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Soluble and immobilized graphene oxide activates complement system differently dependent on surface oxidation state

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ABSTRACT

Graphene oxide (GO) is believed to become applicable in biomedical products and medicine, thereby necessitating appropriate safety evaluation dependent on their applications and the route of administration. We have examined the effect of GO form (in solution versus immobilized) and oxidation state on two related elements of innate immunity: the complement system and interleukin-6 (IL-6) release in human blood. In solution, there was a decrease in GO-mediated complement activation with decreasing surface oxygen content (and altered oxygen functionality), whereas with immobilized GO complement response were reversed and increased with decreasing oxygen content. GO solutions, at concentrations below complement activating threshold, did not induce IL-6 release from human blood leukocytes, and further dampened lipopolysaccharide-induced IL-6 release in the whole blood. The latter effect became more profound with GO's having higher oxygen content. This protective role of GO solutions, however, disappeared at higher concentrations above complement-activating threshold. We discuss these results in relation to GO surface structure and properties, and implications for local administration and development of GO-based implantable devices.

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1. Introduction

Carbon-based nanomaterials like carbon nanotubes, graphene and its derivative graphene oxide (GO) are receiving considerable attention in nanoengineering due to their high specific surface area, remarkable thermal conductivity and mechanical strength, and exceptional optoelectronic properties [1–5]. An increasing focus is also seen on biomedical applications of graphene-based nanomaterials including engineering of bioelectronics and biosensing devices, local and systemic drug delivery, multimodal imaging and artificial implants [6–10]. One of the most common ways of producing graphene is by chemical conversion of graphite into dispersible, but insulating, graphite oxide through harsh oxidation. Upon purification, the graphite oxide can then be exfoliated into GO [11–13], which can then be partly converted back into graphene in solution under reducing conditions [14,15]. The level of oxidation may differ between different GO preparations, which will yield differences in surface characteristics and solubility, where it is generally perceived that oxidation improves compatibility with the immune system [10,16].

On introduction into the body, GO may be sensed by elements of the immune system, and particularly by components of the complement system. This consists of a network of over thirty plasma and membrane-bound proteins, and is the first line of the body's defence that orchestrates immune responses against foreign intruders, including polymers and engineered nanoparticles [17–21]. Such responses lead to opsonization processes that drive phagocytic clearance, and anaphylatoxin release, where its uncontrolled production may initiate adverse infusion-related reactions [22,23]. Briefly, a complement response is initiated by one of three different pathways (classical, lectin and alternative) on binding of complement pattern recognition molecules (e.g., C1q, mannose binding lectins, ficolins, and properdin) to the intruders' surface either directly or indirectly through protein (e.g., antibody) priming [22,23]. Many surfaces, especially those with heavily hydroxylated domains, may further increase the turnover of the alternative

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pathway on binding of the third complement protein (C3), (e.g., in the form of $C3{\cdot}H_2O$ and nascent C3b) [24].

To our knowledge, only one study has assessed GO-induced complement activation, based on C3 cleavage and elevation in levels of C3a; a 9 kDa fluid-phase anaphylatoxin, which is released from the N-terminus of C3 by C3 convertases [25,26]. Although GO, and to some extent PEGylated GO, generated C3a in human serum, liberated C3a was suggested to adsorb to the surface of GO [25]. This led the authors to suggest that PEGylated GO may prevent pro-inflammatory responses through C3a binding, and even attenuate C3a-related consequences from other nanomaterials. C3 cleavage is a central step in complement cascade. This results in generation of the C3b fragment, which is not only an opsonic molecule, but it is further required to activate the fifth complement protein (C5) through the assembly of C5 convertases, thus triggering the terminal phase of complement cascade and formation of the lytic C5b-9 complexes [23]. C5 activation generates C5a, which is a far more potent anaphylatoxin than C3a, capable of modulating the function of a variety of immune and vascular endothelial cells either directly or in cross-talk with Toll-like receptor (TLR)-2, -4 and -9, thereby stimulating the production of various cytokines, and notably interleukin-6 (IL-6) [27,28].

Accordingly, we now assess the effect of GO on C5 cleavage and activation of the terminal phase of the human complement system. Our approach examines the effect of GO oxidation state and form (in solution versus an immobilized form) on complement responses. Furthermore, a number of studies have shown that GO may modulate immune responses in macrophages and related cells directly, and with varying conclusions on cytokine release and cell viability [29–36]. These variations and inconsistencies may have been due to differences in species used and/or GO characteristics including the oxidation state. As such, we further study the effect of GO oxidation state on pleiotropic IL-6 cytokine responses in the human whole blood with fully functional complement system.

2. Materials and methods

2.1. Reagents

Sterile, endotoxin free Dulbecco's phosphate buffered saline (PBS) and zymosan A from *Saccharomyces cerevisiae* were from Sigma (Poole, Dorset, UK). Lipopolysaccharide (LPS) with known endotoxin units was obtained from Genscript (Piscataway, NJ, USA), and used within one week after hydration. MicroVue C5a EIA and SC5b-9 Plus EIA kits were from Quidel (San Diego, CA, USA). The IL-6 ELISA kit was from eBioscience (San Diego, CA, USA). For collection of human whole blood, collection tubes containing lepirudin (a recombinant hirudin) was used (Roche, Basel, Switzerland). All other chemicals were of Analytical Grade and obtained from Sigma, and used as received.

2.2. GO synthesis and characterization

GO synthesis followed the modified Hummers method. Briefly, 0.50 g graphite (Alfa Aesar, natural, 98% metal basis, 325 mesh, 41.6 mmol) and 0.50 g sodium nitrate (5.88 mmol) was mixed with 23.0 mL concentrated sulfuric acid (0.43 mol) in an ice bath, and stirred until a homogeneous mixture was obtained. This was followed by slow addition of 3.0 g potassium permanganate (19.0 mmol). The reaction mixture was then transferred to a 35 °C water bath and left stirring for 1 h after which 40 mL water was added slowly. The temperature was then raised to 90 °C, and the reaction was quenched by addition of 3.0 mL hydrogen peroxide (97.9 mmol). The graphite oxide solution was then filtered, and washed with 100 mL warm water. To fully exfoliate into GO the sample was subjected to several centrifugation steps and ultrasound. Accordingly, the product was first washed twice with Milli-Q water by high-speed centrifugation at 6797 *g* for 15 min, after which all non-oxidized graphitic material was removed by repeated low speed centrifugation at 106 *g* for 2 min until all visible particles were cleared. Then the solution was treated with 30 min ultrasound to fully exfoliate the graphite oxide into GO. To ensure a more homogeneous size distribution, the solution was treated with one 6797 *g* centrifugation step for 15 min to remove all small debris from the sonication step, followed by a 664 *g* centrifugation step for 10 min. The mass concentration of the GO solution was found to be 0.156 mg/mL by UV-VIS spectroscopy using an extinction coefficient of 53 mL/(mg·cm) for the peak at λ~230 nm, as previously determined [37].

Reduced GO was prepared by heating 18 mL GO solution to 60 °C, where pH was stabilized to 10 with sodium hydroxide to ensure a stable solution of reduced GO. 20 μL of a 1/10 diluted hydrazine monohydrate solution was then added to initiate a mild reduction process. From these reduced GO solutions, immobilized GO was obtained by filling wells on a Nunc PolySorb 96-well plate with 350 μL solution, allowing the solvent to evaporate over-night under nitrogen flow.

AFM was performed on a Veeco Nanoscope® V, PicoForce™ Force spectroscopy control module in tapping mode. X-ray photoelectron spectroscopy analysis was carried out in a Kratos Axis $Ultra^{DLD}$, fitted with a monochromatic $Al_{K\alpha}$ X-ray source ($h\nu = 1486.6$ eV, power = 150 W). A pass energy of 20 eV was used for high resolution scan, and of 160 eV for wide spectrum. Binding energy calibration was done using the Si 2p peak from the SiO_2 substrate fixed at 103 eV. Data analysis was performed using the CasaXPS software with its associated relative sensitivity factor library.

2.3. Complement activation

Human serum was acquired from healthy donors by venipuncture according to local approved protocols. Blood tubes without additives were used, and serum was prepared as previously described and stored in aliquots at −80 °C [17]. Clotting was induced gently and below room temperature to avoid spontaneous complement activation or destabilization of complement proteins. To activate complement, 40 μL undiluted serum was mixed with 10 μL of the sample reagent in Eppendorf tubes to reach a final GO concentration of 31 μg/mL, unless stated otherwise, followed by incubation for 30 min at 37 °C. No visual aggregation was observed after the incubation. To assess complement activation by immobilized GO, 90 μL of neat serum was added to either uncoated microtiter wells or wells coated with GO. Immediately after incubation, samples were transferred to ice and diluted appropriately with a protein-stabilizing solution provided with the ELISA kit, and supplemented with 25 mM EDTA to stop further complement development. After centrifugation, the complement markers SC5b-9 and C5a (and C4d in selected cases) were quantified from the supernatant using ELISA according to earlier protocols [17,21]. To assess the complement-activating potential of the serum, zymosan was used as a positive control at an incubation concentration of 0.1 mg/mL [17,21]. For assessing the role of alternative pathway in GO-mediated complement activation, serum was supplemented with a final concentration of 10 mM EGTA and 2.5 mM Mg^{2+}. For simultaneous measurement of C5a and IL-6, C5a was measured from the supernatant on samples prepared for IL-6 after finished incubation (see below). To investigate the binding of complement activation products to GO, serum was first activated

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