Graphene-Wrapped Copper Nanoparticles: An Antimicrobial and Biocompatible Nanomaterial with Valuable Properties for Medical Uses

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ABSTRACT: The great demand for antibacterial, biocompatible, and easily manufactured nanostructures has led to the design and development of graphene-wrapped copper nanoparticles (CuNPs) supported on Si wafers. In this study, we investigated the antibacterial properties of graphene/CuNPs nanostructures against Gram-positive and Gram-negative bacteria. Additional experiments regarding graphene/CuNPs nanostructures behavior against mouse fibroblast cell line L929 indicated their biocompatibility and consequently render them as model biomaterials for medical uses. Biofunctionalization of graphene/CuNPs nanostructures with a high-molecular-weight protein (green fluorescent protein), which retains its functionality after a “tight binding” on the nanostructure’s surface, opens the way for attaching and other proteins, or biomolecules of great biological interest, to prepare novel biomaterials.

1. INTRODUCTION

Biosafe and simultaneously antibacterial nanomaterials used in biological processes are in great demand for a variety of applications such as tissue and genetic engineering, cell differentiation and growth, etc. Within this context, graphene exhibits an extraordinary combination of properties that include high electrical and thermal conductivity, biofunctionalization versatility due to its entire exclusive sp2 bonding (in particular, because of its electrical conductivity as well as the availability of active p, electrons, graphene is sensitive to the biochemical environment and so can be modulated by certain chemical species that can bind on its surface), and foremost being the thinnest (0.345 nm) stiff and mechanically stable substance ever produced. On the other hand copper nanoparticles (CuNPs) are established as one of the most efficient antimicrobial materials. As a lucky coincidence, copper is also the most efficient catalyst for the growth of graphene by chemical vapor deposition (CVD). Consequently, the investigation of graphene-wrapped copper nanoparticles, which have the additional asset of larger specific surface area compared to plane graphene, is emerging as promising biomaterials for the immobilization of biomolecules, and as biofunctionalized surfaces with DNA, peptides, and proteins. However, studies about graphene biocompatibility are controversial, and only a few of them investigate the in vitro cytotoxicity of graphene-wrapped copper nanoparticles. For example, Szunerits et al. proposed that graphene can be placed at the forefront for different applications in biomedical, device development, diagnosis, and healing, while toxicity in eukaryotic cells are actually dependent on various factors such as the chemical composition, size, surface and shape, and even graphene edges, which result in the formation of pores in bacterial cell walls and cause subsequent osmotic imbalance and, consequently, cell death.

In this work, we provide a systematic study of the functionalization of graphene-wrapped copper nanoparticles, grown by a combination of electrodeposition and CVD, with a fluorescent protein to access the ability to retain its tertiary structure and remain functional. Finally, we explore the antibacterial activities toward Gram-positive and Gram-negative bacteria and the cytotoxic effects against mouse fibroblast cell line L929. The proposed fabrication method, which is based on the electroplated Cu firmly supported on Si wafers without any prior smoothing or polishing process, and the subsequent wrapping of the supported nanoparticles by...
conventional low-pressure chemical vapor deposition (LPCVD) of graphene provide very high throughput and scalability and excellent reproducibility.

2. MATERIALS AND METHODS

2.1. Materials. Ammonium fluoride (NH₄F) copper sulfate (CuSO₄·5H₂O), sodium potassium tartrate (KNaC₄H₄O₆·4H₂O), methanol, nitric acid, sulfuric acid, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxy succinimide (NHS), Luria-Bertani broth (LB), and agar were purchased from Sigma-Aldrich Reagent Company. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Invitrogen. Penicillin−streptomycin, fetal bovine serum (FBS), 0.25% (w/v) trypsin−0.03% (w/v) ethylenediaminetetraacetic acid (EDTA) solution, and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Gibco BRL (Carlsbad, CA). Green fluorescent protein (GFP) was overexpressed in BL21(DE3) cells using the pT77 plasmid vector. The protein was purified with Ni-NTA column (Qiagen).

2.2. Substrate Preparation by Galvanic Deposition. A thin layer of Cu was electroplated on a SiO₂/Si substrate. The Si substrate had a native oxide layer of around 10 nm. No seed layer was sputtered for the deposition of Cu on Si in this study. The electrodeposition was done in a plating bath with the following additives in the aqueous solution: ammonium fluoride (NH₄F 40%) 50% vol, copper sulfate (CuSO₄·SH₂O) 0.01 M, ascorbic acid (C₆H₈O₆) 0.01 M, sodium potassium tartrate (KNaC₄H₄O₆·4H₂O) 0.005 M, and methanol 30% vol (percentages are referred to the final solution volume). The anode was made up of a pure copper wire dipped into the solution. Stirring the solution continuously on a magnetic stirrer, the substrates were dipped in the solution that served as the cathode. A constant voltage of 10 V DC was applied, and the current density was kept at ~4 A/dm². The deposition was carried out at different times of 2, 3, 5, 7, 10 min, etc.

2.3. Synthesis and Characterization of Graphene−Copper Nanoparticles. The LPCVD reactor used in the present study consisted of a quartz tube coupled with a furnace, a rotary pump for evacuating, carbon source gases, and pressure sensors. A detector measured the temperature inside the chamber in which the substrates were loaded. Two gases were used during the graphene synthesis: argon as the precursor and acetylene as the carbon source gas. First, the substrates were loaded on an alumina boat and introduced into the chamber and the chamber was evacuated (~10⁻³ mbar). Once the base pressure was attained, the chamber was heated up to a higher temperature to anneal the Cu films to enable large descent terraces of Cu. After annealing, a mixture of C₂H₂ and Ar was introduced into the chamber for 6 min for the graphene growth and then again cooled back to room temperature under an Ar flow. For the present case, the optimum C₂H₂/Ar ratio was 15:100. In addition, the growth temperature was 950 °C. The base pressure was maintained at 6.3 × 10⁻³ mbar and the working pressure at 1.3 mbar. The described LPCVD processes result in growing bilayer graphene cages around the copper nanoparticles, and it was validated by Raman spectroscopy.

For obtaining morphological, microstructural, and analytical information, scanning electron microscopy (SEM) pictures were taken at every step of the sample fabrication using a high-resolution scanning electron microscope with a W filament (JEOL JSM-6390LV) and equipped with both energy-dispersive spectroscopy (EDS) and wavelength-dispersive spectroscopy (WDS) with an Oxford INCA microanalysis suite.

2.4. Treatment of Nanostructures for Protein or Peptide Binding. The samples were suspended in concentrated sulfuric acid and nitric acid mixture (3:1, v/v) and stirred for 2 h. Then, the surfaces were washed with deionized water until no acid was detected.

The process for covalently binding the green fluorescent protein (GFP) to the surface of the nanostructure is detailed as Figure 1. Figure 1. Schematic representation of GFP biofunctionalized in graphene−copper nanoparticles. Graphene/CuNPs/Si (A) is biofunctionalized according to the procedure; free carboxyl groups are formed on the surface of the nanoparticles (B) and activated via EDC/NHS reagent (C) to biofunctionalize GFP via peptide bonds (D).
follows: First, the carboxylic nanoparticles were suspended in 2 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). Then, 5 mM N-hydroxy succinimide (NHS) was added, and in the final step, GFP was added to a final concentration of 50 nM. The mixture was continually stirred for 4 h at 4 °C. The suspension was washed successively with water to remove excess EDC, NHS, and byproduct urea. In the final step, the washed samples were dispersed in deionized water for fluorescent observation (Figure 1).

The images were taken on a Nikon Eclipse 80i confocal microscope of the Veterinary Faculty of the Aristotle University of Thessaloniki.

2.5. Evaluation of Antibacterial Activity with Spread-Plate Method. The antibacterial activity of graphene/CuNPs/Si was tested against Escherichia coli and Staphylococcus aureus strains. The inoculum for the antibacterial assay was prepared from the actively growing organisms in the logarithmic phase. More specifically, the inoculum of E. coli and S. aureus were prepared from an overnight culture grown aerobically in a sterilized Luria–Bertani broth (0.5% w/v yeast extract, 1.0% w/v tryptone, and 1.0% w/v NaCl, pH 7).

To assess the antibacterial effect of graphene-wrapped nanoparticles, the spread-plate method was used. This method measures the living cells capable of forming colonies in a sample using a suitable agar medium. The assumption made in the particular counting process is that each living cell can grow and divide to give a colony. Thus, the number of colonies is a reflection of the number of cells. For the experiment, 0.1 mL of the bacterium (e.g., E. coli) was first ejected onto the studied surfaces and then incubated for 1 h at 37 °C. The surfaces were transferred into 10 mL of LB medium and stirring was continued for 10 min. A volume (usually 0.1 mL or less) of the appropriately diluted culture was spread on the surface of LB, agar culture plates. All experiments were conducted under aseptic conditions. The dishes were incubated overnight until the colonies appeared, and the number of colonies was measured. If the colonies cannot be measured, appropriate dilutions are made.

2.6. Evaluation of L929 Cells Viability with MTT Assay. The eukaryotic cells used were normal L929 mouse fibroblasts. The growth culture was made with DMEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (PS) antibiotic in 100 mm dishes in 5% CO2 and 37 °C.

To measure the cellular mitochondrial activity, the MTT assays were employed as previously described by Mosmann (1983) with minor modifications. Briefly, the samples were placed in 24-well plates and then cells were seeded at 2 × 104 cells/well. L292 cells were incubated in DMEM/10% FBS/1% PS for 48 and 72 h at 37 °C, 5% CO2. After exposure, the test medium was discarded, and cells were incubated with 1 mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (at a final concentration of 5 mg/mL diluted in PBS 1x) for 4–6 h at 37 °C, 5% CO2, until the formazan crystals were formed. Subsequently, the MTT solution was discarded and 1 mL of isopropanol was added to each well to solubilize the formazan salts and give a violet color to the solution. The solution was transferred to 96-well plates in triplicates. Optical density (OD) was measured with a microplate reader (ELISA reader BioTek) at 570 and 630 nm. Cell viability for each treatment was calculated as the ratio of the mean OD of replicated wells relative to that of the control (only cell culture medium added).

3. RESULTS AND DISCUSSION

3.1. Surface Morphology. Scanning electron microscopy images show graphene domains grown with LPCVD at 950 °C for 6 min on top of three different types of electrodeposited CuNPs (Figure 2). The CuNPs in this process formed on circular shape grains after annealing. The grains with size in the range of 150–700 nm, and the graphene domains are formed around the electroplated CuNPs.

3.2. Biofunctionalization of Green Fluorescent Protein (GFP). Biofunctionalization of nanomaterials with biomolecules, like proteins, antibodies, and nucleotide sequences, is very useful because of their broad spectrum of uses in biosensing, implantology, catalysis, etc. Until now, biofunctionalization of graphene, was possible mainly with noncovalent modifications. Covalent biofunctionalization is obtained with microwaves or surface treatment techniques. We propose a novel covalent modification of graphene-wrapped copper nanoparticles that is easy to use, up scalable, and, most importantly, able to retain the biological properties and the functionality of the selected biomolecule. This method is based on the use of the reagents EDC/NHS. After the treatment of the surfaces with H2SO4/HNO3 acid mixture (3:1 v/v ratio), free carboxyl groups are formed. These carboxylic groups are activated with the EDC/NHS reagent and can form peptide bond, a strong covalent bond, with the desirable protein.

Green fluorescent protein (GFP) was used as a control protein. Concerning its characteristics, it is a small (27 kDa), naturally fluorescent, a nontoxic protein with the ability to enter the nucleus, fill the cytoplasm of the cell, and diffuse into small cytoplasmic extensions. Most importantly, GFP requires no substrate but fluoresces simply in response to ultraviolet (UV) or blue light. Its fluorescence property is directly related to the natural tertiary structure of the protein and therefore to the functionality of the protein.
During the antibacterial test, the appropriate dilution was used. Copper Nanoparticles (graphene/CuNPs/Si) were used as control surfaces. Copper exhibits great antibacterial properties. Silicon and hydrophobicity.33 The most prevalent mechanism suggests that graphene destroys the outer membrane by extracting large amounts of phospholipids and provokes the production and release of reactive oxygen species, thus leading to oxidative stress.16

The results show that the pretreatment of the surfaces with the H$_2$SO$_4$/HNO$_3$ acid mixture (3:1 v/v ratio) is necessary to functionalize the protein in graphene-wrapped copper nanoparticles. Furthermore, the procedure does not affect the three-dimensional conformation of the protein because it retains its fluorescence and remains functional. Consequently, other proteins with biological interest can be functionalized in graphene-wrapped copper nanoparticles with this method.

### 3.3. Antibacterial Activity of Graphene-Wrapped Copper Nanoparticles

A desirable property of some nanomaterials is their antibacterial activity, particularly in medicinal uses. The antibacterial effect of graphene nanomaterials is an upcoming field that has been studied since 2010. Many studies have demonstrated that graphene and its derivatives have potent antibacterial properties, but the mechanism of action is not clearly elucidated due to different properties of the derivate used, i.e., surface charge, roughness, and hydrophobicity.33 The most prevalent mechanism suggests that graphene destroys the cellular membrane by extracting large amounts of phospholipids and provokes the production and release of reactive oxygen species, thus leading to oxidative stress.16

Two different bacterial strains, a Gram-negative (E. coli) and a Gram-positive (S. aureus), were selected. The selection was based upon the different membrane structure. The Gram-positive bacterial envelope consists of the peptidoglycan cell wall and the inner membrane, while the Gram-negative one has three layers in the envelope, the outer membrane, the peptidoglycan cell wall, and the inner membrane.34 Surfaces used were 0.5 cm and accessed with the spread-plate method. It is known that silicon shows low antibacterial effects, while copper exhibits great antibacterial properties. Silicon and CuNPs/Si nanostructures were used as control surfaces. During the antibacterial test, the appropriate dilution was found to be $10^{-4}$, and the results in Table 1 refer to this dilution.

Graphene/CuNPs/Si nanostructures exhibit antibacterial properties due to the morphology of the nanomaterial. Also, exposed copper in some regions reinforces the antibacterial activity. The antibacterial effect seems to be greater in S. aureus because of the cell membrane structure, which does not consist of an outer lipopolysaccharide cell wall in their envelope. The Gram-negative bacteria, like E. coli, have two lipid bilayers and thus more difficult to be destroyed. Graphene reaches the outer membrane and destroys it, but it cannot easily pass through the inner membrane.

### 3.4. Cytotoxic Effect of Graphene-Wrapped Copper Nanoparticles on L929 Cells

The assessment of the biocompatibility of the inspecting nanomaterial and their possible toxic effects on in vitro cellular models is considered very significant. With this aim, L929 cells were selected as a human model. Figure 4 shows the effects of the graphene-wrapped copper nanoparticles on the viability of L929 cells after 48 and 72 h in culture. As observed, graphene-wrapped copper nanoparticles show cytotoxicity to some extent and particularly a 15% decrease in cell viability. SiO$_2$/Si substrates as well as the surfaces of copper (Cu), electroplated on the SiO$_2$/Si substrates, do not show cytotoxicity. The cell viability remained stable with time.

According to Fujita et al., exfoliated graphene decreases euarkytotic cell viability, and the authors suggest that its cytotoxicity should be considered.35 Nevertheless, according to Muzi et al., multilayer graphene does not demonstrate cytotoxicity to cell models.36 This explains the fact that our nanostructure, which consists of more than two layers, does not show a considerable percentage of cytotoxicity.

Nevertheless, according to the bibliography, MTT has the same limitations in giving a positive result and other cell viability methods like WST-8, trypan blue, and LDH assay should also be used.38

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**Table 1. Average Number of Colonies of the Bacterial Strains E. coli and S. aureus**

| samples                  | number of colonies of E. coli | number of colonies of S. aureus |
|--------------------------|------------------------------|---------------------------------|
| Si                       | 53                           | 22                              |
| CuNPs/Si                 | 7                            | 0                               |
| BLG/CuNPs/Si             | 37.7                         | 15.3                            |

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**Figure 3.** Confocal microscopy images of graphene-wrapped copper nanoparticles (graphene/CuNPs/Si) being treated with 50 nM GFP, (a) without any surface pretreatment, (b) addition of NHS/EDC reagent without any pretreatment with H$_2$SO$_4$/HNO$_3$ acid mixture, (c) pretreatment with H$_2$SO$_4$/HNO$_3$ acid mixture and then the addition of NHS/EDC reagents.

**Figure 4.** Cell viability diagrams with maximum absorption at 570 and 630 nm after 48 and 72 h.
4. CONCLUSIONS

In summary, we have successfully produced nanostructures of graphene grown on electrodeposited CuNPs (BLG/CuNPs/Si) using the CVD method. BLG/CuNPs/Si can be biofunctionalized with proteins with an easy and low-cost method. The process does not affect the tertiary structure of proteins and leaves them fully functional. BLG/CuNPs/Si show significant antimicrobial effects against both Gram-positive and Gram-negative bacteria. The results are greater against Gram-positive bacteria. Finally, the prepared nanostructures show lower cytotoxicity compared to exfoliated graphene. Further cell viability tests, like WST-8, trypan blue, and LDH assay, should be conducted because of the limitation of the MTT assay.

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Notes
The authors declare no competing financial interest.

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