers the membrane look turbid sometimes. The number and the size of the pores can be changed with increase in the content of the PVP of the blend of polymers.

The mechanism of the bactericidal activity of silver containing polymers like polysulfone is based on the release of silver ions (Ag+) through interaction with a liquid watery phase (Kumar & Munstedt, 2005) . The distribution of nanosilver particles on the membrane should be uniform. Therefore, in order to produce distributions of nanoparticles, where the composition of the alloy is uniform, a technique such as high energy ball milling is necessary. Since we do not have this particular facility as yet, we plan to prepare the materials by this technique using external facilities.

The use of metal nanoparticles for water disinfection is relatively new concept. High reactivity due to the large surface to volume ratio, nanoparticles is expected to play a crucial role in water purification. Several investigations have been carried out on the bactericidal effect of nanoparticles. Extensive work has already been done on the utility of different types of nanomaterials for destroying bacteria (Stoimenov *et al*., 2002). Internationally, the effort to develop nanoscale filters for water purification is a very active area of research, though to our knowledge a cost effective nanofilter that removes bacteria is not available. In India, there is a recent research publication from IIT- Madras on water filtration using nanoparticle coated polyurethane foam (Jain & Pradeep, 2005).

More recent techniques involve passing the sample through capillaries (Rotem *et al.,* 1979), membranes (Divizia *et al.,* 1989 a,b), hollow fibres (Belfort *et al.*, 1982) with pore sizes that permit passage of water and low molecular mass solutes but exclude viruses and macromolecules, which are then concentrated on the membrane or fibre. Most laboratories now use membranes or fibre system with cut off levels of 30-100 kDa. Compared to traditional water filtration media such as cellulose ester membrane, silver nano filters offer significant performance benefits including better biological control / defense, low operational costs achieved from reduced maintenance cycles, lower power consumption due to lower pressure drop profile, improved water recovery, improved recycling rates, reduced chemical inputs, reduced maintenance and replacement cycles (Guide standard protocol for testing microbiological water purifier 1987).

In future, particle size distributions in filters as well as concentration of nanoparticles in the immobilization medium will be optimized. The potability of drinking water and toxicity of nanometal ions in water will be tested according to the specifications of the Bureau of Indian Standards. Further study on the contaminant level of silver in filtered water and their damage to water filter equipment are under way.

Conclusion

Polysulfone membranes with Silver nanoparticles and ceramic nano filter are found to be effective against diarrhoegenic *E. coli* isolated from a clinical sample. Purification of water by conventional means can be quite expensive. The application of cost effective nanofilters in water purification and water disinfection will decrease the water borne pathogens in contaminated water and therefore reduces the water borne diseases. The effectiveness of nanoscale silver based filtration units for the removal of bacterial pathogens in contaminated water and testing of water quality was established as demonstrated by the results.

Indian Journal of Science and Technology Vol. 5 No. 2 (Feb 2012) ISSN: 0974- 6846

Acknowledgement

We thank Dr. M. Ponnavaikko, Vice Chancellor, SRM University and the staff of the department of Nanotechnology, SRM University.

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