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Research Article

Silver Nanoparticles Crosslinked with Chitosan–Montmorillonite Nanocomposites for Antibacterial Activities

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Abstract: In this present work, preparation of silver nanoparticles using Aloe Vera extract (A-AgNPs) crosslinked with chitosan (CS)-montmorillonite (MMT) nanocomposites called as (A-AgNPs-CS/MMT) by employing the solution casting method. Further the formation of nanocomposites A-AgNPs-CS/MMT was investigated using UV-visible spectrophotometer, XRD, FTIR, TEM, TGA and DLS. The AgNPs-CS/MMT exhibited good antimicrobial activity against both Gram-negative and Gram-positive organisms. It could be seen that AgNPs-CS/MMT exhibited excellent antimicrobial activity.

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1. Introduction

With nanotechnology, a large set of materials and improved products rely on a change in the physical properties when the feature sizes are shrunk. Nanoparticles for example take advantage of their dramatically increased surface area to volume ratio (Florence et al., 2010; Nguyen et al., 2010; Doll et al., 2013). The biological and medical research communities have exploited the unique properties of nanomaterials for various applications. Terms such as Biomedical Nanotechnology, Nanobiotechnology, and Nanomedicine are used to describe this hybrid field. Functionalities can be added to nanomaterials by interfacing them with biological molecules or structures. The size of nanomaterials is similar to that of most biological molecules and structures; therefore, nanomaterials can be useful for both in vivo and in vitro biomedical research and applications. Thus far, the integration of nanomaterials with biology has led to the development of diagnostic devices, contrast agents, analytical tools, physical therapy applications, and drug delivery vehicles (Rajeshkumar et al., 2017).

Silver nanoparticles (AgNPs), bound to a suitable antibody, are used to label specific molecules, structures or microorganisms (Doll et al., 2013; Xu et al., 2013). AgNPs tagged with short segments of DNA can be used for detection of genetic sequence in a sample. In recent decades, silver and some of its compounds such as silver nitrate (AgNO_3) have attracted the attention of researchers due to their inhibitory effects to Gram negative bacteria (Luderer et al., 2013; Doll et al., 2013; Xu et al., 2013; Yun et al., 2013; Moretti et al., 2013; Deng et al., 2013; Satyavani et al., 2011; Kim et al., 2007). Silver, in its many oxidation states has long been recognized as having an inhibitory effect towards many bacterial strains and microorganisms commonly present in medical and industrial

processes. One of the most powerful naturally occurring antiseptic materials with a severe bactericidal effect and believed low toxicity towards mammalian tissue, the deposition and insertion of silver ions to various products has been a highly appealing approach for the prevention of biofilm formation (Mei et al., 2017; Kang et al., 2017).

This paper highlights the design and development of novel plant mediated silver nanoparticles crosslinked with Chitosan-montmorillonite nanocomposites that target gram negative and positive bacteria and inhibit their growth, though we have tested for them against gram positive bacteria also.

The present study highlights the design and development of novel plant mediated silver nanoparticles embedded with Chitosan-montmorillonite nanocomposites that target gram negative and positive bacteria and inhibit their growth, though we have tested for them against gram positive bacteria.

2. Materials and method

Chitosan was purchased from HiMedia. Doxorubicin was purchased from Hindustan scientific, Cuttack, India. MMT was purchased from Southern Clay, USA. All other reagents and chemicals were used as the analytical grade. Millipore water was used in the entire experimental work.

2.1. Synthesis of AgNPs

AgNPs were synthesized by green synthesis method using medicinal plant leaf extract of Aloe Vera (AVE) in 100 ml of water containing 10 mM AgNO_3 at 37 °C for 12 h. The colour change from colourless to brownish color can be attributed to the formation of AgNPs in the reaction mixture (Tippayawat et al., 2016). The prepared silver nanoparticles was called A-AgNPs

2.2. Preparation of CS/MMT nanocomposites

The CS/MMT films were prepared using CS, MMT. CS solution was prepared by dissolving CS in a 2% (v/v) aqueous acetic acid solution, followed by centrifuging to remove the insoluble material. MMT was first swelled in 2% (v/v) aqueous acetic acid solution and then added to CS solution with continuous stirring (300 rpm) at 60°C for 4 h (Kasirga et al., 2012). CS/MMT film was prepared using different ratios of CS and MMT as shown in table 1. These were coded as CS, CS/MMT-1, CS/MMT-2, CS/MMT-3, CS/MMT-4 and CS/MMT-5 representing different wt % of MMT content (1%, 2%, 3%, 4% and 5 %,) respectively.

2.3. Preparation of A-AgNPs crosslinked with CS/MMT

A-AgNPs (5ml) were mixed in CS/MMT (2:4) solution and incubated on a magnetic stirrer at 100 rpm overnight. Free CS/MMT particles were removed by centrifugation at 5000 rpm for 25 minutes. Crosslinked A-AgNPs with by CS/MMT nanocomposites were collected by ultra-centrifugation at 35000 rpm for 25 minutes at 4 °C.

3. Characterization

3.1. UV–visible spectral analysis

The UV–visible spectrum from 200 to 800 nm of AVE, A-AgNPs, A-AgNPs-CS/MMT with agar medium were recorded in an EPOCH™ Multi-Volume Spectrophotometer System (Biotek Instruments, Inc., Mumbai, India). A-AgNPs displayed maximum absorbance at 453nm with predomination of smaller size particles.

3.2. X-ray Diffraction (XRD)

D8 Advance Diffractometer (Bruker, U.S.A.) equipped with a CuK α radiation source ($\lambda = 0.154$ nm) was utilized for X-ray diffraction. The diffraction data were obtained from $2\theta = 1^{\circ}$ – 80° .

3.3. FTIR analysis

500 μ L of the synthesized AVE, A-AgNPs, A-AgNPs-CS/MMT was lyophilized for 30 minutes, and the pellet thus obtained was resuspended in chloroform.

3.4. Transmission Electron Microscopy (TEM)

TEM sample was prepared on 400 mesh carbon-coated copper grid.

3.5. Dynamic light scattering (DLS)

A-AgNPs was used to measure the hydrodynamic diameter and laser Doppler anemometry was used to determine zeta-potential (mV). The DLS and laser Doppler anemometry analyses were performed using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The DLS measurements were carried out at a wavelength of 532 nm at 25°C with an angle detection of 90°.

3.6. Antibacterial assay

Three Gram-negative bacteria *E. coli* and *S. aureus* were grown overnight in LB medium and spun at 5,000 rpm for 5 minutes. The pellet was washed with phosphate-buffered saline and resuspended in LB medium. The antibacterial activity was tested using agar well diffusion method. One 100 μ L of the suspended culture was spread uniformly on LB plates. The solid medium was then gently punctured with the help of cork borer to make wells, and 50 μ L of the synthesized A-AgNPs, CS/MMT and A-AgNPs-CS/MMT (150 μ g/mL) was added in the respective well. The plates were incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well. Ampicillin (5 mg/mL) was used as positive controls, while sterile water served as a negative control (Abudunia et al., 2017).

3.7. Statistical analysis

Data were expressed as mean $\pm$ SD or SEM. Statistical analysis was done by one-way ANOVA where appropriate with Graph Pad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

Table.1. Sample preparation of A-AgNPs-CS-MMT nanocomposite

Sample	CS (in gms)	MMT (wt%)	AgNPs (ml)
CS	2	0	0
CS/MMT-1	2	1	0
CS/MMT-2	2	2	0
CS/MMT-3	2	3	0
CS/MMT-4	2	4	0
CS/MMT-5	2	5	0
A-AgNPs-CS/MMT	2	4	5

4. Result

4.1. UV–visible spectra

The UV–visible spectra of the AVE, A-AgNPs, A-AgNPs-CS/MMT in water are presented in fig. 1. The absorption peak at 262, 671 were observed for AVE extract. At same time 458 was observed for A-AgNPs 1:7 (AgNO₃:AIE) which was also clearly observed in F-A-AgNPs-CS/MMT in water and in agar medium. This data shows that the biological media to be used for the antibacterial studies did not change the stability of the A-AgNPs. The stability of the A-AgNPs was determined by measuring the absorption spectrum at 24-h intervals for 90 days. AVE and CS/MMT solutions did not show any peak at 453 nm (Ashraf et al., 2016).

4.2. XRD

In fig. 2, the XRD pattern of CS exhibits broad diffraction peaks at $2\theta = 5^\circ$ and 22° which are typical finger prints of semi-crystalline CH. The A-AgNPs showed a crystalline nature in the region of $2\theta = 7^\circ, 11^\circ, 21^\circ, 35^\circ, 51^\circ, 64^\circ, 74^\circ$. The CS/MMT was showed an semicrystalline structure. However, the XRD pattern of A-AgNPs-CS/MMT showed peak at $2\theta = 5^\circ, 21^\circ, 47^\circ$ and 49° due to A-AgNPs along with the CS/MMT. The increase in ordering

of polymer composite with the addition of A-AgNPs indicates that the structure of A-AgNPs was strongly influenced and crystalline (Velu et al., 2017; Sahoo et al., 2012).

4.3. FTIR:

FTIR analysis was performed to characterize any chemical changes that occurred during the synthesis of A-AgNPs. Fig. 3 shows the FTIR spectra of AgNO₃, CS, MMT, AVE, CS/MMT and CS/MMT-A-AgNPs. Importantly, the characteristic peaks seen for CS/MMT alone are also evident in the sample of CS/MMT-embedded A-AgNPs. By contrast, the signature peak (1279 cm^{-1}) of AgNO₃ corresponding to the nitrate group is not evident in the A-AgNP sample (Velu et al., 2017; Sahoo et al., 2012).

Figure. 1. UV–visible spectrum of AVE, A-AgNPs, CS/MMT, A-AgNPs-CS/MMT in water, Arrow indicates the characteristic absorbance peak of A-AgNPs change in different samples.

Figure.2 XRD pattern of the AVE, A-AgNPs, CS/MMT, A-AgNPs-CS/MMT

Figure 3. Characterization of CS/MMT-embedded A-AgNPs: Fourier transform infrared spectra of AgNO₃, AVE, A-AgNPs, CS, CS/MMT, and CS/MMT-embedded A-AgNPs

4.4. TEM and DLS

TEM was performed on the A-AgNPs to visualize their morphology. The images demonstrated that particles are spherical and polydispersed with a size ranging from 40 to 80 nm in fig 4a. TEM analysis was carried out at 1.4 Å resolution, which is optimal for NP size analysis. Next, DLS analysis revealed that the formulated A-AgNPs had an

average diameter of 132.4 ± 1.3 nm in fig 4b (Velu et al., 2017; Elemike et al., 2017).

4.5. TGA

The thermal stability of A-AgNPs, CS and CS/MMT-A-AgNPs were analyzed using TGA. The attachment of A-AgNPs to CS/MMT-A-AgNPs is quite evident as the weight losses are increasingly noticeable fig. 5. In the scan, CS starts

to degrade near 250 °C. This is attributed to the degradation and deacetylation of CS (Aldosari et al 2013). Water evaporation occurred in all scans of CS derivative near 90°C and is in agreement with the literature (Sahoo et al., 2012). A rapid degradation is observed for CS/MMT-A-AgNPs derivative near 450 °C which was attributed to the presence of silyl groups of the hybrid derivative. The presence of one endothermic for all peaks indicated that all samples were homogeneous (Sahoo et al., 2012).

4.6. Antibacterial activity

The antibacterial activity of the synthesized CS/MMT, A-AgNPs and CS/MMT-A-AgNPs were investigated using the agar well diffusion method and standard dilution micro method. The results for the inhibition zones and their average values obtained from agar well diffusion assay are shown in fig6. In liquid growth experiments, 43%, 80% and 91% growth inhibition were observed when *E. coli* (10^6 CFU/mL) was treated with 150 µg/mL concentration of CS/MMT, A-AgNPs and CS/MMT-A-AgNPs for 4 hours, and a decline in

Figure 4. Size-distribution analysis by DLS (a) TEM of A-AgNPs (b)

Fig.5: TGA curves for CS/MMT and CS/MMT-embedded A-AgNPs

Figure.6 Antibacterial activity of CS/MMT-A-AgNPs against gram-positive *Staphylococcus aureus* and gram-negative *Escherichia coli*.

CFU per milliliter was seen from 1.54×10^7 (untreated control) to 1.42×10^6 (nanocomposite treated), while in the case of Gram-positive *S. aureus* (10^6 CFU/mL), treatment with 150 μ g/mL of CS/MMT, A-AgNPs and CS/MMT-A-AgNPs for 4 hours exhibited a 30%, 39% and 58% growth inhibition with CFU per milliliter reducing from 8.6×10^7 (untreated control) to 4.64×10^7 (nanocomposite treated). Therefore, it was apparent that all bacterial cultures used in this study were inhibited by CS/MMT-A-AgNPs. Gram-negative bacteria were highly suppressed by the AgNPs compared to Gram-positive bacteria. Additionally it was clearly identify that CS/MMT-A-AgNPs is more antimicrobial activities in comparison with CS/MMT and A-AgNPs (Elemike et al., 2017).)

5. Discussion

Currently, many methods have been reported for the synthesis of A-AgNPs using chemical, physical, photochemical, and biological routes. The chemical and physical methods of nanosilver production are often expensive and also involve the use of toxic and hazardous chemicals, which may pose potential environmental and biological risks (Marulasiddeshwara et al., 2017; Park et al., 2014). To reduce the dreadful effects of chemical synthesis and to obtain novel nanoparticles at a cheaper rate, green synthesis is increasingly being explored. An ecofriendly solvent system and availability of biocompatible reducing and capping agents are the basic requirements for green synthesis.

In this study suggest that CS/MMT-A-AgNPs have antimicrobial properties. Recently, researcher reported that plant mediated AgNPs is able of reducing tumor-cell growth and antimicrobial activities in vivo and in vitro (Osváth et al., 2015; Sayed et al., 2015; Yang et al., 2014; Li et al., 2014; Hashemi et al., 2017; Battogtokh et

al., 2017; Boyles et al., 2015). In the present report, we have exposed for the first time that CS/MMT and CS/MMT-A-AgNPs exhibit biomedical applications and determined the mechanism of action in different approaches (Muddineti et al., 2015; Duan et al., 2014; Amanulla et al., 2017; Bao et al., 2015).

Plant extract are easy to culture using relatively inexpensive and simple nutrients under varied conditions of temperature, pressure, pH, etc. In this work, alover extract was possessed the ability to synthesis of silver nanoparticles (A-AgNPs). AVE was able to accumulate AgNPs in medium under various conditions, and process optimization was carried out with respect to time, temperature, pH, and concentration of silver salt. A-AgNPs and CS/MMT-A-AgNPs were characterized using UV/vis spectroscopy, TEM, XRD and FTIR. The plant mediated synthesis of A-AgNPs and CS/MMT-A-AgNPs exhibited good antimicrobial activity against both Gram-negative and Gram-positive organisms activity. This paper we prepared CS/MMT-A-AgNPs which showed remarkable stability, uniform, excellent surface charge, excellent size and good physicochemical properties distribution throughout the experiment analysis period in fig.1,3,4,5 (Velu et al., 2017; Elemike et al., 2017; Elemike et al., 2017). This report highlights, for the first time, the use of A-AgNPs embedded with CS/MMT. These nanoparticles exhibit excellent antibacterial activity. Finally, we have drawn a scheme of the mechanism of action and signalling for CS/MMT-A-AgNPs in fig.7. This data was suggested here that CS/MMT-A-AgNPs may be suitable for antibacterial activity.

Conclusion

We report here a simple, rapid, and efficient method for the synthesis and extracellular accumulation of A-AgNPs using alover leaf

extract. The synthesized A-AgNPs are spherical in shape and have an average particle size of 40-80 nm. We have demonstrated the potential of these A-AgNPs for antimicrobial properties. They exhibited excellent broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria. The data provide strong evidence that CS/MMT-A-AgNPs should be studied further as potential novel antibacterial agents to treat gram positive and gram negative bacteria with an interesting mechanism of action.

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Conflict Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Figure.7 scheme of the mechanism of action and signalling of CS/MMT-A-AgNPs for antibacterial activities

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