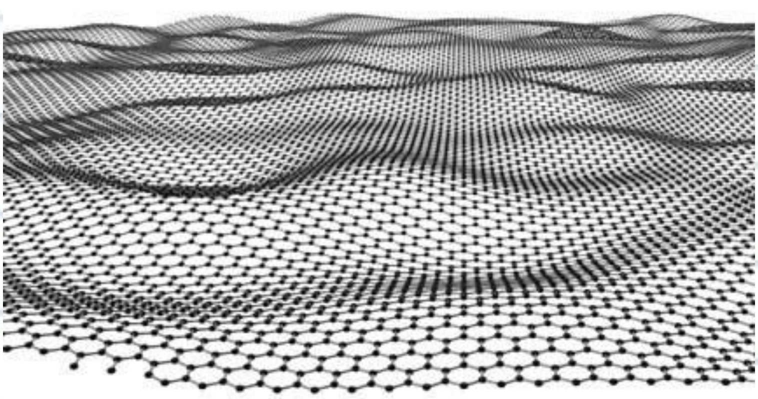
DEGREE FINAL PROJECT

ANTIMICROBIAL ACTIVITY OF GRAPHENE AND ITS VIABILITY



Scopes: **Physical chemistry**

Microbiology

Public Health

Pharmacology

Toxicology

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ABSTRACT

Graphene has burst onto scene as the last nanomaterial within the emerging field of nanotechnology. Its special properties such as high conductivity, huge strength and flexibility, as well as a large surface that can be functionalized modifying these characteristics, have made appear in numerous studies. These emphasize applications in several scopes like electronics to form the chips next generation, the energy storage using in solar panels, and biomedicine. The applications in this last field are very wide as phototherapy, detection of compounds, formation of bio-images, drug delivery, and as antibacterial agent.

Due to the big problem with current antibiotic resistance, the viability of this material as an alternative has been proposed, owing to the appearance of articles that indicate an antibacterial effect of graphene or graphene oxide, in front of a big number of bacteria such as *E. Coli* or *S. Aureus*. It seems that the mechanism of action is based on physical damage with consequent destabilization of the membrane, or oxidative stress among others. In addition, another advantage is to exhibit a low toxicity in the case of graphene oxide, and an apparent biocompatibility.

This project tries to make an overview of this material and its global impact and its feasibility, through a review of various methods of synthesis, biocompatibility and toxicity. It also reviewed the various published articles on antibacterial activity and its possible mechanism of action, proposing from here, practical applications of this property, for example, its use in wound healing, water disinfection or improvements in prosthetics.

It is hoped that this review provides valuable insight, stimulates broader concerns, and spurs further developments in this promising field.

RESUM

El grafè ha sigut l'últim nanomaterial en aparèixer en escena dintre de l'emergent camp de la nanotecnologia. Les seves particulars propietats com una gran conductivitat, elevada duresa i flexibilitat, així com una gran superfície que pot ser funcionalitzada modificant aquestes característiques, l'han fet aparèixer en multitud d'estudis. Destaquen les aplicacions en distints àmbits com l'electrònica per formar la següent generació de xips, el magatzem d'energia utilitzant-lo en plaques solars i la biomedicina. Les aplicacions en aquest últim, són molt amplies com fototeràpia, la detecció de compostos, la formació de bio-imatges, el transport de fàrmacs i com a agent antibacterià.

A causa del gran problema amb les resistències a antibiòtics actual, s'ha plantejat la viabilitat d'aquest material com a alternativa, degut a la aparició d'articles que indiquen un efecte antibacterià del grafè o òxid de grafè, davant un gran número de bacteris com *E. Coli* o *S. Aureus*. Sembla ser, que el mecanisme d'acció es basa en un dany físic amb conseqüent desestabilització de la membrana, o estrès oxidatiu entre altres. A més a més, un altre dels avantatges és presentar una toxicitat baixa en el cas del òxid de grafè, i una aparent biocompatibilitat.

Aquest estudi intenta fer una visió general d'aquest material així com el seu impacte global, la seva viabilitat a través de la revisió dels diferents mètodes de síntesi i biocompatibilitat així com toxicitat. També fa una revisió als diferents estudis publicats sobre l'activitat antibacteriana i el seu possible mecanisme d'acció, plantejant a partir d'aquí, aplicacions pràctiques d'aquesta propietat, tal com el seu ús en bandatges, desinfecció d'aigua o la millora en pròtesis.

S'espera que aquesta revisió proporcioni informació valuosa i una consciència més amplia així com estimular nous desenvolupaments en aquest prometedor camp.

FIELDS OF INTEGRATED STUDY

The fields integrated in the present project are:

- **Physical chemistry**: the main field, basic to understand all the processes that are performed at nano scale, the structure of graphene and how it affects to the antibacterial activity and mechanisms of action. Also helpful to explain, the diverse biomedical applications of this material.
- **Microbiology**: useful to comprehend the different studies of antibacterial activity of graphene (made with different strains of bacteria), and its functionalization with other antibacterial agents.
- **Public health:** basic to figure up the actual problem of antibiotics, the main reason of this project, proposing graphene as an alternative. Also helpful, in the study of economic and environmental viability of this material in the different ways proposed.
- **Pharmacology:** important to explain the mechanism of action of graphene, and the functionalization with other drugs.
- **Toxicology:** essential in the analysis of how graphene affects the cells, to confirm this material as a real alternative in the antibacterial treatments.

1. INTRODUCTION

Nanotechnology is an innovative science in constant growth that has risen a lot of interest for many reasons: the high number of structures that were synthesized, the techniques designed and/or improved for its characterization and the multiple applications on a wide range of scientific fields that are summarized in Figure 1. [(1)(2)(3)]

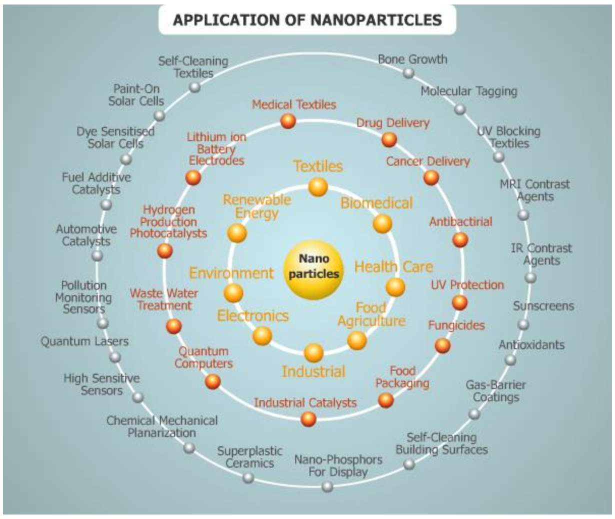

Figure 1 : Applications of nanoparticles [(2)]

Specifically in the biomedical field, working at small scale, allows to study and operate in processes, like bacterial growth or cellular delivery, with a high precision and functionality. [(4,5)]

One of the most interesting type of nanoparticles that has been developed, are the carbon-based nanomaterials, among which stand, carbon nanotubes, nanodiamonds, buckminsterfullerene or the latest nanomaterial to burst onto the scene, **graphene**.[(6)]

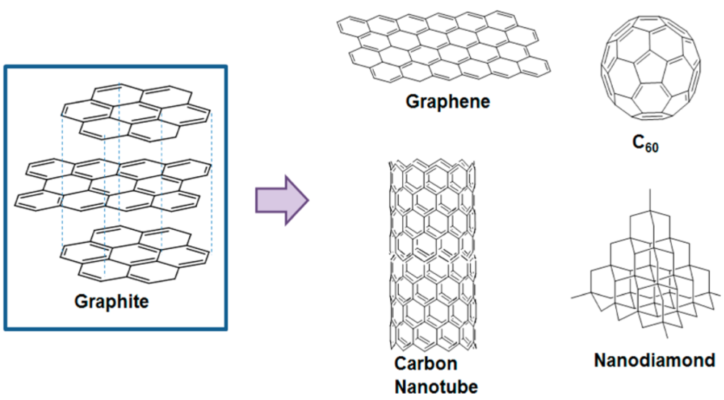

Figure 2 : Various types of carbon-based nanomaterials [(6)]

These structures that differ on their hybridization: a) graphite with a sp^2 (angle between atoms: 120 ^{o}) that forms hexagonal structures with covalent bonds, constituting parallel sheets (with a distance of 0,335 nm approximately) united with Van der Walls forces; b) carbine (not introduced earlier, because of its low knowledge) has a sp (angle between atoms: 180^o) that form linear or cyclic structures, that its believed to be more hard than graphene or graphite; c) diamond with a sp^3 (angle between atoms: 109,5 ^{o}) that forms pentagonal and three-dimensional structures; and d) nanotubes and fullerenes that combine sp^2 + sp^3 and that is the reason why they form curved structures (incorporate hexagons and pentagons).[7]

Graphene is (as proposed in 1986) each one of the sheets that form graphite and until 2004 it was believed that it was impossible to isolate, due to its thermodynamic instability. This year, Konstantin Novoselov and Andrew Geim, isolated the graphene in Manchester University, merit that gave them the Nobel Prize in Physics in 2010. It presents a large number of properties and, consequently, very interesting applications, reason why it has emerged as one of the materials of the future.

1.1 CHARACTERISTICS OF GRAPHENE

Graphene is strictly a two-dimensional material, a sheet of carbon just one atom thick of approximately 0,33 nm, but the IUPAC actual definition is: "*Infinite alternant polycyclic aromatic hydrocarbon, with only six-member carbon ring*."[7,8] In this way, it also refers to groups of several layers, finding with the mono-, bi-, multi- (<10) sheets; it is not the same, four layers of graphene, than four of graphite that keep the Van der Waals forces.

Its structure, gives extraordinary properties, being the most relevant: [7,9,10]

- Completely transparent and so dense, that can sustain current densities six orders of magnitude higher than that of cooper.
- 200 times more resistant than steel, and harder than diamond.
- More flexible than carbon fibers, and as light as it.
- Completely impermeable that makes it an excellent option for creation of separation membranes.

- Good conductor of heat and electricity
- Supports ionizing radiation.
- Shows a large and nonlinear diamagnetism.
- Has high electronic mobility and low noise.
- Practically, it has not Joule effect (kinetic energy loss of the electrons, in the form of heat.
- Has Hall quantic effect; this means that conductivity of graphene cannot be 0.
- Its large surface area (2360 m^2/g) can be functionalized with various agents, and also can be incorporated into other materials to form graphene-based material to be used in a variety of applications.
- Hydrophobic, not dispersible in water and highly reactive.

We can find graphene in a multitude of forms such as, fibers or nanoribbons, due to its structural diversity, depending on its purity, presence of functional groups, 5 or 7 carbon rings instead of 6, etc. This gives a wide range of different properties and possible applications. One of the most interesting forms is the **graphene oxide (GO)**.(7,11)

Figure 3 : One of the principal differences, Graphene is hydrophobic, while GO is hydrophilic.(12)

GO is graphene functionalized with oxygenated groups and presents interesting properties, some of them very different to graphene: (7,9)

- As graphene, it has small size, large surface area, exceptional strength and versatility; it forms impermeable membranes and can be functionalized with other chemical groups due to its hydroxyl, epoxy and carboxyl groups on its surface, for example, hydration with polymers, gold and magnetic nanoparticles used in biomedical and biotechnological applications.

- It is non-conductor, but upon reduction, it behaves as semiconductor or semimetal like graphene.
- It is amphiphilic, as surfactants. Stable colloidal suspensions can be formed due to its hydrogen bonds between water and polar functional groups on GO surface.
- A low energy is required for electron movement, a property important in Biosensor and Bioimaging applications.
- It presents fluorescence quenching ability, intrinsic photoluminescence and it is suitable for surface enhanced Raman scattering (SERS). This property, makes ideal in biosensors, like detection of metal ions (Hg^{+2}, etc) reducing the background signal.
- It is hydrophilic, that makes biocompatible, and dispersible in most solvents. (Figure 3)
- Its cost is lower than graphene.
- It has been shown, that GO (but also graphene) has **high antibacterial activity**, the main property studied in this project.

1.2 CURRENT STATUS

Graphite has had high utility throughout history being a material that takes part in our lives in a lot of sectors like transport and construction among others. But the discovery of the possibility of the separation of graphite in different sheets giving graphene, and the repercussion this has reached, has supposed a scientific and technologic new revolution.

Therefore graphene, as its predecessors (fullerenes, nanotubes), have risen a big wave of optimism. Among all the possible forms, graphene presents more practical applications and manufacturing processes show fewer problems. Despite of this, one should be cautious with this initial expectation and enthusiasm, because so much needs to be developed and studied in a way to produce graphene in a big scale and with low cost, or toxicity in living organisms.

According to the articles and patents published up to now, the interest of graphene is growing very fast. In 2009, this material was the subject of about 2.000 articles of investigation and 550 patents; one year later these numbers increased in more than 3.000 articles and more than a thousand patents and since 2012, a total of 125.117 articles have been published and 31.300 patents have been submitted, concluding that the number of studies performed of graphene have grown exponentially, proportionally to the interest that it is awakening. The principal country with the large number of patents is China followed by EEUU and United Kingdom, and about the companies, *Samsung* shows the highest figures, and in the second place it is found *IBM*. [7,13]

Examples of these patents are: the creation of a graphene chip by IBM 10.000 times more potent and 4 times faster than the actuals made of silicon[14]; NOKIA has promote a prototype of a flexible mobile phone[7]; or also in the field of synthesis like a new method of obtaining of GO from pre-graphite materials studied by the *Consell Superior d'Investigacions Científiques* (CSIC) (patent number: WO2014140399 A1).[15]

The Pentagon has assigned 3 million $ to Princeton University for studying the combination of graphene and fuel to optimize the motors of supersonic airplanes. In this way, Europe has intended to invest 1.000 million € during the next ten years for financing projects aimed to develop applications of the material (known as the *Graphene Flagship Program*), and other countries like United Kingdom, Sweden, Denmark, Holland and Germany have also created their own programs. [13]

About the actual market, as it can be seen in Figure 4, the electronic and energetic sectors are the largest consumers, but it is believed that others like defense or health will make a high increase. The material is reported to have reached 9 million $ on 2012.[11]

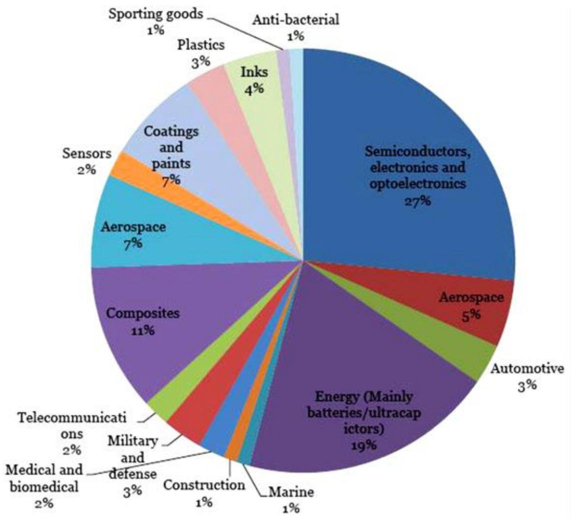

Figure 4 – Use of Graphene in % for the final users or sectors.(13)

The price of graphene is still very high at present. Sheets can be obtained with a cost that goes from 300 € to 1.000 € depending on the quality and the size. However, price is expected to decrease because carbon is an affordable material, there will appear new methods of synthesis and the demand will increase.(13)

So, to sum up, it is a quite young material, and many experts say that there will not be any commercial graphene-based product until 2020 (being optimist). Due to the many scopes of application the following section deepens in the subject.

1.3 APPLICATIONS OF GRAPHENE

This promising material, with very interesting properties as previously commented, has caught the attention of many sectors, emerging a lot of applications.

It has been exposed that the easiness to convey data at great speed and its high energy storage capacity as well as its low Joule effect, could gave to graphene a key role in the development of the next generation chips and batteries, as an alternative to silicon and lithium, likewise the upcoming mobile phones and computers (quantic computers). Also, related with this property, it is believed that it can play a crucial role in the progress of photovoltaic cells.(7,16)

Moreover, in the field of sensors large efforts are being made like the one that NASA has developed for detection of atmospheric oxygen or the one that *The film Electronics* from Oslo, has achieved, a Temperature sensor made of graphene(7); but also for detection of contaminants or biomolecules like glucose, cholesterol, haemoglobin or DNA. [7,17]

However, the last emerging field, with promising results, is the health. Its small size, chemical purity, large surface area and easy functionalization possibility, has opened the door to the use of graphene in drug and gene delivery.[18,19] The excellent mechanic properties of the material suggest it is application in regenerative medicine and tissue engineering. The conductivity, resistance and low thickness makes graphene an ideal support for the detection of biomolecules in transmission electronic microscopy (TEM).[7]

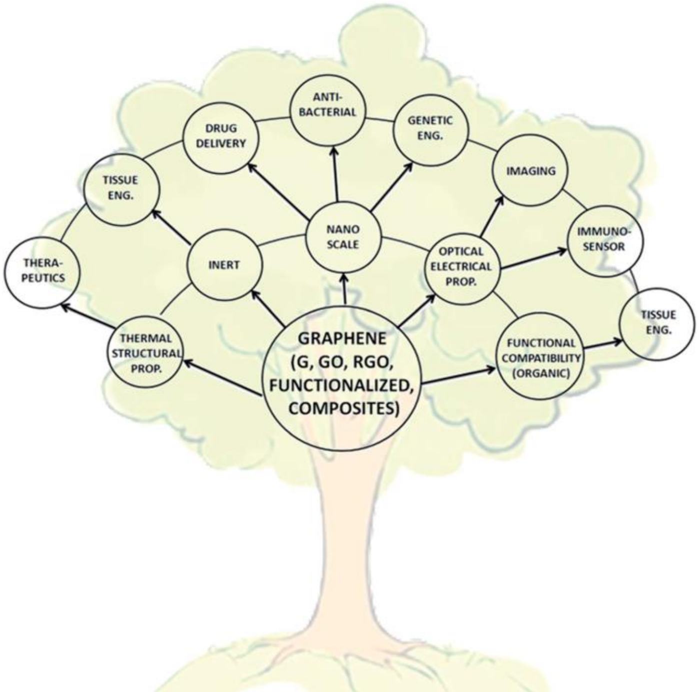

Figure 5 - Unique properties of graphene and its derivatives together with the application opportunities [20]

It should be noted that, these applications can be used in graphene oxide, that as commented previously, the reactive COOH and OH groups facilitate the conjugation with various systems, such as polymers, biomolecules (ligands, DNA, proteins, quantum dots, Fe_3O_4 nanoparticles, and others) imparting GO with multi-functionalities and multi-modalities for diverse biological and medical applications, decreasing the cost of production. Also the hydrophilic character of GO, turn this variant of graphene into a better option in biomedical applications for its biocompatibility.

Hybridization of GO with gold, polymers and magnetic particles provide carbon-related nanocomposites with a large number of applications such as:

- Phototherapy: nanoparticle mediated therapy is an attractive therapeutic modality for the ablation of tumors in combination with conventional treatments such as surgery.(21) The formation of hyperthermic temperatures at targeted site is a significant novel approach for cancer therapy, where we can find a) **photothermal therapy** that employs photosensitizing agents (like the proposed GO) taken up by cells, which generate heat from light absorption leading the cell to death, and allowing treatment for cancer and not-cancer diseases, such as Alzheimer's disease (AD)(22), or b) **photodynamic therapy** where a photosensitizer (for example GO loaded with hypocrellin B with a non-covalent method) is exposed selectively to a particular wavelength transferring the energy of photon to surrounding oxygen molecules to produce highly reactive oxygen species (ROS) resulting in toxicity to the targeted issue.(23)

- Bio-imaging: High light transmittance, photoluminescence, and high charge mobility make GO an important material for magnetic resonance imaging.(24,25) Also, recent researches have designed smaller GO sheets with a size of 10 nm or less, known as graphene quantum dots (GQDs) that exhibit intrinsic fluorescence and can be used for bioimaging that compared with other QD types, show excellent biocompatibility, physiological solubility, and low cytotoxicity and can be used directly for intracellular imaging without the necessity surface processing or functionalization.(26)

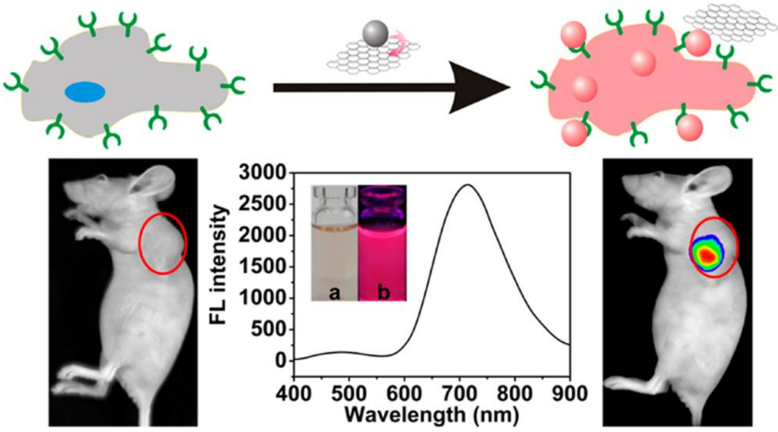

Figure 6 – Transferrin-functionalized gold nanoclusters/graphene oxide nanocomposite was fabricated as a turn-on near-infrared (NIR) fluorescent probe for bioimaging cancer cells in small animals.[25]

- Bio-sensing: GO has been considered as biosensor because of its superior mechanical strength, low density, and high heat conductance, fluorescence quenching phenomenon and intrinsic photoluminescence, as discussed before.[9] Using hybridization chain reactions metal ions can be detected [27], or other molecules like DNA [28], proteins [29], chiral amino acid [30], etc. Furthermore, virus can be detected with a GO-based immune biosensor and can be expanded to a GO microarray format for multiple pathogen analysis. [31]

- Drug and gene delivery: It has been observed that functionalized GO can be used for controlled loading and target delivery of drugs like doxorubicin (a widely used cancer drug) for selective killing of cancer cells [18], and genes. [19]

- Antibacterial action: GO has shown an intrinsic antibacterial activity, the main theme of this thesis and that will be discussed extensively in Results.

2. OBJECTIVES

The present report intends to give an overview of graphene, one of the most recent nanomaterials described up to now.

The different topics evaluated are:

- Study of graphene characteristics and its present situation.

- Review the advantages of graphene and its derivatives with regard to other carbon-based nanomaterial.
- Appraise the economic impact to see the importance it has and the potential of this material.
- Global revision of the applications, deepening on the antibacterial activity.
- Evaluate the graphene or derivatives, as an alternative to the current problem of antibacterial drugs resistance, considering its toxicity, biocompatibility and economic viability.
- Assess the potential uses of the material, from the study of its antibacterial activity viability study.

3. MATERIAL AND METHODS

This thesis is a bibliographic review of actual situation of graphene and its biomedical applications, focusing on the antibacterial activity of this new emerging material.

Firstly, a global vision of this two dimensional nanostructure was done to understand the principal characteristics and the nanomaterials current scene concentrating on the last carbon-based nanomaterial that burst onto scene, the graphene. The information sources have been books, monographs and articles that make a review of the different studies that have been done up to now.

Afterwards, in order to deepen into one of the most interesting biomedical properties discovered, its intrinsic antibacterial activity, the research has been focused on the selection and analysis of many specific articles that described different studies in the field of synthesis, distribution, biocompatibility, toxicity and antibacterial activity as well as the possible mechanisms of action.

The research of the documents was done through multiple online portals, besides research of books in the library registry portals. This was basically managed through the CRAI platform from the UB (resource center for research and learning) search tools, which enables to access to different data base like Pubmed, Scifinder, Scopus among others.

4. RESULTS

The results of this project come from the bibliographic compilation of the studies of toxicity, biocompatibility, synthesis and antibacterial activity of graphene to evaluate the viability of this material as an alternative to overcome the actual antibacterial resistance problem as previously commented on the objectives section.

4.1 SYNTHESIS AND BIOCOMPATIBILITY

4.1.1 Synthesis and economic viability

As it is known, graphene is each one of the sheets that form graphite, so **graphite** is the principal raw material. However, this one it is not unique, because one can find different types of graphite with various percentages of carbon (between 80-90%) and distinct number and nature of inorganic impurities. Therefore, it is important to know its origin because it determines its characteristics. Thus, we can differentiate between natural and synthetic graphite: the first type comprises crystalline flakes, crystalline vein and amorphous graphite, being the crystalline flakes the most used in graphene preparation; while the synthetic includes the pyrolytic and polygranular graphite, that are obtained from an organic precursor (carbon or petroleum derivatives) treated with temperatures on the order of 1.000 ^{o}C in an inert atmosphere (known as carbonization process) which subsequently makes a graphitization at temperatures superior to 2.500 ^{o}C.(7)

The main producers of this raw material are China, South Korea and India, in this order. In Europe there is a certain problem of availability because no veins have been found in recent years. However, it is a salvable problem considering the synthetic type and the fact that graphite it is not the only option to obtain graphene, although it is the most economic and the one that generates more variety.(7)

For the graphene obtaining, it is necessary to distinguish between the top-down (it is set up from materials with bigger dimensions) and bottom-up methods (it is set up from small molecules).

Top-down methods [32–34]:

- Mechanical or micromechanical cleavage (Figure 7.a): This was the method used by K. Novoselov and A. Geim.[35] It consists on pushing graphite, usually high oriented pyrolytic graphite (HOPG), made from the thermal decomposition of hydrocarbons under pressure. This material is placed on a surface (generally made of silica: Si/SiO_2), followed by the adhesion of a scotch tape which is slowly removed, leaving behind sheets of graphene on the surface with high structural quality and size (approximately 200 nm). The problem of this method is that, the sheets have to be located manually with an optic microscope, resulting on a very low output. Besides, the sheets cannot be positioned in a controlled way. It is estimated that the commercial price of a gram of graphene with this technique surpasses the Europe GDP (gross domestic product) of one year, making the process non-viable to the industry production.
- Exfoliation on liquid phase (Figure 7.d): It is based on the increase of the distance between the sheets (using polymers, surfactants or small molecules like potassium) that reduces the interaction, making possible to disjoin with a later ultrasonication. One should remark that the graphene oxide (GO) has the triple of distance compared with graphene because of its functional groups and it is hydrophilic, making easier to exfoliate. [36] Therefore, oxidize graphite with acids (like sodium nitrate, sulfuric acid, and potassium permanganate, known as Hummers method) obtaining graphite oxide with a first exfoliating the bulk material and then reducing the individual sheets back to graphene, seems to be an ideal route to synthesis graphene.

  This process generate high outputs at a very low cost, but long times of sonication are required, producing small sheets (known as nanoribbons, that can also be synthetize by “unzipping” carbon nanotubes) and structural defects. The highest concentration of few-layer graphene that one can reasonably achieve is less than 0.1 mg/mL of solvent. However, new variants of this method are being studied, like the one developed in the Trinity College, which allows a direct exfoliation without a previous expansion, generating high quantities with a reasonable quality.[37]

- Anodic bonding (Figure 7.b): The graphite joins a glass, where a high voltage is applied, and then the graphene sheets get hooked on it because of the electrostatic interaction. It is widely used in the microelectronics industry.
- Photoexfoliation or laser ablation (Figure 7.c): The exfoliation can be also done with a laser beam, but the energy has to be controlled for avoiding damage on the sheets. It is best implemented in inert or vacuum conditions since ablation in air tends to oxidize the graphene layers. This method is on its first steps and needs further development.

Bottom-up methods [32–34]:

- Thermic decomposition of silicon carbide (Figure 7.e): It consists on a thermic treatment of silicon carbide crystals that evaporates the silicon atoms, making a partial graphitization of the superior sheet. This technique needs to be improved, because there is not good control on the number of sheets stacked, the separation and the transference of these ones to other substrates, so crystal size, could be a major cost impediment for large scale production.
- Molecular growth (Figure 7.f and 7.i): Small organic molecules like benzenes or polyaromatic hydrocarbons, are deposited on a substrate making possible to control the structure and functional groups and being able to produce small sheets of graphene (also known as nanographene).
- Chemical Vapour Deposition (CVD) (Figure 7.g): Is a process widely used to deposit or grow thin films, crystalline or amorphous, from solid, liquid or gaseous precursors of many materials. In the case of graphene production, hydrocarbons are decomposed at high temperatures above a metallic substrate (like Nickel or copper), where the atoms are restructured forming graphene sheets. This method allows obtaining high quality sheets, but it is a very slow process.
- Epitaxial growth (Figure 7.h): It is a similar technique to the CVD, but in this case, molecular beams are used on a surface (silicon carbide, alumina, mica…).

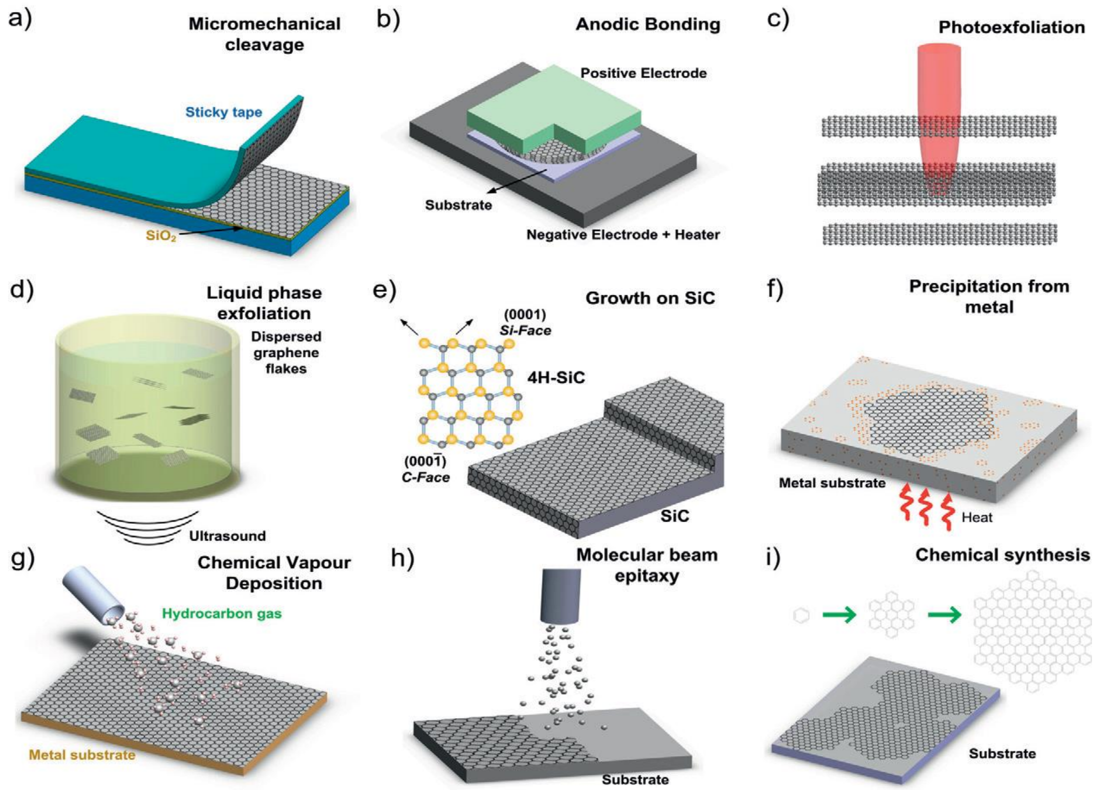

Figure 7 - Schematic illustration of the main graphene production techniques.[33]

Production method		Advantages	Disadvantages
Top - down	Mechanical exfoliation	- Simple, inexpensive - Usable with many different layered materials - Suitable for demonstrations	- Too inefficient for mass production - Yields mainly few-layer to many-layer graphene - Dimensions constrained by size of initial crystal
	Liquid-phase exfoliation	- Simple and inexpensive - Faster than mechanical exfoliation - Less damaging than chemical exfoliation	- Dimensions constrained to size of initial crystal - Not high-yielding
	Anodic bond	- Optimal for electronical devices	- High voltage is needed
	Photoexfoliation	- Inexpensive	- Energy of the laser beam has to be controlled - Vacuum conditions are needed
Bottom - up	Termic descomposition	- Large-area single layer and few layer graphene - Growth directly on insulating substrate - Easily transferrable to arbitrary substrates	- Requires specialized equipment and apparatus - Very expensive to produce - Single crystal size limited to SiC terrace size
	Molecular growth	- Atomically precise - Simple chemical process	- Very low yield - Field is too new for confident predictions
	CVD	- Arbitrarily large area graphene - Self-limiting to a single or few layers - Large single-crystal growth - Easily transferrable to arbitrary substrates	- Still somewhat expensive compared to GO - Transfer can contaminate and damage graphene sheet
	Epitaxial growth	-	Similar to CVD

Table 1 – Comparison of the different methods of graphene synthesis

As mentioned before, GO is the best option for antibacterial uses mainly for its hydrophilic property, so it is needed to comment also on the preparation of this material:[7,9]

1. Graphite is oxidized in acid medium, with strong oxidants. There are several methods:
   a. **Brodie, Staudenmaier and Hofmann**: uses a combination of potassium chlorate ($KClO_3$) and concentrated nitric acid (HNO_3) as the oxidants.
   b. **Hummers and Offeman**: involves treatment of graphite with potassium permanganate ($KMnO_4$), sodium nitrate ($NaNO_3$) and concentrated sulphuric acid (H_2SO_4). It is the most used, because the solution is more acid, which facilitates the next step.
   c. **Marcano**: Exclude the $NaNO_3$ of the Hummers method, increasing $KMnO_4$, and a mixture H_2SO_4/H_3PO_4. These changes improve the oxidation process. The reaction is not exothermic and produces no toxic gas, making useful for biomedical applications.

2. The graphite oxide is dispersed and exfoliated on water or other solvents (graphite oxide is hydrophilic like graphene oxide). The water molecules are intercalated between the sheets increasing the distance, so put through an ultrasonication or a mechanical agitation, can produce GO on water suspension, powder or layer form. The times of sonication or agitation are important parameters to control, to avoid a decrease of the sheets size.

It is also possible, to produce GO from rGO (reduced graphene oxide) with a thermic or chemical oxidation followed by a centrifugation and vice versa.

A good thermal control is highly needed in all of these methods.

4.1.2 Biocompatibility and toxicity

Carbon-based materials studies show currently contradictory results. While carbon nanotubes (CNTs) have been found to be highly cytotoxic in human and bacteria cells, graphene and GO showed reduced toxicity. This problem can be overcome by surface functionalization with biocompatible polymers (such as polyethylene glycol (PEG) or dextran) that appeared to produce no detectable toxicity as shown in cellular and animal tests.[9] Taking into consideration that rGO (similar to graphene) has a significant higher cytotoxicity than GO,[38] it is clear that the oxygenation of GO plays a major role in reducing the degree of toxicity. Highly oxygenated GO and a reduced number of carboxylic acid groups (-COOH) at the periphery of the nanosheets exhibits diminished toxicity,[9] as tested in in-vivo studies on mice which eliminated this compound in one week without any toxic effects appreciable.[7]

This low or no detectable observed toxicity of graphene and GO is affected by the size, the structure of the sheets and the solute which is dispersed. It has been proved in amyotrophic lateral sclerosis cells, human fibroblasts, human lung cancer cells and cancerous human liver cells.[7] In summary, the major possible health risk of GO comes from: a) size distribution, b) dose to target tissue, c) surface treatment, and d) surface charge, by making 1) damage of mucus, 2) cell division, 3) penetrating the phospholipid bilayer, 4) skin permeation, 5) crossing the blood-brain barrier, 6) crossing the placenta and 7) glomerular filtration. [39]

There are two principal entry routes for GO into the human body: the inhalation that presents high risk, and dermal penetration. Various factors influence the pulmonary toxicity of GO, such as particle size and surface area. Duch et al.[40] observed that GO directly administrated into the lungs of mice presents high toxicity causing severe and persistent lung injury.

About the biocompatibility, as commented above, the superficial functionalization is a key factor, because it can decrease the high hydrophobic interaction of the graphene with the cells and tissues.[7] GO as an amphiphilic and hydrophobic material, exhibits excellent stability when tested in biological solutions and has been proved (with a size around 100 nm) to be stable in living cells and in mice, and when it is functionalized with, for example, PEG, it acquires high stability in biological solutions as Robinson et al. [41] strongly demonstrated. But this stability it is not always needed, for instance, for bone reparation we look for stability over time, while in bactericidal insulation bands it is no important because it would be removed periodically.

The cell uptake efficiencies are significantly different in the diverse carbon nanomaterials, showing GO the lowest cell uptake ratio among all of them, as proved on HeLa cells. [42] Hu et al.[38] demonstrated that GO were inside the endosome of the cytoplasm suggesting that the internalization is via endocytosis.

In conclusion, GO nanosheets are relatively biocompatible nanomaterials with mild cytotoxicity.

4.2 ANTIBACTERIAL ACTIVITY AND ITS MECHANISM OF ACTION

Due to the vast inappropriate and overuse of the antibiotics, microorganisms have begun to develop resistance to the commonly used antimicrobial agents, resulting in prolonged illness, disability, and death. Without effective antimicrobials for prevention and treatment of infections, medical procedures such as organ transplantation, cancer chemotherapy, diabetes management and major surgery become very high risk, increasing the cost of health care with longer stays in hospitals and more intensive care (use of more expensive drugs, additional test…) required.

4.2.1 Current antibacterial resistance problem

The present situation is critic; the antibiotic resistance is present in all countries, treatments in *K. pneumoniae* and *E. coli* are ineffective in more than a half of patients in some countries. Treatment failure with third generation antibiotics has been confirmed in at least 10 countries, resistance to first-line drugs to treat infections caused by *S. aureus* is widespread, 480.000 people develop multi-drug resistant Tuberculosis each year and is starting to complicate the fight against HIV and malaria, as well.[43]

Therefore, for all this reasons, development of the new and effective antimicrobial agents seems to be necessary and nanotechnology in pharmaceuticals and microbiology display promising applications to overcome this problem.

4.2.2 Carbon-based materials antibacterial activity

As previously commented, carbon-based nanomaterials such as fullerenes, carbon nanotubes (CNTs) and graphene oxide or reduced (GO or rGO) show potent antimicrobial properties, but as discussed earlier GO seems to be the best option for its better biocompatibility (has the lower cytotoxicity), properties like functionalization and best cost production. Chen et al. [44] results, indicated that disperse GO showed the highest antibacterial activity, followed by rGO, graphite and graphite oxide, so different forms of graphene interact in a different way with pathogenic bacteria, and the degree of dispersion is largely dependent. However, as nanowalls, rGO showed stronger antibacterial activity than GO, because of it has more sharpened edges and better charge transfer, two of the mechanism of bacteria cell damage.

Furthermore, a combination with other antibacterial substances produces synergetic effect, overcoming the limitations of the individual components like stability and dispersion. These graphene-based nanocomposites used for their antibacterial activity, can contain:[45]

- **Metals**: such as silver (Ag) that can destroy bacterial cell membranes and penetrate the cells to inactivate enzymes and cause cell death. Bare silver nanoparticles aggregate when they come in contact, reducing the antibacterial activity. To overcome this problem, nanocomposites were fabricated with graphene using reductants (for example $NaBH_4$), making the dispersion stable, and showing synergistic effect and antifungal activity.
  Cooper (Cu) is also an efficient inexpensive antibacterial agent, thanks to its redox properties that lead to oxidation of lipids and proteins and a subsequent cellular damage, but aggregation is also the limiting factor, that can again be overthrown by anchoring in the graphene surface.
  Other metals like gold (Au) and lanthanum (La) have been combined with rGO or GO to prepare antibacterial materials, showing the mix with lanthanum also an anticoagulant activity aside from antibacterial activity.
- **Metal oxide**: when a light energy greater than the bandgap energy from a metal oxide semiconductor (like TiO_2, ZnO, SnO_2 and Fe_3O_4), illuminates it, the electron-hole pairs will diffuse to its surface, generating positive holes that can react with water to produce hydroxyl radicals besides that negative electrons can combine with oxygen to form superoxide anion. This reactive oxygen species (ROS) can effectively inactive bacteria. TiO_2 is the most promising

candidate because of its inherent excellent chemical stability and photocatalytic efficiency, and the combination with graphene could significantly inhibit the charge carrier recombination rate from the ultraviolet (UV) to the visible (VIS) region, indicating its potential for indoor air disinfection.

- **Polymers**: Due to the strong interplanar interactions of graphene it presents poor solubility and processability that limit the antibacterial application. This problem can be overthrown by the incorporation of this material into a polymer matrix, for example poly-N-vinyl carbazole (PVK), chitosan (CS) and poly-L-lysine (PLL). PVK is an excellent candidate due to its excellent electronic and mechanical performance, ease of thin film fabrication, corrosion resistance and use as a polymer dispersant, that combined with GO, the antibacterial effects were up to 57% in biofilms and 30% in planktonic cells, more high than GO alone.

Also multicomponent composites combining the advantages of graphene, nanoparticles, and polymers, were fabricated, showing a prominent synergistic antibacterial effect.

4.2.3 Mechanism of action

About the mechanism of action of the antibacterial activity of graphene, different processes were described, but the full method has not been completely understood. Diverse studies(45), have shown distinct mechanisms:

- **Physical damage**: is the most important. The sharps edges of graphene acts like a knife that could cut through the bacterial cell membrane to form pores, which subsequently led to osmotic imbalance and bacterial death. The density of their edges was considered to be one of the key factors. Huang et al.(38) reported that *E. coli* growth can be effectively inhibited by destroying their membrane integrity, as confirmed by scanning electron microscopy (SEM).
- **Destructive extraction of phospholipid molecules**: experimental and theoretical approaches found that *E. coli* lost their integrity, leading to the loss

of cytoplasm by three distinguishable stages: swing, insertion and extraction of lipid molecules.

- **Antibacterial trapping**: the bacteria are trapped in the graphene sheets when they aggregate in the bacterial suspension, happening to be inactive for their isolation, and cannot proliferate. Large GO, showed stronger antibacterial activity due to the ability to fully cover bacteria, block the active sites and decrease the viability of bacteria.
- **Photothermal ablation**: is an effective strategy to eliminate trapped bacteria considering the excellent near-infrared (NIR) absorption properties and intrinsic thermal conductivity of graphene. If a targeting substance, such as antibody-functionalized, is integrated, it can improve the efficiency of ablate bacteria, treating the targeted region and minimizing cellular damage. Szunerits et al. [46] designed and rGO-PEG-coated gold nanorods to selectively kill the uropathogenic *E. coli* UTI89, with a result of a killing rate of 99% of pathogens in 10 min.
- **Oxidative stress**: charge transfer, ROS generation or direct oxidation of cellular components has also been implicated in graphene-induced bacterial damage, as commented before.

All of these mechanisms depend on the physicochemical characteristics of graphene, such as lateral size, number of layers, surface charges, surface functional groups, and oxygen contents. For example, larger GO nanosheets have stronger antibacterial activity. Also it is time and concentration dependent.[45]

4.2.4 Studies on bacteria

Graphene (or similar like rGO) and GO have shown inhibitory effect on the growth of *E.coli*, *S. aureus*, *P. aeruginosa*, *X. perforans*, *C. albicans* and *B. subtilis* as Table 2 summarized.[45] Also Ag-Carbon nanocomplexes present efficient inhibitory activity against other pathogens like *B. cepacia*, methicillin-resistance *S. aureus*, multidrug-resistant *A. baumannii*, K. pneumoniae and *Y. pestis*.[47]

Formulations	Size	Toxicity	Function	Advantages	Disadvantages
rGO GO GO	From nm to μm Lateral dimensions 500, 200, and 50 nm	*E. coli*: 90% viability loss *E. coli*: 98.5% viability loss *E. coli*: 90.9%, 51.8%, and 40.1% viability loss	Cell membrane damage due to contact interaction Insertion/cutting of cell membranes and destructive extraction of lipid molecules	Longer and lasting antibacterial activity, physical damage, shows little bacterial resistance	Tendency to agglomerate, thus reducing their antimicrobial activities by altering their surface and edge properties
GO	0.753–0.010 μm^2	*E. coli*: larger sizes present stronger activities	Trap bacteria and isolate them from their environment	Physical damage	Bacteria can be reactivated after removing the sheets
rGO	No data	*S. aureus*: 99.6% viability loss *E. coli*: 99.9% viability loss	Photothermal ablation upon NIR laser irradiation	Can focus on a targeted area for effective treatment	Limited deeper tissue penetration
GO rGO	0.525 μm 3.40 μm	*P. aeruginosa*: 87% viability loss *P. aeruginosa*: 86% viability loss	ROS generation	ROS is a broad-spectrum bactericide	Antibacterial properties will be considerably inhibited when basal plane is masked
GO–AgNPs	AgNP: 18 and 5 nm	*X. perforans*: 99% viability loss	The adhesion of bacteria to GO–Ag composites is increased, and the size, distribution, and aggregation of AgNPs are controlled.	Synergistic antimicrobial effect; silver ions and AgNPs have excellent antibacterial activity	High toxicological risk
GO– AgNPs CNSs–AgNPs	AgNP: 30–50 nm	*C. albicans*: 38.9% viability loss *C. albicans*: 78.6% viability loss	Controlled release of silver ions		
rGO–Cu	CuNPs: 10–50 nm	*E. coli*: 99.990% viability loss *S. aureus*: 99.581% viability loss	Increase the stability of copper nanoparticles and control the release of Cu^{2+}	Synergetic antimicrobial effect; copper is of low cost	High toxicological risk
rGO–Au	AuNPs: 8–45 nm	*S. aureus* and *P. aeruginosa*: 100% viability loss *B. subtilis*: 99.76% viability loss *E. coli*: 97.47% viability loss	Oxidation stress on both the antioxidant systems and membrane	Synergistic antimicrobial effect; Good biocompatibility	High costs
GO–TiO_2	3D ordered porous structure 1.6-nm thickness	*P. aeruginosa* and *E. coli*: 7.5-fold stronger than bare TiO_2 film	TiO_2 can generate ROS when illuminated and graphene can improve the efficiency of photoinactivation	Synergistic antimicrobial effect; TiO_2 possess excellent chemical stability and photocatalytic efficiency	Low photoinactivation efficiency in visible region
GO–ZnO	ZnO: 4 nm	*E. coli*: about 200-fold stronger than ZnO NPs	GO facilitated the dispersion of ZnO NPs and enabled close contact with bacteria, thus increasing the local zinc concentration and the bacterial membrane permeability.	Synergistic antimicrobial effect; low cytotoxicity	Low photoinactivation efficiency in visible region
GO–PVK	Average grain size was about 160 nm	*E. coli*, *C. metallidurans*, *B. subtilis* and *R. opacus*: 57% (biofilms) and 30% (planktonic cells) higher than GO alone	Encapsulate bacteria to reduce their microbial metabolism	Form well-defined and homogenous coating on various surfaces	Difficult to completely eradicate bacteria
GO–PLL	No data	the bacterial inhibition is much higher than PLL alone	The composite possesses a positively charged high surface area and tends to strip the bacterial outer membrane	Promote the growth of human cell	Limited stability

Table 2 – Different antibacterial activity studies of graphene. [(45)]

5. RESULTS DISCUSSION

In the view of the results, graphene has been postulated as a potential effective antimicrobial substance, although it needs to be more studied. This property has been under debate, with several groups claiming that GO has little antibacterial activity, but most of the current findings and advances support its properties. With regard to its viability low toxicity and high biocompatibility are needed while high purity seems not required. Therefore, GO appears to be the best option of the graphene-derivatives, to be used as antibacterial agent, having the advantage of being easy and cheap to synthesize, facts that will decrease its production cost. Due to the interest that has awakened, several methods of production have been described and are still new under development. Another attractive factor is that it is a carbon-based material, one of the most common elements in earth. Also, the functionalization with other substances with antibacterial activity looks like a very good approach to increase its activity by synergy, representing as an alternative to natural or synthetic antibiotics.

Considering all of this, many practical applications emerge such as the capacity to inhibit the growing of bacteria on its surface and the excellent mechanic properties as well as its non-conductor nature (is an insulating material). These properties lead to think in the development of films that can be used in food packaging to prevent the rot, or in wound dressing. In this aspect Yang et al.(48) have developed, a combination of silver, rGO and a polymer (PDDA) with a high adsorptive capacity that allows to capture the bacteria and increase the antibacterial activity. The in vivo studies showed that the nanomaterial could promote the regeneration of epidermis. On another hand, graphene-based nanocomposites in the form of fabric or hydrogel are excellent candidates for wound dressing, just like Fan et al.(49) achieved. Briefly, this group prepared a GO-based cotton fabric (flexible, foldable and reusable) that showed excellent antibacterial activity, good laundering durability with minimal skin irritation and no cytotoxic effect on human fibroblasts. Interesting is the fact that the fabric was found to inactivate over the 90% of the bacteria after being washed 100 times; furthermore this combination improves its electrical conductivity and self-cleaning properties. Wang et al.(50) prepared an hydrogel with polymer mixed with GO-Ag, that demonstrated to have an excellent antibacterial activity and accelerate the wound-healing rate, with an exceptional water-maintaining capacity, biocompatibility and extensibility, basic properties for wound care. This hydrogel could also be used as a disinfectant agent, for example for the sanitary furniture. In the same way, for the reasons commented before, graphene-based nanoparticles could be used to keep the material in aseptic conditions, for instance surgical instruments, or condoms making them thinner but very strong.(51,52) Also the combination with prosthesis, can be a solution to the infection problems that presents, for the formation of biofilms in the metal surface, preventing this, in addition to an increase in hardness and flexibility.(53)

Another important application of graphene for its antibacterial properties, is water purification. A nanocomposite made of rGO-Ag was prepared, with a minimal power consumption of a 1,5 V battery. This antibacterial device can be used to rapidly purify drinking water. In addition, GO conjugated with peptide made an effective separation and inactivation of methicillin-resistant *S. Aureus* pathogens from water. [(45)]

Moreover, graphene-based nanocomposites can promote the growth of human and mammalian cells, which makes them suitable as tissue engineering scaffolds.

By last, graphene-based delivery systems has been designed to improve therapeutic efficacy, with advantages such as convenience, high utilization rate and reduced toxicity to transport drugs including antibiotics, because of its rich surface chemistry, high aspect ratio and ability to cross the plasma membrane. Ma et al. [(54)] prepared biocompatible GO-modified with PSA (polysebacic anhydride) functionalized with levofloxacin showing a significant increase in effective release time compared to the pure PSA. This can be useful to treat various topical bacterial infections as a single medication, increasing patient compliance due to its prolonged action, or for example optical drops for eye infections due to its hydrophilic nature, but it is possible to extend to other administration routes.

For all of these reasons and examples, I believe in graphene-derivatives as a possible solution or alternative to the actual antibacterial global problem.

6. CONCLUSIONS

It is sure that nanotechnology will help to overcome many problems of the current society. Within this science in constant growth, the carbon-based materials are the most outstanding, highlighting graphene as the newest.

This nanomaterial has shown a lot of interesting properties that can be modified with processes like oxygenation, obtaining Graphene Oxide (GO). Due to its particular characteristics a large number of applications have appeared in the fields of electronics, energy storage, communication and biomedicine.

In this review, graphene antibacterial activity, its mechanism of action and the viability has been discussed. The results shows that GO presents less toxicity and best biocompatibility that graphene, due to its oxygenation, so seems to be the best candidate to be used as an antibacterial agent. Furthermore, its synthesis is easier and cheaper, and the preparation of GO by chemical processing routes may be able to produce large amounts, although chemical details including the oxidation mechanism need to be more fully understood. The Marcano method is suggested as the best process for biomedical applications for its improvement on oxidation and the fact that the reaction is not exothermic and produces no toxic gas.

The antibacterial activity of GO, is dependent on multitude factors such as concentration, time, lateral size, number of layers, surface charges, surface functional groups, and oxygen contents. In addition, the impurities generated during the GO synthetic process due to careless washing, like sulfur and manganese, and the oxygenated groups that tends to dissociate, can slightly reduce the pH and disturb the bacterial microenvironment and inhibit their proliferation. This leads to think about the fact that no good GO quality is required, which facilitates the synthesis.

Several mechanism of action have been reported, being the physical damage the most relevant, but they have not been completely understood, thus, in depth studies are needed to comprehend the mechanisms and factors influencing the antibacterial activity of graphene-based materials.

Furthermore, graphene can be used as a support to disperse and stabilize various nanomaterials with antibacterial activity such as metals, metal oxides and polymers, and as a nanocarrier for controlling the delivery of antibiotics and improving the therapeutic efficacy, leading to higher antibacterial action, due to the synergistic effect.

The studies in diverse bacteria indicate that this antibacterial activity has also been under debate, with several groups claiming that GO has little effect, but most of the current findings and advances support its antibacterial activity on bacteria like *E. coli* or *S. aureus*.

Some of the latest developments in the practical antibacterial applications of graphene materials have also been reviewed. Wound healing, food packaging, prosthesis, water purification or tissue engineering, are examples of the promising uses of this material as an antibacterial agent.

In conclusion, graphene-based materials presents a huge potential as a solution to the current antibiotic resistance global problem, and as an alternative to conventional chemical antibiotics, being renewable, easier to obtain, and cheaper, with little bacterial resistance, but more studies in body distribution, toxicity, biocompatibility, degradation or galenic forms among others are clearly needed.

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