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Review

Synthesis, Characterization and Bioapplications of Pristine Graphene: A Review

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Abstract: Graphene, ostensibly the strongest material to date, has been a topic of interest for engineers, scientists and researchers since its first isolation through mechanical exfoliation through forming mechanical cleavage of a graphite. The thickness of the sp^2 hybridized carbon framework is only one atomic layer. The unique physical, electrochemical and optical properties, such as room temperature hall effect, large surface area, and excellent electrical conductivity caused from high electron mobility, make graphene become an attractive material in the fields of nanoelectronics, biosensing, biomedical engineering and related applications. The high demand of this material has led to the development of many synthesis methods and different characterization methods, and towards ensuring lower defects, high quality and large-scale production. In this paper, a comprehensive overlook of the electronic, physical and optical characteristics of graphene, synthesis methods, characterization techniques, and integration level applications are reviewed with solely focusing on pristine graphene.

***Keywords*:** graphene; structure and properties; synthesis; characterization; biosensing; biomedical applications; integration level application

1. Introduction

Carbon has been a topic of interest in the area of manufacturing and material science for a long time. It makes sense as we are practically surrounded by carbon and carbon allotropes, and the interest is reflected in scientific community with numerous publications and patents. Diamond and graphite are the two 3D structures found in the nature that are different forms of carbon. From early 16th century, the use of carbon can be found in form of graphite to manufacture writing pencils [1, 2]. The concept of one-dimensional(1D) or zero-dimensional(0D) carbon allotropes came much later, around the last decades of 20th century [1]. The ground-breaking discovery came very recently when through implementing mechanical exfoliation. Utilizing adhesion of a scotch tape, a single layer of carbon atom was successfully isolated [3] and the existence of monolayer pristine graphene became a reality from theory.

Since the discovery of monolayer graphene, it has been a desirable material for modern chemistry, physics and many other fields of science. The development of the synthesis processes, characterization methodologies and a great many applications represent the stirring progress. This progress is reflected through the number of research articles and publications. So, the increasing number of publications and research articles suggest that graphene may has the power the revolutionize the industry as we know it with its unique and superlative characteristics. Numerous thermal

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applications, energy storing devices, coatings, sensors, electronics, fuel cells have been proposed and on the verge of marketing that are solely based on graphene. Graphene offers a unique nanostructure that makes it suitable for polymer composites, conductive coatings and inks, batteries for fuel cells, catalysts, and ultracapacitors [4–6]. This shows the demand of graphene as a topic of interest. The review on graphene, its properties, synthesis, characterization and application have been brought about with the assistance of bibliometric data of published articles from 1947 to 2019. Since 2004, the number of graphene-related publications in healthcare and biomedicine has substantially increased and the use of graphene in health and life-science applications has been steadily growing as shown in Figure 1. In the inset of Figure 1, the number of publications published in journals on biomedical and its related sectors are shown.

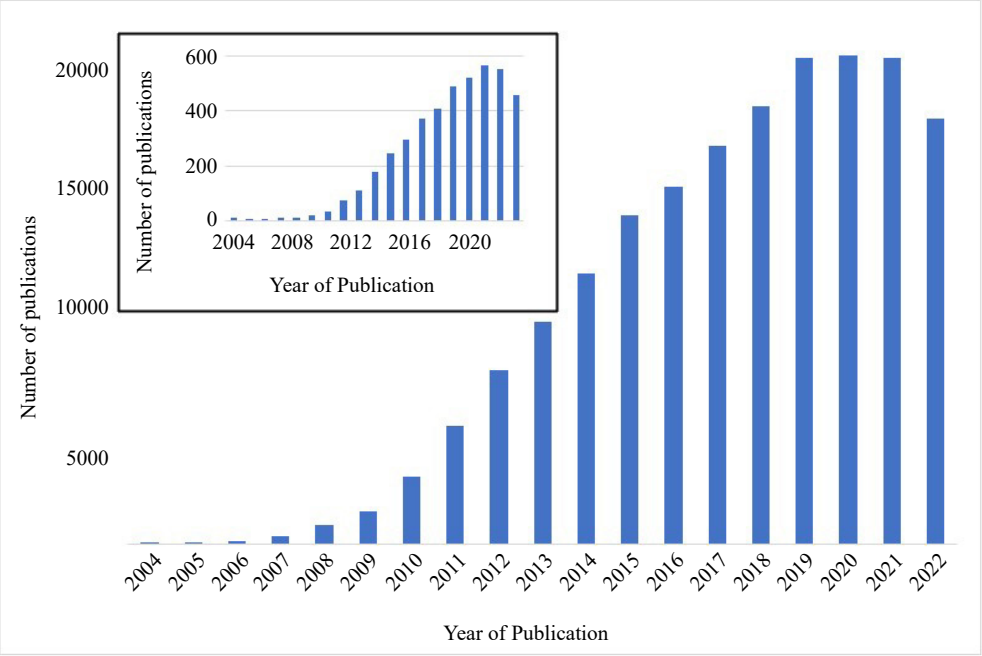
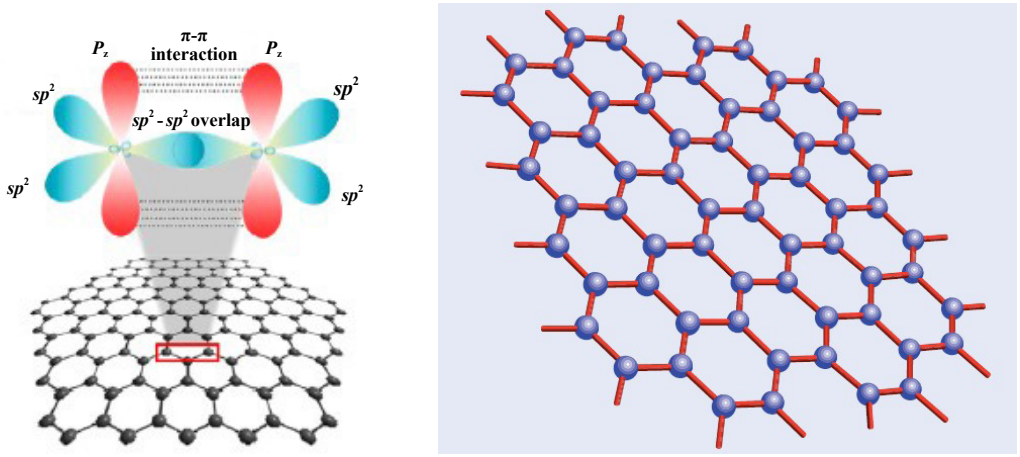


Figure 1. The post-2004 timeline representing the number of publications related to graphene published in journals (Source: Scopus.com, searched with keyword "Graphene" within "Article title, Abstract, Keywords"). The post-2004 graphene-related publications on biomedical area published in journals (Source: Pubmed.gov, searched with keywords "Graphene, Biomedical" in advanced search option) (Inset)

2. Properties of Graphene

Graphene is defined as a single atomic layer allotrope that has covalently bonded sp^2-hybridized carbon atoms arranged in a lattice structure that is similar to honeycombs. The hybridization of the carbon atoms causes this hexagonal lattice formation because of the bonds among the carbon atoms. In a three-dimensional construction, a carbon atom forms two kinds of bonds with three other carbon atoms that are the closest neighbors of the carbon atom, and two other neighboring carbon atoms that are vertically positioned with the carbon atom. As we are considering the single sheet of carbon atoms, except the carbon atoms at the very edge, all other carbon atoms are surrounded by three carbon atoms which are 1.42 Å apart. The bonds include a sigma (σ) bond that is formed with the three neighboring atoms and the overlap of the rest of the p-orbitals. This causes the carbon atoms to form half-filled π bands but only one sigma bond is formed with one neighbor carbon atom while the pi (π) bond is delocalized out of the plane. The delocalized π bonds lead to π electrons that gives graphene its unique physical and chemical characteristics [4, 5].



(a) (b)

Figure 2. (a) Structure of a pure-arranged carbon atom or pristine graphene with sp^2-hybridization (Reprinted from [9]) (b) The representation of carbon atoms in a honeycomb lattice (Reprinted with permission from [10])

The pristine graphene structure is specified and distinguished. In Figure 2a the array of 2D and sp^2- hybridization of carbon atoms that are laid out in a hexagonal lattice pattern held by covalent bonds. Figure 2b shows the representation of the hexagonal pristine graphene lattice. Pristine graphene shows many desirable properties such as large thermal conductivity (3000-5000 Wm^{-1} K^{-1} [11], high charge mobility (230,000 $cm^2V^{-1}s^{-1}$) while the percentage of visible light absorption at 2.3%, the incredible high strength at almost 130 GPa, thermal conductivity at 3000 WK^{-1} m^{-1}, Young modulus of 1 TPa and high theoretical specific surface area of 2600 m^2 g^{-1} are among the numerous characteristics that make graphene so attractive to researchers and engineers[6–10, 12, 13].

In addition, pristine graphene is a zero-bandgap semiconductor with a symmetrical structure that enables the electrons to move effortlessly and very quickly. The conduction band and the valence band meet at the Dirac point[11, 14, 15]. The bandgap of graphene is tunable by varying electric field as necessary. One very unique characteristic of graphene is the distinctive characteristics of its charge carriers that act as Dirac fermions or massless relativistic particles, which are appraised as electrons without the rest of its mass that has a speed of $1/300^{th}$ of the speed of light. These particles can be defined with three-dimensional (3D) Dirac equation. Under a magnetic field, the properties of Dirac fermions act anomalously in comparison to electrons, such as strange integer quantum Hall effect (IQHE) can be observed at ambient room temperature[16–19].

3. Graphene Synthesis

Due to the excellent properties of a single layer graphene, the synthesis of graphene is of paramount importance as the demand is increasing rapidly. Graphene production should focus on producing monolayer, defect-free, and target-scaled graphene. Fabrication or extraction of graphene according to the preferred size and quality can be defined as the synthesis of graphene. In the field of biosensor engineering, several types of graphene and graphene-based materials have been used and found useful. There is still a lack of a single method of fabrication to fulfil the demand for all potential applications and uses. So, different methods have been devised to get definite and desirable properties suitable for various applications of graphene and graphene-based materials.

In this following review section, pristine graphene, its synthesis and characterization processes are mainly focused on.

3.1 *Exfoliation methods*

Among the graphene synthesis processes, different types of exfoliation processes are utilized. These are the least expensive and can produce high quality graphene. This technique is the most cost efficient yet offers least defects and

highest electron mobility. To match with the high demand, exfoliation processes can come in handy as the processes are capable of producing graphene at a large scale. Exfoliation processes are usually top-down process, in which by applying transverse or longitudinal stress, a single layer or multiple atomic layers of carbon can be separated from graphite structures by weakening the van der Waals force, which is weak itself and that holds the atomic layers together. Different exfoliation techniques are discussed below:

3.1.1 *Mechanical exfoliation*

Theoretically monolayer graphene has existed for a very long time and can be traced back to an article by Wallace in 1947 [20]. But graphene wasn't considered as an alternative for existing silicon based semiconducting materials because of its instability and rolling over tendency. With the repetition of exfoliation, mechanically, with a scotch tape on a highly ordered pyrolytic graphite (HOPG), a single atomic-layer of carbon was separated and deposited on a silicon dioxide substrate (Figure 3a) [21, 22]. This top-down process is capable of producing graphene sheets of different thicknesses as necessary. Besides highly ordered pyrolytic graphite, single crystal graphite or natural graphite can be used for peeling off or mechanical exfoliation [4].

Additional plasma cleaning and heat treatment of the substrates enables better exfoliation process resulting in monolayer graphene with a dimension of a couple of hundred microns that might be single crystal. [23] This exfoliated graphene offers the best electrical properties. Mechanical exfoliation process have given birth to several corollary concoction procedures such as utilization of polydimethylsiloxane(PDMS) stamps [24] and ball milling [25].

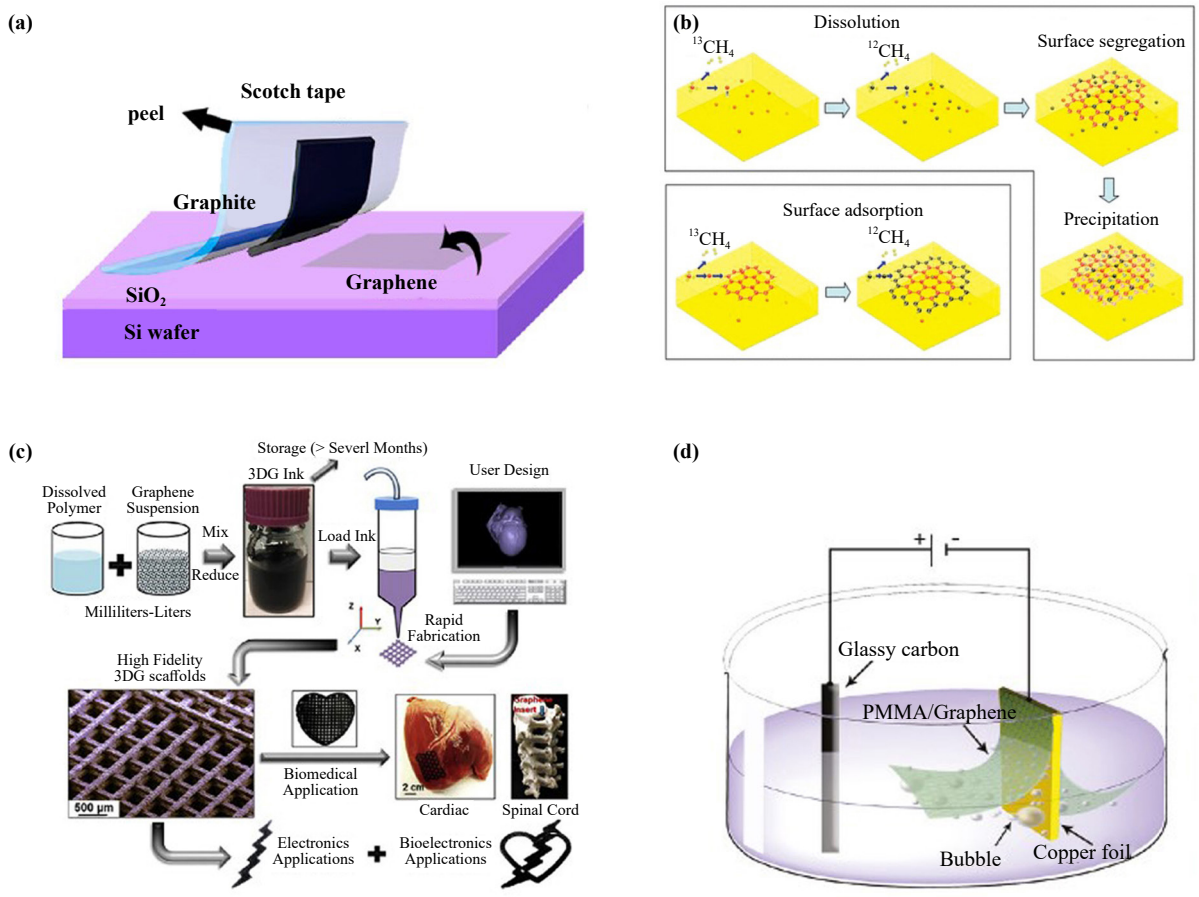


Figure 3. (a) Schematic diagram showing mechanical exfoliation of graphene using a scotch tape and graphite on a silicon dioxide substrate (Reprinted from [9]). (b) Deposition of graphene mixed with isotopes through CVD (Reprinted with permission from [19]. Copyright 2009 American Chemical Society). (c) Schematic diagram of graphene synthesizing through 3D printing with graphene suspension mixing with polymer solution and the possibilities (Reprinted with permission from [26]. Copyright 2015 American Chemical Society). (d) Schematic diagram of electrochemical cell separating graphene film with the help of hydrogen bubbling (Reprinted with permission from [27]. Copyright 2011 American Chemical Society)

3.1.2 *Chemical exfoliation*

Chemical exfoliation process is done through two separate steps. The main objective is to reduce the interlayer van der Waals forces. This can be done through extending the interlayer distance with the help of Graphite Intercalation Compounds (GICs) [28]. The GICs are synthesized by intercalation of alkali metal ions such as potassium, cesium and NaK_2. Then alkali metal-ions are exposed to acid-intercalated exfoliated graphite that are put in Pyrex sealed tubes for GICs to be produced [29]. Rapid heating and sonication method were used to yield the GICs exfoliated in graphene. This results in a single or a few layers of graphene. Through direct exfoliation and noncovalent functionalization followed by graphene solubilization in water utilizing potassium salt of coronene tetracarboxylic acid, monolayer graphene-coronene tetracarboxylic acid composites was produced. Then it is sonicated in ionic liquids and functionalized graphene can be developed as the final product [29].

3.1.3 *Electrochemical exfoliation*

Among the exfoliation processes, electrochemical exfoliation process is the most environmentally safe and produces high quality graphene. This process utilizes a solution with ions in it and the product of the process varies with the characteristics of the electrolyte. The electrolyte properties depend on the necessities of the oxidizing environment as well as the dimensions of the intercalating ion. Two electrodes are usually used: one was made of graphite or Highly Ordered Pyrolytic Graphite (HOPG), and the other one was made of Cu, Pt, graphite or HOPG. [30] After applying a definite voltage, the intercalating ions of the solution exfoliates the graphite in the anodic few layer graphene (AFLG) through penetration between the sheets. Positive and negative voltages are applied in this process, while the positive charge exfoliates the graphene, and the negative charge returns the ions in the solution.

3.1.4 *Liquid phase exfoliation*

Liquid phase exfoliation mechanism represents a top-down exfoliation process. This process uses ultrasonic energy to create micro-cavitations as well as alleviating the source material that is the bulk graphite into smaller particles and leaner film of graphene. The exfoliation process depends on the comparative energy of van der Waals between the layers and the solvent-graphene repulsion. When the energy is balanced between the solvent surface energy with dispersed graphene energy, the exfoliated graphene, that was already coated by the surfactant, stabilizes itself. Liquid phase exfoliation technique is designed to exfoliate graphite into solubilized graphene. Several recent articles discuss liquid phase exfoliation processes using organic, namely aromatic solvents such as hexa-fluorobenzene, octafluorotoluene, pentafluorobenzonitrile, pentafluoropyridine, N-methylpyrrolidone (NMP), γ-butyrolactone (GBL), N, N-dimethylacetamide and 1,3-dimethyl-2-imidazolidinone (DMEU). [9, 25, 31].

The mechanisms of this procedure are simple, such as cavitation, exfoliation and intercalation, making liquid phase exfoliation process a popular choice. A pattern of graphene can be exfoliated through dispersion of graphite, which was prepared through sonication and centrifugation. The exfoliated layer of graphite shows high throughput while in production. There are some limitations too. Excessive ultrasonication can contaminate the graphene with nano sized impurities. As graphene characteristics are dependent on thickness, if the thickness gets over acceptable range through exfoliation. This cost efficient technique of graphene synthesis has been utilized in synthesizing graphene for developing transparent electrodes, sensors having high surface area, electrocatalytic activity, optical limiters, and polymer based composite mechanical reinforcements [9].

3.2 *Chemical Vapor Deposition*

Chemical Vapor Deposition (CVD) is the most popular method of graphene fabrication at industrial level. The synthesized graphene deposited through CVD provides the highest quality, lowest cost and the number of layers of the graphene can be precisely controlled, satisfying with requirements for different applications of graphene. Even though the quality of graphene is of the premium quality, however, the electronic and structural characteristics offered are of lesser quality than mechanically exfoliated graphene. CVD graphene can ensure the uniform thickness of the single atomic layered graphene and can be produced in required dimensions.

Briefly, CVD graphene is fabricated through vapor deposition method. A gaseous thermally decomposed precursor

is brought into exposure of a substrate causing the deposition of the product on the substrate surface. CVD is usually done at a high temperature but if the situation requires, with the help of plasma the temperature can be reduced. At a high temperature, the precursor gases or gaseous reactant used are ethane, methane, acetylene, benzene or propane [2, 32]. The heated substrate reacts with the precursor gases and single or multiple layers of carbon get deposited on the substrate by design. The deposition of graphene can be controlled through gas flow rate, reaction time, temperature and pressure. The single to few layer graphene films are usually deposited on transition metal substrates such as, Ni, Pd, Ru, Ir and most used Cu [32–35]. In Figure (3b), upon cooling the deposited carbon, a single layer or multiple layers of film can be got accumulated on the metal substrates. A minor number of carbon isotopes also get deposited on the substrate as byproducts or impurities. The most optimized condition among the reactant gases and metal substrates is the use of Cu foil with a low ratio of hydrogen to methane at a high pressure. Even though the development of graphene layers gets slower, the quality increases of the accumulated single or multi-layered graphene [36]. Using the plasma enhanced CVD (PECVD) processes can significantly improve the graphene quality and growth rate. PECVD processes doesn't rely on catalyst for the growth or deposition of carbon but the accumulation can be controlled through controlling process parameters such as vacuum, plasma wattage and pressure [37].

3.3 *Epitaxial Growth on Silicon Carbide*

Epitaxial growth means regular growth of one material over another, namely on different substrates. Among the graphene synthesis processes, epitaxial growth on silicon carbide is considered quite difficult because regulating the number of layers, coordinated with temperature and defects or disorder in the lattice. Silicon carbide is heated up to 1250–1450 °C with specified conditions such as ultrahigh vacuum [9]. Silicon has a higher vapor pressure, when the wafer is applied heat to, the silicon ions sublimate leaving the carbon atoms behind. Specified conditions are required to ensure the honeycomb lattice of the carbon atoms. Upon removal of graphene from the wafer, it can be transferred on to a required surface. Some unique properties are added to graphene by the synthesis process, such as, breaking translation symmetry to hybridize of electronic states at Dirac points and breaking the equivalence atomic sublattices [38, 39]. The energy bandgap becomes cause of some change in electronic properties or electronic induce off state of graphene which makes it suitable for graphene transistor and sensor devices. However, the quality of epitaxial graphene isn't always up to the mark as many defects can be associated with the graphene, reduced mobility as well as the process itself is not cheap [40].

3.4 *Unzipping of Carbon-Nanotubes*

In this graphene synthesis process, without transferring or growing on any kind of substrate, pristine graphene films can be obtained. The Multi Walled Carbon Nano-Tubes (MWCNT) are graphene ribbons that are developed through several chemical, lithographic, synthetic or CVD procedures [41]. Pristine graphene film can be extracted through unzipping the MWCNTs. The unzipping of MWCNTs can be done in several ways through chemical or exfoliation processes, such as through intercalation of Li and ammonia, acid exfoliation and rapid heating [42]. plasma etching [43], and chemical redox reactions [4, 41].

3.5 *Recently Developed Methods*

As introduced above, these are the traditional or relatively common synthesis methods of pristine graphene. In the recent years, novel methods are developed to synthesize pristine graphene. These methods are suitable for both large- and small-scale production, non-destructive, economic and more environment friendly. The novel techniques are introduced below.

3.5.1 *3-Dimensional Printing*

Instead of mass producing the same architectural pattern on graphene or graphene synthesizing, it will be more convenient and efficient if the thin-film graphene can be produced depending on individual demand and requirements, such as 3D printed "On Demand" electronic devices [44]. Additive manufacturing can print out 3D graphene-based

composites that will cut down the resource wastage, expense and increase the quality of the manufactured product.

Using the 3D printable graphene ink (3DG ink), the 2D deposition process of graphene is similar to traditional 3D printing as they follow the same four categories - Photopolymerization, Extrusion, Powder-based and Lamination. In figure (3c), the fabrication process of graphene through 3D printing is depicted through diagrams. Direct Ink Writing (DIW), Fused Deposition Modeling (FDM), Stereolithography (SLA), Selective Laser Sintering (SLS) are different printing techniques to print 3D graphene[26, 45]. For each requirement, the design of operation or the printing parameters can be changed resulting in changes of characteristics that can be intrinsic or structural. Rapid prototyping can be cost and resource-efficient in this way. Among the printing techniques, DIW is the most discussed one though the technique requires complicated post-treatment steps. In comparison, SLS is one of the easiest and most simple printing techniques and it shows unique characteristics such as controllable pore structures, extremely high mechanical strength and free-standing capability that results from the interaction between graphene and laser. The 3D printing techniques have the potential to revolutionize the graphene synthesis scenario [45].

3.5.2 *Bubbling transfer*

Usually in traditional graphene transfer processes, chemical etchants are used to etch away the substrate that can cause damage to the transferred graphene. As the substrate is etched away, it can't be reused. Inert or valuable the substrate has to be sacrificed. An electrochemical delamination process that utilizes hydrogen bubbles to separate monolayer or few-layer graphene from its substrate has been recently being used. This technique is nondestructive and economic. Unlike the conventional etching processes, which can be time consuming and sometimes quite expensive besides being harmful to substrate. Bubbling transfer is used to bypass this step and to minimize the time required. In this process using electrochemical cells, as shown in figure (3d), hydrogen bubbles are generated from the electrodes that provides tender yet continuous force on the surface of the graphene that is in touch with the substrate, causing the graphene film to detach from the substrate. The substrate then can be re-used, and this process saves time and money. A supporting scaffold of PMMA is used to ensure the intactness of the graphene sheet. The transferred graphene shows high crystal quality, lowest wrinkle height and greater carrier mobilities. As PMMA coating is already an important part of graphene transfer process, no extra steps are needed for the delamination of the graphene film [27, 46]. For a delamination process, Cu electrode/graphene was charged as cathode at -5V and aqueous solution of $K_2 S_2 0_8$(0.05 mM) used as electrolytic solution [27]. In some cases, etching can't be done where the substrate is inert or expensive such as Pt, the substrate/graphene is polarized as cathodes and electrolytic solution such as NaOH aqueous solution is utilized [46]. The electrolytic cleaning for removing PMMA residues after thermal annealing, which utilized a similar process, were also reported [47].

Table 1 summarizes the properties, advantages, disadvantages, production sizes and area of applications of the above-mentioned synthesis processes for the ease of the readers.

Table 1. Summary of mainstream methods for graphene synthesis and fabrication

Classification	Methods	Production Sizes	Properties	Application	References
Top-down approach	Mechanical exfoliation	Small scale (500 Å to 10 µm)	Excellent electrical properties, Graphene flakes, Varied thickness distribution, Low exfoliation efficiency, Insolubility, Low cost	Research, Prototyping	[3, 18, 23], [48]
	Chemical exfoliation	Small scale (500 Å to 1500 Å)	Low-cost Lowest number of defects Highest electron mobility	Polymer composites, Conductive coatings and inks, Fuel cells batteries	[4, 49]
	Electrochemical exfoliation	Small scale	Eco-friendly technique Produces high quality graphene	Catalysts, Ultracapacitors	[4]
	Liquid-phase exfoliation	Large scale with less than 10 nm thick	Low cost High throughput during production	Conducting additives, Fillers in batteries, Composite coating and inks, Transparent electrodes, Sensors, Optical limiters, Mechanical reinforcements for polymer-based composites	[9, 48]
Bottom-up approach	Chemical vapor deposition	Both small and large scales	Large size with continuous graphene films; Monolayer, Higher quality and uniformity, Transfer is required and may cause defects and residues, High energy consumption, High cost, Electrical properties significantly dependent on the amount of crystalline boundaries, needs a metal surface (namely Copper) at high temperature to transfer onto	Transparent conductive films, Coating, Electronics, Bioelectronics, and photonics	[48, 50]
	Epitaxial growth on silicon carbide	Large scale	High quality films with unified crystallographic orientation, Fine monolayer controllability, Suppression of wrinkle formation on a wafer scale	electronics and radio frequency transistors	[48, 51], [52]
Additive approach	3D printing	Large scale (50×50 micron^2)	Cost reduction On demand manufacturing	Electrical and electromechanical devices, Biomedicine, Sensors	[44]
Separation	Bubbling transfer and delamination	Large and small scale	Large single-crystal grains, nondestructive transfer, no etching, time saving, economic, scalable, substrates reusable	Electronics, spintronics, sensors, batteries, supercapacitors	[27, 46]
Self-assembly in 2D	Molecular assembly	Monolayer, small-scale film	Self-assembly in 2D	Nanoelectronics devices	[48]
	Unzipped multi-walled carbon nanotubes	Moderately small and large scale	Straight edges, gradually transform semiconductors to semimetals with the increase of width, different scales of graphene can be produced	Opening possibilities for substrate, Free graphene synthesizing, Electronics and composite materials	[4, 41]
	Reduced graphene oxide	Both small and large scales	Water-soluble, Small-size sheets, Highly disordered and defects	Compositions, Conducting additives, Thermal dissipation films	[50]

4. Characterization Methods

In the post-silicon age, Graphene is considered as a promising alternative for an excellent electronic material because of its potential for implementations in electronic devices such as field-effect transistors and transparent electrodes etc. Because of the increasing interest, the research on graphene is a swiftly developing sector. Thus, structural and electronic properties of graphene such as extremely high mechanical strength, thermal conductivity, transport energy gaps and absorption coefficient of lights, have piqued the interest of scientists and researchers. To ensure the quality of graphene, proper characterization processes are necessary. Some principal characterization methods are discussed below.

4.1 *Raman Spectroscopy*

Among the non-destructive characterization techniques, Raman is the most popular and comprehensive

characterization technique. It gives versatile information, that can be compared to fingerprints in terms of uniqueness, like quality of graphene, number of layers, induced strain, discrepancy of lattice and existence of defects [2]. Initially Raman was discovered based on absorption and emission of phonons (Stokes and anti-Stokes, respectively) by C.V. Raman and his colleagues back in the 1920s. Since then, it is widely used for achieving information on catalyst structure determination, in-situ reaction monitoring, investigation of structure and quality of carbon-based materials as it provides both qualitative and quantitative data.

In case of graphene identification and experimentation, among the phonons of the Brillouin zone center depicted by Γ, the E_{2g} phonons are the main determinant of Raman spectra. The G peak (1580–1605 cm^{-1}) and the 2D peak (2640–2680 cm^{-1}) are the predominant peaks for Raman spectra. In figure 4, using the laser with ~532nm wavelength on SiO_2/Si substrate, the Raman spectra of monolayer graphene can be seen. It shows G-band and 2D-band that represents the carbon allotrope characteristics of monolayer graphene ~1580 cm^{-1} and ~2690 cm^{-1} respectively. From the spectra, different bands represent different information such as tangential stretching mode of highly oriented pyrolytic graphite (HOPG) through G band, sp^2 hybridized carbon atom disorder and second order scattering process through 2D band [53–56].

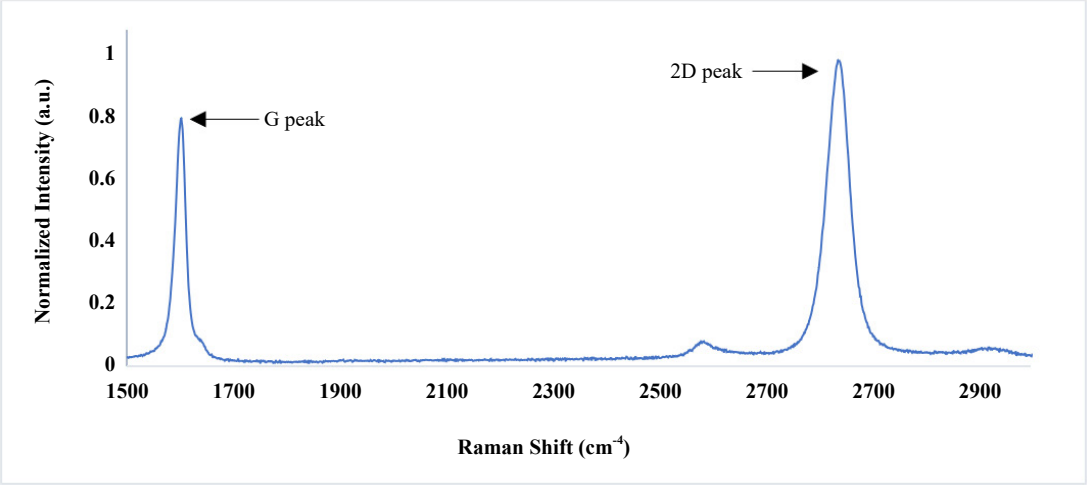


Figure 4. Raman spectra of monolayer graphene on a silicon dioxide substrate

On a silicon-dioxide substrate, the transferred graphene from store-brought (Graphene supermarket), was subjected under Raman spectroscopy and the Figure (3) spectra was achieved. From the figure, the steep 2D peak and its comparison of intensity with the G peak proves the existence of monolayer graphene and the G peak confirms the existence of graphene [57]. Also, the smaller D peak coupled with a large ratio of 2D/G peak intensity signifies the good quality of the transferred graphene. From all types of synthesized graphene, the CVD grown graphene gives the best 2D/G intensity ratio and the relatively low D peak proving it to be the best quality and larger sized graphene available [58]. The difference between monolayered and multi layered such as bi-, tri- or several layered can be identified from the peak intensities too, but this can be done properly when the layers have a number less than five [59].

4.2 *X-ray Photoelectron Spectroscopy*

By measuring the kinetic energy of the exerted electrons from surface of the specimen that are energized through radiation from an X-ray beam, the X-ray Photoelectron Spectroscopy (XPS) spectra can be acquired. The principal use of XPS is for catalytic research. Through XPS, chemical and electronic state of the elements of the surface can be characterized. Each element and elemental composition cause different spectra that can be identified. Elemental composition of the elements of the surface can also be identified as XPS is a surface -sensitive technique [58–60]. Even though XPS is a powerful quantitative spectroscopic technique, it is not considered as a principal technique to

characterize graphene. It is tough to exact the position of intrinsic graphene carbon 1s line that is utilized to measure the dopant or foreign elements, making the acquiring measurement difficult from XPS survey spectra.

Graphene oxide can also be characterized with XPS as well as to get information on graphene quantum dots [63]. When used alongside TEM, Raman, AFM or cyclic voltammetry, functionalized graphene nanosheets can also be analyzed [64]. But there are different models of XPS that are suited for different tasks, so it's upon the user to choose the correct model. The use of XPS is still unpopular, as the information extraction through experimentation, designing of experiment, data collection and interpretation need skill and the user have to go through a steep learning curve. But the significance of this characterization process can be realized as most all publications that work on doped-graphene catalysis utilize XPS. XPS is used as the first step to validate their results as well as to get information on heteroatoms [62].

4.3 *Atomic Force Microscopy*

Atomic Force Microscopy (AFM) is widely used to determine thickness and layer number because of the reliability it offers. It has different modes and working processes for different materials and surfaces. AFM utilizes a sharp tip that is around 5–10 nm in radius and is attached through a cantilever. Through measuring the sensitive changes in amplitude, phase and frequency of the vibration that are caused form the heterogeneity of the sample surface, the information collected from the tip is processed and the topographical image of the surface is generated. From the topographical 3D image, the thickness of the graphene film can be measured. The acceptable thickness that has been used for a single atomic layer of graphene is 0.35 nm and by using this value the layer number of the graphene can also be determined [65]. Surface roughness of a surface can provide several useful information such as active sites, defects, contaminations, and residues. For example, during graphene transfer from Cu onto desired substrate, such as silicon dioxide, the PMMA residues can be identified through AFM [47]. This information was used to increase the integrity of the graphene through electrolytic cleaning as described in Figure 5.

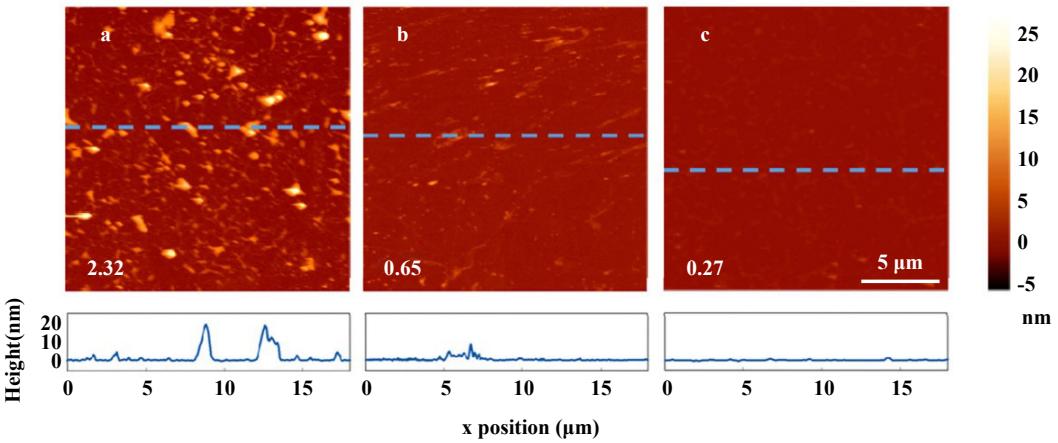


Figure 5. AFM images of multiple stages of cleaning process of the graphene. (a) shows the surface of the graphene just after transferring, (b) shows the surface of the graphene after annealing to remove the PMMA residue and (c) shows the reduction of PMMA residue through electrolytic cleaning. (Reprinted from [47])

The principal concern with AFM is that the tip requires to be in perfect contact with the surface to get the best results but that is a rare occurrence as it is tough to ensure proper contact. Besides, the surface needs to be flat otherwise the result will be erroneous. Also, as the single layer graphene is not always uniform in thickness, so measuring the layer number isn't always correct from the measurement. Different factors, such as surface chemistry and image feedback locales, also has effects on the results [66].

4.4 *Transmission Electron Microscopy*

Transmission Electron Microscopy (TEM) is based on electron transmission through a sample. When the electrons are excited through applying a very high voltage, transmitted through the sample and the return signal is processed and projected on a viewing screen for monitoring. For this reason, a thinner sample gives better result than a thick sample. TEM is usually used to measure discrete flakes from solution through measuring lateral size, thickness, crystal structure characteristics, and chemical composition. The atomic structure and interface of graphene can be obtained when the thin film is exposed under TEM. The surface defects and elemental mapping to observe distribution can be identified from graphene-based catalysts [65, 66]. The numbers of layers can also be determined from dark lines by TEM for graphene characterization.

4.5 *Polarized Optical Microscopy*

Among the non-destructive processes, opposed to the destructive methods such as TEM, polarized optical microscopy is the easiest. For graphene characterization, this straight-forward and efficient characterization technique relies on contrast to identify graphene layers and substrates. Optical images taken through this technique show the graphene structure, its shape, and the size.

4.6 *Ultraviolet-Visual Spectroscopy*

Ultraviolet-Visual (UV-vis) spectroscopy works through measuring the absorption and reflectance in a specific spectrum of radiation. The spectrum used in UV-vis spectroscopy is the ultraviolet spectral span. Through increased enervation of the beam, the absorbance of the sample also enhances. For graphene, UV-vis spectroscopy is used for characterization of the optical transparency of a graphene sample through measuring the absorbance or transmittance properties. The number of absorption or transmittance bands can be correlated to the transition of electrons to the excited state form the lowest energy state and it produces an absorption or transmittance band. So, through a thorough observation of the UV-vis spectrum, the characteristics of the graphene layers can be examined [67, 68]. The layer numbers of graphene can also be verified through examining differences in optical transmittance.

Besides pristine graphene, UV-vis spectroscopy is the most widely used characterization technique to ensure the proper synthesis of graphite oxides. Through absorbance spectra acquisition, the UV-vis spectroscopy can differentiate between bonds that shows the successful synthesis of the graphene oxide products [69,70]. Though this characterization method is hindered by insufficient oxidation of graphite that causes the unsatisfactory reaction performance of the product. Therefore it is customary to check for oxidation level of the exfoliated graphite oxide before utilizing it for synthesis of catalysts [71].

4.7 *X-ray Crystallography*

X-ray Crystallography (XRD) is, as the name suggests, a characterization technique that is used for observing crystallinity. It is also used to study the atomic positioning, crystallite size, imperfections, and other structural characteristics of a material. XRD instruments utilizes different optical phenomenon such as transmission, absorption, refraction, scattering and diffraction [61, 72]. The distinct property that differentiates between materials is the diffraction spectrum. To characterize graphene, XRD is not a suitable characterization method to characterize monolayer graphene but can be of assistance to determine the existence monolayer graphene.

A brief comprehension of the most used and above stated characterization techniques with their relative advantages, disadvantages and properties are summarized in table 2.

Table 2. Principal characterization methods of graphene

Methods	Properties	Disadvantages	References
Raman Spectroscopy	determine the number and orientation of layers, in-situ reaction monitoring during transferring, exam the quality and types of edge, study the effects of perturbations (functional groups, doping, strain etc.)	Missing comprehensive quantitative understanding of the peaks, deconvolution of 2D bands relation with spectra, lack of defect level estimation	[62, 73, 74]
X-ray Photoelectron Spectroscopy (XPS)	Investigate surface properties, doping and charge transfer, confirming existence of foreign atoms	Limited information offered on heteroatoms, require specific skills on experimental design, complex data collection procedure	[62, 75, 76]
Atomic Force Microscopy (AFM)	Determine the number of layers of the graphene, film thickness, and surface roughness	Destructive in contact tapping mode, inaccuracy of results due to thickness, needs experience to extrapolate data from AFM images, specimen needs to be flat, cleanness of the specimen heavily affects the result	[62, 77]
Transmission Electron Microscopy (TEM)	Giving information on the structure, number of layers and morphology of the graphene sheets through generating atomic level image	Obtain best results only for thin samples, need additional characterization approach (i.e., Raman results) to validate	[77]
Polarized Optical Microscopy (OM)	Simple technique, determination of number of layers through contrast imaging, non-destructive	Generate low resolution images	[2, 62, 77]
UV-vis Spectroscopy	Identify Reduced Graphene Oxide from Graphene Oxide, determine graphene layer	Prone to faulty results for the specimen slightly contaminated	[77]
X-ray Crystallography (XRD)	Crystallinity and crystallite size, atomic arrangement	limited uses, and uncommon technique for characterization	[77]

5. Graphene-based Biosensing

The applications of graphene are versatile and spread across healthcare and biosensing sectors, biological and biomedical engineering, and energy sector. For example, the desirable properties of graphene cause the biosensors to be lighter and smaller. The charge carrier mobilities measured from isolated graphene, suspended graphene devices, and CVD grown graphene on silicon dioxide substrate have shown significantly higher than other bulk nanomaterials. Also, graphene shows lower intrinsic noise, ambipolar field-effect characteristics, and large detection area than other nanomaterials. The most significant property of a single layer graphene in case of biosensing is the higher sensitivity than most other bulk nanomaterials [78].

The use of biosensing is so versatile and widespread. So, for this portion, biosensing from specifically human bodily fluids are discussed. In the complex biological fluids, the distinguishable changes in biomarkers can be the savior for diseases like cancer. Graphene based medical diagnostic devices and tests are up for these. The properties of the devices like selectiveness, rapidness and most importantly highly sensitiveness towards the slightest of changes in biomarkers make them more suitable for point-of-care diagnostics than conventional systems.

5.1 *Common Types of Graphene Biosensors*

The most common biosensor that are used are the electrochemical biosensors. Here are some examples of the electrochemical biosensors implementing graphene, to detect D-dimers in human blood serum that causes different diseases, such as Venous Thromboembolism (VTE), Deep Vein Thrombosis (DVT) and Pulmonary Embolism (PE), graphene microelectrodes combined with lipid bi-layer membranes or BLMs have been used [79]. The use of lipid film enhances the quality of graphene biosensors in terms of reproducibility, high selectivity, reusability, quick response time, longevity when unused, and high sensitivity. A new kind of electrochemical biosensor that utilizes 3D graphene as the surface electrode that immobilizes Hb on the surface of the electrode with a chitosan film shows promise at detecting blood hemoglobin level. Furthermore the process shows the direct electron transfer of Hb [80]. Graphene based surface plasmon resonance (SPR) was utilized to detect folic acid protein from blood serum. Folic acid is a cancer biomarker and is extremely important to diagnose carcinogenic symptoms for providing treatments accordingly. Biomarkers like folic acid exist in extreme low concentration that requires maximal sensitivity and selectivity towards specific compounds. Graphene based SPR chips were successful in detection of folate biomarker detection in serum [81]. In

figure (6), for thrombin detection graphene promoted 3, 4, 9, 10- perylene tetracarboxylic acid (PTCA) nanocomposites were synthesized to manufacture an electrochemical aptasensor via covalent bonds (figure 6a) [82]. To detect the same compound, another approach was described through well-dispersed graphene oxide on gold films. The released amount of thrombin was detected with surface plasmon resonance and impedance spectroscopy (figure 6b) [83]. Graphene quantum dot modified electrode, fabricated through incubation of graphene with the help of pyrolytic graphite electrode can be also used to identify the existence of thrombin (figure 6c) [84]. Finally, magneto-controlled bioelectronics approach can also be utilized to detect thrombin (figure 6d) [85].

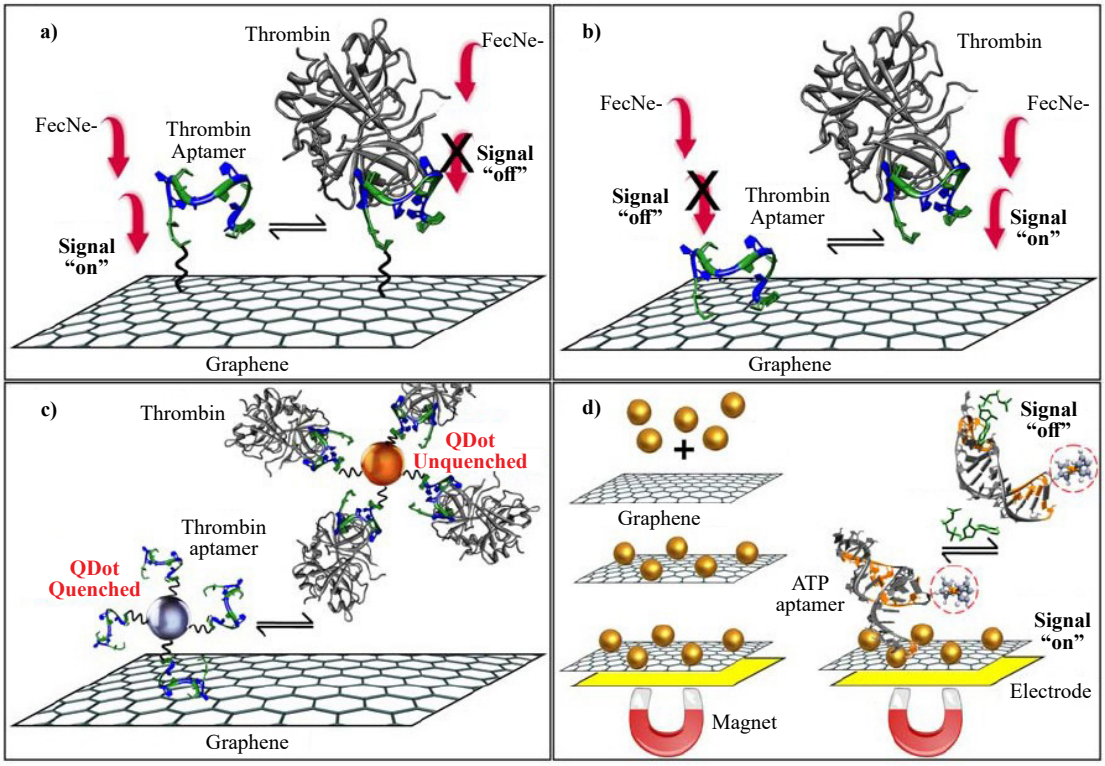


Figure 6. Schematic diagrams of four different approaches to fabricate a graphene based impedimetric electrochemical sensing platform to detect thrombin with (a) covalent aptamer immobilization (b) non-covalent aptamer immobilization (c) graphene quantum dots and (d) magnetic-nanoparticles-graphene bioelectronics (Reprinted from [86])

Graphene based field-effect transistor (GFET) is a viable option for biosensing as well. The traditional methods of sensing can be accurate and specific objective oriented, but at the same time, they are slow, need high concentration and large volume of samples, and quite expensive. Graphene based FETs cover all those shortcomings from the conventional biosensing methods, and adds stability, tunable sensitivity, and biocompatibility [78]. For example, to electrically identify and determine immunoglobulin E (IgE) protein concentration from blood, a label free immunosensor based G-FET was used with An IgE aptamer as the bioreceptor [87]. To detect vascular endothelial growth that works as an angiogenesis biomarker for cancer, nitrogen doped graphene grown on Cu substrate showed high sensitivity (100 fM LOD). The graphene field effect transistor aptasensor was based on converted polypyrrole precursor and doped with nitrogen. Additionally, the biosensor showed high performance, better reusability, ruggedness, bendability, and flexibility [88]. For diabetes diagnosis, flexible CVD grown graphene-FET gives higher sensitivity (3.3–10.9 mM LOD). The GFET, functionalized by linker molecules to immobilize enzymes inducing catalytic response of glucose, has the potential to be applied in wearable, portable and implantable glucose level monitoring devices [89].

5.2 *Integrated Graphene Biosensor*

FETs are recently integrated into microfluidic platforms in handheld, on-site, point-of-care (POC) miniaturized medical devices and tests due to their smaller size, simplicity and microfabrication compatibility. They provide better quantitative, accurate and reliable test result because of the chance of using multiple sensing elements. When coupled with microfluidics system, FET functionality is greatly enhanced thanks to the automated sampling at a small volume, pre-separation and detection of multiple analytes. This automated system is making the necessity of laboratory facilities and skilled operators obsolete. Among the categories of microfluidics systems, polymeric based system, inorganic and paper-based system and the paper-based microfluidics system are being introduced to point-of-care (POC) diagnostic systems because of their lower costs and environment friendliness [90].

A protein biomarker responsible for multiple cardiovascular diseases like hemostasis, thrombosis, and tumor growth, can be used to detect thrombin. The biosensor should have a limit of detection in picomolar level, and the detection range of concentration should be up to micromolar scale [91]. A miniaturized and mobile biosensing module fulfills this long list with integrated microfluidic GFET chip. Furthermore, enhancement of performances regarding detection limit, sensitivity and real time monitoring was observed. This technology is much more cost-efficient, dependable, and user friendly [92].

Lower material and manufacturing cost are the key advantages of disposable integrated lateral flow devices. An integrated self-powered lateral flow device that utilizes capillary force with functionalized polymer-based microfluidics was proposed to separate plasma from human blood from the minimal sample volume and biosensing. The integrated device has the potential to detect multiple biomarkers simultaneously with functionalization of different bioreceptors for analytes, was reported for having flexibility fabricated on desired substrates and by employing various sensing methods [93]. For example, to detect immunoglobulin IgG directly from plasma, a lateral flow device (LFD) integrated GFET was utilized[93]. The configuration of the device is shown in figure (7a). As buffer solution 0.01×PBS was used and to replicate the amount of IgG present in undiluted plasma normally, different concentrations of IgG was injected in the buffer solution and the drain current was observed closely. Decrease of drain current can be noticed with the increase of concentration of IgG in the figure (7b). An example was shown in figure (7c) where a drop of 0.18 μA was observed upon adding plasma. This proves the existence of IgG present in the plasma is causing the drop of drain current and the estimated concentration found from the calculation was well within the natural range of concentration of IgG found in human plasma [94].

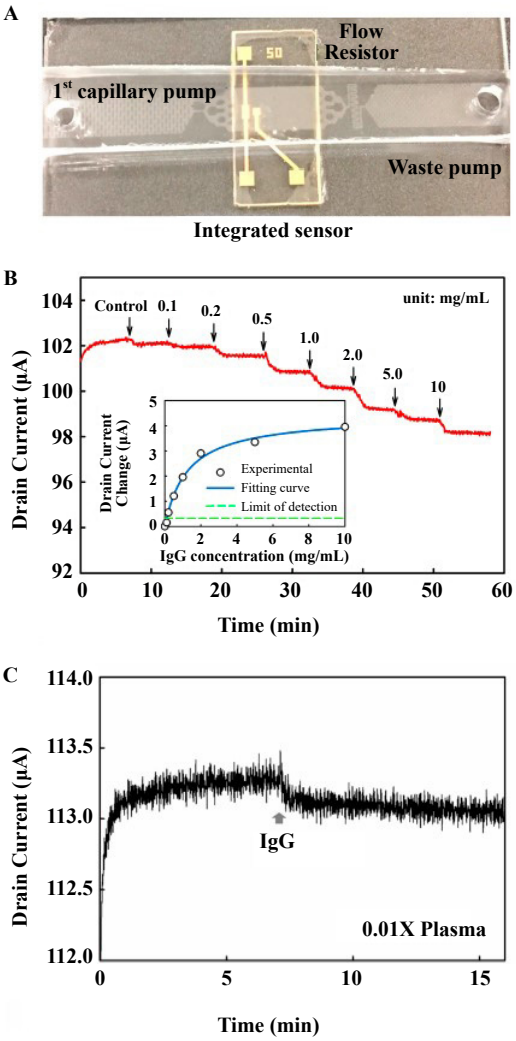


Figure 7. (a) The nanoelectronics biosensor integrated LFD used in detecting IgG from human plasma. (b) The response of drain current to increasing IgG concentration in the buffer solution. (c) Detection of IgG from human plasma (Reprinted from [93])

To identify cervical cancer, a paper-based biosensor was utilized to detect human papillomavirus. A graphene-polyaniline (G-PANI) electrode was printed by inkjet printing. An anthraquinone-labeled pyrrolidinyl peptide nucleic acid (acpcPNA) probe (AQ-PNA) was used to modify the electrode. A synthetic 14-base oligonucleotide target, in companionship of an amino acid on graphene electrode, that is negatively charged, causes surface engineering, identifies the primary stages of cervical cancer through electrostatic attraction. To detect the cancer, the measurement of the electrochemical signal response of the AQ label was taken and compared with a sequence that has been identified from human papillomavirus (HPV) type 16 DNA [95]. An ultrasensitive poly-l-lysine (PLL)-functionalized graphene field-effect transistor (PGFET) biosensor is used to detect breast cancer miRNAs and viral RNA that can indicate the existence of SARS-CoV-2 virus in 20 minutes using minimal volume of human serum and throat swab samples [96]. Gold nanoparticles patterned on a single layer graphene was also reported for glucose biosensor to detect blood glucose level [97].

In paper-based microfluidic electrochemical immune-devices, the use of graphene on biosensor surfaces increases electron transfer, and the detection signals get enhanced when coupled with controlled radical polymerization reaction called Activator Generated by Electron Transfer- Atom Transfer Radical Polymerization (AGET ATRP). This proposes new handheld compact technologies with swift results, more cost efficiency, and environment friendly point-of-care diagnostics [98].

6. Conclusion and future perspectives

At present, Graphene can be considered as the hottest topic of interest in condensed matter physics, materials science, and engineering. The unique properties of graphene, synthesis processes, characterization methods, and integration level bio-applications are addressed in this review article. The exceptional electrical, mechanical, optoelectrical and thermal properties of the graphene make it very suitable for applications spread across various areas of interests. The conventional synthesis processes of graphene along with some novel methods are presented. The methods presented in this review paper show the promise of producing scalable, defect free and high quality of graphene with tunable properties. However, graphene synthesis methods are not still ready to handle industrial level production as not all the methods are scalable and cost effective. Transferring of graphene onto different substrates are also important as it directly affects the quality of graphene and the interim connection between synthesis and application. But the synthesis process optimization is dictated by the objective or the applications the device are intended to perform. Sometimes, to avoid synthesizing graphene or to produce commercial-ready graphene, graphene transfer is implemented but improperly transferred graphene can cause damages to the film or introduce defects and impurities that can negatively impact the applications. The process of transferring graphene can be avoided through utilizing transfer free graphene growth processes. Though the transfer free methods are still in the primary stages and need extensive research and development for these to be viable for industrial level productions and applications.

In this review article, the characterization methods such as Raman, AFM, TEM, XPS, UV-vis spectroscopy, X-ray Crystallography and optical microscopy are reviewed. These are the main and most used characterization methods to investigate the properties of graphene. Both the synthesis and characterization methods are summarized through Tables 1–2 for the ease of the readers. The applications covered the integration level implementation of graphene in biosensor devices namely for human body fluids. But to properly understand the full extent of data gathered and presented here, deep mining over the presented data is necessary as well as skills to take full advantage of the synthesis and characterization tools.

In the recent times, despite the hardships in synthesis and transfer of graphene, nanomaterials based on graphene is used in different categories of biomedical and biological research areas that include but not limited to photodynamic and photothermal therapies, tissue engineering, regenerative medicine and so on [99]. The increasing popularity has opened up new possibilities such as bioimaging, photothermal therapy and gene drug delivery [100]. The near future of graphene seems bright as scientists and researchers are consistently in search of greener technologies. Graphene, combined with other materials that shows greater wettability, flexibility has a huge potential for implementation in multifunctional smart materials. Better biocompatibility, self-folding properties and environmental sensitiveness will make graphene the material of choice. But we are yet to see these results as most of the nanomaterial-composite and pristine graphene based devices are not clinical-trial worthy yet [99]. Upon solving the problems and when coupled with other smart technologies, graphene has the potential to revolutionize the whole biomedical sector beyond our imagination.

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Conflict of interest

There is no conflict of interest for this study.

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