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Antifungal Properties of Graphene Silver Nanocomposites against *Candida Sp.*

Mansi

Assistant Professor at Swami Vivekananda Group of Institutes, Rajpura, Chandigarh, India

Abstract: ***The irrational use of antibiotics has made the disease causing microbes resistant to a majority of the antibiotics. This might be because of excessive use of antibiotics or because administration of sub – lethal doses. This development of antimicrobial resistance (AMR) has become a matter of concern as its occurrence is increasing worldwide. The disease causing microbes are continuously developing new ways to survive in the presence of antimicrobial agents. They adapt to every new antibiotic that is employed for killing them. This has caused an increase in medical complications. Researchers and clinicians have been facing issues due to AMR. So, the discovery of new antimicrobial agents is needed in order to kill resistant cells and to prevent the progression of AMR. Various nanoparticle conjugates have been studied for their use as agents to combat antimicrobial resistance. These compounds can be used in places where antimicrobial materials are required. These nanoparticle composites have applications in various fields, for example – nanofillers in polymers, cancer therapeutic drugs, killing various gram positive bacteria, gram negative bacteria and clinically significant fungi, Candida albicans. Candida albicans is an opportunistic pathogenic yeast. It is commonly found in the human gut microflora. It is detected in the gastrointestinal tract and mouth in 40–60% of healthy adults. In immunocompromised patients, it causes oropharyngeal or thrush candidiasis. Use of silver – graphene nanocomposites has been found to show decrease in its growth. This decrease depends on exposure time and concentration of nanoparticles along with which they are incubated. This is studied in order to find the minimum inhibitory concentration of the said nanoconjugates for the particular microorganisms.***

Keywords: Antimicrobial resistance, Nanoparticles, Nanoconjugates, Graphene, Nanofillers, Nanocomposites

1. Introduction

Over 1·6 million people have been estimated to lose their lives due to fungal diseases every year. Nearly 1 billion people suffer from cutaneous fungal infections. The diagnosis of fungal diseases requires specialised laboratories and trained personnel. Treatment of fungal infections is difficult as compared to bacterial and most viral infections (Cole *et al*.2017). Kingdom Fungi includes microorganisms which are remarkably diverse eukaryotes, nearly 1.5 million species. The menace they cause in the form of diseases is very high. So, studying them becomes all the more important. Though their role as food and providers of diverse pharmaceuticals has been known to the common public, there are a lot of species that are disease causing. They might be present on the human body as commensals, but a drop in the immune – capacity of the host turns them into pathogenic forms. Various antifungal agents have been studied and are constantly being studied in order to combat the menace caused by fungi. The most commonly found type of invasive candidiasis is called candidemia. According to a recent survey and estimation facilitated by the Leading International Fungal Education (LIFE) portal organised a survey and estimations were made based on that survey. It estimated that the global occurrence of invasive candidiasis to be 750, 000 cases annually, i. e., 2.1 to 21 cases per 100, 000 people around the world (Bongomin *et al*., 2017).22% of the healthcare associated blood infections were found to have been caused by *Candida sp.* via a survey carried out in the USA in 2011 (Magill *et al*., 2014). In 2015, a study carried out in Taiwan and Korea stated that *Candida sp.* cause 12% of the blood infections and 31% of the urinary tract infections in ICU patients in Taiwan. The same study stated the percentages to be 13% for blood infections and 23% for urinary tract infections in Korea (Chiang *et al*., 2018). In India, candidemia occurrence has been found to be 1.17% of the ICU patients (Tan *et al*., 2015). These statistics intrigue the researchers in order to find newer cures for the fungal infections. Antimicrobial resistance is an issue of worldwide concern. Resistant microbes have been found to have evolved diverse intrinsic resistance mechanisms by virtue of chromosomal mutations (Humphreys and Fleck 2016). These mechanisms include:

a) Limited diffusion of antimicrobial agents to the organism's cell due to glycocalyx of the biofilm matrix (Dhakal, McDaniel, and Sharma 2016).
b) Degradation of the antimicrobial agents by the components of the biofilm matrix, both cells and polymer (Brown, Aldrich, and Gauthier 1995).
c) Production of enzymes that degrade the antimicrobial compound, this is studied basically by degradation of pollutants by microbes (J. F. Ma *et al*., 1998).
d) Decreasing cellular activity of the cells present at the surface of the biofilm so that the antimicrobial agent does not affect the cells of the inner layers (Gilbert *et al*., 1989).

a) Adaptations at the genetic level, like *mar* operon of *E. coli* ensures multidrug resistance (Maira - Litrán, Allison, and Gilbert 2000).
b) Efflux pumps in the membrane pump out a broad range of dissimilar compounds (Nikaido 1996).
c) Outer membrane prevents entry of hydrophilic antimicrobial agents using the phospholipid bilayer and hydrophobic antimicrobial agents using the outer membrane proteins (Cloete 2003).

According to the 2016 WHO report, AMR infections would be the main cause of mortality as compared with the death toll attributable to other diseases (Humphreys and Fleck 2016). It would result in 10 million deaths per year by 2050 until and unless proper measures are implemented (Bryan - Wilson 2016). Moreover, the mutated genes that cause AMR also pose a very high threat of being propagated across

strains and species using horizontal gene transfer as a weapon, thereby preceding an overuse of antibiotics in livestock because of lessened drug effectiveness (de Kraker, Stewardson, and Harbarth 2016). A breakthrough of antimicrobial agents is the primary goal sketched in a plea for global cooperation in suppressing the AMR crisis.

Maintenance of a global momentum in the fight against AMR requires a uniformity in the estimates being reported and providing different estimates for the same metric must be avoided. This can be achieved only by having a consistency in methodology and analytical approaches, which makes it urgent to determine the most suitable methods, models and evaluation systems for AMR burden estimates (Midega 2020).

Rationalising and enabling the development of new antimicrobial agents is an important but costly option (S. Harbarth, Theuretzbacher, and Hackett 2014), Also, resistance has been observed against these compounds very quickly. Most classes of antibiotics have been found to be of no use because of emergence of resistance against them within an average span of five years. Mostly the national strategies against AMR identify the urgency of requirement of newer antibiotics and alternative solutions in order to avoid AMR. These solutions include probiotics, prebiotics, drugs that target bacterial communication or virulence, bacteriophage based therapies, phage enzymes, strategies that harness the power of the immune system, such as antibodies or vaccines. Vaccination may not work against all bacterial strains, but it shows great promises against some, such as uropathogenic E. coli (UPEC) strains responsible for the majority of urinary tract infections (Stephan Harbarth *et al*., 2015).4

The world is on high alert and is preparing to respond to AMR, a lot has to be done in order to increase the global access for vaccines that have already been licensed due to their positive impacts in reducing AMR. Although it is quite unlikely to have vaccines against all pathogenic microbes which contribute to AMR, the possibility for vaccines that are in later stages of development are reassuring in fighting AMR as soon as their effectiveness has been tested and implemented. The human body counters a lot of foreign agents on a daily basis and there is no limit on the number of vaccines that can be given to an individual. So, more and more vaccines can be developed in the hope to fight maximum number of pathogens, thus reducing the need for more antimicrobial agents. Yet, the fact that microbes keep evolving cannot be ignored because this might render vaccines useless after some years. The importance of vaccines in global healthcare systems is noteworthy and ignoring this aspect of fight against AMR might become a matter of concern for most countries around the world (Jansen and Anderson 2018). In India, over the counter selling of antibiotics were banned in 2014 in order to limit the unmonitored use of antibiotics based on the Global Risk Report on the World Economic Forum (Frey and Smith 2014). WHO released a global action plan on AMR which included some key objectives and the means to reach those objectives ("Global Action Plan on Antimicrobial Resistance" 2015). Based on this action plan, all the countries of the world devised their own action plans to fight the menace of AMR.

Table

Objectives of Global Action Plan by WHO	
Improving awareness and understanding of AMR	Communication, education and training
Strengthening knowledge and evidence based on AMR	Surveillance and research
Reducing the incidence of infections	Sanitation, hygiene and infection prevention measures
Limiting the emergence and spread of AMR	Optimal use of antimicrobial medicines in human and animal health
Developing new tools to fight AMR	New medicines, diagnostic tools, vaccines and other interventions

Global Action Plan on Antimicrobial Resistance" 2015)

The strategies that were considered to be most effective in fighting AMR are now becoming obsolete. So, a global action has to be taken in order to contain the situation of AMR. The use of antimicrobials in livestock and resistance that circulates in humans and environment is not well understood. This is because we do not have sufficient data to support the link between antibiotic use and resistance genes (Berglund 2015). Further, the study of "cross resistance" and "co - selection" mechanisms is also necessary. The management of invasive fungal infections is limited to verified agents from five well – known classes of antifungal agents. The existing agents show disadvantages such as dose - limiting toxicity, cross - reactions, limited routes of administration and the emerging resistance to the traditional antifungal agents. So, newer antifungal agents need to be developed (Gintjee, Donnelley, and Thompson 2020). Medicinal and aromatic plants (MAP) are conventional systems used in medicine which have been used for treatment of many diseases since the old times on the basis of their antimicrobial, antioxidant and immunomodulatory properties. These plants also offer maximum protection to human health. Thus, the present era also demands the use of historic herbs in order to fight AMR through natural means (Gupta and Birdi 2017). The advent of nanotechnology has led to the emergence of various types of nanoparticles, including silver nanoparticles, graphene oxide nanoparticles, lanthanum hydroxide nanoparticles, etc. These nanoparticles are being seen as alternative therapeutic reagents in place of antimicrobial compounds against which resistance has been observed in the past. These particles can be synthesized using chemical reduction as well as green synthesis. Silver (Ag) is a very appealing material due to its distinguishing properties like good conductivity, chemical stability, catalytic activity and antimicrobial activity (Frattini *et al*., 2005). Silver nanoparticles are being used as an important component of antimicrobial agents due to the antimicrobial effect of silver ions (Sharma, Yngard, and Lin 2009; Sondi and Salopek - Sondi 2004; W. Huang *et al*., 2020; Dimkpa *et al*., 2012). Silver nanoparticles show cytoprotective activity for HIV - 1 infected cells (Sun *et al*., 2005). Silver nanoparticles are used in the field of medicine along with water and air filtration (Russell and Hugo 1994; Lee *et al*.,

2007; Alt *et al*., 2004). Amalgam materials are formed by using Ag nanoparticles as a base and conjugating them with other nanoparticles or amphiphilic hyper branched macromolecules (Aymonier *et al*., 2002). These are created for using them as a component of surface coatings because of their antibacterial activity. The surfaces which are coated with Ag nanoparticle – 6 embedded paint based on vegetable oil have shown excellent antimicrobial properties (Kumar *et al*., 2008). Incidences of various water related diseases like diarrhoea and dehydration can be reduced by simply improving the microbial quality of the drinking water. Carbon filters can be employed for reducing the bactericidal activity of Ag nanoparticles (Das *et al*., 2011). The use of Ag coated activated carbon filters (ACF) can efficiently remove bio aerosols. Numerous commercially available products such as wound treating creams have Ag nanoparticles as key antimicrobial agents. Ag in wound dressings is present in the form of nanocrystals. These wound dressings are used for treating ulcers. Silver sulfadiazine is a compound that is used in creams for treating burn wounds (Das *et al*., 2011).

Graphene oxide – silver nanoconjugates Graphene oxide and silver nanoparticles are conjugated by use of various chemical methods in order to create antimicrobial materials. The methods for synthesis of AgNPs include physical, chemical and biological processes. Amid the chemical processes, chemical reduction is the simplest method which is based on the silver salt solution reduction by using a suitable reducing agent (Faramarzi and Sadighi 2013; Shah *et al*., 2015; Kharissova *et al*., 2013; Scala *et al*., 2019). This is a decently cost effective and often used method. Silver ion (Ag+) reduction results in formation of silver atoms (Ag0). These atoms then cluster into oligomeric clusters which leads to formation of colloidal AgNPs. Though these colloids have a low stability in liquid form, the formation of aggregates occurs by the virtue of the high surface area of the nanoparticles. During the synthesis of AgNPs, certain stabilizing agents have a very important role as they ensure a controlled size and a well – defined shape of the nanoparticles hence synthesized. The structure, shape and size of the nanoparticles formed in a process determine the physicochemical properties of the nanoparticles. Therefore, many studies show the size and shape of nanoparticles affect the antimicrobial activity. Graphene oxide is the oxidized counterpart of graphene. It as a suitable material that can be used to disperse and stabilize the silver nanoparticles as it combines large specific surface area and many oxygenated functional groups. Moreover, graphene oxide gives stable dispersion in aqueous medium. The oxygen rich groups present on the surface of graphene act as an anchoring site where the metal nanoparticles get attached. The graphene oxide sheets work as an anchor for growth and stability of the silver nanoparticles. The synthesis of these nanoconjugates has been done by using various approaches by various types of reducing 7 agents. Furthermore, the use of a stabilizing agent is considered vital for preventing the clustering of the silver nanoparticles and for controlling the structure (Cobos *et al*., 2020).

Characterization of GO – AgNPs

Fourier transform infrared spectroscopy (FTIR), ultraviolet–visible absorption spectroscopy (UV–vis), X - ray photoelectron spectroscopy (XPS), Raman spectroscopy, X - ray diffraction (XRD) and transmission electron microscopy (TEM) have been used for evaluating the structure and morphology of the nanoconjugates (Cobos *et al*., 2020).

Minimum inhibitory concentration

The concentration of an agent at which the growth of the target organism gets reduced by half as compared to the control is considered as the minimum inhibitory concentration. In order to find the minimum inhibitory concentration of an agent, the target organisms are incubated with serially diluted concentrations of the agent for prolonged times, generally 72 hours. The stock solution of the antifungal agent is prepared first. Meanwhile, the target organism is grown on suitable optimised media. Then, following the CLSI guidelines, broth dilution method is used to calculate the MIC of the agent (Pfaller *et al*., 2002).

Various studies on silver nanoparticles

In recent years, many scientists have been keen on studying the impact of silver nanoparticles on microbes. Here, the effect of silver nanoparticles on *Candida albicans* is being considered. In a study by Vasantharajan *et al*, the leaf extract of the plant *Costeus igneus*, also known as the insulin plant, which is known for its anti – diabetic action, was used to study inhibition of growth of *Candida albicans*. It was observed that the size of zone of inhibition on PDA plates increased as the concentration of the extract being used was increased (Vasantharaj, Sathiyavimal, and Hemashenpagam 2015). In order to support the fact that the leaf extract had silver nanoparticles, Packialakshmi *et al*. demonstrated that the silver nanoparticles produce black colour in aqueous medium because of excitation of surface plasmon vibrations (Packialakshmi, Suganya, and Guru 2014). The same colour production was also seen in case of *Costeus igneus*. Panacek *et al*. used low concentrations of silver nanoparticles and concluded that a significant decrease in growth was observed, that too without causing any toxicity to human cells (Panáček *et al*., 2009). The antifungal activity of silver was also observed on contact basis with the cells of the fungus. The ions enter the cells and hijack the cell coverings, viz. the cell membrane and the cell wall. This leads to a resultant damage to the cell as a whole (Rai and Bai 2011).

Graphene alone as an antifungal agent 10

Li *et al*. and Al - Thani *et al*. demonstrated lack of antifungal activity of graphene against *C. albicans (*C. Li *et al*.2013; Al - Thani, Patan, and Al - Maadeed 2014). It has been found that graphene oxide was effective against *C. albicans* when incubated for 24 hours. There was a significant decrease in growth of the fungus due to the presence of graphene oxide in the growth medium (Di Giulio *et al*., 2018). Asadi Shahi *et al.* found that graphene oxide – fluconazole conjugates were able to inhibit *C.* albicans growth and that their capacity increased due to synergistic effect (Asadi Shahi *et al*., 2019). This compound was found to be an apt candidate for therapeutic usage, yet, detailed in vitro and in vivo studies are needed in this arena. Another study involved in this arena used fluorescein isothiocyanate and polyethylene glycol in combination with graphene oxide for stimulation of pro - inflamatory and reparative macrophages (Diez - Orejas *et al*., 2018). The effect of stimulated macrophages was found to be more than the normal macrophages on *C.*

albicans. They also demonstrated that the presence of intracellular graphene oxide increases the killing capacity of the macrophages. So, it was found that this material can be used to increase the fungicidal activity of macrophages for future research in biomedical treatments (Diez - Orejas *et al*., 2018).

Various studies on silver – graphene conjugates

M. camomile plant extract was used to synthesize silver nanoparticles grown on magnetic graphene oxide. This conjugate showed significant decrease in the growth of *C. albicans.* Even very low concentration of the nanoconjugate was able to produce stark inhibition of growth. Moreover, this nanoconjugate could be used repeatedly with no loss in its inhibitory property. This study proved helpful in understanding the fact that plant extracts could be a very useful and sustainable source of raw material for creating graphene silver nanoconjugates (Ocsoy *et al*., 2017). Low concentrations of silver graphene nanoparticles were found to decrease cell concentration of *C. albicans.* The increase in concentration did not produce any significant change in the growth curves (Peng *et al*., 2017). This inactivation of cells at low concentration has been hypothesized to be a result of formation of reactive oxygen species by stimulation from the nanocomposites under aerobic conditions (Lu *et al*., 2013; R. S. Huang *et al*., 2015). Shen *et al*. have shown the effect of silver – chemically converted graphene (CCG) nanoparticle composites as very potent antimicrobial agents against organisms like *Colibacillus*, *S. aureus* and *C. albicans (*Shen *et al*., 2010).11

Candida albicans showed a significant decrease in growth after 24 hours of incubation with GO (Di Giulio *et al*., 2018). A major virulence factor of *Candida albicans* is the ability to form biofilms. A biofilm is a densely packed community of cells which is formed by cellular adhesion to a suitable surface. *C. albicans* biofilms are naturally resistant to the conventional antifungal therapeutics. They also cause trouble for the host immune system. Therefore, biofilm – associated infections have been a challenge for researchers and clinicians (Gulati and Nobile 2016). In a study on formation of biofilms on medical devices, it was found that the biofilm formation of *C. albicans* can be prevented by using graphene oxide in combination with curcumin and polyethylene glycol. It was observed that the medical device could be coated with the above combination to reduce *C. albicans* adhesion and biofilm formation (Palmieri *et al*., 2018). GO–AgNP nanohybrids have been efficaciously synthesized using environment friendly one - step approaches without the use of stabilizers. The concurrent reduction of silver nitrate (AgNO3) and graphene oxide (GO) in the presence of ascorbic acid brought about the decoration of partially reduced graphene oxide with uniformly distributed AgNPs. The size of the silver nanoparticles is governed by using the concentration of the silver salt and temperature. The effect of concentration of nanoparticles on *C. albicans* has been found to be of more significance at lower temperature. The concentration of silver ions in the reaction mixture is directly proportional to the temperature of reaction. It is also directly proportional to the size of the nanoparticles that get attached to the surface of graphene oxide. GO dispersion alone did not have antimicrobial activity against *C. albicans* over a wide range of concentration that had been tested. On the other hand, the nanohybrids showed species - specific antimicrobial activity. GO–AgNP nanohybrids produced dose and time – dependent toxicity for many drug resistant microbes (Cobos *et al*., 2020). These nanohybrids have been found to be quite effective against *C. albicans*. These findings suggest that GO – AgNP nanoconjugates are effective antifungal agents.

Lanthanum hydroxide - graphene nanoconjugates GOs combined with La (OH) 3 nanoparticles (La[at]GO) are an efficient antibacterial agent that can overpower the completion of AMR by extracellular bactericidal mechanism. Case in point, a library of La[at]GO nanocomposites had been made for examining the bactericidal effects of these composites in resistant strains. The effect of La[at]GOs on evolution of AMR was assessed by a chronological selection of resistant cells by subminimum inhibitory concentration (sub - 12 MIC) incubation with nanocomposites along with whole - genome sequencing of the acquired strains. Synchrotron, mass spectrometry and super resolution microscopy based methods were used to examine changes in cellular morphology and cellular components and to decode.

2. Conclusion

Now, apart from *Candida albicans*, non – *albicans Candida* are also causing many diseases. Some of these include *Candida auris, Candida glabrata, Candida krusei, Candida parapsilosis,* etc. (Clinical Practice of Medical Mycology in Asia, Arunaloke Chakrabarti, 2020). The menace caused by multi – drug resistant *C. glabrata* is of key importance now because it has been found to be resistant to newer azoles and Amphotericin B also (Pfaller *et al*., 2010; Alexander *et al*., 2005). It has been observed over the past 20 years that there has been a decrease in clinical isolates of *Candida albicans* and an increase in clinical isolates of *Candida glabrata* and *Candida parapsilosis (*Pfaller *et al*., 2019). Tackling these species has become an important task for researchers as these species show antifungal resistance. So, nanoparticles can be used in this aspect. The use of nanoparticles in combating drug resistant yeasts is a novel approach. GO–AgNPs nanoconjugates have been found to possess the highest activity against *C. albicans*. This class of nanohybrids is used as antimicrobial fillers in the preparation of polymer nanocomposites that have antimicrobial properties, which have applications in different fields. These nanocomposites could also be considered non - toxic agents for use in cancer therapy (Cobos *et al*., 2020). The antifungal activity of graphene and its different variants has been promising. Due to these reasons, Graphene – Silver nanoconjugates can be chosen to study their effect on non – *albicans Candida*. This is seen as a novel approach because not much has been reported in this field.

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