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# Do CVD grown graphene films have antibacterial activity on metallic substrates?

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ABSTRACT

Accurate assessment of the antibacterial activity of graphene requires consideration of both the graphene fabrication method and, for supported films, the properties of the substrate. Large-area graphene films produced by chemical vapor deposition were grown directly on copper substrates or transferred on a gold substrate and their effect on the viability and proliferation of the Gram-positive bacteria *Staphylococcus aureus* and the Gram-negative bacteria *Escherichia coli* were assessed. The viability and the proliferation of both bacterial species were not affected when they were grown on a graphene film entirely covering the gold substrate, indicating that conductivity plays no role on bacterial viability and graphene has no antibacterial activity against *S. aureus* and *E. coli*. On the other hand, antibacterial activity was observed when graphene coated the copper substrates, resulting from the release of bactericidal cupric ions in inverse proportion to the graphene surface coverage.



## 1. Introduction

Graphene is a two-dimensional crystal composed of hybridized-$sp^2$ carbon atoms in a hexagonal lattice structure that possesses many exceptional properties. *Inter alia*, it is a zero-gap semiconductor, mechanically hard, extremely flexible, chemically inert, impermeable to any atom or molecule, and optically transparent [1]. Such properties allow the development of biomedical devices with the proviso that the observed or suspected interactions between biological systems and graphene are well understood. In this context, a few toxicity studies have recently been conducted on graphene, in particular regarding its antibacterial activity, which have led to conflicting results in some cases [2–8]. One of the main reasons of the controversy is the nature and the properties of the various graphene-based materials. Graphite, graphene oxide, reduced graphene oxide or chemical vapor deposition (CVD) produced graphene can lead to dramatically different results when their impact on biological systems is studied. Most of the studies have been carried out on graphene oxide [9–11], which requires a subsequent reduction step for converting it into graphene [12]. The proliferation of L-929 cells [2] or neuroendocrine PC12 cells [3] on graphene paper resulting from the reduction of graphene oxide suggests the biocompatibility of this material. On the contrary, other results have shown that, on such a paper, the membrane

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integrity of *Escherichia coli* (*E. coli*) DH5 strain was lost, which induced a bactericidal effect [4]. Graphene oxide nanowalls, because of their very sharp edges, damage cell envelopes during the contact interaction with both Gram-negative bacteria *E. coli* and Gram-positive bacteria *Staphylococcus aureus* (*S. aureus*) and exhibit an even stronger antibacterial activity when they are in a reduced form [5]. Recently, this phenomenon was explained by the penetration of graphene oxide nanowalls in the membranes of *E. coli* resulting in the extraction of phospholipids and thereby in a reduction of the bacterial viability [6]. The antibacterial activity towards *E. coli* was also shown to depend on the type of graphene materials, i.e. increased activity from graphite to reduced graphene oxide [7]. Conversely, Ruiz et al. demonstrated that graphene oxide supports *E. coli* growth and therefore does not possess antibacterial properties [8]. These conflicting conclusions most likely come from the structural and chemical characteristics of the studied graphene materials, mainly graphene oxide nanowalls, which have sharp edges and must undergo a reduction step involving the presence of chemical functions on their surfaces. The antibacterial activity of structurally flat graphene films, on other hand, is an interesting avenue to investigate.

Very recently, large-area graphene films produced by CVD on Cu or Ge, or transferred to $SiO_2$ were reported to possess an antibacterial activity that was thought to be related to the electronic properties of the substrate [13]. The antibacterial mechanism was hypothesized to involve electron transfer from the microbial membrane, via graphene, to the substrate which acts as an electron pump [13]. In the present study, we provide experimental evidence that rules out the electron transfer model as a mechanism explaining the presumed antibacterial activity of CVD graphene films. This calls for revisiting the problem in order to clarify the role of the substrate conductivity, keeping in mind that any study on antibacterial activity must meet the required standards in biology in order to be convincing [14]. For this purpose, we elaborated CVD graphene films on copper (Cu) and gold (Au) substrates. Either entirely or partially covering films were grown on Cu surfaces whereas entirely covering films were transferred on Au surfaces. The antibacterial activity of the different samples was tested on the Gram-positive bacteria *S. aureus* and the Gram-negative bacteria *E. coli*.

## 2. Experimental details

### 2.1. Graphene samples

The graphene samples were grown by atmospheric pressure CVD on Cu foil pieces [15,16] (~1 $cm^2$; 99.9% purity; 50-μm thick) with dilute $CH_4$ (95:5 Ar:$CH_4$) as carbon precursor, using a hotwall furnace and a quartz reactor. The Cu foils were cleaned by sonication in acetone and isopropanol, and Cu was de-oxidized with acetic acid. The synthesis was next conducted at a temperature of 1050 °C for 1 h under flows of Ar (500 sccm), $H_2$ (20 sccm), and dilute $CH_4$ (0.2 sccm for partial coverage and 1 sccm for full coverage). After graphene growth, the quartz tube was extracted rapidly under flows of Ar and $H_2$ in order to maintain the integrity of graphene [16]. Just after the synthesis, some Cu foils were covered by poly(methyl methacrylate) (PMMA) and etched by aqueous ammonium persulfate for subsequent transfer on Au substrates. After a few hours in the solution, the Cu foils were completely etched. The PMMA/graphene stacks were then copiously rinsed in distilled water in order to remove contaminants arising from the Cu etching step. Next, they were scooped from the solution with Au substrates and left to dry in air. After a second coating of PMMA [17], the samples were soaked in acetone for several hours to dissolve PMMA, rinsed in isopropyl alcohol and finally gently blown dry with nitrogen.

In order to verify the quality and the number of layers of the graphene films, they were transferred on $Si/SiO_2$ wafers and analyzed by Raman spectroscopy (see Fig. S1 in the Supplementary information). Raman spectroscopy was performed at room temperature with a LabRam Horiba spectrometer at a laser wavelength of 514 nm. Fig. S1a shows optical microscope image of a graphene layer after transfer on a $Si/SiO_2$ piece. The main background layer is typically monolayer graphene (Raman spectrum in Fig. S1b: 2D peak position = 2693 $cm^{-1}$; G peak position = 1590 $cm^{-1}$; $I_{2D}/I_G$ = 2.7; full width at half maximum = 33 $cm^{-1}$), with some bilayer islands (Raman spectrum in Fig. S1c: 2D peak position = 2713 $cm^{-1}$; G peak position = 1592 $cm^{-1}$; $I_{2D}/I_G$ = 0.9). In addition, the 2D peak of the bilayer graphene (full width at half maximum = 53 $cm^{-1}$) can be fitted by four lorentzians (see Fig. S1c), thereby revealing that the analyzed bilayer graphene islands are AB-stacked. All these values are in good agreement with the literature [18,19] and our previous work [16]. The defect-related band (located at ~1350 $cm^{-1}$) is barely visible, testifying to the good quality of the transferred graphene films.

It is noteworthy that graphene oxide may lead to bactericidal effects [10]. In order to ensure that this effect can be excluded in our case, we have assessed the possibility of reactive oxygen species generation by the graphene sheets. We inspected graphene on Cu foils by X-ray photoelectron spectroscopy (see Ref. [16] for more details) and found that the corresponding graphene film was not oxidized (within the detection limit of the equipment), as illustrated by C 1s spectrum in the Supplementary information Fig. S2 (in accordance with our previous results [16]). Therefore it is very unlikely that our graphene sheets generate reactive oxygen species.

### 2.2. Bacterial live/dead analysis by flow cytometry

After sterilization in a 75% v/v ethanol solution, triplicates of each surface (1 $cm^2$) were placed in a 24-well plate (NuncTM). In order to reduce water evaporation from the bacterial solution, the remaining wells and the empty spaces between them were filled with deionized water. Sixty microliters of a bacterial suspension containing $6 \times 10^6$ CFU/ml of *S. aureus* (ATCC 25923) or $2 \times 10^7$ CFU/ml of *E. coli* (ATCC 25922) were poured onto the surfaces and incubated for 24 h at 37 °C. In order to assess bacterial viability, bacteria were diluted in a 0.85% NaCl solution and stained with the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes®) according to the manufacturer protocol. 50,000 events per experimental condition were acquired with a BD FACSCalibur™ flow