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RESEARCH ARTICLE

STUDY OF EGGSHELL MEMBRANE EMBEDDED WITH BIOSYNTHESIZED SILVER NANOPARTICLES FOR THEIR ANTIBACTERIAL ACTIVITY

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ABSTRACT

Facile, efficient, and robust immobilization of metal nanostructures on porous bioscaffolds is an interesting topic in materials chemistry and heterogeneous catalysis. This paper reports an *in situ* method for the synthesis and immobilization of small silver nanoparticles (AgNPs) at room temperature on natural eggshell membrane (ESM). The eggshell membrane is the clear film lining the eggshell, visible when one peels a boiled egg, which presents interwoven fibrous structure and can be used as a unique protein-based biotemplate. Many chemical and physical techniques are available for the synthesis of antimicrobial silver nanoparticle (AgNPs), but the green synthesis is the most emerging method of synthesis. Here the silver nanoparticles were synthesized using *Ocimum sanctum* leaf extracts which act as both reducing and capping agent. Visual color change, UV spectroscopy, scanning electron microscopy and transmission electron microscopy unambiguously identifed the presence of AgNPs on ESM. Besides the antibacterial activity of these ESM's was seen through the zone of inhibitions observed for the test samples against plates of Escherichia coli. Such egg membranes can be used against skin wounds like that in burns wherein the AgNPs can prevent the bacterial infections and the presence of ESM will also help the recovery of the skin as the amino acid composition of the egg membranes is quite close to that of human skin and other human tissues.

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INTRODUCTION

Silver nanoparticles (AgNPs) with zero, one, or two-dimensional nanostructures such as nanoparticles, nanowires, and nanocubes have been the subject of focused research due to their great biocidal potential. Many techniques are known to be available with the evidence of publication for the synthesis of AgNPs. Silver nanoparticles have unique properties which help in molecular diagnostics, in therapies, as well as in devices that are used in several medical procedures. The major methods used for silver nanoparticle synthesis are the physical and chemical methods. The problem with the chemical and physical methods is that the synthesis is expensive and can also have toxic substances absorbed onto them. To overcome this, the biological method provides a feasible alternative. The major biological systems involved in this are bacteria, fungi, and plant extracts. Biological generation of AgNPs, especially using plant extract is known to produce large quantities of nanoparticles that are free of contamination and have a well-defined size and morphology which is important for diagnostic application (Hutchison *et al*., 2008; Amit Kumar Mittal *et al*., 2013). In most of the therapeutic applications, it is the antimicrobial property that is being majorly explored, though the anti-inflammatory property has its fair share of applications. So here we have used a unique green synthesis method of synthesizing AgNPs by using leaf extract of *Ocimum sanctum* i.e. Tulsi and eggshell membranes (ESM).

ESM is bilayered membrane which contains some proteins which are antimicrobial in nature (due to presence of lysozymes). It is used in remedy of some diseases from ancient era. It can be easily obtain from an eggshell which is waste product (ESM History, 2014; Wong *et al*., 1984). The composition of ESM is mainly water-insoluble glycoproteins such as collagen (types I, V and X), and amino acids like glycine, alanine and uronic acid. Wong *et al*., reported the presence of Type I and Type V proteins in the inner and outer shell membranes whereas Arias et al., observed the presence of Type X collagen in the egg shell membrane.

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A few known major applications of ESM are as a matrix for the recovery of heavy metals, as a template for hierarchically ordered macroporous materials, and for biosensing of enzyme immobilized Au-ESM42–45 (ESM History, 2014; Wong *et al*., 1984; Arias *et al*., 1991; Moulton *et al*., 2010). ESM possess not only antimicrobial property but also ability to produce metallic nanoparticals

MATERIALS AND METHODS

Preparation of *Ocimum sanctum* leaf extract

10 g of *Ocimum sanctum* (Tulsi) leaves were taken. They were surface sterilized and finely chopped. They were then boiled in 50 mL of deionized water and stirred for 1 h. The mixture was then cooled, filtered through filter paper and stored at 4^0C for further use. (Debajit Borah *et al*., 2013)

Separation of egg membranes from egg shells

Eggshell membrane is visible as a clear film lining the eggshell, on boiling the egg. Thus 4 eggs were boiled and the ESM was simply peeled off. (Hincke *et al*., 2000)

Synthesis of AgNP'S

5 mL of the leaf extract was added in 45 mL of 10^{-3} M silver nitrate ($AgNO_3$) solution. Also 0.35g of separated ESM was added to reaction mixture. In the reference blank only the 10^{-3} M $AgNO_3$ solution was replaced by distilled water. The reaction was carried out at room temperature for 2 to 3 hours. The change of colour from yellow to reddish brown indicates the formation of Ag nanoparticles. (Charusheela Ramteke *et al*., 2013)

Analysis of bioreduced AgNP's

The initial synthesis was monitored by color change at 1st and 3rd hr. UV- vis analysis of the reaction solutions was carried out at room temperature on "Shimadzu UV-1800"double beam UV-visible spectrophotometer at a resolution of 1 nm. UV-VIS spectrophotometric analysis was carried out at 3rd hr using cuvette of path length 10 mm to confirm the presence of AgNPs. The measurements were carried out as a function of reaction time at room temperature. (Jin Zhang *et al*., 2014) Nano-silver samples for TEM were prepared by placing a drop of silver nanoparticle solution on carbon coated copper grids and allowing for the complete evaporation of water. The shape and size of the formed nano-silver was analyzed from the conventional TEM micrographs recorded at 120kv on JEM 2100, JEOL, Japan. The surface properties of the egg membranes were also studied using scanning electron micrographs performed on high pressure Hitachi SEM S3400N. For the SEM characterization the ESM samples were mounted on a double faced conductive adhesive tape. Images were obtained at an accelerating voltage of 15-kv. (Miao Liang *et al*., 2014; Dong Yang *et al*., 2003)

Assessment of antibacterial activity

In order to examine the antibacterial activity of the AgNPs on selected bacteria (*E. coli*), the well diffusion and a mimic of standard disc diffusion was carried out using egg membranes containing AgNP's.100ml of nutrient agar (HiMedia) was prepared in sterile flask. A fresh loopful of culture of *E.coli* was inoculated in the nutrient agar flask under sterile conditions. Pour plate technique was performed for having 6 petri dishes. Well diffusion: Holes were punched on the solidified agar plates using gel punch.45µl of AgNP containing solution was added to the wells. Disc diffusion: A test strip of egg membranes containing AgNPs was placed onto solidified agar plates using sterile forcep .Both the plates were placed in refrigerator for 15mins to facilitate diffusion. Antimicrobial activity was demonstrated by zone of inhibition on and around the test strips.

RESULTS AND DISCUSSION

Formation of AgNPs by reduction of silver nitrate during exposure to Tulsi leaf extract and ESM can be easily monitored from the change in colour of the reaction mixture. Silver nanoparticles bear a characteristic yellow brown colour due to the excitation of surface plasmon vibrations. The change in color of the reaction mixture from 0th hour and after 3 hours is presented in Figure 1 and 2 respectively, indicating the formation of AgNPs.

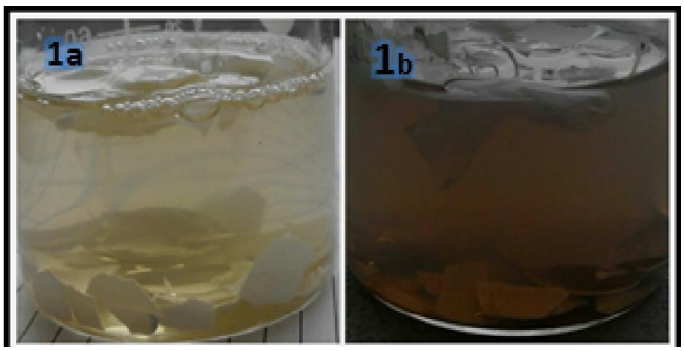


Fig. 1. Colour change in reaction mixture at 0 hours. (a) Control-ESM+ 5ml *O.sanctum* extract+45ml D/W water. (b)Test- ESM+ 5ml *O.sanctum* extract+45ml AgNO3 solution (1mM)

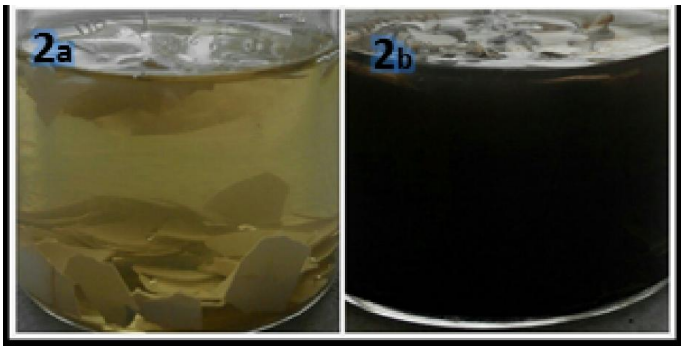


Fig. 2. Colour change in reaction mixture at after 3 hours. ; (a) Control-ESM+ 5ml O.sanctum extract+45ml D/W water. (b)Test-ESM+ 5ml *O.sanctum* extract+45ml AgNO3 solution (1mM)

This formation indicates that silver ions in reaction medium have been converted to elemental silver having the size of nanometric range. Natural antioxidants have been reported to have strong reducing ability. As Tulsi possess a potent antioxidant activity, we can attribute the reduction process to

the presence of high quantity of antioxidants in the leaves extract. Also the composition of ESM and the aldehyde moieties on its surface can contribute to reduction of silver ions. (Dong Yang *et al*., 2003) A simultaneous colour change of the membrane from white to yellow was noticed, suggesting the adsorption of Ag^0 on the membrane surface.

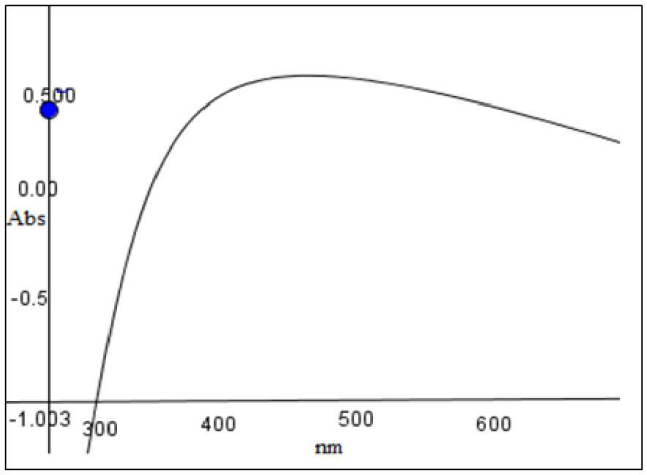


Fig. 3. UV-Vis spectrum image showing peak at 469 nm indicates the formation of AgNPs by aqueous extract of O. sanctum

Figure 3 gives a UV-Vis absorption spectrum for the AgNP samples synthesized using *O.sanctum* leaf extract and ESM membranes with its peak at 469nm.It is observed that the silver surface plasmon resonance (SPR) occurs at 450 nm and steadily increases in intensity as a function reaction time. The reduction of silver ions was quite rapid. More than 70% of the reaction was complete within 60 minutes of the reaction time. Generally, biosynthetic methods are considered as time consuming when compared with chemical methods.

As given by the studies of Ramtheke *et al.,* reaction time of at least 12 hours is required in plant-mediated nanomaterials synthesis. However, the time consumed in the present study for the reaction to complete is several fold lesser than reported. Such alacrity in reaction time can be the outcome of potent antioxidant activity of the Tulsi extract, which makes the reaction much more efficient than others. (Govindaraju *et al*., 2010; Mandal *et al*., 2006; Huang *et al*., 2007; Ankamwar *et al*., 2005; Li *et al*., 2007; Song and Kim, 2008; Jain *et al*., 2009). According to previous literature, this metallic Ag+ reducing ability of plant-extracted polyphenol is attributed to the multiple hydroxyls that can chelate with the Ag^+ ions and reduce the chelated Ag^+ into Ag^0 in situ.

The difference in the peaks as reported in different papers is due to the influence of concentration of reducing agent, size and shape of the nanoparticles formed. The SPR peak of AgNPs are influenced by especially for the NPs with small diameters. Therefore, the morphology and size distribution of AgNPs cannot be deduced simply through the SPR peak, but they should be examined further by TEM analysis. We obtained the absorption maximum at 469 nm which is close to the values of surface plasmon resonance of silver nanoparticles. The plasmon band is broad due to the presence of components in Tulsi extract which are also being read in the spectrophotometric range (Huang *et al*., 2010; Bulut and Ozacar, 2009; Guo *et al*., 2011; Moulton *et al*., 2010; Dadosh *et al*., 2009; Peng *et al*., 2010). Yet the absorption max at 469nm confirms the presence of AgNP's.

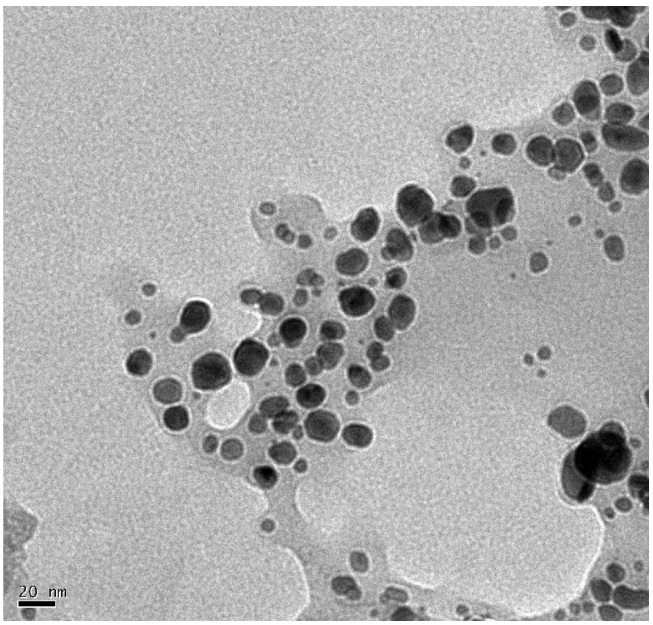


Fig. 4. TEM images of AgNPs synthesized with O.sanctum leaf extract

TEM and SEM were also used to investigate nanoparticle morphology. In the TEM analysis, a heterogeneous population constituting of nanostructures morphologically different in size and shape can be observed. The histogram of particle size reveals dimensions ranging from 5nm to 30nm with a maximum frequency of 15nm.

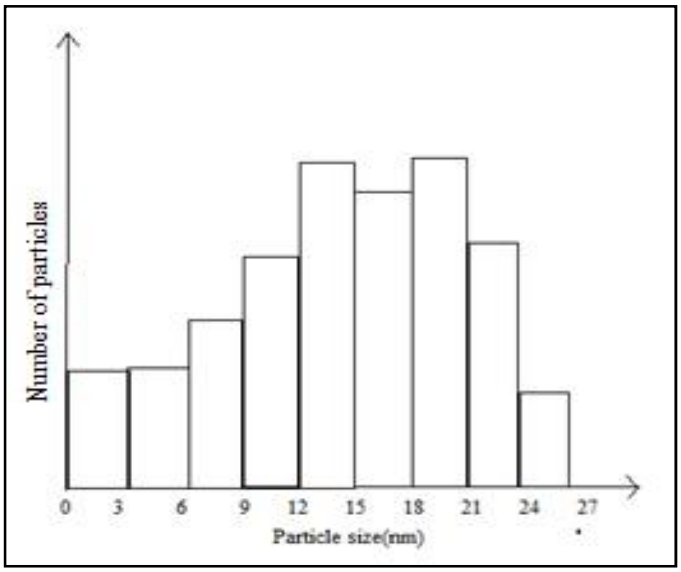
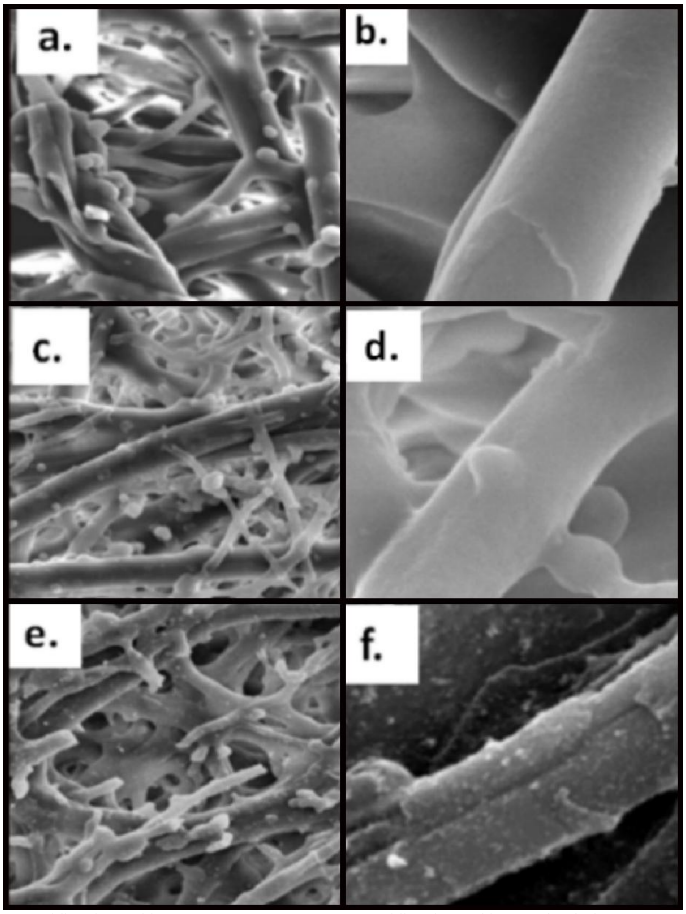


Fig. 5. Frequency distribution histogram for particle size of AgNPs

The result was in agreement with that obtained by DLS analysis. The TEM results indicate that the formed small-sized NPs are predominantly spherical and well-dispersed without agglomeration in the aqueous solution.

SEM analysis was performed to characterize the morphology changes of ESM. Figure 6a, b shows the typical SEM images of the natural ESM at different magnifications. The biomembrane exhibited a structurally macroporous network constructed of interwoven and coalescing protein fibers with a diameter ranging from 0.5 to 2.0µm.

ESM was seen with good permeability that allows reactants to contact the inner fibers sufficiently. . The high magnification image of ESM+ *O. sanctum* presented in Figure 6d and 6e reveals that the surface of the fibers is slightly rougher than that of the natural ESM, indicating the successful grafting of plant extracts and thus some of the biomolecules onto the fiber surface. The inherent interconnected fibrous structure of ESM is still well preserved, and the synthesized AgNPs can also be well observed in Figure 6e and 6f as microdots evenly distributed on the ESM surface.



Magnification of image a, c, e = 20µm, Magnification of image b, d, f = 3µm

Fig. 6. SEM images of natural ESM (a, b), *O.Sanctum*+ESM (c ,d), and AgNPs + *O.sanctum* + ESM (e, f) at different magnifications

The SEM result which picturised the presence of AgNPs on the surface of ESM's .This further opened gates to assess the antibacterial activity of AgNPs on impregnated on ESM against bacterial cultures.

Figure 7 shows the well diffusion image of the AgNP solution against a *E.coli* plate. The assay pictured here give the zones of inhibition and the test is called the Kirby-Bauer Test. (Hirotaka Koga and Takuya Kitaoka, 2010)

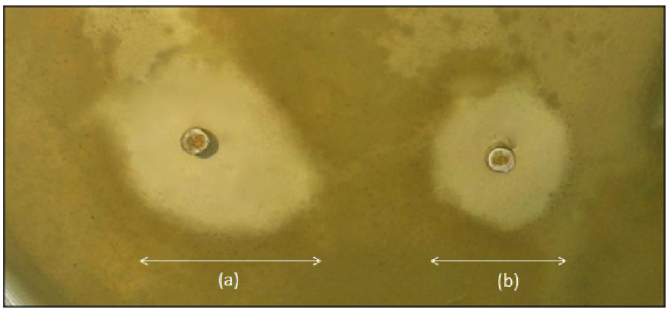


Fig. 7. Plates showing diameters of zones of inhibition against *E. coli.* (a) AgNPs +*O.sanctum* + ESM = 2.1 cm ; (b). Tulsi extract+ water control =1cm

It is used clinically to measure the antibiotic resistance. In the present study the zones of inhibition measured for AgNP solution was 2cm and for *O. sanctum* extract was 1cm respectively. Both the samples are known to possess antibacterial activity, but that seen from AgNP synthesized from the *O. sanctum* extract is significantly greater than that of *O. sanctum* solution. Thus biosynthesized AgNPs are twice more effective than the crude plant extract.

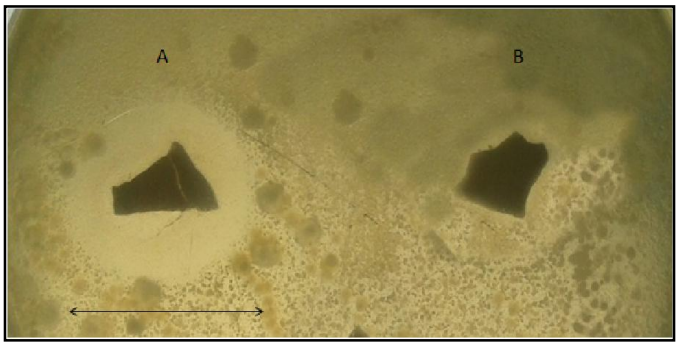


Fig. 8. The zone of inhibition were seen as: (A)Test sample: ESM dipped in ESM + *O.sanctum* +AgNO3 solution= 2.3cm (B)Control sample: ESM dipped in ESM + *O.sanctum* +water = none

The antibacterial properties of the AgNP impregnated ESM's, from the diameters of inhibition obtained after they were incubated on a *E.coli* plate for 24 hours at 37^0C.The ESM dipped in *O. sanctum,* to surprise gave no zone of inhibition while the ESM's present in AgNP solution gave a zone of inhibition measuring 2.3cm. This result indicates that the AgNP's impregnated on the ESM's are infact responsible for the antibacterial activity and not merely the ESM or *O.sanctum* extracts adhered to the surface of ESM.

This bioadsorptive potential of ESM to accommodate metallic nanoparticles can be used in case of wound dressing were in the ESM can act as natural bioscaffold with a synergistic effect of antibacterial activity and also the wound healing potential due to the presence of collagen proteins in the ESM. (Sukumaran Prabhu and Eldho K. Poulose, 2012) As exact mechanism of their action still not clearly known there are some theories which explains their mode of action. Silver nanoparticles have the ability to anchor to the bacterial cell wall and subsequently penetrate it, thereby causing structural changes in the cell membrane like the permeability of the cell membrane and death of the cell.

(Sondi and Salopek-Sondi, 2004) The formation of free radicals by the silver nanoparticles may be considered to be another mechanism by which the cells die (Danilcauk *et al*., 2006; Kim *et al*., 2007). Antimicrobial effect of AgNPs is efficiently seen against gram negative *E.coli* (Lee *et al*., 2007; Shah *et al*., 2008; Wang *et al*., 2008; Zheng *et al*., 2010) and gram positive *B*. *subtilis*. Hence, it is beneficial to use ESM's with AgNPs in burn treatments, as they are majorly effective against *E.coli* which is generally found on burn wounds (Abdelrahm *et al*., 2014). Besides as reported by Debojit et.al the biosynthesized AgNP's can be also effective against drug resistance bacterial infection.

Advantages of this method of nanoparticle synthesis over other methods include cost effectiveness, easy scale up for large scale synthesis and eco-friendly method for biomedical applications. The major benefit is that the green synthesis using *O.sanctum* always takes place extracellularly and the reaction time is very short compared to that of microbial synthesis.

Conclusion

In conclusion, we demonstrated that *O.sanctum* leaf extract and ESM had reductive properties to synthesize AgNPs. Such ESM's bear many potential applications where treatment of bacterial infection is a critical task for antibiotics too. Our future scope includes a design of ESM bandage carrying silver nanoparticles that can act against bacterial burn infections. The AgNPs could be tuned through controlling the concentration of $AgNO_3$ and the *O. sanctum* extract. This method is low-cost, simple and straightforward for large scale production of AgNPs. More importantly, these prepared AgNPs are known for higher activity and stability in antibacterial activity. The *in vivo* tests for wound healing properties of such membranes are in progress and present a novel way for preliminary treatment of open wounds.

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