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Synthesis and Characterization of Alginate Nanoparticles Encapsulating Selenium and their Antimicrobial Evaluation

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ABSTRACT

Biofilm interfere in antibiotic therapy, undergo gene transformation to become highly virulent, develop quorum sensing and protect bacteria from host immunity. To combat these biofilms the encapsulation method of nanoparticles formation using PEG can be used as they are better alternative to chemical and physical methods. In the present study, the antibiofilm and antioxidant properties of the selenium nanoparticles produced by using probiotics have been evaluated. TEM images of the prepared selenium nanoparticles showed a uniform distribution and their spherical morphology. Due to the small size of the nanoparticles, these could be easily taken up by the bacterial and fungal biofilm leading to their disruption. Nanoparticles showed inhibition of biofilm in the evaluated microbes as evident by decrease in protein and carbohydrate content.

Keywords: selenium, nanoparticles, bacterial biofilms, antimicrobial activity

Biofilms are universal, complex, interdependent communities of surface associated microorganisms enclosed in an exopolysaccharide matrix (Costerone *et al*. 1999). Biofilms create an environment that enhances antimicrobial resistance through lateral gene transfer within the mucopolysaccharide through a process of qourom sensing. Biofilm growth poses a major clinical threat in patients using indwelling devices like catheters and implants posing a constant source of recurrent and resistant infections. Various strategies are being explored to control biofilm formation including biological interventions such as pilicides which are compounds designed to inhibit pili which are extracellular fibers of bacterial cells helping in binding and colonization, enzymes which degrade EPS of biofilm, inhibiting quorum sensing by using different compounds, using low intensity electrical current, surface coating in catheter tubes and using bacteriophages (Vasanthi *et al*. 2014). Chemical methods include antimicrobial coatings of antibiotics, biocides and ion coatings on surface of indwelling devices which prevent biofilm formation by interfering with the attachment and expansion of immature biofilms. Mechanical methods include generation of hydrophobicity, surface roughness and surface charges.

Nanoparticulate formulations hosting antimicrobial compounds including those of metals present a promising approach to combat biofilms owing to their small size and high penetration potential. Several different metals are used to produce nanoparticles which include gold, silver, alloy, selenium nanoparticles using the microorganisms. Selenium is a trace element commonly found in the materials of the earth's crust. It occurs in different forms as red amorphous selenium (Se^0), highly water soluble selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) and as gaseous selenide (Se^2). Elemental selenium is considered as the least toxic of all selenium forms. Normally Se is available as selenate and selenite oxoanions (Deepa and Ganesan, 2015).

In the present study selenium has been encapsulated in polyethylene glycol nanoparticles and evaluated as an antibiofilm agent against the sessile oppurtunistic bacteria and fungi.

MATERIALS AND METHODS

All reagents used in the study were of analytical grade.

Micro-organisms: *E.coli* (MTCC 118), *Staphylococcus* aurens (MTCC 96), *Psuedomonas aeroginosa* (MTCC 2488), *Klebsiella pneumoniae* (MTCC109), *Bacillus subtilis* (MTCC 121) and *Candida albicans* (MTCC 183) were obtained from, MTCC, IMTECH, Chandigarh.

Methods for detection of biofilm (Christensen *et al*. 1995):

Tube method: 10 ml of Trypticase soy broth with 1% glucose was inoculated with a loopfull of test organism from overnight culture on nutrient agar individually. Broths were incubated at 37^0 C for 24 hours. The cultures were decanted and tubes were washed with phosphate buffer saline pH7.3. The tubes were dried and stained with 0.1% crystal violet. Excess stain was washed with deionized water. Tubes were dried in inverted position and observed for biofilm formation. Biofilm Production was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined.

Congo Red Agar Method (CRA): Prepared 250 gm nutrient agar medium and Congo red stain as concentrated aqueous solution separately and autoclaved at 121 C for 15 minutes. Then it was added to with sucrose at 55°C. Plates were inoculated with test organism and incubated at 37°C for 24 to 48 hours aerobically. Black colonies with a dry crystalline consistency indicated biofilm production; weak producers usually remained pink, though occasional darkening at the centre of colonies was observed.

Characterization of biofilms: The biofilms were characterized for their protein and carbohydrate content, pre- and post-treatment with selenium nanoparticles.

Carbohydrate content: For determination of carbohydrate content, modified DNSA (3,5-dinitrosalicylic acid) method was employed (Miller, 1959).

Protein content: For determination of protein content in biofilms, Doumas method (1981) was employed.

Preparation of nanoparticles: Nanoparticles containing sodium selenite were prepared by using Nano precipitation method (Peltonen *et al*. 2002). Drug was dissolved in water, and then cosolvent (acetone) was added into this solution. A cosolvent was needed in order to make the inner phase more homogeneous. The biopolymer (sodium alginate) was used and dissolved in chloroform. This solution was added to drug solution to form dispersion. The dispersion was added to ethanol and separated by centrifugation. The transmission electron microscopy of selenium nanoparticles is shown in Fig. 3(a,b). It indicated that selenium nanoparticles have a discrete spherical shape without aggregation. The particles size of the nanoparticles varied somewhat among the formation due to variation in the composition of formations.

Nanoparticle characterization

Samples for transmission electron microscopy (TEM) analysis were prepared by drop coating selenium nanoparticles solution on to carbon coated copper TEM grids. The films on the TEM grids were allowed to stand for 2 minutes. The extra solution was removed using blotting paper and the grids were dried prior to measurement.

Biofilm Inhibition assay (O'toole *et al.* 2000)

Biofilms were cultured in microtitre plates stained with crystal violet and different concentrations of selenium nanoparticles were incubated with sessile cultures of bacteria and fungi. The disruption of biofilm was studied by reading the decrease in intensity of the dye at 570 nm.

Antioxidant activity assay

The antioxidant activity of the selenium nanoparticles was tested using Ferric thiocyanate (FTC) method (Kikuzaki and Nakatani, 1993). The FTC method was used to measure the amount of peroxide at the beginning of the lipid peroxidation, in which peroxide react with ferrous chloride and form ferric ions. The ferric ion then combines with ammonium thiocyanate and produce ferric thiocyanate. The substance is red in colour. The thicker the colour, higher the absorbance.

RESULTS AND DISCUSSION

Biofilm Formation and Characterization: Different bacterial strains were used for the production of biofilm i.e. *E. Coli, Pseudomonas aeruginosa and Staphyloccus aureus* as these are known biofilm producers.

1. Congo Red Agar method: It is an efficient method to determine the biofilm formation of different micro-organisms. Colonies of different colours were observed as shown in Fig. 1(a), 1(b) and 1(c).

Black colonies – Strong biofilm formation,Red colonies –Moderate biofilm formation Pink colonies – Weak biofilm formation

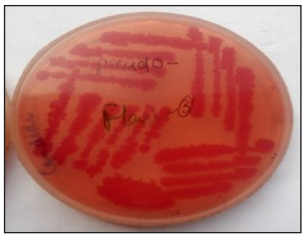


Fig. 1(a) *E. coli*

Fig. 1(b) *Pseudomonas aeruginosa*

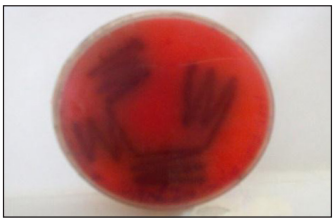
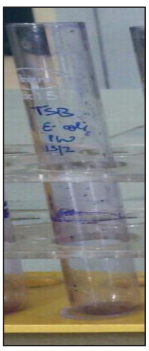


Fig. 1(c) *Staphylococcus aureus*

2. Tube method: Biofilm Production was considered positive when a visible film lined the wall and bottom of the tube. Tubes containing bacterial cultures were stained with crystal violet. Then, tubes were examined. Results are shown in Fig. 2(a), 2(b) and 2(c).

2(a) *E. coli*

2(b) *Pseudomonas*



2(c) *Staphylococcus*

Biofilm ring formed in tubes when stained with 0.1% crystal violet.

These results are nearly identical as reported by Vasanthi *et al.* (2014) that all the microorganisms used are biofilm producers as depicted by both the methods used for biofilm formation and detection.

Synthesis and characterization of Selenium nanoparticles

Sodium alginate encapsulated Selenium nanoparticles were prepared. Due to greater stability and due to their easier manufacturing these offer advantages like increased bioavailability, site specific drug delivery, sustained release of drug over longer period of time, retention of dosage from entire length of gastrointestinal tract and convenient to patient`s due to reduction in frequent dosing. TEM images of the prepared selenium nanoparticles showed a uniform distribution and spherical morphology. The depicted nanospheres in the TEM images were ranging in size from 3-8 nm in diameter which characterizes its morphology.

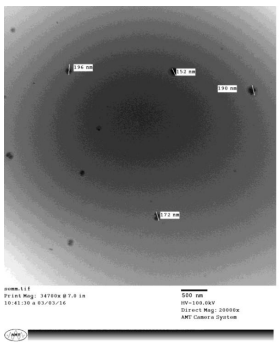


Fig. 3(a): TEM image of Selenium particles

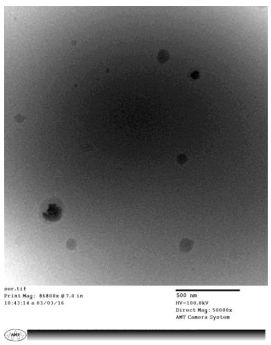


Fig. 3(b): TEM image of Selenium particles

Fig. 3(a) and 3(b) shows selenium nanoparticles in the range of 3 nm to 8 nm on scale, respectively. The Transmission Electron Microscopy (TEM) is one of the most widely used methods for the morphological characterization of nanoparticles. TEM is a vital characterization tool for imaging nanomaterials to obtain quantitative measures of particle, size distribution and morphology. The nanoparticles obtained in the present study were of relatively smaller size than that reported by Eszenyl *et al*. (2011) who synthesized Nano selenium using *Lactobacillus* spp. and obtained nanoparticles with the sizes of 100-200 nm.

Antioxidant Activity

The antioxidant activity of the selenium nanoparticles was determined with the oxidation of linolenic acid for five days. Ascorbic acid was used as a control against the Se NPs. Red colour was formed which increases gradually over time. The absorbance of selenium nanoparticles were observed as function of time. Unlike ascorbic acid, nanoparticles inhibited linolenic acid oxidation at higher rates and the activity gradually increases over time.

Table 1: Antioxidant activity for different concentrations of nanoparticles as compared to ascorbic acid P< 0.01, value is very significant p< 0.001, value is highly significant, P< 0.0001, value is very highly significant

Samples	Day 1	Day 2	Day 3	Day 4	Day 5
Ascorbic	0.000 ± 0.000	0.0013 ± 0.00044	0.0013 ± 0.00044	0.0013 ± 0.00044	0.0013 ± 0.00044
Acid					
NPs	0.214 ± 0.0333**	0.250 ± 0.03***	0.389 ± 0.06**	0.51 ± 0127***	0.68 ± 0.069***

Ferric thiocyanate method employed the lipid peroxidation to form ferric ions which in turn produced ferric thiocyanate to give the resultant colour red. This colour was measured at 500 nm for five days after every 24 hour until the colour reached maximum. Ascorbic acid was used against the Se NPs. Red colour was formed which increased gradually over time. The absorbance of selenium nanoparticles were plotted as function of time. Unlike ascorbic acid, nanoparticles inhibited linolenic acid oxidation at higher rates and the activity gradually increases over time. The absorbance values with respect to days of incubation are given in details in graph. On 6th day, inhibition of lipid peroxidation by 100µl/ml of sample was calculated to be 57.5 ± 0.052% and 55.2 ± 0.0085%. Similar results were reported in silver nanoparticles, having antioxidant activity of 44 ± 0.95% at same concentration, by in Bathamizh *et al.* (2013).

Biofilm Inhibition: The selenium nanoparticles were incubated with microbial biofilms at different concentrations in 96 well tissue culture plates to evaluate their biofilm scavanging activity.

A concentration dependent antibiofilm activity can be observed upon incubation of selenium nanoparticles with bacterial biofilms. This can be attributed to antioxidant potential of selenium and high penetration and small size of nanoparticles.

Table 2: Biofilm inhibition assay at different concentrations of selenium nanoparticles

Micro-organisms	Conc. of NPs (mg/ml)	Mean % Inhibition
E. coli	Ampicillin (2µg/ml)	63.69 ± 0.37
	Se NPs (1mg/ml)	67.38 ± 0.96*
	Se NPs (1.5 mg/ml)	71.75 ± 0.16*
Pseudomonas	Ampicillin (2µg/ml)	52.43 ± 0.25
	Se NPs (1mg/ml)	55.03 ± 0.38
	Se NPs (1.5 mg/ml)	58.76 ± 0.41*
Staphylococcus	Ampicillin (2µg/ml)	46.54 ± 0.39
	Se NPs (1mg/ml)	76.58 ± 0.41**
	Se NPs (1.5 mg/ml)	80.87 ± 0.33**

*P< 0.1, *value is significant; p<0.001, **value is highly significant; P<0.0001, ***value is very highly significant.*

In recent years, the utilization of metal ions and metal nanoparticles has emerged as an alternative to the use of organic compounds as antimicrobial agents (Duran *et al*. 2013). Indeed, a widespread antimicrobial activity is often a common trait of nanomaterials, mainly due to the high surface to volume ratio of their constituent particles which results in a high reactivity. This opens a new perspective for these nanoparticles in terms of coating agents in medical devices and health-related products to prevent bacterial infections.

Evaluation of Biochemical composition of Biofilm matrix: Biofilms are universal, complex, interdependent communities of surface associated micro-organisms. The organisms are enclosed in an exopolysaccharide matrix occurring on any surface, particularly aquatic and industrial water systems as well as medical devices. Se NPs reduces their protein content.

Estimation of Biofilm Disruption

Due to the action of nanoparticles on the biofilms, the protein and carbohydrate content which forms the matrix of biofilm is altered. As shown in table 3 and table 4 disruption of biofilm was accompanied with a decrease in the amount of protein and carbohydrate associated with sessile bacteria. The major functional categories of genes up regulated in biofilms are those implicated in transcription regulation, protein synthesis, amino acid synthesis, cell wall synthesis, efflux pumps and adhesions (Bouza *et al*. 2014).

Table 3: Effect of selenium nanoparticles on disruption of biofilm proteins

Micro-Organisms	Protein content before NP's action	Protein content after NP's action (1mg/ml)	Protein content after NP's action (1.5mg/ml)
E. Coli	3.4mg/ml	1.76mg/ml	1.5mg/ml
Pseudomonas	3.8mg/ml	2.25mg/ml	0.8mg/ml
Staphylococcus	4.6mg/ml	1.7mg/ml	1.3mg/ml

Estimation of protein content

Estimation of carbohydrate content by DNSA method: As nanoparticles act on the biofilm, carbohydrate content can be changed. Therefore carbohydrate estimation was done by using DNSA method which determines the remaining carbohydrate content of the biofilm after the activity of selenium nanoparticles. The intensity of the red colour determines the content of the carbohydrate in the biofilm.

Estimation of Carbohydrate content

Table 4: Effect of selenium nanoparticles on disruption of biofilm carbohydrates

Micro-Organisms	Carbohydrate content before NP's action	Carbohydrate content After NP's action (1mg/ml)	Carbohydrate content after NP's action (1.5mg/ml)
E. coli	1.58 $\pm$ 0.04 mg/ml	0.86 $\pm$ 0.02 mg/ml	0.78 $\pm$ 0.04 mg/ml
Pseudomonas	0.88 $\pm$ 0.02 mg/ml	0.75 $\pm$ 0.03 mg/ml	0.68 $\pm$ 0.03 mg/ml
Staphylococcus	2.33 $\pm$ 0.02 mg/ml	1.64 $\pm$ 0.06 mg/ml	1.24 $\pm$ 0.05 mg/ml

CONCLUSION

Selenium nanoparticles synthesized using biological method present a low cost and effective strategy to combat the growth of biofilms which are a major cause of antimicrobial therapy. These nanoparticles offer a dual benefit of better penetration in biofilm due to small size and antioxidant activity responsible for biofilm disruption owing to the presence of selenium.

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