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***Short communication***

# Graphene derivatives potentiate the activity of antibiotics against *Enterococcus faecium*, *Klebsiella pneumoniae* and *Escherichia coli*

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**Abstract:** Antibiotic resistance in bacteria is developing at a faster rate than new antibiotics can be discovered. This study investigated the antimicrobial activity of several carbon-based derivative compounds alone and in combination with clinically relevant antibiotics against key ESKAPE pathogens *Enterococcus faecium*, *Klebsiella pneumoniae* and *Escherichia coli*. Three compounds, graphite, graphene and graphene oxide, in conjunction with ciprofloxacin (CIP), chloramphenicol (CHL) and piperacillin/tazobactam (TZP) were examined using fractional inhibitory concentration (FIC) testing. CIP combined with graphene demonstrated additive antimicrobial activity against *E. faecium* compared to individual application. Furthermore, CIP supplemented with graphene, graphene oxide or graphite showed additive activity with ∑FIC values of 1.0 against *K. pneumoniae*, whereas only TZP showed ∑FIC values <1.0 with graphene oxide. For *E. coli*, the antibiotic activity of CIP was enhanced with graphene, graphene oxide or graphite, whereas only graphite and graphene enhanced the activity of CHL and TZP respectively. Graphite and graphene oxide caused significant antagonism (∑FIC > 4.0) in conjunction with TZP against *E. coli*. In conclusion, the results demonstrate the potential to supplement clinically relevant antibiotics with carbon-based graphene, graphene oxide derivative or graphite for use as an additive supplement for novel systemic or topical treatment solutions against key priority pathogens.

**Keywords:** graphene; ESKAPE pathogens; synergy; antibiotics; chloramphenicol; ciprofloxacin; piperacillin-tazobactam

## 1. Introduction

Bacterial resistance to antibiotic treatments has become a significant global challenge [1], with more successful strategies being urgently required [2]. It has been suggested that combining graphene and derivatives with antibiotics might provide a novel approach to treating the most serious of resistant bacterial infections [3,4].

Graphene is a single layer of carbon atoms arranged into a honeycomb lattice [5]. The crystalline structure is held together by $sp^2$ hybridisation of the carbon atoms [6]. Graphene is a two-dimensional structure which can be manipulated to form different carbon allotropes such as fullerenes (zero-dimensional) through wrapping, nanotubes by rolling (one-dimensional) or stacked into graphite (three-dimensional) [5,7]. Graphene oxide is produced by attaching functional groups to graphene sheets via oxidation. Epoxide and phenol hydroxyl groups attach to the basal plane whilst the edges of the graphene sheet are covered in carboxylic groups [8]. Graphene compounds have high surface energies that allow for strong absorption of ions and molecules which alter the bacterial microenvironment. Slight pH changes via hydroxyl and carboxyl dissociations change the environment and therefore bacterial proliferation is affected [9].

The *in vitro* antimicrobial properties of graphene and derivatives have been well established [4]. There are three main proposed mechanisms of antimicrobial activity of graphene and derivatives. Firstly, graphene-based compounds have nanoknives which physically disrupt the bacterial membrane via sharp edges causing leakage of intracellular substances; membrane integrity is lost and cell death occurs [8,9]. Secondly, oxidative stress via the generation of reactive oxygen species dependent/independent pathways may disrupt bacterial metabolism and cellular functions leading to cell death through apoptosis [9]. More specifically, inducible oxidative stress is the mode of action that is attributed to graphene [8]. Finally, a thin flexible barrier is created by the graphene lateral two-dimensional structure which wraps/traps the bacterial cell membrane preventing nutrient acquisition and disruption of optimum physiochemical growth condition [4]. This results in a decrease in cell viability and metabolic activity [9]. Indeed, the sheeted structure of graphene oxide can intertwine with the bacterial cell, reducing membrane accessibility [10]. These modes of action are very distinct from those of traditional antibiotics which have clearly defined target sites within the bacterial cell [11].

Despite the well-established antimicrobial properties of graphene and derivatives, so far these have been unsuitable for medical use due to low efficacy [4]. The high concentration of compound required to achieve sufficient *in vivo* antimicrobial activity is likely prohibitive when considering the requirements for clinical applications. To address this, some studies have conjugated graphene and derivatives with metals [12,13], natural products such as curcumin [14] and antibiotics [15] to study potential synergist effects. However, there is a dearth in knowledge regarding the choice of suitable antibiotic combinations, which promote synergy and avoid antagonism.

In this study, three clinically relevant antibiotics with different modes of antimicrobial activity were selected. Ciprofloxacin (CIP), a fluoroquinolone, inhibits nucleic acid synthesis by inhibiting the activity of DNA Gyrase and Topoisomerase IV [16]. Chloramphenicol (CHL) inhibits protein synthesis by binding to the 50S ribosomal subunit which prevents the activity of peptidyl transferase [17]. Piperacillin is a β-lactam which inhibits the action of penicillin binding proteins which disrupts cell wall synthesis [18,19]. This is used in combination with tazobactam as piperacillin/tazobactam (TZP), a β-lactamase inhibitor which is designed to reduce resistance

generation [20]. Utilising the very distinct antibacterial properties of each antibiotic compared to those of graphene, graphene oxide and graphite, and using antimicrobial screening methods, we identified that combination therapy may provide a novel treatment option against well-characterised representative type strains of three ESKAPE pathogens, *Enterococcus faecium*, *Klebsiella pneumoniae* and *Escherichia coli*.

## 2. Materials and methods

### *2.1. Bacterial strains*

*E. faecium* strain NCTC 7171 was cultured using Columbia Blood agar (Oxoid, UK) supplemented with 5% horse blood (TCS Biosciences, UK) or Brain Heart Infusion (BHI) broth (Oxoid, UK) with agitation and incubated in anaerobic conditions at 37 ℃ for 24 h. *K. pneumoniae* strain NCTC 9633 and *E. coli* strain NCTC 10418 were cultured using Nutrient agar or broth (Oxoid, UK) and incubated in aerobic conditions at 37 ℃ for 24 h.

### *2.2. Antimicrobial compounds*

Graphene, graphene oxide (aqueous solution) and graphite were supplied by Manchester Metropolitan University (UK) and prepared in distilled water. All antibiotics were obtained from Sigma-Aldrich (Poole, UK) with CIP solubilised in 0.1 M hydrochloric acid, CHL in 95% ethanol and TZP (manufacturer pre-prepared) in distilled water.

### *2.3. Minimum inhibitory concentration (MIC) assay*

MIC values were determined for each antibiotic and graphene derivative by using a 96 well microbroth dilution assay [12]. Briefly, 0.15% (w/v) tetrazolium blue chloride (TBC) (Sigma-Aldrich, UK) was added to approximately $1.0 \times 10^9$ colony forming units per mL of each bacterial inoculum (*E. faecium*, *K. pneumoniae* and *E. coli*) in 2× concentrated media. Aliquots of 100 μL culture were mixed with equal volumes of respective antimicrobial compounds and serially diluted sequentially to a final ten fold dilution. Ethanol (95%) and hydrochloric acid (0.1 M) solvent controls were included. Plates were incubated in aerobic or anaerobic conditions at 37 ℃ for 24 h. All experiments were conducted with $n = 3$. MIC values were recorded as the lowest concentration with no visible colour change.

### *2.4. Fractional inhibitory concentration (FIC) assay*

FIC values were determined to identify synergistic antimicrobial activity between each antibiotic and carbon-based supplement against each bacterial species as described by Sopirala et al. (2010) [21]. Briefly, similar methods were employed as described above, however, 50 μL of each compound at twice concentration were added to the starting well before serial dilution prior to incubation and MIC determination. FIC for each antimicrobial compound was determined using the equation sum FIC = [($MIC_{compound}$ with antibiotic/$MIC_{compound}$ alone) + ($MIC_{antibiotic}$ with compound/$MIC_{antibiotic}$ alone)], where compound relates to the carbon supplement and antibiotic

relates to CIP, CHL or TZP. The fractional index thresholds used were ≤ 0.5 indicating synergy, > 0.5 ≤ 1 additivity, > 1 ≤ 4 indifference and > 4 antagonism [21].

## 3. Results

The fractional inhibitory concentration (FIC) was calculated to analyse synergistic relationships between each compound combined with selected antibiotics against all three bacterial strains. The fractional inhibitory concentration index analysis revealed additive, indifferent or antagonistic effects. Additive activity was observed when CIP was combined with graphene, graphene oxide or graphite against *K. pneumoniae* (Table 2) and *E. coli* (Table 3), but only graphene demonstrated additive effects (∑FIC = 0.56) against *E. faecium* (Table 1).

All CHL combinations with graphene, graphene oxide and graphite resulted in indifferent activity against *E. faecium*, *K. pneumoniae* and *E. coli* within ∑FIC range 1.01–2.95 (Tables 1–3), with the exception of CHL which when supplemented with graphite demonstrated additive interactions against *E. coli* (∑FIC = 1.00) (Table 3)

**Table 1.** FIC analysis of CIP, CHL and TZP in combination with graphene, graphene oxide and graphite against *E. faecium*. The fractional index points used were ≤ 0.5 synergy, > 0.5 ≤ 1 additivity, > 1 ≤ 4 indifference and > 4 antagonism. (A) denotes carbon-based compound as shown, (B) represents antibiotics ciprofloxacin (CIP), chloramphenicol (CHL) and piperacillin/tazobactam (TZP). All MIC values are in mg/L. ∑FIC, sum of the fractional inhibitory concentrations. Values are representative of three independent biological repeats.

| Compound (A) | Antibiotic (B) | MIC (A) | MIC (A+B) | MIC (B) | MIC (B+A) | ∑FIC | Interaction |
|---|---|---|---|---|---|---|---|
| Graphene | CIP | 500 | 31.3 | 0.62 | 0.31 | 0.56 | Additive |
| | CHL | 500 | 7.81 | 0.16 | 0.47 | 2.95 | Indifferent |
| | TZP | 500 | 250 | 2.22 | 42.5 | 19.6 | Antagonistic |
| Graphene oxide | CIP | 292 | 52.1 | 0.62 | 0.52 | 1.02 | Indifferent |
| | CHL | 292 | 5.21 | 0.16 | 0.31 | 1.96 | Indifferent |
| | TZP | 292 | 52.1 | 2.22 | 8.85 | 4.17 | Antagonistic |
| Graphite | CIP | 250 | 62.5 | 0.62 | 0.63 | 1.27 | Indifferent |
| | CHL | 250 | 3.91 | 0.16 | 0.23 | 1.45 | Indifferent |
| | TZP | 250 | 15.6 | 2.22 | 2.65 | 1.26 | Indifferent |

**Table 2.** FIC analysis of CIP, CHL and TZP in combination with graphene, graphene oxide and graphite against *K. pneumoniae*. The fractional index points used were ≤ 0.5 synergy, > 0.5 ≤ 1 additivity, > 1 ≤ 4 indifference and > 4 antagonism. (A) denotes carbon-based compound as shown, (B) represents antibiotics ciprofloxacin (CIP), chloramphenicol (CHL) and piperacillin/tazobactam (TZP). All MIC values are in mg/L. ∑FIC, sum of the fractional inhibitory concentrations. Values are representative of three independent biological repeats.

| Compound (A) | Antibiotic (B) | MIC (A) | MIC (A+B) | MIC (B) | MIC (B+A) | ∑FIC | Interaction |
|---|---|---|---|---|---|---|---|
| Graphene | CIP | 417 | 0.98 | 0.01 | 0.01 | 1.00 | Additive |
| | CHL | 417 | 3.91 | 0.23 | 0.23 | 1.01 | Indifferent |
| | TZP | 417 | 5.21 | 0.67 | 0.89 | 1.34 | Indifferent |
| Graphene oxide | CIP | 500 | 0.98 | 0.01 | 0.01 | 1.00 | Additive |
| | CHL | 500 | 5.21 | 0.23 | 0.31 | 1.36 | Indifferent |
| | TZP | 500 | 3.26 | 0.67 | 0.56 | 0.84 | Additive |
| Graphite | CIP | 500 | 0.98 | 0.01 | 0.01 | 1.00 | Additive |
| | CHL | 500 | 8.45 | 0.23 | 0.51 | 2.23 | Indifferent |
| | TZP | 500 | 5.21 | 0.67 | 0.89 | 1.34 | Indifferent |

**Table 3.** FIC analysis of CIP, CHL and TZP in combination with graphene, graphene oxide and graphite against *E. coli*. The fractional index points used were ≤ 0.5 synergy, > 0.5 ≤ 1 additivity, > 1 ≤ 4 indifference and > 4 antagonism. (A) denotes carbon-based compound as shown, (B) represents antibiotics ciprofloxacin (CIP), chloramphenicol (CHL) and piperacillin/tazobactam (TZP). All MIC values are in mg/L. ∑FIC, sum of the fractional inhibitory concentrations. Values are representative of three independent biological repeats.

| Compound (A) | Antibiotic (B) | MIC (A) | MIC (A+B) | MIC (B) | MIC (B+A) | ∑FIC | Interaction |
|---|---|---|---|---|---|---|---|
| Graphene | CIP | 250 | 0.98 | 0.01 | 0.01 | 1.00 | Additive |
| | CHL | 250 | 1.63 | 0.08 | 0.10 | 1.26 | Indifferent |
| | TZP | 250 | 0.98 | 0.17 | 0.17 | 1.00 | Additive |
| Graphene oxide | CIP | 333 | 0.98 | 0.01 | 0.01 | 1.00 | Additive |
| | CHL | 333 | 1.95 | 0.08 | 0.12 | 1.51 | Indifferent |
| | TZP | 333 | 208 | 0.17 | 35.4 | 209 | Antagonistic |
| Graphite | CIP | 333 | 0.98 | 0.01 | 0.01 | 1.00 | Additive |
| | CHL | 333 | 1.30 | 0.08 | 0.08 | 1.00 | Additive |
| | TZP | 333 | 417 | 0.17 | 70.8 | 418 | Antagonistic |

However, for TZP the synergistic effects were more diverse across the three target bacteria. TZP used in combination with graphene resulted in additive interactions against *E. coli* (∑FIC = 1.00) (Table 3), indifferent activity against *K. pneumoniae* (∑FIC = 1.34) (Table 2) and antagonistic effects against *E. faecium* (∑FIC = 19.6) (Table 1). For TZP supplemented with graphene oxide, additive interactions were observed against *K. pneumoniae* (∑FIC = 0.84) (Table 2), whereas antagonistic effects occurred with this combination against *E. faecium* (Table 1) and *E. coli* (Table 3). When TZP was combined with graphite, indifferent activity was observed against *E. faecium* (∑FIC = 1.26) (Table 1) and *K. pneumoniae* (∑FIC = 1.34) (Table 2), whereas for *E. coli*, this combination was the most antagonistic (∑FIC = 418) (Table 3).

Hydrochloric acid and ethanol solvent controls were used for MIC and FIC assays and these showed no effect on bacterial growth (data not shown).

## 4. Discussion

Three antibiotics were combined with carbon-based compounds to determine synergistic antimicrobial activity against three key priority pathogens. The antimicrobial activity of CIP was most potentiated by the addition of graphene, where additive activity was observed against *E. faecium*, *K. pneumoniae* and *E. coli*. The addition of adjuvants such as graphene, which enhance antibiotic action, permits lower levels of antibiotic usage overall [22]. For *E. faecium*, there was an observed one-fold less concentration of CIP required to inhibit bacterial growth in the presence of graphene. Given CIP targets bacterial nucleic acid synthesis and graphene has other reported mechanisms of antimicrobial action [4], it is thought that combinatorial therapy may help reduce the risk of antimicrobial resistance. Given graphene is thought to assist with membrane perturbation [3], it could be suggested that graphene works in combination with CIP by facilitating entry into the bacterial cell thereby exposing target sites for CIP.

The combinations of CIP with graphene oxide or graphite also showed additive activity against both *E. coli* and *K. pneumoniae* but not the Gram-positive *E. faecium*. This may indicate that these carbon-based derivatives are more active against the outer membrane of Gram-negative pathogens. Graphene and graphene oxide enhanced the antimicrobial activity of TZP against *E. coli* and *K. pneumoniae* respectively, but were both antagonistic for TZP targeting of *E. faecium*. This is likely attributed to the mechanism of activity of TZP and the physiology of the Gram-positive bacteria. TZP localises to the bacterial cell wall where, through the action of the β-lactam piperacillin, will inhibit the action of penicillin binding proteins to prevent cell wall crosslinking and formation [18,20,23]. Graphene is thought to provide a film that encapsulates the bacterial cell [9], which may inhibit TZP from accessing the cell wall of *E. faecium*. Significant antimicrobial antagonism was observed when TZP was combined with graphene oxide and graphite against *E. coli*. Such phenomenon have been reported previously where vancomycin demonstrated highly antagonistic activity against *E. coli* when combined with other cell wall inhibitors such as TZP [24]. The mechanisms of antimicrobial action for graphene oxide and graphite are less clear [4] but these may either interact with TZP reducing effectiveness or prevent uptake of TZP into the *E. coli* cell. Further work is necessary to confirm such interactions.

## 5. Conclusion

This is the first report where graphene and derivates potentiate the activity of specific antibiotics (CIP, TZP and CHL) against representative examples of both Gram-positive and Gram-negative bacteria. Other studies have demonstrated the antibacterial activity of graphene conjugates [13,14,15] and this study builds upon these advances by determining the potential for antibiotic-graphene synergistic activity. This may help inform future rational drug design, such as the addition of graphene to CIP for use against *E. faecium*, *K. pneumoniae* or *E. coli*. Using combination therapy where the antimicrobial agents have significantly different antibacterial mechanisms of activity may help reduce the risk of resistance evolution [11,22] and provide valuable solutions to treat the most serious of antibiotic resistant infections.

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## Conflict of interest

All authors declare no conflicts of interest in this paper.

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