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Review Article

Self-Assembling Property of Graphene Derivates Chemico - Physical and Toxicological Implications

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Abstract

This work starts after seeing a recent open letter for transparency related production a quality control technique of m RNA vaccine first signed by Tarro G, Luisetto M and Monsellato ML and an Editorial recognized by IMA Marijnskaya academy: Graphene and Derivates: Physico-Chemical and Toxicology properties in the m-RNA Vaccine Manufacturing Strategy Needed specific proof of absence for the regulatory aspects (accepted for publication). Other relevant evidences come from the work of Giovannini et al. related Dark Field microscopy assay of the blood of 1086 symptomatic subjects after vaccination with two types of mRNA vaccine of great interest on this field the work of Campra P and Young RO, Young Me Lee or Ki-Yeob J.

The Aim of this work is to investigate the self-auto assembly properties of graphene and derivates in order to Find relationship in some biotechnological application like mRNA vaccine.

After a review part an experimental hypotesys project will be submitted to the researcher to produce a global conclusion. The recent evidences published in last period induced the idea to more deeply study these properties for

The clinico-toxicological aspects involved.

Keywords: Self-assembling; Graphene; Graphene GO; Chemico-physicial property; Toxicology; Clinical effect; Biopharmaceuticals; mRNA vaccine

Introduction

Related various and recent evidence Campra P, Young RO, Young Me Lee, Ki-Yeob, Giovannini et al. and review works Luisetto M, Tarro G, it is interesting to observe the self-assembling properties of graphene and its derivates and their implication in clinico-oncological and toxicological field.

The characteristic pattern of this innovative material used in many biotechnological applications related to their specific chemico-phisical properties are reported in various relevant literatures. As reported in article "Bio-pharmaceutical manufacturing large scale production process: The graphene-derivates role and mRNA vaccine":

"Used in many bio-medical and other fields like bio-sensors, in water purifying, to remove heavy metals procedure, in diagnostic field but also in extraction, purifying DNA, RNA and other bimolecular, carrier, adjuvant, antibacterial and other biological and industrial use". In literature it is also possible to see in example: Materials today. New graphene-based material self-assembles into vascular structures. 19 March 2020 "Self-assembly is the process by which multiple components spontaneously organize into larger, well-defined structures. Biological-systems rely on this process to controllably assemble molecular building blocks into complex and functional-materials exhibiting remarkable properties such as the capacity to grow, replicate and perform robust functions.

"There is a relevant great interest to develop materials and fabrication processes that emulate those from nature. The ability to build robust functional materials and devices through the self-assembly of molecular components has until now been limited" said team member Yuanhao Wu, who is also at the Univ. of Nottingham-Queen M. Univ. London. "This research introduces a new method to integrate proteins with graphene oxide GO by self-assembly in a way that can be easily integrated with additive manufacturing to easily fabricate various biofluidic devices that allow us to replicate key parts of human tissues and organs in the lab" (Figure 1 and 2).

"Reactivity control of the graphene is an important problem because chemical functionalization can modulate graphene's unique mechanical, optical, and the electronic properties. Using systematic

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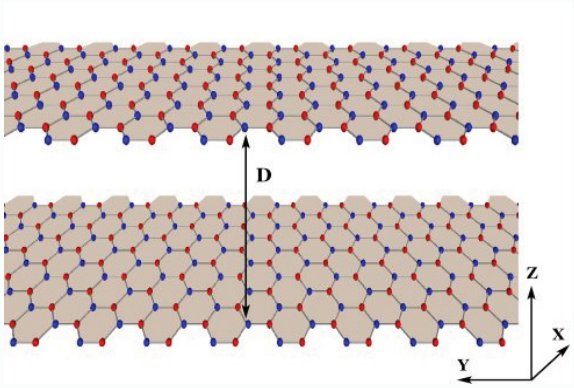

Figure 1: Color online Schematic depicting 2 undoped and un-strained freely suspended graphene layers separated by a finite distance (D).

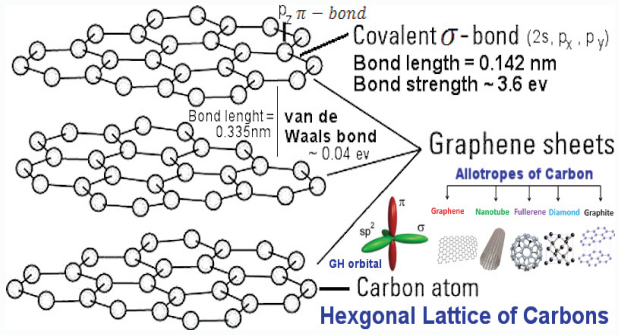

Figure 2: Chemical reactivity of graphene on 2-dimensional (2D) hetero-structures.

optical research studies, we demonstrate that van der Waals VDW interaction is the dominant factor for the chemical reactivity of graphene on 2-dimensional (2D) hetero-structures. A significant enhancement in chemical stability of graphene is obtained by replacing the common SiO_2 substrate with 2D crystals such as an additional graphene layer, WS2, MoS2, or h-BN. Our theoretical/experimental results show that its origin is a strong van der Waals VDW interaction between graphene layer and the 2D substrate. This results in a high resistive force on the graphene to-ward geometric lattice deformation. We demonstrate that chemical-reactivity of the graphene can be controlled by the relative lattice orientation with respect to the substrates and thus can be used for a wide range of applications including hydrogen storage".

Self-assembly is a process mechanism by which a disordered system of pre-existing components forms an organized structure or pattern like a consequence of specific, local interactions among the components themselves, without external direction. When the constitutive components are molecules, the process is named molecular self-assembly. Regarding the self-assembly process in nano science, it is possible to see (Figure 3).

Related graphene materials

"Graphene self-assembly GSA represents a promising and interesting method for microelectronic applications. Recently in last year's, graphene micro-patterns (consisting of crossed stripe of single- and 2-layer graphene) have been fabricated by means of the (evaporation-induced) self-assembly technique".

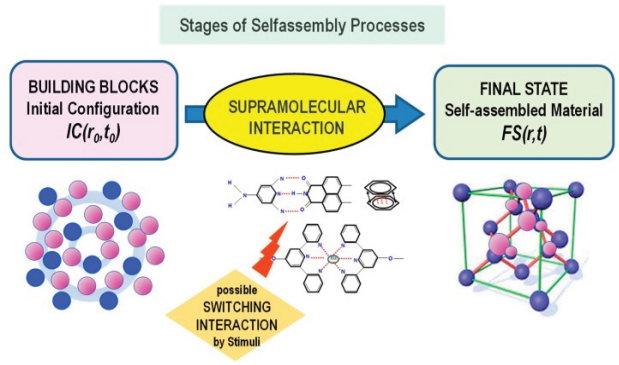

Figure 3: Conceptual scheme indicating the main stages of the self-assembly process in nanoscience.

Chinese chemical letters

"The research works on the properties of Graphene Oxide (GO) in various media has become one of the hottest topics since GO is now the main- principal raw material for graphene-based advanced materials. In this research work, the γ-ray radiation chemistry effect of GO nano-sheets and their self-aggregation behavior in t-butanol/water medium were investigated. The results show that GO nano-sheets are reduced and hydroxy-alkylated simultaneously by alcohol free radicals produced by the radiolysis of t-butanol/water solution under γ-ray radiation. The radiation-modified GO nano-sheets will self-assemble into a self-standing graphene hydrogel when the pH of solution is lower than 2. A hydroxyl-functionalized free-standing graphene-aerogel is further obtained simply by freeze-drying. This work provides not only a general Self-Assembly (SA) mechanism of GO nano-sheets in strong acidic alcohol/water media under a high energy radiation, but also a facile and economical preparation method for hydroxy-alkylated graphene-based aerogel" (Figure 4).

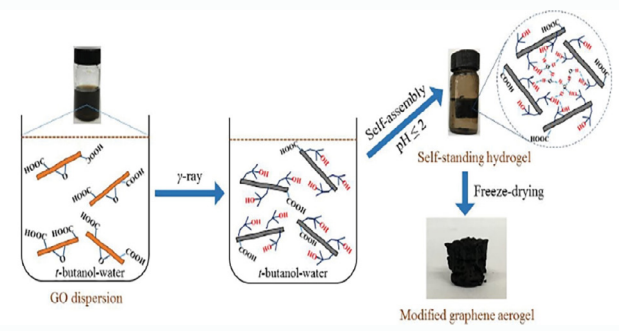

Figure 4: Facile and economical preparation method for hydroxy alkylated graphene-based aerogel.

"Graphene derivatives, like Graphene Oxide (GO) and Reduced Graphene Oxide (RGO), have been great widely used as promising 2-dimensional nano-scale building blocks due to their interesting properties, cost-effective production, and a good processability. Understanding the intrinsic self-assembling, colloidal, and rheological features of the graphene derivatives is of critical importance to establish the formation-structure-property relationship of graphene-based materials".

Acta biomaterialia

"In this research study we explore the use of Graphene Oxide

(GO) as like nano-filler for the reinforcement of FEFK. FEFK (β-sheet forming self-assembling peptide) hydrogels. Our results obtained confirm the presence of strong interactions between FEFK-FEFK and GO flakes with the peptide coating and forming short thin fibrils on the surface of the flakes. These strong interactions were found to affect the bulk properties of hybrid hydrogels. At the pH 4value electrostatic interactions between peptide fibres and the peptide-coated GO flakes are thought to govern the final bulk-properties of the hydro-gels while at pH 7, after conditioning with the cell culture -media, electrostatic-interactions are removed leaving the hydro-phobic interactions to govern hydrogel final properties. The GO-F820 hybrid hydrogel, with mechanical properties similar to the NP, was shown to promote a high cell-viability and retained cell metabolic activity in 3D over the 7 days of culture and shown to harbour significant potential as an injectable-hydrogel scaffold for the *in-vivo* delivery of NP-cells" (Figure 5).

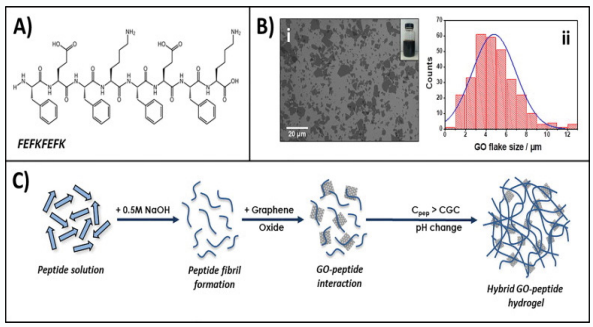

Figure 5: Chemical structure of FEFKFEFK peptide (A), SEM image of GO flakes (Bi) and flake size distribution (Bii). Bottom: Schematic representation of the formulation route used to prepare peptide/GO hybrid hydrogels (C).

Self-assembly can be classified as either static or dynamic. In static self-assembly, the ordered state forms as a system approaches equilibrium, reducing its free energy. In dynamic self-assembly, patterns of pre-existing components organized by specific local interactions are not commonly described as "self-assembled" by scientists in the associated disciplines. These structures are better described as "self-organized", although these terms are often used interchangeably.

"Due to its amphiphilic property, (GO) can achieve a variety of nanostructures with different morphologies (in ex. membranes, hydrogel, crumpled particles, hollow spheres, sack-cargo particles, Pickering emulsions) by self-assembly. The self-assembly is mostly derived from the self-concentration of GO sheets at various interfaces, including liquid-air, liquid-liquid, liquid-solid interfaces".

"In this research work, a 2-dimensional self-assembled Magnetic Nano Particle-Graphene Oxide (MNP-GO) nano composite is reported for the detection of DNA. Single-Stranded DNA (ssDNA) coils, generated through a Rolling-Circle Amplification (RCA) reaction triggered by the hybridization of target oligos and pad-lock probes, have a strong interaction with MNP-GO nano-tags through several mechanisms including π–π stacking, hydrogen bonding, van derWaals VDW, electrostatic, and hydrophobic interactions. This interaction leads to a hydrodynamic size increase (or aggregation) of MNP-GO nano tags, which can be detected by a simple optomagnetic setup. Due to the high shape anisotropy, MNP-GO nano-tags provide stronger opto-magnetic signal than individual MNPs. The avoidance of DNA probes (short ssDNA sequences as the bio-sensing receptor) provides easier material preparation and lower measurement cost.

From real-time measurements of the interactions between MNP-GO and RCA products amplified from a highly conserved E. coli 16S rDNA sequence, a limit of detection of 2 pm was achieved with a total assay time of 90 min. Even if the non-specific binding force between GO and ssDNA is much weaker than the specific base-pairing force in a DNA duplex, the proposed method provides a detection limit similar to DNA probe-based magnetic bio-sensors, which can be ascribed to the abundant binding sites between GO and ssDNA. For target concentrations higher than 100 pm, the MNP-GO nano tags can be applied for a qualitative naked eye detection strategy based on nano tag-ssDNA flocculation" (Figure 6).

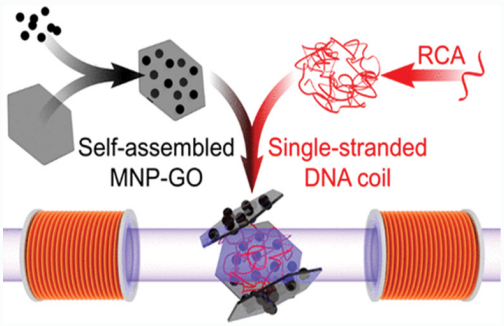

Figure 6: Abundant binding sites between GO and ssDNA.

Material and Methods

Whit an observational point of view various relevant literature and figure are reported and analysed. After this review part an experimental project hypotesys will be submitted to researcher in order to produce a global conclusion related the topics of this works. All literature comes from biomedical or other scientific or technological involved database.

Results

Carbon

"(GO) is a unique 2-dimensional (2D) material with interesting physical/chemical properties. GO can be considered as a 2D amphiphilic conjugated polymer, consisting of hydrophilic oxygenated groups and hydrophobic conjugated graphitic- domains. The diverse chemical groups endow graphene oxide with high chemical activity to react with other molecules and form new species with graphitic framework. The amphiphilic properties of GO sheets provide them the abilities to self-assemble into 3-dimensional (3D) structure or reduced GO (rGO) gels with porous micro-structures. The pre-condition of these promising properties of GO is its excellent solution-like dispersibility in aqueous or non-aqueous media. These liquid media facilitate the exfoliation of GO into single-layer sheets and provide the exfoliated GO sheets with specific chemical environment for functionalization/processing. It is essential to understand the solution-based chemical behaviour of graphene oxide, which is important for better application of the GO. In this review work, we outline the solution-based chemistry of GO mainly in terms of the molecular structure, dispersibility in solvents, solution properties and related processing of GO sheets. This review work aims to systematically present physical/chemical behaviours of GO in solvents including aqueous and non aqueous solvents, which is helpful for better understanding and application of GO graphene oxide materials" [1].

Co-assembly

"We used GO sheets of 2 different average lateral sizes, including

the larger GO (GO-L) measuring 10.5 μm ± 4.5 μm and smaller GO (GO-S) of 2.3 μm ± 0.9 μm, both exhibiting a typical hydrophobic-surface and negatively charged carboxylic- groups on their periphery (We chose ELRs as the protein component because of their modular and disordered nature and the possibility to exhibit different molecular conformations at the different temperatures. The ELK1 sequence is a 51.9 kDa molecule consisting of 24 repeats of single-block made of 4hydro-phobic pentapeptides (VPGIG) and a positively (+) charged (VPGKG) one. This relatively simple molecular design offers an accessible transition team (Tt) of 30°C (at 2% ELK1 in MilliQ water) with clearly different ELR conformations above or below it, as well as medium molecular-weight to enable both cooperative interactions between its charged and hydro-phobic segments as well as with an ionic edge and hydrophobic surface of the GO. ELRs with similar molecular weight but different levels of charge and hydrophobicity, as well as a single repeat of an individual block of each of these three ELRs, was used as a control.

Figure 1: Molecular building blocks (and rationale) for When an ELK1 solution at its Tt (30°C) is immersed in a larger volume of a GO Graphene Oxide solution, a multilayered membrane of up to 50 μm in thickness develops at the interface around the immersed drop maintaining both solutions separated. This kind of membrane consists of layers made from both Graphene Oxide (GO) sheets and ELK1, with GO sheets being present throughout the cross-section of the membrane and ELK1 gradually decreasing in concentration from the inside (ELK1- side) to the outside (GO side) of the membrane. Multi-layered structures are known to emerge from diffusion-reaction mechanisms. We have previously demonstrated that with co-assembling PAs with ELRs, it is possible to trigger a diffusion-reaction mechanism, which generates multi-layered membranes capable of exhibiting dynamic-properties. The same In Similar way, by touching any surface within the first few seconds of formation, the ELK1-GO membrane adheres, spontaneously and reproducibly opens, and can be manipulated to grow into a tubular structure with spatiotemporal control. In this case, the underlying ELR-GO mechanism of interaction and supramolecular assembly lead to the growth of a material with remarkably enhanced properties" (Figure 7) [2].

Toxicity studies of six types of carbon nano particles in a chicken-embryo model

"In the present research study, the toxicity of 6 different types of Carbon Nano Particles (CNPs) was investigated using a chicken-embryo model. Fertilized chicken eggs were divided into this following treatment groups: placebo, diamond-NPs, graphite NPs, pristine graphene, small graphene oxide, large Graphene Oxide GO, and reduced graphene oxide. Self-assembly of CNPs with albumin amino-acids AA by non-covalent bonds is very efficient, implying that CNPs can be effectively transported into embryos. According to Szmidt et al, lower concentrations (50 and 500 μg/mL) of graphene penetrate the embryo more efficiently than the higher concentrations, due to different NP-dispersion levels. These results were explained by the natural tendency of CNPs to agglomerate when they are coated by albumin-proteins that surround the embryo. In the present research study work, we also administrated CNPs to egg-albumin, which gets progressively consumed by the embryo during the development process and is ultimately fully absorbed, ensuring that the whole dose was delivered during embryo-genesis" (Figure 8) [3].

"In blood, non-covalent adsorption occurs through weak van der Waals VDW forces, hydrophobic, electrostatic, and π–π stacking

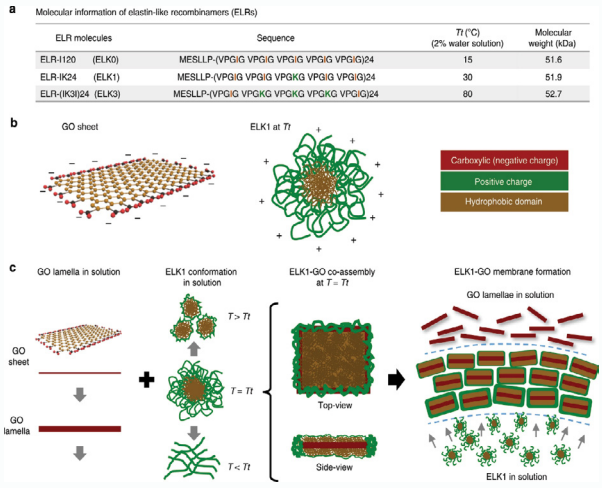

Figure 7: a) Table summarizes the key information of the three Elastin-Like Recombinamers (ELRs) used in the study comprising similar molecular-weight but different levels of hydrophobicity (VPGIG) and positive charge (VPGKG). b) Illustrations of the molecular-structure of a GO sheet and the supra-molecular organization of ELK1 at its transition temperature (T) (30°C) indicating both the charged (red and green) and hydrophobic (brown) segments. c) Schematic of the proposed mechanism of formation illustrating the molecular-supra-molecular conformation of the GO and ELK1 before and after the co-assembly at the ELK1's Tt as well as their interaction for membrane formation.

interactions. The sp2 hybridized honey-comb carbon- lattice of rGO and GO is hydrophobic and, interacts with the hydrophobic -regions of proteins, according to the protein geometry. The basal plane of the GO is also enriched with π electrons, making π–π stacking interactions possible. At the same time the oxygen groups of GO, whose composition is strictly dependent on preparation and storing conditions, allow further hydrogen bonds and electrostatic bonds. These electrostatic bonds are strongly influenced by the charge on proteins and by pH ionic strength of the buffer. Bonding on GO. Can also be mediated by van der Waals VDW interactions. While the electrostatic interactions are more pronounced on GO both van der Waals WdW and electrostatic interactions play a major role in the adsorption of proteins on RGO due to the increase in the non-functionalized area on the surface. In the following other sections, we will show how functionalization of the GO surface alters protein adsorption and consequently BC-properties".

Effects of bio-coronated GO materials on the blood components

BC composition directly influences interactions with the other blood components. The presence of antibodies, complement and clotting factors in the nano-particle BC may activate clotting and coagulation cascades. The BC coating can promote phagocytosis and elimination from the circulation.

We will first consider data on the GO interaction with the Red Blood Cells (RBC), in Table reported. An intravenously IV injected nano material is likely to interact first with RBCs rather than other cells, due to their abundance in blood. Hemolysis represents the damage to RBCs that leads to the leakage of hemoglobin into the blood-stream. After hemolysis, the nano-material may adsorb released hemoglobin HB and/or adhere to cell debris, which can increase its likelihood of elimination by macro-phages. Although the literature is contradictory regarding the GO effects on RBC, when BC is introduced into the

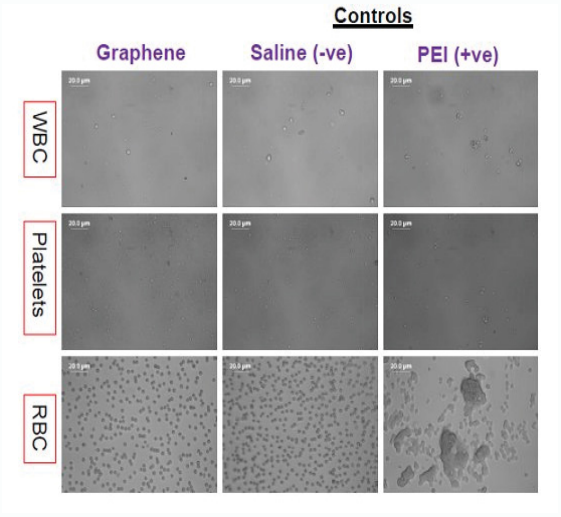

Figure 8: Blood cell aggregation on incubation of graphene with blood from Blood Compatibility and Bio medical Applications of Graphene.

framework the results become clearer. Due to the sharp edges of GO and RgO, hemolytic- effects might be expected in vivo, possibly caused by nano-material blades disrupting cell- membranes, as reported for the GO interactions with the bacteria" (Figure 9) [4].

"Graphene and derivatives are emerging as attractive and interesting materials for the biomedical applications: like anti-bacterial, the gene delivery, contrast imaging, and anticancer therapy applications. It is of fundamental importance to study the cyto-toxicity and the biocompatibility of these materials as well as how they interact with immune system [5]. The present research study was conducted to assess the immuno-toxicity of Graphene Oxide

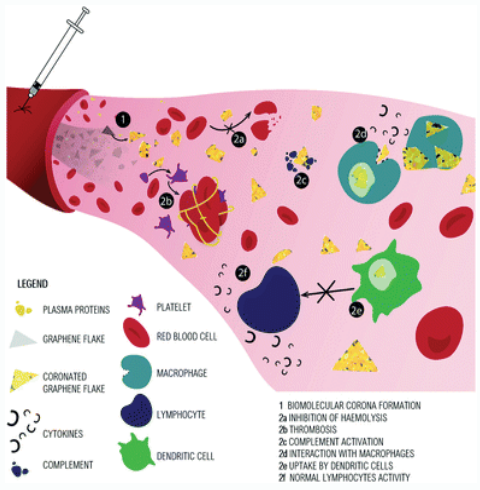

Figure 9: Main results of G.O interaction with the blood components are summarized in this illustration of the injection of GO Graphene Oxide flakes in the blood-stream. The formation of the BC (1) prevents the hemolysis of red blood cells RBC (2a). Thrombosis (2b) and interaction with the comple-ment-proteins (2c) are ascribed to GO. In (2d) some of the possible fates after macro-phage encounters are shown: extra-cellular blocking or intra-cellular uptake. The release of cytokines occurs when macro-phages uptake GO. Aggregates of GO in macro-phage cytoplasm induce the production of the pro-inflammatory cytokines. Dendritic-cells fail to present antigens to lym-phocytes when they uptake GO (2e). Lymphocyte activity is not inhibited, and BC protects lymphocytes from apoptosis (2f).

(GO) and vanillin-functionalized GO (V-rGO) on THP-1 cells, a human acute monocytic leukemia cell line. The synthesized GO and V-rGO was characterized by using various analytical techniques. Various concentrations of GO and V-rGO showed toxic effects on THP-1 cells such as the loss of cell viability and proliferation in a dose-dependent manner. Cyto-toxicity was further demonstrated as an increased level of lactate dehydrogenase, loss of Mitochondrial Membrane -Potential (MMP), decreased level of ATP content, and the cell death. Increased levels of Reactive-Oxygen Species ROS and lipid-peroxidation caused redox imbalance in THP-1 cells, leading to increased levels of malondialdehyde and decreased levels of anti-oxidants like glutathione, glutathione-peroxidase, super oxide dismutase, and catalase. Increased generation of ROS and reduced MMP with simultaneous increases in the expression of pro-apoptotic genes and down regulation of anti-apoptotic genes suggest that the mitochondria-mediated pathway is involved in GO Graphene Oxide and V-rGO-induced apoptosis. Apoptosis was induced consistently with the significant DNA damage caused by increased levels of 8-oxo-dG and up regulation of various key DNA-regulating genes in THP-1 cells, indicating that GO and V-rGO induce cell death through oxidative stress. As a result of these events, G.O and V-rGO stimulated the secretion of various cytokines and chemokines, indicating that the graphene materials induced potent inflammatory responses to THP-1 cells. The harshness of V-rGO in all assays tested occurred because of better charge transfer, various carbons to oxygen ratios, and chemical compositions in the rGO. These research findings suggest that it is essential to better understand the parameters governing GO and functionalized GO in immuno-toxicity and the inflammation. Rational design of safe GO-based formulations for various applications, including nanomedicine, may result in the development of risk -management methods for people exposed to graphene and graphene family materials, as these nano-particles can be used like delivery agents in various biomedical applications" [6].

"Nanomedicines are being developed to treat various diverse diseases; inadvertent or un-intended health effects have to be considered, especially for those targeting cancers. For the cancers, occurrence of metastasis hints an advanced phase of cancer progression, and nanomedicines per se should be evaluated for their effects on existing metastatic tumors and triggering the metastases. Graphene-based 2D nano-materials, such as (GO), due to its unique characteristics, have been extensively studied for biomedical applications including the cancer therapy. The potential effect of GO on metastasis has not been determined yet. We found that low-dose GO could induce significant morphological and structural changes of the cellular membrane within the cancer cells, suggesting an epithelial-mesenchymal transition, with enhanced invasion/migration and the alterations of representative EMT indicators in GO-treated cells. These changes resulted in enhanced lung-metastasis of cancer cells in various kinds of metastasis models. The mechanistic investigations unveiled that GO Graphene Oxide increased the protein levels of the TGF-β receptor, leading to a constitutively activated TGF-β-Smad 2/3 signaling path-way that drives the EMT. Our findings enhance the understanding of the un-intended side and detrimental effects of GO nano-sheets in increasing the progression of metastatic-tumors. So, the likelihood of pro-EMT effects upon low dose GO exposure should be considered when developing GO nanomedicines [7].

"A high dose of GO that forms aggregations can block the pulmonary blood-vessels and result in dyspnea and platelet PTL thrombi were observed at high concentrations of 1 and 2 mg/kg body weight *via* intravenous IV injection" [8].

"GO has abundant surfaces oxygen-containing groups like epoxide, hydroxyl, and carboxylic- groups; it can be prepared through the oxidative intercalation and exfoliation of graphite on a mass scale. Owing to the enriched surface functionalities, the GO is water-soluble and chemically versatile. The surface functional-groups can also provide plenty of reaction sites for linking the nano particles, proteins, enzymes, peptides, bacteria, cells, nucleic acids through the covalent and non-covalent binding. GO Graphene Oxide has been used as a matrix for protein immobilization in different biotechnological applications such as fluorescence or electrochemical based sensors, labeling and imaging, therapy, and targeted delivery. Non-Covalent interaction (Physical adsorption). Non-covalent protein adsorption into solid supports represents the most simple and desirable strategy of physical immobilization. The mechanisms of proteins adsorption on GO Graphene Oxide are a kind of non-covalent self-assembly including weak Van der Waals VDW forces, hydrophobic, electrostatic, and π-π stacking interaction. These types of attractions between the proteins and Graphene Oxide GO involve solution phase incubation, or direct sonication, followed by a washing step to remove the un-bound proteins. The non-covalent bonds responsible for the interaction between GO Graphene Oxide and proteins vary depending on the surface properties of Graphene Oxide, such as morphology and hydrophobicity" [9].

"Although information on the *in vitro* and *in vivo* nano toxicity of graphene nano materials has been increasingly published in the last several years, a complete picture on the bio-compatibility of graphene nano-materials has not been established. The successful applications of graphene nano-materials in nano bio-technology and medicine as well as their effective translation into real clinical utility hinge significantly on a thorough understanding of their nano toxicological profile [10]. Of all aspects of biocompatibility, the hemocompatibility of graphene nano materials with the different blood constituents in circulatory system is one of the most important elements that need to be well elucidated. Once administered into the biological systems, graphene nano materials may inevitably come into contact with the surrounding plasma proteins PP and blood-cells. Crucially, the interactions between these kinds of hematological entities and graphene nano-materials will influence the overall efficacy of their biomedical applications. As such, a comprehensive understanding of hemotoxicity of the graphene nanomaterials is critically important. he *in vitro* evaluations of the potential cytotoxic effects of graphene nanomaterials have been actively conducted on different human cell-lines, such as human fibroblasts, human umbilical vein endothelial cells, normal human lung-cells (BEAS-2B), human lung cancer cells (A549), human hepato carcinoma cells, He La cells, and the human breast cancer cells MCF-7 [11,12]. A majority of these investigations have demonstrated the time and dose dependent cytotoxicity of graphene nanomaterials. Various *in vitro* experimental and theoric investigations have attributed the cytotoxicity of both the graphene and its oxygenated derivative GO on the mammalian cells and bacteria to cellular membrane penetration, followed by phospholipid molecule extraction from the lipid bilayer [13-16].

GO has been demonstrated to possess a high loading capacity for albumin ALB and fibrinogen FIBR in a recent work. Reference Kenry, Loh and Lim. While numerous studies have reported observations on graphene nanomaterial-induced a protein conformational change, the under-lying mechanisms are still poorly understood GO have a surface area of 25 nm^2 and randomly decorated hydroxyl and epoxy groups on its surface. A carboxyl group was attached to the GO edges. While having the same surface area, in comparison to GO Graphene Oxide, the rGO model possesses fewer oxygenated functional groups [17-19]. G.O nano-sheets have been reported to possess a strong thrombus-inducing potential and considerable thrombogenecity. They could trigger the activation of platelets PTL and their strong aggregatory response similar to that evoked by thrombin, an active physiological platelet agonist. The platelet activation by GO was suggested to be extensively dependent on the surface charge distribution of GO graphene oxide as it was revealed that, in contrast to G.O, rGO with reduced surface charge density was less capable in activating and aggregating platelets PTL [20]. The prothrombotic characteristic of GO nano sheets was further verified through the occurrence of significant pulmonary thromboembolism after their intravenous IV administration in mice" (Figure 10) [21].

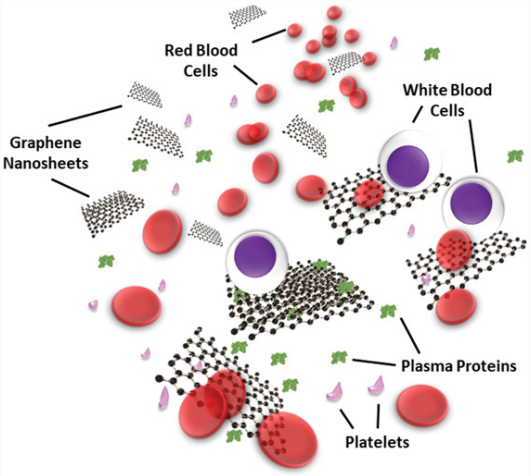

Figure 10: Nano-bio interactions of graphene nano materials with various blood plasma proteins and cells.

"This critical review work aims at giving insights in the spontaneous tendency of the proteins and their constitutive parts to adsorb on Graphitic Nano-Materials (GNMs) through non-covalent interactions occurring in their interfaces" [22].

"This review work collects studies on the toxic effects of GFNs in various organs and cell models. We also point out that various factors determine the toxicity of GFNs including lateral size, surface structure, functionalization, charge, the impurities, aggregations, corona effect. Various typical mechanisms underlying GFN toxicity have been revealed, for instance, physical destruction, oxidative stress, DNA damage, inflammatory response, apoptosis, autophagy, and the necrosis. In these kinds of mechanisms, (toll-like receptors-) TLR-, TGF-β and TNF-α dependent-pathways are involved in the signalling pathway network, and oxidative stress plays a crucial role in these kind of pathways" [22].

"Toxicity of graphene family nano particles, the dose, shape, surface-chemistry, exposure route, and purity play important roles in differential toxicity of GFNs. Different various authors have used various toxicity tests to evaluate the toxicity of GFNs. Studies have been conducted to find out the toxicity of GFNs on different cellular/animal models, including stem cells, He La cells, HepG2 cells, bacteria, Drosophila melanogaster, Zebra-fish, marine organisms, rats, mice, and mammalian cells. Cytotoxicity tests indicated that the RgO can damage cells with direct contact. In this part of the paper, an attempt

has been made to compile the recent and up-to-date research studies related to toxicological aspects of GFNs to different models" [23].

"The peculiar features of these cases were the availability of macroscopic and microscopic autopsy findings. The main macroscopic finding was that venous-thrombosis was much more widespread and catastrophic than diagnosed by imaging during the life. Microscopic findings showed vascular thrombotic occlusions occurring in the microcirculation of multiple organs and increased inflammatory infiltrates [24]".

"Postmortem investigations of fatalities after COVID-19 vaccination are particularly relevant with regard to the detection of anaphylaxis, VITT, and myocarditis.

Vaccine-induced Immune Thrombotic thrombo-cytopenia (VITT)

VITT is characterized by thrombo-cytopenia, combined with thrombosis in most cases. Thrombosis can occur in the both the arterial and, more common, venous system. A distinctive feature of VITT is thrombosis in un-usual locations. These include CVT, as well as splanchnic-venous thrombosis [25].

"In this research work, for the first time, we studied the in vitro and in vivo interactions of a relatively new derivative of graphene, Graphene-Nanopores (GNPs) in the mammalian systems, to systematically elucidate the possible mechanism of their toxicity over time. Heart tissue showed chemodectoma, toxic myocarditis, reddish brown atrophy; yellowish- brown pigments suggesting lipofuscin granules as remnants of the cell organelles and cytoplasmic-material" (Figures 11-14).

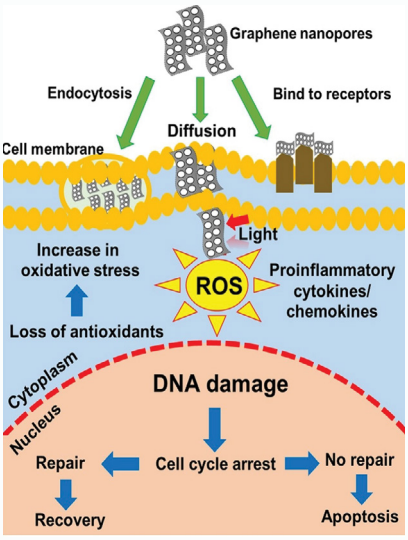

Figure 11: The *in vitro* and *in vivo* interactions of a relatively new derivative of graphene, Graphene-Nanopores (GNPs) in the mammalian systems, to systematically elucidate the possible mechanism of their toxicity over time.

"RBCs were exposed to 3 different forms of GQDs (non-functionalized, hydroxylated, and carboxylated GQDs) at various conc. (0 μg/mL, 500 μg/mL, 750 μg/mL, and 1000 μg/mL) and incubation times (0 h, 1 h, 2 h, 3 h, or 4 h). The rheological characteristics of the RBCs were measured using microfluidic laser diffractometry and aggregometry. The hemolysis rate and rheological-alterations of the RBCs were insignificant at a concentration less than 500 μg/mL. Carboxylated-GQDs were observed to have more substantial hemolytic activity and caused abrupt changes in deformability and aggregation of the RBCs than the non-functionalized or hydroxylated-GQDs at concentrations >750 μg/mL. Our findings indicate that hemorheological assessments could be utilized to estimate the degree of toxicity to the cells and to obtain useful information on safety sheets for the nano-materials".

Experimental project hypotesys

In order to verify *in vitro* the self- assembling property of graphene GO it is necessary to test. Hundered human blood speciman with added graphene GO a concentration similar to as reported in literature. Hundered human blood specimen with no added (control group). This entire sample must to be sended to various certified and indepened analytical laboratory and tested using blind.

If possible, send some sample also at various university centres. With various methods (microscopic cytology, dark field microscope analysis, RAMAN destructive methods, microscope RAMAN et other useful).

Results: The result must be reported in a table in two columns:

a) Sample + graphene

b) Control

Object of the search: self aggregates of graphene

Time of observation: T 1H after collecting sample and added graphene, at 4 H, after 24 H, the after 1 week (needed to use anticoagulant that non produce interference with graphene GO).

At time after 1h after the graphene addition coagulative test must to be performed (DD, fibrinogen and other as well as emocromocitometric assay (platelets, RBC, microscope essay).

To verify if there is difference between the group a and b in significative way $p<0.005$.

Discussion

In the literature reported it is clear the self-assembling properties of graphene derivates as well as clear is the effect that this products and aggregate produce on blood.

The same it is clear by scientific literature the pro-coagulant effect of spike protein during the pathological process in covid-19 disease. Because there a now adays debate about the presence or absence of graphene derivates in some vials of COVID-19 new vaccine it is crucial to think at what can happen when these 2 toxic molecules act in the same moment (spike protein and graphene aggregates) in a human body.

Because many biotechnological processes in last decades see the introduction of innovative material like graphene derivates this molecule must to be analytically secluded for release of biopharmaceuticals and so for the COVID-19 vaccine.

Thrombosis effects can be increasing when 2 different stimuclyactinsimultaney ways like a Synergic effect. And what can be the global effect in an unbalanced blood system like VITT? The clinical effect of this poisonus association must to be deeply more investigated for public safety need.

Conclusion

Because in toxicology are well known various situation of combined toxic effect by multiple chemical dangerous exposure It is

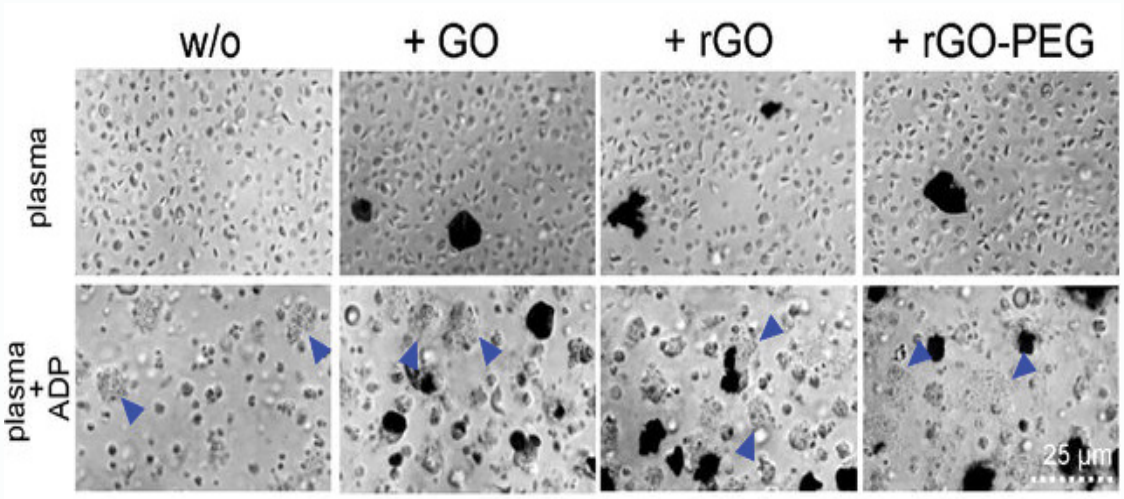

Figure 12: The effect of G.O, rGO, and rGO-PEG on the platelet activation: Graphene nano derivatives were co-incubated with platelet PTL rich plasma at 50 µg/mL. Platelet activation was induced by the addition of 2 µmol/mL of Adenosine Diphosphate (ADP). The Platelet PTL aggregates are pointed by the blue arrows. One representative picture reflects the results of 3independentexperiments.
G.O: rGO Reduced Graphene Oxide; rGO-PEG: Pegylated Reduced Graphene Oxide; ADP: Adenosine Diphosphate

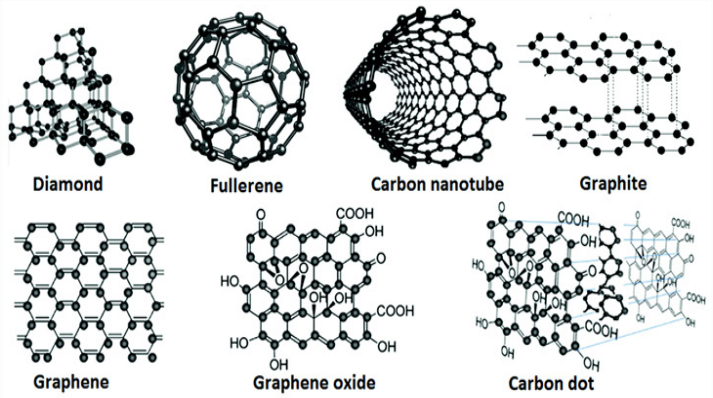

Figure 13: Structures.

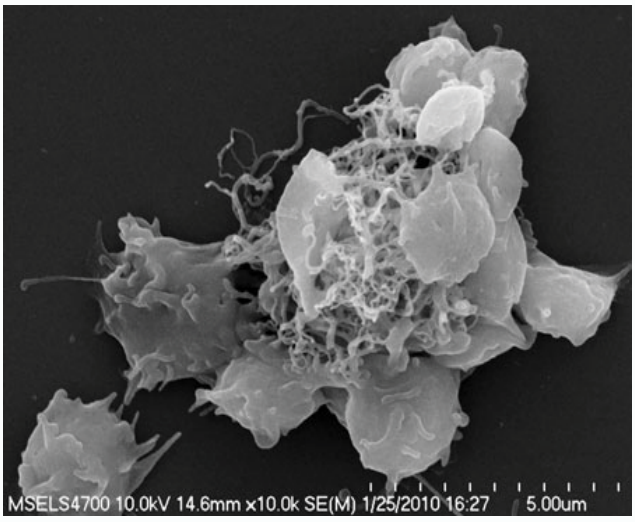

Figure 14: Scanning electron microscopy image shows platelet PTL activation by multi-walled CNTs (M60).

needed to verify the clinical effect of the self-assembling graphene GO effect added to spike protein using *in vitro* sample (Animal model and sample from humans' specimens: subjects volunteers).

The experimental project submitted can help for this scope. It is also of interest to verify if the cumulative effect of this two substantia Graphene GO and SPIKE protein. Show and added toxic effect (sinergic) or this is greater than the single molecule acting alone and the kinetic related.

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