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Cytotoxicity And Anti Microbial Analysis Of Graphene Oxide Decorated Silver Nanoparticles

Research Article

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Abstract

Silver is known to have antimicrobial properties since decades.Graphene has various biomedical applications.Utilising plant extract for synthesis of nanoparticles appear promising and ecofriendly. Synthesis of graphene coated silver nanoparticles was done by utilising plant extract of Ocimum sanctum Linn and Andrographis paniculata as reducing agents.The characterisation was done by UV-vis spectrophotometer Analysis, Transmission electron microscopy, FTIR Analysis andX-ray Diffraction assay. Antimicrobial activity was assessed by measuring zone of inhibition(mm). Assessment of cytotoxicity was done by Brine shrimp lethality assay and MTT assay.The synthesised silver nanoparticles with an average size of 11-20 nm were uniformly dispersed on graphene sheets.The nanoparticles exhibited minimal cytotoxicity and good antimicrobial properties.Combination of Ocimum sanctum Linn and Andrographis paniculata were effective to be used as reduced agents for the synthesis of graphene oxide decorated silver nanoparticles.The synthesised nanoparticles appear as promising agents to be used as antimicrobial agents with minimal cytotoxic effects.

Keywords: FTIR; Graphene Oxide; Silver Nanoparticles; 3T3-L1; XRD.

Introduction

Silver is well known for its antimicrobial activity against bacteria and fungi [1]. Silver nanoparticles tend to have better antimicrobial properties owing to their large surface area.But it has been observed in the recent years that the antimicrobial properties gets reduced as these silver nanoparticles tends to agglomerate [2]. Panacek et al also observed that when silver nanoparticles were used solely they were not able to exert their antimicrobial effect due to agglomeration which occurs as a result from the resistance of some bacteria [3]. Graphene is a monolayer of carbon atoms which is tightly packed and arranged in a two-dimensional honeycomb network of sp2hybridized carbon atoms [4]. It has been the topic of research in the field of biomedicine in recent years owing to its excellent biological properties such as drug delivering capabilities,antibacterial,properties biosensing, anticancer activity [5-8]. Many researchers have found out that as graphene is a multilayered material it can act as a matrix and can compensate for the lack of stability of silver nanoparticles [9, 10]. Graphene oxide exerts its antimicrobial activity by acting as a knife and mechanically disrupt bacterial membranes which results in leakage of bacterial cytoplasm and ultimately cell death [11]. According to Zhu et al there are hydroxyl, epoxide and carboxyl functional group present on graphene oxide so when silver is combined with graphene oxide there are many binding sites for available for silver ions [12]. Liu et al also confirmed that when graphene oxide was combined with silver nanoparticles it induces binding capability of silver nanoparticles and enhanced antimicrobial activity compared to GO or Ag NPs when used solely [13]. Many chemicals have been utilised for the reduction and stabilisation of these metal based

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nanoparticles. As there can be ill effect to the environment by utilising chemicals for nanoparticles synthesis, now a days biological methods have been utilised for nanoparticles are they tend to be more biocompatible, non toxic and environment friendly. Ocimum sanctum Linn is a very common and small herb found in all regions of India and is used for various medicinal purpose.It is used very commonly for the treatment of cold and cough.It also has antioxidant,cardioprotective and hepatoprotective properties. It also helps in boosting immunity [14]. Andrographis Paniculata is a herb which belongs to the family of Acanthaceae and is widely cultivated in south Asia.Its leaves and roots have been used for different medicinal purpose traditionally.It is known to have Anti-inflammatory, Antidioarrhoel, Antiviral, Antimalarial, Hepatoprotective, Cardiovascular, Anticancer properties [15]. Previously our team has a rich experience in working on various research projects across multiple disciplines [16-30]. Now the growing trend in this area motivated us to pursue this project.Our present work was focused on synthesis of graphene oxide decorated silver nanoparticles by adapting an ecofriendly technique and to evaluate their cytotoxicity and antimicrobial properties.

Materials and Methods

Preparation of plant extract

Leaves of Andrographis paniculata and Ocimum sanctum Linn were collected freshly and dried in shade for three days.After that they were powdered coarsely. 0.5g of A Paniculata and 0.5g of O sanctum Linn leaves powder was weighed and dissolved in 100ml of distilled water and mixed well.After that the solution was boiled for 5 minutes at 60-800 C using a heating mantle. The boiled extract was filtered through Whatman No.1 filter paper, and the supernatant was used.

Synthesis of Silver nanoparticles

10 ml of pure plant extract was added into the 90 ml of 3 mM of silver nitrate solution and mixed well. Then the solution was kept in magnetic stirrer for further mixing. The color change was noted and the nanoparticles formation was monitored. UV Spectrophotometer Analysis was done to confirm the synthesis of silver nanoparticles. Then the solution was centrifuged at 8000 rpm for 10 minutes. The solution was then filtered using Whatman No.1 filter paper.The prepared solution was then stored in the refrigerator for further use.

Synthesis of graphene oxide nanoparticles

0.6g of graphite nano powder (Sisco Research Laboratories, Maharashtra, India.) and 0.2 g of sodium hydroxide (MERCK, Mumbai, India.) was dissolved in 50 ml of distilled water, to this 50ml of plant extract was added and mixed well. Then the solution was kept in magnetic stirrer for further mixing. The colour change was noted and the nanoparticles formation was monitored. UV Spectrophotometer Analysis was done to confirm the synthesis of reduced graphene oxide nanoparticles. Then this solution was centrifuged at 8000 rpm for 10 minutes. The solution was then filtered using Whatman No.1 filter paper. The prepared solution was then stored in the refrigerator for further use.

Synthesis of graphene oxide coated silver nanoparticles

50 ml of biosynthesised silver nano solution and 50 ml of biosynthesised graphene nano solution was added together and mixed well. To achieve homogenous mix the solution was kept in magnetic stirrer for 7-8 hrs. The colour change was noted and the nanoparticles formation was monitored. UV Spectrophotometer Analysis was done to confirm the synthesis of silver coated reduced graphene oxide nanoparticles. Then this solution was centrifuged at 8000 rpm for 10 minutes. The solution was then filtered using Whatman No.1 filter paper. The prepared solution was then stored in the refrigerator for further use.

Characterization of GO-Ag nanoparticles

UV-vis spectrophotometer Analysis: The reduction of the Graphene oxide coated silver ions in solution was monitored by periodic sampling of the solution and subjecting the solution to spectrophotometer. (UV-1800 series).

Transmission electron microscopy: Transmission electron microscopy was done to confirm the size and shape of newly synthesised Graphene oxide coated silver nanoparticles.

FTIR Analysis andX-ray Diffraction assay: The chemical functional groups of Graphene oxide coated silver nanoparticles were analysed by FTIR Analysis using Fourier transform infrared spectrometer (Perkin Elmer, USA) and X-ray Diffraction assay was performed using X-ray Diffractometer (Bruker, Germany) toobserve the crystal structure of newly synthesised nanoparticles.

Antimicrobial activity test

100 ml of Muller-Hinton agar was prepared, sterilized and poured onto the petriplates. The plates were allowed for solidification. After solidification plates were swabbed with Enterococcus faecalis. The strains of E Faecalis were maintained at 4°C and were isolated from the patients. After swabbing on each plate four wells were formed using a T shaped well cutter.In the first three wells the test suspension was loaded in the concentration of 25µl, 50µl, 150µl respectively. In the fourth well a standard antibiotic in the concentration of 30 µl was loaded and the plates were incubated at 370 C for 24 hrs and zone of inhibition was measured after incubation.

For Candida albicans , 20ml of Rose Bengal was prepared , sterilised and poured on to a petri plate and allowed for solidification. After solidification plates was swabbed with Candida albicans. After swabbing on each plate four wells were formed using a T shaped well cutter.In the first three wells the test suspension was loaded in the concentration of 25µl, 50µl, 150µl respectively. In the fourth well a standard antibiotic in the concentration of 30 µl was loaded. The plates were incubated at 37^0C for 48 hrs and zone of inhibition was measured after incubation.

The microbiological procedure was repeated three times for each microorganism.

Assessment of Cytotoxicity (Brine shrimp lethality assay)

The artemia tank was filled with 6 L of distilled water, to that 50 g of iodine free salt was added and mixed well using a spatula.2

capsules containing 15g of brine shrimp eggs were added to the tank and left undisturbed for 5 mins for proper soaking in salt water.After that airline tip was placed inside the artemia tank and the aeration level was increased to maximum level according to the manufacturers' instructions. After 24 hrs of incubation , the naupliiwere hatched out from the brine shrimp eggs and observed using a stereomicroscope. Five tubes were taken and filled with 3ml of artificial sea water. 10 nauplii were added in each test tube respectively. Test solution was loaded in the concentration range of 10µl, 20µl, 30µl, 40µl, 50µl. A control tube was prepared by adding 3ml of artificial sea water, 10 nauplii. The tubes were kept for 24 hrs incubation. After incubation, the live and dead nauplii were counted and lethality was assessed.

MTT assay

The cytotoxicity effect of Graphene oxide coated silver nanoparticles was assessed by MTT assay which determines the cell viability and characterises the cytochemical demonstrationof succinic dehydrogenase produced by the cells [31]. Adipose tissue cell line of mouse (3T3-L1)was utilised. Briefly cells were seeded onto 96-well microplates at a density of 1×10^4 cells/100µL per well and were incubated with Graphene oxide coated silver nanoparticles at the concentrations of 10 to 50 µg/mL for 48-hours. The medium was then removed, and 100µL of MTT solution (0.5mg/mL MTT in PBS) was added. Then the cells were incubated for 4 hours in CO_2 incubator and the solutions turned into purple colour indicated formation of formazan. The MTT-purple formazan productions were dissolved in 0.1N isopropanol/hydrochloric acid (HCl) and optical densities of the solutions were measured by absorbance at 570nm in an ELISA plate reader.

% Inhibition= Absorbance of control- Absorbance of Sample/ Absorbance of control X 100

Result and Discussion

In the present work we tried to synthesise nanoparticles by combining graphene oxide with silver nanoparticles to achieve graphene coated silver nanoparticles. The present study appear to be first of its kind as the combination of leaf extracts of Andrographis paniculata and Ocimum sanctum Linn to synthesise graphene oxide coated silver nanoparticles have not been reported previously in the literature. The graphene oxide coated silver nanoparticles were biosynthesised using herbal plants and characterisation was done using UV Vis Spectrophotometry, Transmission electron microscopy, FTIR Analysis and X-ray Diffraction assay.

UV Spectrophotometer Analysis

The peaks were observed at 295nm and 430 nm which confirmed the successful reduction of graphene oxide and silver nanoparticles utilising plant extract.(Figure 1)

Transmission electron microscopy

The presence of Ag nanoparticles on the surface of almost transparent GO sheets was clearly visible. There was formation of few numbers of AgNPs on surface of GO nano-sheets. AgNPs were well distributed on surface of GO nanosheets with a majority of spherically-shaped nanoparticles of diameter 11-20 nm. (Figure 2)

FTIR Analysis

The Fourier transform infrared (FTIR) spectra of Ag/rGO showed absorption peaks at 1301cm-1 corresponding to the N =O (Nitro group) bending vibration, 2092 cm^{-1} corresponding to C =C (alkynes) stretching vibration, 2360 cm^{-1} corresponding to nitrile group C =N stretching vibration, 2841 cm^{-1} correspond-

Figure 1- UV-vis spectrophotometer Analysis.

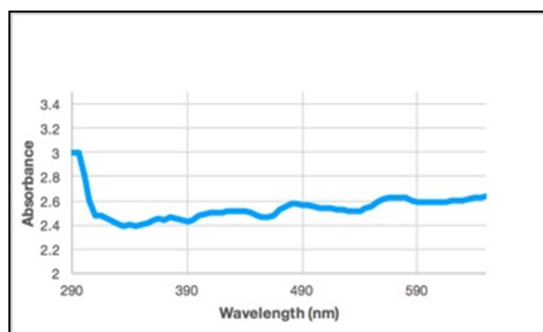
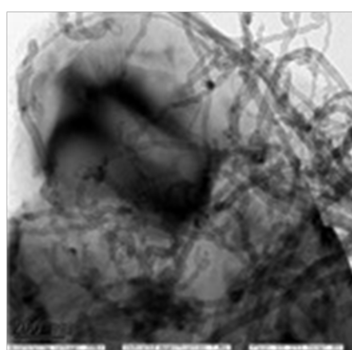


Figure 2. Transmission electron microscopy.



ing to CH2 stretching vibration, 3124 cm^{-1} corresponding to O-H stretching vibration. The relative decease in the intensity of broad band at 3761 cm^{-1} for the hydroxyl group is attributed to successful reduction of graphene oxide. The minor peak observed at 752 cm-1 may be attributed to the phytocompounds present in the plant extract. (Figure 3)

X-ray Diffraction Analysis

In X Ray Diffraction analysis of Silver coated graphene oxide nanoparticles the initial peaks were observed at Two theta value of 26.02° (2θ=26.02°) corresponding to (002) plane which is a usual 2θ value of graphene oxide as confirmed in previous studies [32]. The peaks observed at 28.01° and 32.21° can be attributed to crystalline and amorphous organic phases of the plant extract.2θ values of 38.17°corresponding to (111) and 44.54°corresponding to (200) crystalline planes of silver nanoparticles respectively [33]. From the XRD analysis it can be inferred that at the initial phase of reaction there was reduction of graphene oxide by plant extract and reduced graphene oxide sheets were formed.At the later phase of reaction silver nanoparticles were formed.So there was successful formation of graphene oxide coated silver nanoparticles. (Figure 4)

Antimicrobial activity

At all the concentrations of graphene oxide coated silver nanoparticles, zone of inhibition was observed. For *E Faecalis* and *Candida Albicans* the maximum zone of inhibition was 14mm and 13 mm respectively at the concentration of 150 µL. (Figure 5)

Brine Shrimp Lethality assay

Graphene oxide coated silver nanoparticles showed no cytotoxicity at concentration of 10 µL, 20 µL. As the concentration was increased the mild cytotoxic effects were evident. (Figure 6)

MTT assay

At 50 µg/mL concentration around 86% of the cells were viable suggesting minimal cytotoxic effect of Graphene oxide coated silver nanoparticles. (Figure 7)

In a study done by Chao Li et al when Graphene oxide was used solely against Candida Albicans and Candida tropical it was not proved to be effective [34]. According to Akhavan et al graphene takes several hours to completely inactivate the bacteria [35]. Das et al incorporated silver nanoparticle with reduced graphene oxide and found this combination to be more effective as compared to when used solely [36]. Cui et al. investigated the inhibitory effect against C. albicans of GO and the GO–Ag composite and found that GO was lacking anti fungal properties and anti fungal property was found to be better in the GO–Ag composite [37]. Similarly Jaworski et al. studied the antimicrobial activity of graphene oxide, silver nanoparticles and combination of both and found better antimicrobial activity with the combination [38].

Figure 3. FTIR Analysis.

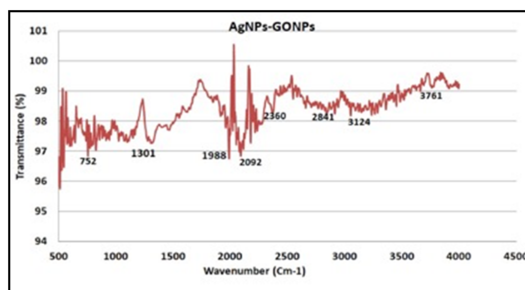


Figure 4. X-ray Diffraction Analysis.

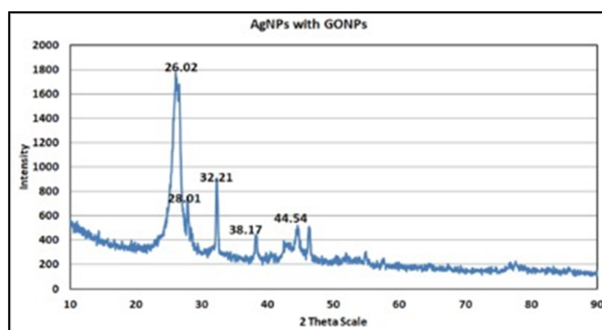


Figure 5. Antimicrobial activity.

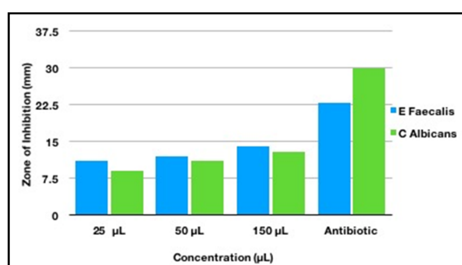


Figure 6. Brine Shrimp Lethality assay.

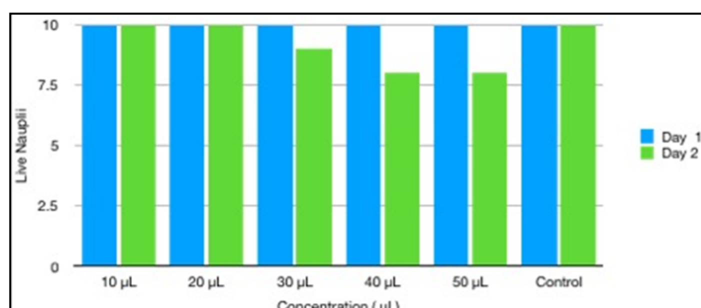
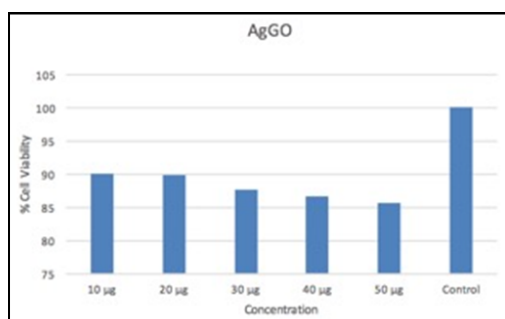


Figure 7. MTT assay.



When silver nanoparticles are used solely they are not stable in aqueous suspensions and have a tendency to aggregate, which limits their applications. GO is known to have an active role in the enhancement of the stability of the AgNPs, and acts as a platform to prevent their agglomeration. The possible formation mechanism of the GO–AgNP hybrids is through electrostatic interactions between the negatively charged oxygen-containing functional groups on the graphene oxide surface and the free silver ions, which are then reduced by the reducing agent, leading to the formation of AgNPs attached to the GO surface [39]. There are few major concerns related to graphene toxicity also. The toxicity depends on surface functionalization, coating, size, the administration routes, dosage, time of exposure and type of material with which these are combined [40]. We utilised Brine shrimp lethality assay and MTT assay to assess the cytotoxicity of the newly synthesised nanoparticles [41]. Silver coated graphene oxide nanoparticles showed minimum cytotoxic effects. Our institution is passionate about high quality evidence based research and has excelled in various fields [42-52].

Conclusion

According to the results of the present study Graphene oxide coated silver nanoparticles showed good anti microbial properties and minimum or no cytotoxic effects at lower concentration. More precise assays can be utilised for assessment of cytotoxicity. Future perspective of the present work is to conduct animal studies and test these nanoparticles in clinical conditions.

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