Antimicrobial Properties of Graphite and Coal-Derived Graphene Oxides as an Advanced Coating for Titanium Implants

Daniel Jankus

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Roop L. Mahajan, Committee Chair

Ranga Pitchumani

Shivakumar Ranganathan

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ABSTRACT

Prosthetic joint infection (PJI) poses a significant risk to implanted patients, requiring multiple surgeries with high rates of reinfection. The primary cause of such infections is otherwise innocuous bacterial species present on the skin that have survived sterilization protocols. Antibiotic drugs have significantly reduced efficacy due to the lack of vasculature in the newly implanted site, allowing microbes to form biofilms with even greater resistance. Graphene oxide (GO) is known to have good biocompatibility while providing drugless antimicrobial properties. The focus of this study is on the development and characterization of a robust coating for titanium alloy implants to promote bone regeneration while inhibiting microbial biofilm adhesion to the implant surface. The novelty of this study is the use of proprietary coal-derived graphene oxide (c-GO) in a biomedical application. c-GO has been demonstrated to have a greater number of functional oxygen groups to promote cell adhesion, while also maintaining thinner layers than possible with graphite exfoliation methods. As an alternative to powerful antimicrobial drugs, it was hypothesized that an advanced coating of graphene-oxide would provide a defensive, passively antimicrobial layer to a titanium implant. While GO is typically quite expensive, the newly developed process provides an economical and environmentally friendly method of producing GO from coal (c-GO). The result is a coating that is inexpensive and capable of halving the biofilm formation of MRSA on titanium-alloy surgical screws in addition to providing improved bone cell adhesion and hard tissue compatibility.

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GENERAL AUDIENCE ABSTRACT

Any time a patient receives implantation surgery, there is a chance of microbes entering the body. These are typically naturally occurring skin flora, harmless but opportunistic. On the surface of implants within the body, these bacteria can form colonies called biofilms, leading to severe and potentially deadly infections, called prosthetic joint infection (PJI). PJI often requires multiple surgeries to remedy, but rates of reinfection are relatively high. As with any surgery, patients are given antibiotic drugs, but implants to not receive blood flow as the body normally would, reducing the effectiveness of antibiotics. Once biofilms are formed, the bacteria become even hardier and resistant even to powerful antibiotics. Graphene oxide (GO) is a carbon material known to have good biocompatibility (i.e., non-toxic) while providing antimicrobial properties. The focus of this study is on the development and characterization of a robust coating for titanium alloy implants to promote bone healing while reducing microbial biofilm colonization on the implant's surface. The novelty of this study is the use of proprietary coal-derived graphene oxide (c-GO) in a biomedical application. c-GO has been demonstrated to have a different chemical makeup than graphite-derived GO, which may improve its efficacy as an antimicrobial coating. As an alternative to powerful antimicrobial drugs, it was hypothesized that a coating of graphene-oxide would provide a defensive, passively antimicrobial layer to a titanium implant. While GO is typically quite expensive, the newly developed one-pot process provides an economical and environmentally friendly method of producing GO from coal (c-GO). The result is a coating that is inexpensive and capable of halving the biofilm formation of MRSA on titanium-alloy surgical screws in addition to providing improved bone cell adhesion and hard tissue compatibility.

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ATTRIBUTION

Rusha Pal and Dr. Muhammad Seleem aided in the writing and research presented as part of this thesis. They both served as co-authors on this paper and assisted in the execution of the research experiments written herein. A brief description of their contributions is included here:

Chapter 3.1: Materials – Antimicrobial Testing

Chapter 3.3: Microbial Activity Testing

Chapter 3.3: GO Solution Minimum Inhibitory Concentrations

Chapter 4.1: Results for Biofilm Inhibition with MRSA

Chapter 4.2: Results for Biofilm Inhibition with *S. epidermidis*

Chapter 4.3: Results for GO Solution MIC

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1 Introduction

1.1 Motivation and Background

Prosthetic joints are largely a success in modern medicine but are not without long-term implications. Of total hip and knee implants, 5-10% of are revised within 7 years, requiring additional surgery and physical therapy for proper patient recovery. Prosthetic joint infection (PJI) and subsequent osteomyelitis are common reasons for revision, occurring in 2% of patients and accounting for 30% of revisions [1] [2]. Even with multiple surgeries and antimicrobial treatments, PJI has a recurrence rate of 16% and mortality rate of 2.5% [1]. To reduce the rates of PJI, researchers have investigated drug-eluting implant materials to prevent biofilm growth [1] [3]. *In vivo* studies are largely successful, but not without their shortcomings. As in any drug-related treatment, there is a chance of antimicrobial resistance [4]. Additionally, the drug attachment tends to weaken structures, especially load-bearing surfaces, and does not provide a benefit to bone regeneration [1] [2] [5]. Furthermore, many implant failures are due to weakening of bone caused by stress shielding and improper bone-implant fusion, so any means of improving this interface is critical to the implant's long-term success [2] [5] [6]. To this end, a means of enhancing existing implantable medical devices to reduce the rates of infection, improve biocompatibility, and facilitate healing must be developed in a manner that is safe, economical, and environmentally friendly.

2 Review of Literature

2.1 Surface Functionalization

Surface functionalization, particularly drug attachment, has been a topic of research in the biomedical field as implants become more prevalent than ever. Suhardi *et al.* [1] and Liang *et al.* [3] focus their research on the storage and dispersion of antimicrobial drugs on the surface of an implant to increase bioavailability at the site of implantation, where they are most needed. With these methods, antimicrobial drugs can be released in slow, steady doses over weeks, helping prevent the onset of PJI. However, other research by Thornes and Bouchier-Hayes has shown that hardy microbes such as *Staphylococcus epidermidis* actually develop antibiotic resistance with bone cements loaded with gentamicin, leading to worse infections with high rates of recurrence [4]. Additionally, Suhardi *et al.*'s research proves only that hard tissue remodeling was not negatively affected by the drug-eluting surface, but it does not aid in the growth of these tissues [1]. This conflicting research and the rise of resistance bacteria demonstrate the need for a non-drug means of fighting microbial infections on joint implants.

2.2 Improving Hard Tissue Compatibility

Increasing the osteogenic (i.e., hard tissue affinity) of implants is another goal of modern research. Albrektsson *et al.* [7] and Carlsson *et al.* [8] have shown extensively that titanium itself presents a surface that aids in bone remodeling, thus its prevalence in the medical field. However, further functionalization can reduce the phenomena known as stress shielding, where the relatively high stiffness and yield strength of metallic implants reduces local bone density and increases porosity, therefore weakening the bone surrounding the implant. To help improve surface adhesion and stress flow, Sun *et al.* [2] and La *et al.* [9] developed coatings that carry bone morphogenic protein-2 (BMP-2). This improves adhesion of apatite to the titanium surface and accelerates the differentiation of osteocytes, therefore increasing the rate and strength of remodeling (a.k.a. healing). However, these surface functionalizations also tend to

indiscriminately promote the growth of undesirable cells, such as microbes [2]. This demonstrates the need for a coating or surface treatment that improves bone growth and adhesion while reducing undesired bacterial colonies.

2.3 Graphene-Oxide in Biomedicine

Graphitic materials have also made their way into the biomedical world. Carbon nanotubes (CNTs) have a promising role in oncology but are also highly cytotoxic to human cells. Graphene oxide (GO) has found its use as both a surface coating and a filler for other materials. Zhou *et al.* [10] Shaui *et al.* [11], Peng *et al.* [12], Saravanan *et al.* [13], and Purohit *et al.* [14], have all developed composite scaffolds utilizing GO to improve both biological and mechanical properties. These scaffolds demonstrate improved high cell viability, cell density, and regrowth of microenvironments in addition to bulk improvement of bone regeneration [15]. However, scaffolds are not always applicable to joints and implants, and lend themselves more to large injuries in the spine, skull, and long bones.

GO itself has been used as a surface coating to enhance bone regeneration without the use of other drugs. Wang *et al.* [6], Jung *et al.* [16], Lee *et al.* [17], Zhao *et al.* [5], Klabacova *eta al.* [18], and La *et al.* [9] have all demonstrated enhanced bone remodeling in the presence of graphitic materials, particularly pure graphene and GO. The main benefit of GO is improved differentiation of bone-marrow derived mesenchymal stem cells (BMSCs), similar to the expensive BMP-2 used by other researchers [6] [16]. Additionally, GO is not as cytotoxic to human cells as other graphitic materials, improving overall safety of such coatings [19]. These studies are not without their drawbacks. Wang and Jung focused on other surface modifications, such as laser microgrooves and BMP-2 attachment, both of which increase the cost of an implant by orders of magnitude [6] [16]. Lee *et al.* and Zhao *et al.* focused on the growth and differentiation of BMSCs, but not the integration of an actual implant into hard tissue [17], [5]. Kalbacova *et al.* showed that graphene and its oxides promote the growth and adhesion of human osteocytes and BMSCs without modification, though this study focuses on graphene-only substrates, rather than coatings [18]. La *et al.*'s coating process uses chemical vapor deposition (CVD) for the coating of GO onto a titanium substrate, which is cost-prohibitive [9]. Additionally, none of these studies address the antimicrobial properties of GO as a coating, and all of them have used commercially available or lab-made GO made with modified Hummer's method.

2.4 Graphene Oxide as a Potential Coating for Bone Implants

The ideal implant promotes osseointegration while inhibiting microbial activity [5] [6]. Titanium alloys are widely used for screws, plates, and whole hip and knee implants due to their inherent corrosion resistance, biocompatibility, and affinity for bone cell adhesion (i.e., osteointegration) [8] [7]. To improve surface bone cell and apatite adhesion and carry antimicrobial drugs, significant and expensive biopolymer functionalization of surfaces is often necessary [1] [2] [3] [4]. These processes are costly and time consuming, and tend to indiscriminately promote cell growth, including microbial biofilms [2] [5] [9]. Recent research into graphene oxide (GO) has shown that this novel material exhibits good biocompatibility in hard tissues while providing both osteogenic and antimicrobial traits [6] [16] [17] [5] [11] [20] [21] [22] [23]. However, current research focuses on one trait or the other in varying concentrations of GO. The goal of this study is to compare the antimicrobial properties of GO derived from traditional modified Hummer's method with a graphite precursor and novel coal-derived processes (c-GO) in a practical application to enhance an existing commercial product. It is hypothesized that the greater number of functional groups and thinner layering of c-GO will provide improved performance in comparison to GO in terms of both antimicrobial and osteogenic properties.

2.5 Differences Between GO and c-GO

The facile method developed to produce c-GO from coal requires fewer volatile chemicals, takes less time, and has higher yields than Hummer's method at the laboratory scale [24]. Additionally, coal is still a cheap and abundant resource compared to graphite, greatly reducing the cost of producing this otherwise expensive material [24] [25]. With a simpler process, and more environment-friendly chemicals, c-GO is well suited for mass production for numerous applications, including biomedical, aerospace, electrical, and heat transfer. This motivates a brief economic analysis on the cost implications of c-GO as a coating for medical implants, typically expensive products from the start.

Modified Hummer's method for creating GO from graphite is an involved multi-step process using strong oxidizers and acids. Graphite powder is mixed with sodium nitrate ($NaNO_3$) then dissolved in sulfuric acid (H_2SO_4). The solution is heated to 66°C, then cooled to 0°C. Potassium permanganate is added to the solution and stirred, then the remaining solution is diluted to produce a solution of graphite oxide. This process produces NO_2 and N_2O_4, both of which are undesirable toxic gasses with negative environmental impacts. The solution must then be exfoliated using an organic solvent such as dimethylformamide (DMF), n-methyl-2-pyrrolidone (NMP), tetrahydrofuran (THF), or ethylene glycol, all of which also present toxicity and flammability hazards [26].

To summarize the one-pot process (PCT/US19/66941, December 17, 2019), 5 g purified coal powder is mixed with 80 mL 16M HNO_3 at 120°C for 5 hours, then cooled. The remaining oxidized coal is washed with DI water, then ultra-sonicated in an ice bath for 2 hours. Finally, the solution is centrifuged at 4,000 rpm for 1 hour to remove unreacted coal particles. The remaining suspension contains c-GO, which can be lyophilized to obtain a powder, or used in aqueous form [25]. The layer height ranges from 0.8 and 2.0 nm with sheet sizes between 300 and 700 nm. The process is summarized in **Figure 1** below, courtesy of Lee and Mahajan [25].

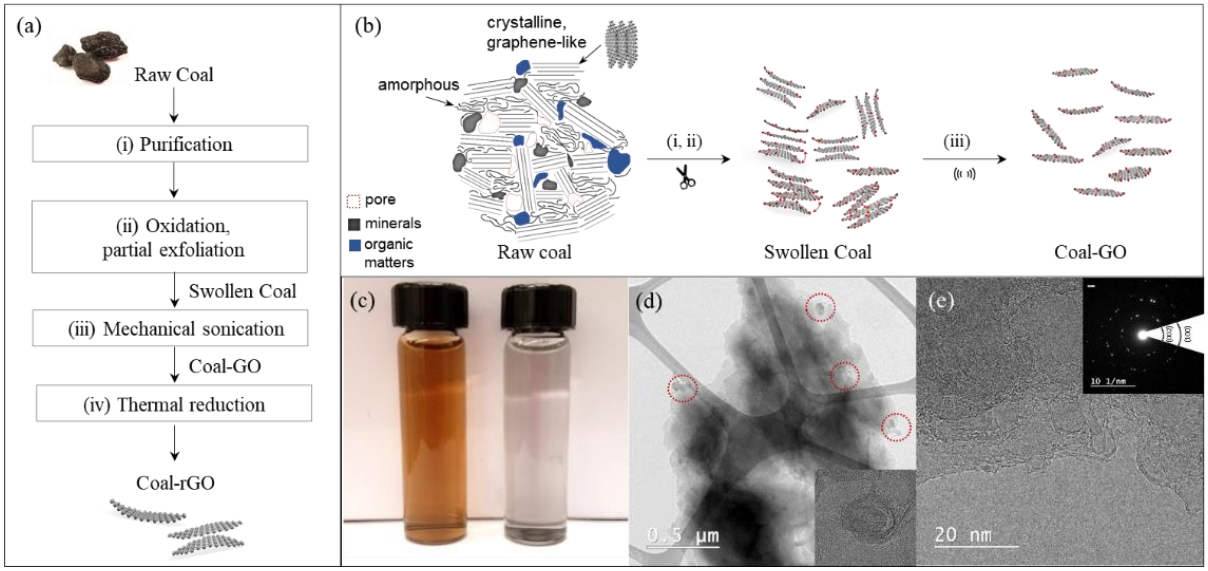

Figure 1: (a) Flow chart, (b) schematic illustration of one-pot process for the GO production from coal, (c) camera images of the Coal-GO and Coal-rGO aqueous solution, (d) TEM image of the Coal-GO (inset: magnification of nanoscrolls), and (e) TEM image of the C *[25]*

3 Experimental Section

3.1 Materials

Screw Coatings

To represent a realistic application of a GO coating for implants, Ti-6Al-4V alloy screws of 2.5mm diameter and 7mm length were selected. These particular screws are chosen for dental surgery and hand repair, both implanted sites subject to harsh conditions and constant microbial attack. The screws were purchased from their manufacturer and shipped in sterile packaging. The manufacturer asked to remain anonymous for this study. For the chemical assembly of the coatings, (3-Aminopropyl)triethoxysilane (APTES, 98% w.t.) and dopamine hydrochloride (DA, 99%) were obtained from Sigma-Aldrich. Both GO and C-GO samples were provided by Professor Mahajan's GrapheneX and Thermal Engineering at Virginia Tech in Blacksburg, VA.

Antimicrobial testing

For microbial activity testing, Methicillin-resistant *Staphylococcus aureus* (MRSA, NRS384), *Staphylococcus epidermidis* (Staph. E, ATCC 35984), and *Pseudomonas aeruginosa* **(***P. aeruginosa*, ATCC 9027*)* were selected due to their medical significance and film-forming capabilities. Microbial species were cultured in general-purpose tryptic soy broth (TSB) and tryptic soy agar (TSA), typical nutrient solutions used for microbial species that have high nutritional requirements or anerobic proliferation.

3.2 GO Coatings

Chemical Assembly of Coatings

Surgical screws of 2.5mm diameter by 7mm length were obtained from the manufacturer, herein referred to as "samples." The method outlined below was found by Wang *et al* to provide a robust coating that is resistant to scratching and flaking [1] [6]. All samples were first immersed in 20% volume HNO_3 solution for 5 minutes to remove the passive oxide layer of the titanium, then rinsed with DI water. All samples were then immersed in 5M NaOH solution for 24 hours with minimal stirring. At this point, 5 samples were set aside to be Control A. The remaining samples were immersed for 30 minutes in 3% weight APTES dissolved in DI water with the pH adjusted to 8.5. After the APTES treatment, the samples moved immediately to a 0.2% weight DA solution with DI water at a pH of 8.44 for 12 hours. At this point, 5 samples were set aside to be Control B. The remaining screws were rinsed with DI water and dried with compressed air. Ten samples each were placed in 1mg/mL GO and C-GO suspensions, respectively, and left to rest at 60°C for 24 hours. After the GO treatment, all samples were rinsed with DI water and dried with compressed air.

Figure 2: c-GO and GO suspensions used for coating

Specimen Sample Groups

Of a total of thirty (30) samples, a total of four (4) test groups were made, called Control A, Control B, Group 1, and Group 2. Control A consisted of 5 total samples that received HNO_3 and NaOH treatment only, used as a clean baseline to compare the performance of other samples. Control B consisted of 5 total samples that received the intermediate APTES-DA treatment to determine if this layer alone had any effect on the antimicrobial properties of the coating. Group 1 consisted of 10 total samples coated with GO, and Group 2 consisted of the final 10 samples coated with c-GO.

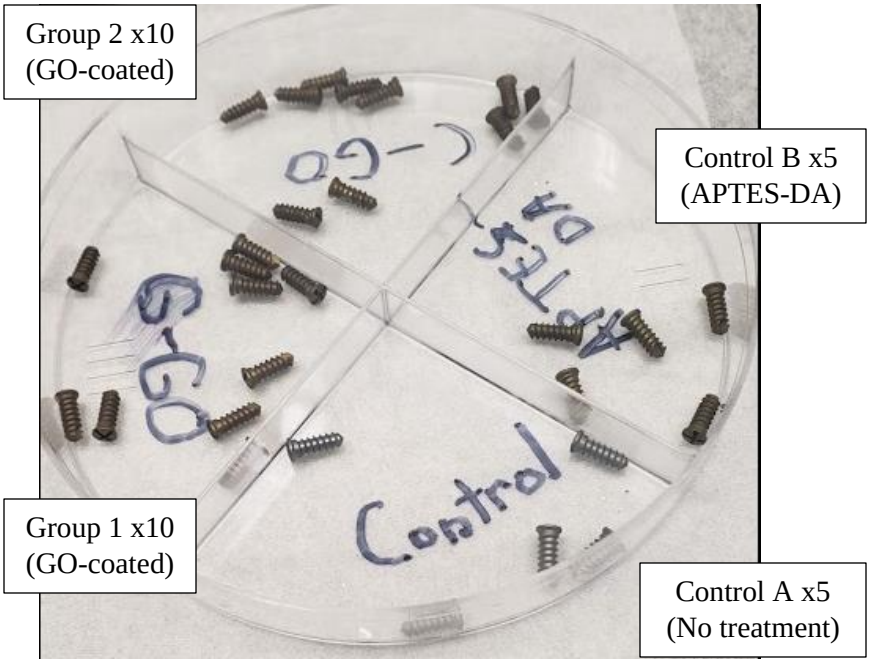

Figure 3: All screw samples after coating. Clockwise from top: Group 2 (c-GO), Control B (APTES-DA, Control A (untreated), Group 1 (GO)

Coating Chemical Confirmation and Analysis

The samples were analyzed using a WITech Alpha 500 Raman microscope with Olympus MPlanFL N 100x / 0.9 objective. An image scan with integration time of 0.2 seconds, excitation wavelength of 633.150 nm, and grating of 300 g/mm was performed on a 50 x 40 µm area of the screws. Single Raman was also performed, using 6 accumulations, integration time of 10 seconds, and the same excitation and grating parameters.

The chemical assembly of the GO coatings proved to be a simple chemical process requiring little specialized equipment. The resultant coatings are nanometer-scale, and do not show significant color change in comparison to the control groups (**Figure 4**).

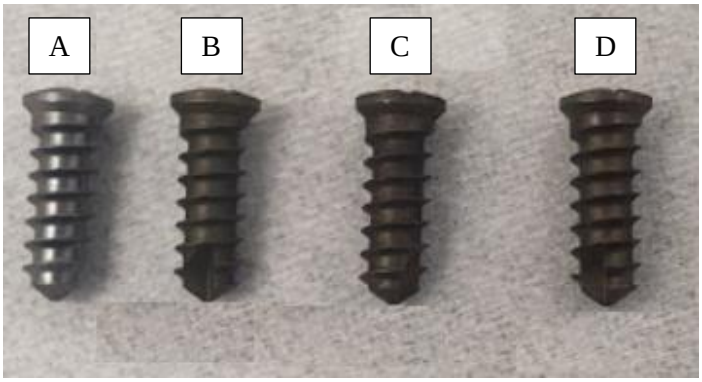

Figure 4: A: Untreated Ti-6Al-4V alloy screws (Control A). B: APTES-DA treated screw (Control B). C: GO coated screw (Group 1). D: C-GO coated screw (Group 2)

The Raman spectra confirm the presence of graphene oxide particles on both Group A(GO) and Group B (C-GO), with major peaks occurring at 1300 and 1600 cm^{-1}, typical of graphene-oxide (**Figure 5**). The C-GO coated screw showed a higher intensity peak, indicating a higher concentration of GO particles present than on the surface of the GO-coated screw. Microscope images show fields of particles on the surfaces. The network appears to be finer for the C-GO samples, while the GO samples display a coarser configuration. Overall, the Raman spectra confirm that GO particles are properly adhered to the screws. At this point no scratch or durability tests were performed, as these were carefully documented by Wang *et al.* [6]. Following the same coating procedure, Wang *et al* shows that APTES plus DA provide an intermediate chemical layer that improves GO adhesion to the titanium alloy substrate than GO alone. Chemical assembly of the coating allows for a more uniform distribution of GO than CVD or electrochemical deposition methods in addition to requiring no expensive equipment. APTES alone was shown to provide a GO layer prone to peeling, an undesirable characteristic in screws especially. Thus, dopamine (DA) is used as a secondary intermediate layer with a critical load (L_c) of approximately 74N. Comparing to a typical anodized aluminum coating with L_c 37N, this proves that such a chemical layup has excellent durability [6]. Durability is an important characteristic for screws especially, which are subject to high traction loads when driven. This is of particular importance due to the fact that graphitic materials such as graphene, GO, and carbon nanotubes can be cytotoxic to human cells if released into the blood, in addition to losing their surface coating benefits.

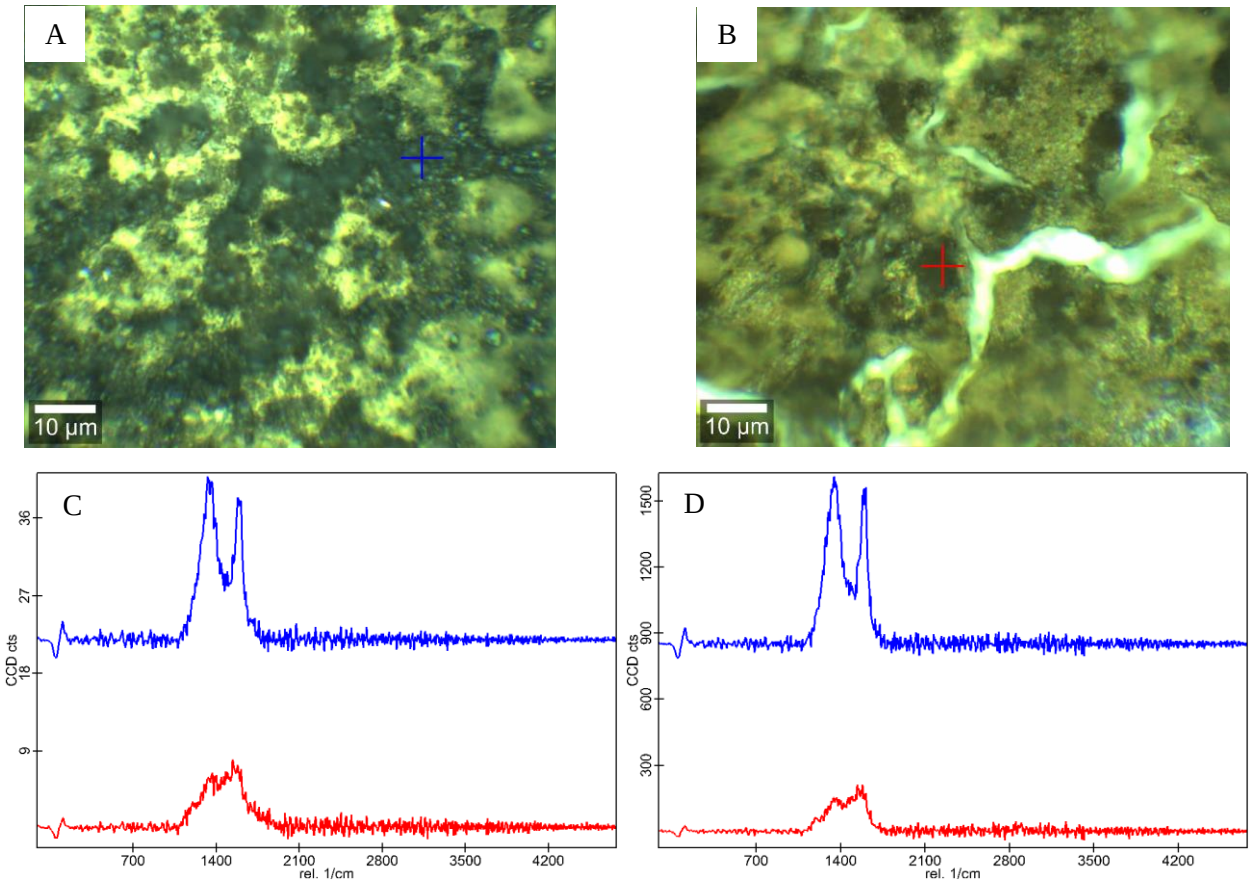

Figure 5: A: Microscope image of c-GO-coated screw. B: Microscope image of GO-coated screw C: Raman average spectral analysis of GO (red) and c-GO (blue) coated screws. D: Raman single-point spectral analysis for GO (red) and c-GO (blue) coated screws.

It is notable that GO and reduced GO (rGO) may be eliminated from the body via endocytosis [10]. The question may be poised, will human cells damage or destroy the GO coating after implantation? An *in vivo* study would be best to determine this, however, one may conjecture that if human leukocytes (immune cells) are reaching the surface of the implant, then there are no bacteria in the way for them to attack first. Therefore, the coating is no longer necessary, and can be absorbed and eliminated. This also means in the case of flaking or other surface damage, released GO can be safely eliminated from the body. An informative set of tests that could be performed in the future is a study of the coating's chemical durability in an environment with equivalent pH to the human body.

3.3 Microbial Activity Testing

Biofilm Inhibition Study

To quantify the antimicrobial affect and film-fighting capabilities of the GO coatings, two common and medically significant species were selected for testing, MRSA and *S. epidermidis*. Biofilm forming clinical isolates were streaked on tryptic soy agar (TSA) and allowed to incubate aerobically at 37°C overnight. A single colony was then isolated from the plate and again incubated overnight in tryptic soy broth (TSB) overnight at 37°C with shaking. The culture was then diluted 1:100 with TSB and 4mL of the solution was transferred to a 6-well tissue culture plate containing the screws, then incubated aerobically for 24 hours at 37°C. The screws were then removed, rinsed with phosphate-buffered saline (PBS), and immersed in 5mL of PBS in 50 mL Falcon tubes. The tubes were vortexed for 30 seconds, followed by 5 minutes of sonication and an additional 30 seconds of vortexing [27]. The remaining fluid was diluted and plated, with colony counts occurring the next day. The goal of this process is to remove any attached microbes from the samples so they can be more easily enumerated. Three samples each of Control A, Control B, Group 1, and Group 2 were tested with each microbe, repeated twice.

GO Solution Minimum Inhibitory Concentrations

The minimum inhibitory concentrations (MICs) of the solutions and the control antibiotics were tested against Methicillin-resistant *Staphylococcus aureus* NRS384, *Staphylococcus epidermidis* NRS101, and *Pseudomonas aeruginosa* ATCC 9027 as per the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) , outlined below. Each strain was cultivated on tryptic soy agar (TSA) plates and incubated for 24 hours in aerobic condition. The clinical isolates were used to prepare a bacterial suspension equivalent to the turbidity of 0.5 McFarland solution, and added to tryptic soy broth (TSB) to attain a bacterial concentration of 5×10^5 CFU/ml. The desired concentrations of each solution and control antibiotic were added to each first well of the 96-well plates. Serial dilution was carried out and the plates were incubated aerobically at 37ºC for 16-20 hours. The maximum concentration that could be produced was The MIC was defined as the lowest concentration of the drugs that inhibited bacterial growth after the incubation period.

3.4 Economic Impact

Comparison of GO and C-GO

The one-pot process produces graphene oxide particles at approximately 40% efficiency (i.e., yield), compared to 5% for Hummer's method [25]. This coupled with fewer expensive chemical reagents and lower temperatures results in a cost of approximated $10 per gram of c-GO, compared to $135 per gram of commercially available GO. This ten-fold reduction in cost eliminates a serious economic barrier in the large-scale use of graphitic materials, such as reinforcement in polymers, hydrophobic coatings for aircraft, and antimicrobial surfaces for medical implants.

Cost Implications of c-GO

For these studies, 10 mg of c-GO at a concentration of 1mg/mL was used to coat 10 titanium screws. The screws were procured from their manufacturer for approximately $3 per unit, and the total coating process cost approximately $0.10 per screw for miscellaneous reagents. The cost of c-GO per screw is $0.01, increasing the cost of each screw by $0.11, or ~3.6% or the original cost. By way of comparison, the exact same coating process using graphite-GO raises the cost per screw by $0.24, or ~7.8% in materials alone. The coating process is comprised of simple chemical baths, an easily automated process. This method could be used to batch-coat mass-produced GO-coated parts for any application, but especially biomedical implants and tools. For all the benefits that graphitic materials can provide, a 3.6% increase in base material cost is a small price to pay. Additionally, these costs are based on the production of laboratory quantities and can be reduced further in an industrial setting.

Drug-eluting implants, silver nanoparticles, and other novel coatings require an intense amount of chemical treatment and expensive materials, such as BMP-2 at a cost of $37 per microgram or functionalized silver nanoparticles at $212 per milligram [1] [2] [3] [4] [25]. Numerous researchers have studied graphitic materials and traditional antibiotic drugs on a multitude of surfaces and materials, but few have considered the enhancement of actual commercially available and well-proven products [2] [19]. This simple, inexpensive coating opens the window of opportunity for wide-spread use of graphene particles.

4 Results and Discussions

4.1 Results for Biofilm Inhibition with MRSA

In terms of antimicrobial performance, the primary goal of the coating is to combat the formation of biofilms, which vastly increase the infection's resistance to antibiotic drugs up to 10000 times for some species [3]. As such, to improve the efficacy of oral or intravenous antibiotics, the GO coating must inhibit the formation of biofilms. The GO coating proved to reduce the formation of MRSA biofilms by 10%, while C-GO was far more effective at 60% in comparison to the untreated Control A group. Compared to Control B, GO coating reduced MRSA biofilm formation by as much as 30%, and C-GO by 50%. The test was repeated to confirm the results with additional samples from the same groups. These results are summarized in **Figure 6** and confirmed to be statistically significant

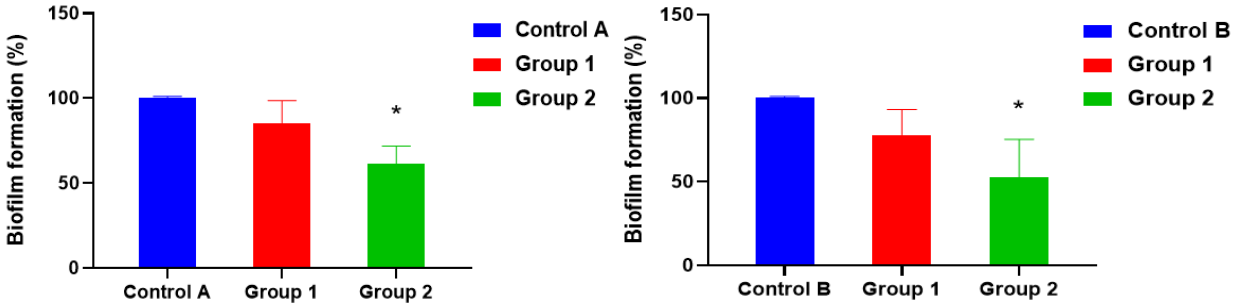

Figure 6: Resultant MRSA biofilms formed on GO and C-GO coated screws compared to Control A (Titanium only, LEFT) and Control B (APTES-DA, RIGHT). Error bars represent standard deviation values. Asterisk (∗) denotes statistical significance (P = 0.002) between control B and Group 2 analyzed via one-way ANOVA with post hoc Dunnet's test for multiple comparisons

The results for MRSA show that both GO and c-GO coatings effectively reduce biofilm formation, which assists the natural immune system in fending off PJI. Coupled with systemic antibiotic drugs such as Linezolid, it is believed that a GO coating could significantly reduce rates of PJI in implanted patients. MRSA is of particular danger in hospitals due to the significant use of antibiotics and the fact that it can live on the skin for years without causing infection. When a patient is opened for implantation surgery, MRSA can reach the wound site, particularly the prosthetic itself, causing PJI. The GO coating, particularly c-GO, will be effective in reducing the chance of this and severity of infection, should contamination occur. MRSA is susceptible to acute oxidative stress, which explains the significant efficacy of the GO coatings observed during this study [28].

4.2 Results for Biofilm Inhibition with *S. epidermidis*

When evaluated for *S. epidermidis* biofilm activity, GO-coated screws were found to have a 25% reduction compared Control A and 15% reduction compared to Control B. C-GO screws did not perform as expected, causing a notable increase of 10% and 50% compared to Controls A and B, respectively. The results are shown in **Figure 7.** As with MRSA, these tests were repeated to confirm the results with additional samples from the same groups. A possible explanation for this discrepancy follows.

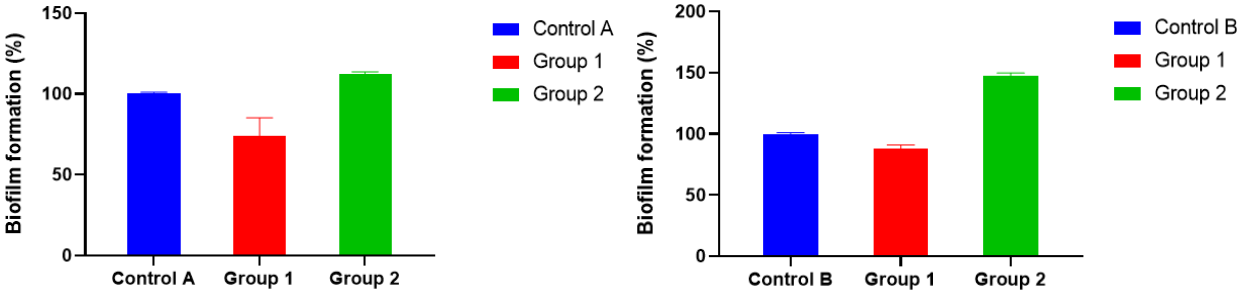

Figure 7: Resultant *S. epidermidis* biofilms formed on GO and C-GO coated screws compared to Control A (Titanium only, LEFT) and Control B (APTES-DA, RIGHT). Error bars represent standard deviation values

S. epidermidis is known to exhibit increased biofilm formation in high-stress environments, which may have caused this adverse effect [29]. The particular strain used (ATCC 35984, NRS101) is known to be extremely hardy and resistant to numerous antimicrobial drugs such as methicillin, erythromycin, and others, and may also be referred to as MRSE. However, the positive effects shown on MRSA biofilm formation dispels the theory that drug-resistant bacteria may be resistant to graphitic materials. Instead, the greater presence of the oxygen groups on graphitic basal planes of c-GO may have triggered a release of nitrate reductase, which contribute to biofilm formation and robustness of the growing colony. This causes the existing colony to slow its metabolic rate and release proteins needed for the formation of biofilm. Once the protective biofilm is formed, the colony can continue to grow anaerobically within, free of external stressors [30]. *S. epidermidis,* like MRSA, lives naturally on this skin, though not all strains for biofilms. As previously mentioned, this opportunistic microbe is of particular danger to implanted patients due to its hardiness and prevalence on the skin.

Overall, the coating of GO proves somewhat effective against both MRSA and *S. epidermidis* in combating the formation of biofilms. c-GO, while vastly more effective against MRSA, was shown to have an unforeseen affect to *S. epidermidis.* This result requires further investigation as to the biological mechanisms at play, and what contribution the c-GO has to these processes. At this point, however, the hypothesis that c-GO would outperform GO in its ability to reduce biofilms, in the case of *S. epidermidis*, is disproven.

4.3 Results for GO Solution MIC

Antimicrobial tests in solutions of GO and C-GO particles proved to be inactive, therefore showing a MIC greater than 512 µg/ml (**Table 1**). This result is highly intriguing, as it corroborates the research of Perreault *et al* regarding the entrapment and inactivation of microbes, but not cell death [29]. The cells avoid growing near the site of the solution due to oxidative stress but are otherwise not inhibited in the surrounding agar dish, showing no noticeable reduction in their colonies. This contradicts other research on the antimicrobial and antiviral properties of GO in solutions and shows that much is yet to be learned about the interactions of graphitic particles with biological specimens. The following table represents the concentration of solutions/control antibiotics that have been found to completely inhibit the bacterial growth under anaerobic conditions. A different test or measure of antimicrobial activity may be needed to categorize GO and c-GO suspensions in the future.

Table 1: Minimum Inhibitory Concentration (MIC) values of solutions/control antibiotics against Gram-positive and Gram-negative bacterial isolates

Table 1: Bacterial isolates	**MIC (µg/ml)**					
	Coal-GO	**Graphite-GO**	**Vancomycin**	**Linezolid**	**Gentamicin**	**Colistin**
MRSA NRS384	>512	>512	1	0.5	NT	NT
Staphylococcus epidermidis NRS101	>512	>512	1	0.25	NT	NT
Pseudomonas aeruginosa ATC 9027	>512	>512	NT	NT	2	0.5

NT= Not tested

5 Conclusions

The coating process of GO onto a titanium substrate was already proven to be simple and robust, and it was confirmed that c-GO could also be applied with the same procedure. This process could easily be scaled to larger components for aerospace and electronics applications [5] [6] [16] [17]. Due to the reduced costs and environmental impacts, c-GO may be a commercially viable material for large-scale coating and reinforcement processes. GO coatings have already been proven to provide osteogenic and osteointegration benefits in hard tissues, improving apatite adhesion and osteocyte proliferation [6] [16]. As an antimicrobial coating, initial results are promising with MRSA, with the GO and c-GO coatings able to reduce biofilm formation. Future work is required to fully characterize the interactions between c-GO and other medically significant microbial strains, such as *S. epidermidis*, particularly their interactions on a chemical level. Follow-on work may include the effect of varying c-GO concentrations during the coating process and *in vivo* studies to compare GO and c-GO in terms of osteogenic benefits, as well as TEM images of biofilms on the substrates to observe the mechanisms of adhesion. The culmination of this study would be *in vivo* testing of these implants to measure metrics of both antimicrobial and osteointegration enhancement, as well as the life cycle and durability of the coating in the implanted environment.

6 References

[1] V. B. D. K. S. e. a. Suhardi, "A fully functional drug-eluting joint implant," *Nat Biomed Eng,* vol. 0080, no. 1, 2017.

[2] A. A. N. D. M. Sun, "Methacrylate Coatings for Titanium Surfaces to optimize biocompatibility.," *Micromachines,* vol. 11, 2020.

[3] J. W. H. L. M. Liang, "Biomaterial surfaces self-defensive against bacteria by contact transfer of antimicrobials," *Biomaterials,* vol. 204, pp. 25-35, 2019.

[4] M. P. B.-H. D. Thornes B, "Development of resistant strains of Staphylococcus epidermidis on gentamicin-loaded bone cement in vivo.," *J Bone Joint Surg Br.,* vol. 84, pp. 758-760, 2002.

[5] Z. C. L. J. Zhao C. Lu X., "The promising application of graphene oxide as coating materials in orthopedic implants: preparation, characterization, and cell behavior.," *Biomedical Mater,* vol. 10, p. 015019, 2015.

[6] H. H. L. Z. S. Y. X. Y. Z. G. Z. X. D. J. Z. S. R. T. Z. Y. Wang C., "Enhanced Osseointegration of Titanium Alloy Implants with Laser Microgrooved Surfaces and Graphene Oxide Coating.," *ACS Appl. Mater. Interfaces,* vol. 11, pp. 39470-39483, 2019.

[7] R. T. A. B. A. T. B. P. Carlsson L., "Osseointegration of titanium implants.," *Acta Orthopaedica Scandinavica,* vol. 57, no. 4, pp. 285-289, 1986.

[8] B. P. H. H. L. J. Albrektsson T., "Osseointegrated titanium implants: Requirements for Ensuring a Long-Lasting, Direct Bone-to-Implant Anchorage in Man," *Acta Orthopaedica Scandinavica,* vol. 52, no. 2, pp. 155-170, 1981.

[9] J. M. P. S. Y. H. J. G. B. S. P. H. C. K. K. B. La WG, "Delivery of bone morphogenic protein-2 and substance P using graphene oxide for bone regeneration," *Int Journal of Nanomedicine,* vol. 9, pp. 107-116, 2014.

[10] Z. K. e. al, "Hierarchically porous hydroxyapatite hybrid scaffold incorporated with reduced graphene oxide for rapid bone ingrowth and repair," *Biomedical Materials,* vol. 10, p. 015019, 2015.

[11] Z. Z. Y. Y. Q. F. P. S. Y. W. H. C. W. G. Q. G. Shuai C., "Graphene oxide assists polyvinylidene fluoride scaffold to reconstruct electrical microenvironment of bone tissue," *Materials and Design,* vol. 190, p. 108564, 2020.

[12] B. R. S. X. Y. W. Y. M. Peng Y., "Self-assembled high-strength hydroxyapatite/graphene oxide/chitosan composite hydrogel for bone tissue engineering.," *Carbohydrate Polymers,* vol. 155, pp. 507-515, 2017.

[13] C. A. V. M. S. T. S. K. S. N. Saravanan S., "Scaffolds containing chitosan, gelatin, and graphene oxide for bone tissue regeneration in vitro and in vivo.," *Int. Journal of Biological Macromolecules,* vol. 104, pp. 1975-1985, 2017.

[14] B. R. S. H. Y. I. G. M. M. N. Purohit S.D., "Development of a nanocomposite scaffold of gelatin-alginate-graphene oxide for bone tissue engineering," *Int. Journal of Biological Macromolecules,* vol. 133, pp. 592-602, 2019.

[15] B.-R. H. H. E. Pahlevanzadeh F., "In-vitro biocompatibility, bioactivity, and mechanical strength of PMMA-PCL polymer containing fluorapatite and graphene oxide bone cements.," *Journal of the Mechanical Behavior of Biomedical Materials,* vol. 82, pp. 257-267, 2018.

[16] H. S. Jung, Y. j. Choi, J. Jeong, Y. Lee, B. Hwang, J. Jang, J. H. Shim, Y. S. Kim, H. S. Choi, S. H. Oh, C. S. Lee, D. W. Cho and S. K. Hahn, "Nanoscale Graphene Coating on Commercially Pure Titanium for Accelerated Bone Regeneration," *RSC Adv,* vol. 6, pp. 26719-26724, 2016.

[17] C. H. Y. X. L. H. S. L. A. L. T. Y. W. T. L. a. K. P. L. W. C. Lee, " Origin of enhanced stem cell growth and differentiation on graphene and graphene oxide," *ACS Nano,* vol. 5, pp. 7334-7341, 2011.

[18] B. A. K. J. K. M. Kalbacova M., "Graphene subtrates promote adherence of human osteoblasts and mesenchymal stromal cells.," *Carbon,* vol. 48, pp. 4323-2329, 2010.

[19] Z. T. H. M. B. E. W. J. J. R. K. J. C. Y. Liu S., "Antibacterial Activity of Graphite, Graphite Oxide, Graphene Oxide, and Reduced Graphene Oxide: Membrane and Oxidative Stress.," *ACS Nano,* vol. 5, pp. 6971-6980, 2011.

[20] H. J. D. A. E. V. K. J. Gurunathan S., "Oxidative stress-mediated antibacterial activity of graphene oxide and reduced graphene oxide in Pseudomonas aeruginosa.," *International Journal of Nanomedicine,* vol. 7, pp. 5901-5914, 2012.

[21] X. Y. Y. C. C. G. L. Y. Z. T. P. Q. Xia M.Y., "Graphene-based nanomaterials: the promising active agents for antibiotics-independent antibacterial applications," *Journal of Controlled Release,* vol. 307, pp. 16-31, 2019.

[22] A. L. B. d. F. A. B. M. A. O. de Moraes, "Graphen oxide-silver nanocomposite as a promising biocidal agent against methicillin-resistant Staphylococcus aureus.," *International Journal of Nanomedicine,* vol. 10, pp. 6847-6861, 2015.

[23] R. L. M. &. S.-Y. Lee and p. pending., "One-pot process for synthesis of graphene and graphene-derivatives from coal". Patent Serial No.: 62/780,502, 17 December 2018.

[24] S. M. R. Lee, *A Facile Method for Coal to Graphene Oxide and its Application to a Biosensor.*

[25] F. K. H. U. T. S. L. W.-W. V. C. Zaaba N.I., "Synthesis of Graphene Oxide using Modified Hummers Method: Solvent Influence.," *Procedia Engineering,* vol. 184, pp. 269-477, 2017.

[26] P. K. J. M. H. A. U. K. O. D. e. a. Trampuz A, "Sonication of removed hip and knee prostheses for diagnosis of infection," *National Engl J Med,* vol. 357, pp. 654-63, 2007.

[27] e. a. Wang K. Raun J., "Biocompatibility of Graphene Oxide," *Nanoscale Res Lett,* vol. 6, 2011.

[28] A. V. S. C. B. M. Martínez S.R., "Oxidative stress respons in reference and clinical Staphylococcus aureus strains under Linezolid exposure.," *Journal of Global Antimicrobial Resistance,* vol. 22, pp. 257-262, 2020.

[29] d. F. A. N. S. E. M. Perreault F., "Antimicrobial Properties of Graphene Oxide Nanosheets: Why Size Matters," *ACS Nano,* vol. 7, pp. 7226-7236, 2015.

[30] C. C.-F. N. C.-Z. M. G.-C. S. P. A. U.-C. S. Uribe-Alvarez, "Staphylococcus epidermidis : metabolic adaptation and biofilm formation in response to different oxygen concentrations.," *FEMS Pathogens and Disease,* vol. 74, 2016.

[31] R. M. Seul-Yi Lee, "A Facile Method for Coal to Graphene Oxide and its Application to a Biosensor," *Carbon,* 2020.