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Original Article

# A promising poly (e-caprolactone)/graphene-based scaffold as an antibacterial in regenerating bone tissue

## Uma promissora estrutura à base de poli (e-caprolactona)/grafeno como antibacteriana na regeneração do tecido ósseo

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**Abstract**

Scaffolds are 3D biomaterials that provide an environment for cell regeneration. In the context of bone remodeling, poly(e-caprolactone) (PCL) combined with graphene has been developed as the scaffold. It is imperative for scaffolds to possess antibacterial properties in order to properly reduce the risk of potential infections.Therefore, this study aims to analyze the antibacterial characteristics of PCL/graphene scaffolds against *Staphylococcus aureus (S. aureus)* and *Porphyromonas gingivalis (P. gingivalis)* in vitro. In this study, five different groups were used, including PCL (K-), Amoxicillin (K+), PCL/Graphene 0.5 wt%, PCL/graphene 1 wt% and PCL/Graphene 1.5 wt%. All experiments were performed in triplicates and were repeated three times, and the diffusion method by Kirby-Bauer test was used. The disc was incubated with *S. aureus* and *P. gingivalis* for 24 hours and then the diameter of the inhibition zone was measured. The results showed that the PCL/graphene scaffolds exhibited dose-dependent antibacterial activity against S. aureus and P. gingivalis. The inhibition zone diameter (IZD) against *S. aureus* of PCL/graphene 1 wt% was 9.53 ± 0.74 mm, and increased to 11.93 ± 0.92 mm at a concentration of 1.5 wt% of graphene. The PCL/graphene scaffold with 1.5 wt% exhibited a greater inhibitory effect, with an IZD of 12.56 ± 0.06 mm against P. *gingivalis*, while the inhibitory activity of the 1 wt% variant was relatively lower at 10.46 ± 0.24 mm. The negative control, PCL, and PCL/graphene 0.5 wt% exhibited no antibacterial activity sequentially ($p = 1$). Scaffolds of poly(e-caprolactone)/graphene exhibited an antibacterial activity at 1, and 1.5 wt% on *S. aureus* and *P. gingivalis*. The antibacterial properties of this scaffold make it a promising candidate for regenerating bone tissue.

**Keywords:** scaffold, bone regeneration, antibacterial, infection, human well-being.

**Resumo**

Andaimes são biomateriais 3D que fornecem um ambiente para regeneração celular. No contexto da remodelação óssea, a poli(e-caprolactona) (PCL) combinada com grafeno foi desenvolvida como suporte. É imperativo que os andaimes possuam propriedades antibacterianas, a fim de reduzir adequadamente o risco de possíveis infecções. Portanto, este estudo tem como objetivo analisar as características antibacterianas dos andaimes de PCL/grafeno contra *Staphylococcus aureus* (*S. aureus*) e *Porphyromonas gingivalis* (*P. gingivalis*) *in vitro*. Neste estudo, cinco grupos diferentes foram utilizados, incluindo PCL (K-), Amoxicilina (K+), PCL/grafeno 0,5% em peso, PCL/Grafeno 1% em peso e PCL/grafeno 1,5% em peso. Todos os experimentos foram realizados em triplicata e repetidos três vezes, sendo utilizado o método de difusão pelo teste de Kirby-Bauer. O disco foi incubado com *S. aureus* e *P. gingivalis* por 24 horas e então mediu-se o diâmetro da zona de inibição. Os resultados mostraram que as estruturas de PCL/grafeno exibiram atividade antibacteriana dose-dependente contra *S. aureus e P. gingivalis*. O diâmetro da zona de inibição (IZD) contra *S. aureus* de PCL/grafeno 1% em peso foi de 9,53 ± 0,74 mm e aumentou para 11,93 ± 0,92 mm na concentração de 1,5% em peso de grafeno. A estrutura de PCL/grafeno com 1,5% em peso exibiu um efeito inibitório maior, com um IZD de 12,56 ± 0,06 mm contra *P. gingivalis*, enquanto a atividade inibitória da variante de 1% em peso foi relativamente menor em 10,46 ± 0,24 mm. O controle negativo, PCL e PCL/grafeno 0,5% em peso não exibiram atividade antibacteriana sequencialmente ($p = 1$). Estruturas de poli(e-caprolactona)/grafeno exibiram atividade antibacteriana de 1% e 1,5% em peso em *S. aureus* e *P. gingivalis*. As propriedades antibacterianas deste andaime o tornam um candidato promissor para a regeneração do tecido ósseo.

**Palavras-chave:** andaime, regeneração óssea, antibacteriano, infecção, bem-estar humano.

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## 1. Introduction

Scaffolds are 3D biomaterials that provide an environment for cell regeneration. In the field of dentistry, they can be used for periodontal regeneration treatment, dentomaxillofacial surgery, endodontic, and pediatric treatment (Budi et al., 2023; Gholami et al., 2021). An ideal scaffold should possess biocompatibility, biodegradability, a porous structure, and robust mechanical properties. Subsequently, poly(e-caprolactone) (PCL) has gained prominence in bone tissue engineering and drug delivery systems due to its biocompatible and biodegradable properties (Anitasari et al., 2023). However, the optimization of PCL often necessitates the inclusion of supplementary components.

Graphene is a 2D carbon nanofiller that has potential in biomedical applications such as drug delivery, tissue engineering, and biosensors because it has good thermal, electrical, and mechanical properties (Williams et al., 2023). Physically, graphene can damage bacterial membranes through its sharp edges, using a "penetration mode" or "insertion mode" mechanism when it comes into direct contact (Cao et al., 2021). Moreover, graphene can induce cellular damage by triggering oxidative stress through the generation of reactive oxygen species (ROS). These ROS can disable proteins and lipids within bacteria and result in cell membrane degradation, consequently impeding bacterial growth (Williams et al., 2023). A study proved that the cytotoxic effect of ROS produced by graphene oxide (GO) and reduced graphene oxide (rGO) against *Pseudomonas aeruginosa* was related to mitochondrial membrane depolarization and dysfunction. The "wrapping" mechanism of graphene can trap bacteria and obstruct the transport of nutrients consumed by bacteria (Cao et al., 2021).

The scaffold development of PCL/graphene showed non-toxic properties, adequate mechanical properties, and suitable pore size and porosity for bone tissue engineering (Anitasari et al., 2023). Subsequently, it has been proven that graphene can increase the attachment and proliferation of human neurons, cardiomyocytes, and several types of stem cells without causing harmful effects on human cell membranes and mitochondria (Malhotra et al., 2022). Another finding also showed that graphene can stimulate bone formation without causing damage to the bones (Chang et al., 2020).

A previous study identified the optimal concentrations for the osteoinduction mechanism in PCL/graphene scaffolds, which are 0.5, 1.5, and 2.5 wt% (Qiu et al., 2024). At these concentrations, a pore size of 100-500 µm is obtained. This investigation also proves that the total porosity of the PCL/graphene scaffold is 85%, while cancellous bone has an average porosity of 79.3%. This favorable environment can induce osteogenesis, and angiogenesis, and stimulate osteoblast migration. Other studies have confirmed the biocompatibility, proliferation, and differentiation experiments, showing that PCL/graphene scaffold concentrations of 0.5, 1.5, and 2.5 wt% are non-toxic, with a cell survival rate exceeding 70%, meeting the basic requirements for human applications (Anitasari et al., 2023).

One of the weaknesses of the scaffold is susceptibility to infection, which can lead to a prolonged inflammatory phase and failure of bone repair (Shuai et al., 2017). Scaffolds containing antibacterial agents not only support tissue regeneration but also prevent detrimental local inflammatory processes (Gholami et al., 2021). *Staphylococcus aureus (S. aureus)* and *Porphyromonas gingivalis (P. gingivalis)* are normal microflora of the oral cavity that can become pathogens and cause infections (Risky et al., 2019). *S. aureus* is commonly associated with implant infections, while *P. gingivalis* is the primary cause of periodontal disease (Johnson and Garcia, 2015). Another study has shown that *S. aureus* and *P. gingivalis* are often found to cause osteomyelitis, periodontitis, and peri-implantitis (Hofstee et al., 2020).

To assess the antibacterial properties of these materials, the inhibition effectiveness, or the ability to inhibit bacterial growth, is commonly evaluated. One of the most common analysis methods of antibacterial activity is the disk diffusion method (Balouiri et al., 2016). The results of the diffusion method test were observed by measuring the diameter of the inhibition zone around the disc using a caliper (Magvirah et al., 2020). This study was conducted to analyze the antibacterial properties of PCL/graphene scaffolds against *S. aureus* and *P. gingivalis* in vitro.

## 2. Materials and Methods

### *2.1. Fabrication of scaffold PCL/graphene*

3D porous scaffolds were produced using a solvent casting and particulate leaching process. Subsequently, PCL (Mn 80,000) from Sigma-Aldrich (St. Louis, Missouri, United States) was used as the matrix material, and sodium chloride (NaCl) as the porogen (Sigma-Aldrich). Graphene was produced by heating a graphite intercalation compound to 700 °C in a common furnace, positioned in front of a fume to avoid nanoparticle inhalation, and left for 60 seconds. An ultrasonication process was used to produce a graphene dispersion in a solution, and PCL was dissolved in chloroform at a 1:10 w/v ratio for 12 hours at room temperature. The PCL solution was added to the NaCl and graphene solution and stirred for 2 hours with a magnetic stirrer. The mixed solution was poured into the mold and allowed to dry at room temperature for 1 day. Chloroform residues were removed over 24 hours in a vacuum oven set at 37 °C. The PCL/graphene scaffold was soaked in deionized (DI) water for 24 hours to remove porogens. The DI water was changed every 2 hours during this period. The PCL/graphene scaffold was then dried in a vacuum oven at 50 °C for 12 hours. A porous scaffold made of PCL/graphene mixture was formed. PCL porous scaffolds containing graphene were prepared with concentrations of 0.5, 1.5, and 2.5 wt% (Qiu et al., 2024).

### *2.2. Preparation of Staphylococcus aureus and Porphyromonas gingivalis strain and culture condition*

Each bacteria culture was obtained from *S. aureus* ATCC 6538 and *P. gingivalis* ATCC 33277 strain using a sterile tube and planted in Brain Heart Infusion Broth (BHIB) media (Oxoid™, CM1135, Thermo). The bacterial cultures on BHIB media were incubated in an incubator at 37 °C for

24 hours. Then the turbidity was observed to be equal to the McFarland standard of 0.5 (1.5 x $10^8$ Colony Forming Unit (CFU)/mL) (Missiakas and Schneewind, 2013).

### *2.3. Preparation of antibiotics paper disc*

In this study, two different kinds of antibiotic paper discs were used, namely amoxicillin (Oxoid™, CT0061B, Thermo) at a concentration of 25 µg and clindamycin (OxoidTM, CT0064B, Thermo) at 2 µg. A sterile paper disc (74146, Sigma) was impregnated in sterilized water overnight at room temperature in a laminar flow hood as the negative control.

### *2.4. Inhibitory zone diameter (IZD) measurement of PCL/graphene scaffolds*

In this study, the inhibitory test was carried out using the disc diffusion method. Mueller Hinton Agar (MHA) (Oxoid™, CM0337, Thermo) was used as the medium for the paper disc test. *S. aureus* and *P. gingivalis* cultures, previously grown in BHIB media and adjusted to the McFarland standard 0.5 were taken using sterile cotton swabs. These bacterial cultures were evenly spread across the entire surface of MHA using the swab method. The smearing process was carried out consistently on the surface of the agar. Bacteria were grown in seven petri dishes containing MHA and marked using a marker on the back of the petri dish. For the positive control group, paper discs containing amoxicillin and clindamycin were placed on the MHA surface that had been inoculated with bacteria. Meanwhile, for the treatment and negative control groups, sterile paper discs were placed on MHA media inoculated with bacteria. Scaffolds of PCL and PCL/graphene with concentrations of 0.5, 1.5, and 2.5 wt% were then cut into diameter 5 mm. These scaffolds were sterilized for 30 minutes by ultraviolet (UV) were used directly for further experiments, and were placed carefully on sterile paper discs on agar media inoculated with bacteria. Subsequently, the process of incubation was conducted at 37 °C for 24 hours. All experiments were performed in triplicates and were repeated three times. The zone of inhibition was measured using a caliper (Akbaş et al., 2023).

### *2.5. Statistical analysis*

Data was expressed as the mean ± standard error (SE). Statistical analyses were performed using the Kruskal-Wallis and Mann-Whitney test to determine the data with SPSS version 21.0 software (SPSS, USA). Significant differences between groups were determined at a level of $p$-value < 0.05.

## 3. Results

### *3.1. Antibacterial effectiveness of PCL/graphene scaffold on S. aureus*

The results of the antibacterial activity testing using the disk diffusion method incubated on *S. aureus* showed that an inhibition zone was formed in the positive control group, where amoxicillin and clindamycin, PCL/graphene 1% and 1.5 wt% were administered. In the negative control group, PCL and PCL/graphene 0.5 wt% scaffolds did not show any ability to inhibit the growth of *S. aureus* (Figure 1). The mean of inhibition zone diameter (IZD) in the group administered with amoxicillin was 16.20 ± 0.35 mm, and 16.10 ± 0.00 mm for the clindamycin disk. In the treatment group, the largest IZD was found on the disk with a PCL/graphene concentration of 1.5 wt%, namely 11.93 ± 0.92 mm (Table 1).

The results of the Kruskal-Wallis test showed a significance value of 0.003, meaning that p-value <0.05. This shows that there is an inhibitory effect on the PCL/graphene scaffold on the growth of *S. aureus* bacteria (Table 1). The difference in mean inhibitory ability between groups was followed by the Mann-Whitney test (Figure 2).

### *3.2. Antibacterial effectiveness of PCL/graphene scaffold on P. gingivalis*

Antibacterial activity testing against *P. gingivalis* showed that there was an inhibition zone on the amoxicillin, clindamycin, PCL/graphene 1%, and 1.5 wt% disks. Meanwhile, in the negative control group, PCL and PCL/graphene 0.5 wt% scaffolds did not show the ability to inhibit the growth of *P. gingivalis* (Figure 1). There was an increasing in the IZD on the amoxicillin disk, clindamycin disk, and PCL/graphene concentrations of 1% and 1.5 wt% on the growth of *P. gingivalis* compared to *S. aureus*.

### *3.3. Inhibition ability related to inhibition zone diameter (IZD)*

The antibacterial strength of the 1 wt% PCL/graphene scaffold falls into the medium category (5-10 mm) against *S. aureus* and *P. gingivalis*. However, increasing the graphene concentration to 1.5% produces a strong IZD (11-20 mm) (Table 2).

## 4. Discussion

Inhibitory activity is one of the important factors that must be studied to determine the ability of a material to inhibit bacterial growth. The diameter of the inhibitory zone, as observed using the disc diffusion method, serves as an indicator of bacterial sensitivity to a disc paper. The diameter of the inhibition zone was measured by taking the vertical and horizontal diameter using a caliper, in units of millimeters (mm).

In this study, it was found that the use of 15 and 1.5 wt% PCL/graphene scaffolds had antibacterial activity. with a graphene concentration of 1.5 wt% led to the absence of growth of S. *aureus* within a zone measuring 11.93 ± 0.92 mm, and a zone of 12.56 ± 0.057 mm for P. *gingivalis*. This suggests that when bacterial cells come into contact with the edges of the paper disc infused with graphene material, damage to the bacterial membrane occurs, leading to the release of bacterial cell molecules and, consequently, bacterial cell dysfunction.

*Staphylococcus aureus* is a round-shaped gram-positive bacterium, which is an opportunistic pathogen (Li, 2018). It is also part of the commensal flora found on the skin

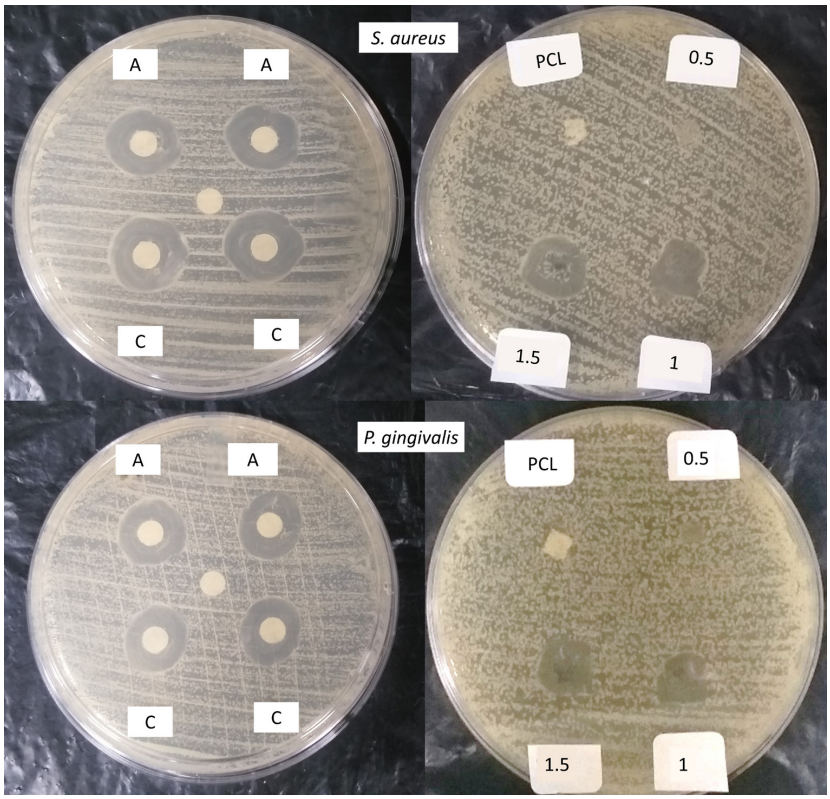


**Figure 1.** Antibacterial activity to inhibit the growth of *S. Aureus* and *P. gingivalis*. (A) inhibition zone diameter on positive control (A = amoxicillin, C = clindamycin) and negative control, PCL, PCL/graphene 0.5, 1 and 1.5 wt%.

**Table 1.** Inhibition zone diameter of antibiotics, PCL and PCL/graphene on *S. Aureus* and *P. gingivalis* culture.

| Groups | IZD *S. aureus* (mm) Mean ± SE | *p-value* | IZD *P. gingivalis* (mm) Mean ± SE | *p-value* |
|---|---|---|---|---|
| K- (disk) | $0.00 \pm 0.00^{a}$ | 0.003 | $0.00 \pm 0.00^{a}$ | 0.003 |
| Amoxicillin | $16.20 \pm 0.35^{b}$ | | $16.70 \pm 0.35^{b}$ | |
| Clindamycin | $16.10 \pm 0.00^{b}$ | | $17.55 \pm 0.93^{b}$ | |
| PCL | $0.00 \pm 0.00^{a}$ | | $0.00 \pm 0.00^{a}$ | |
| PCL/Grap 0.5wt% | $0.00 \pm 0.00^{a}$ | | $0.00 \pm 0.00^{a}$ | |
| PCL/Grap 1 wt% | $9.53 \pm 0.74^{c}$ | | $10.46 \pm 0.24^{c}$ | |
| PCL/Grap 1.5wt% | $11.93 \pm 0.92^{d}$ | | $12.56 \pm 0.06^{d}$ | |

$^{a,b,c,d}$Different superscript letters in the same column mean significant difference at 0.05 levels ($p < 0.05$).

**Table 2.** Classification of antibacterial activity of antibiotics, PCL and PCL/graphene on *S. Aureus* and *P. gingivalis culture* by inhibition zone diameter in milimeter (mm).

| Groups | IZD *S. aureus* (mm) | Category | IZD *P. gingivalis* (mm) | Category |
|---|---|---|---|---|
| K- (disk) | 0.00 | Weak | 0.00 | Weak |
| Amoxicillin | 16.20 | Strong | 16.70 | Strong |
| Clindamycin | 16.10 | Strong | 17.55 | Strong |
| PCL | 0.00 | Weak | 0.00 | Weak |
| PCL/Grap 0.5wt% | 0.00 | Weak | 0.00 | Weak |
| PCL/Grap 1 wt% | 9.53 | Strong | 10.46 | Strong |
| PCL/Grap 1.5wt% | 11.93 | Strong | 12.56 | Strong |

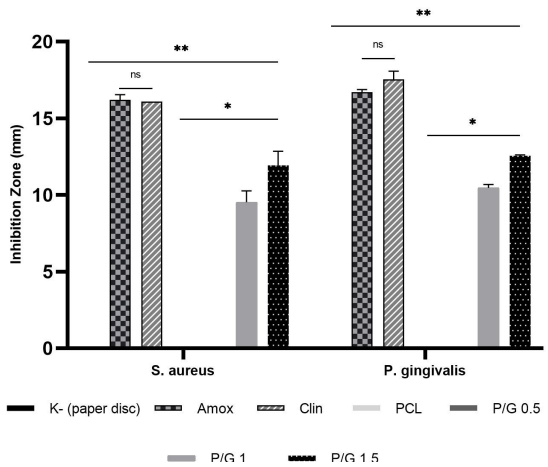


**Figure 2.** Antibacterial activity of PCL/graphene scaffold against *S. aureus and P. gingivalis* by inhibition zone diameter. ns: not significant; *: significant at $p < 0.05$; **: significant at $p < 0.01$.

and mucosal surfaces. *S. aureus* can cause several oral diseases such as periodontitis and osteomyelitis, and systemic diseases such as heart disease, chronic kidney disease, orofacial granulomatosis, and Crohn's disease (Zaatout, 2021). Meanwhile, Phorpyromonas gingivalis is a rod-shaped obligate anaerobic bacterium that is the main cause of periodontitis, and these bacteria primarily inhabit the gingival sulcus around the teeth (How et al., 2016).

Graphene is a two-dimensional carbon nanofiller that has potential in biomedical applications such as drug delivery, tissue engineering, and biosensors due to its good thermal, electrical, and mechanical properties (Williams et al., 2023). In addition, it is known to have good antimicrobial properties and can prevent biofilm formation, especially in Gram-positive bacteria, namely *S. aureus*. The mechanisms underlying these antimicrobial properties are rooted in the physicochemical characteristics of graphene, including its capacity to disrupt bacterial structures upon direct contact, generate Reactive Oxygen Species (ROS), modulate electron transfer, and other mechanisms (Staneva et al., 2021).

One notable feature of graphene is its sharp edge structures referred to as "nanoknives". These edges can damage bacterial membranes upon direct contact with graphene (Staneva et al., 2021). Bacterial membranes play an important role in maintaining cell integrity and structure, as well as regulating the molecules transport into the cells. Lipopolysaccharide (LPS) in *P. gingivalis* plays an important role in pathogenicity by stimulating a prolonged host inflammatory process. This disrupts the wound-healing process, especially in the bone remodeling phase (How et al., 2016). This study used graphene nanoparticle filler which facilitates bonding between the sharp edges of graphene and bacterial cells due to the smaller size of carbon-based materials, improving adhesion with existing bacteria. When the bacterial membrane comes into direct contact with the sharp edges of graphene, it can damage the integrity of the bacterial cell and cause cell death. The results showed that the use of PCL/graphene scaffold 1 wt% and 1.5 wt% has antibacterial activity. Scaffolds containing 1.5 wt% graphene completely inhibited the growth of S. aureus within a zone measuring 11.93 ± 0.92 mm and P. gingivalis with a zone of 12.56 ± 0.057 mm. This means that when bacterial cells come into contact with the edges of graphene-infused discs, it results in damage to the bacterial membrane, leading to the release of bacterial molecules and subsequent dysfunction of the bacterial cells.

According to antibacterial activity criteria (Davis and Stout, 1971), is classified based on the diameter of the inhibition zone, where an inhibition zone diameter > 5 mm is categorized as weak, 5-10 mm is categorized as moderate, 11-20 mm is categorized as strong, and > 20 mm is categorized as very strong. Based on the results of testing the inhibition zone of the PCL/graphene scaffold, show that the scaffold with a graphene concentration of 1.5% produces strong antibacterial activity.

Graphene can produce ROS, which is a key factor in its antimicrobial properties. Superoxidant anions and hydroxyl radicals produced by graphene can affect electron transfer in bacterial cells, and increased markers of oxidative stress have been observed in several studies. The direct interaction between the surface edge of the bacterial cell membrane and graphene leads to oxidative stress, charge transfer, entrapment, and even the phenomenon of cell death (Seifi and Kamali, 2021). This electron transfer occurs from the bacterial cell membrane to the graphene surface, which can damage the bacterial respiratory system.

Furthermore, electrons on the graphene surface increase the production of ROS, which can damage the bacterial membrane structure, causing the death of bacterial cells (Baowei et al., 2021). Oxidative stress experienced by bacterial mitochondria can hinder the transfer of nutrients to cells, thereby causing the death of bacterial cells (Qiu et al., 2024). The transfer of electrons between the bacterial cell membranes of *S. aureus* and *P. gingivalis* influences the increase in ROS production and ultimately causes the death of bacterial cells. Consequently, the utilisation of PCL/graphene scaffolds may result in the formation of an inhibitory zone.

## 5. Conclusion

In conclusion, this study showed that the PCL/graphene scaffold possessed significant antibacterial properties by effectively inhibiting the growth of bacteria. The combination of poly(e-caprolactone) and graphene in scaffolds has shown the ability to kill *S. aureus* and *P. gingivalis* bacteria at 1 and 1.5 wt%. This remarkable antibacterial capability positioned the PCL/graphene scaffold as a promising candidate for applications in bone tissue regeneration.

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