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Fractionation of graphene oxide single nano-sheets in water-glycerol solutions using gradient centrifugation

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ABSTRACT

A centrifugation method for the separation and fractionation of graphene oxide (GO) single nano-sheets in the size range of 150–850 nm is reported. The measured electrophoretic mobility of the fractionated single sheets ranges from −0.2 to −1.4 μm cm/V·s where the interpreted zeta potentials vary from −3 mV to −17 mV with increasing sheet size. The single GO sheets show auto-fluorescence in the visible range of 350–650 nm using an excitation wavelength of 200 nm. Furthermore, the GO nano-sheets functionalized using PEG are found to be non-cytotoxic in in-vitro at concentrations up to 90 μg/ml, with a small reduction in cell viability −10%- at 260 μg/ml. The observed concentration-dependence of the cytotoxicity potentially explains the differing conclusions on cytotoxic potential reported in the literature. The GO nano-sheets therefore have the potential to be used as fluorescent drug delivery carriers of specific size.

1. Introduction

Graphene Oxide (GO) is a two dimensional hydrophilic network made of oxygenated graphene [1]. GO nano-sheets accept a wide range of functional groups that allow the surface chemistry to be modified for a number of potential applications in drug delivery and cellular/organ targeting [2–4]. The combination of these properties with the observed auto-fluorescence makes GO sheets attractive for theranostic delivery systems and signalling materials [3,5,6]. A major body of related literature has been directed towards exploring the chemical and physical properties of this unique material for optoelectronic and biomedical applications [7–10].

A mixture of sheets with different morphologies, sizes and thicknesses are produced when GO is fabricated by a defined chemical oxidation procedure [11,12]. The chemical modification of pure graphene (source material of GO) introduces a number of oxygenated functional groups to render the graphene surface hydrophilic [13] and also makes the GO more chemically reactive [14–16]. The GO is then a two-dimensional (2D) structure with hydrophilic edges and both hydrophobic and hydrophilic regions on the faces due to the presence of carboxylic acid groups on the edges and hydroxyl and graphene groups on the surface [17].

For a given chemical modification, the proportion of hydrophilic functional groups to hydrophobic regions of the graphene oxide structure varies with the sheet size [17,18]. The effective “amphiphilicity” of the GO sheets and interfacial activity is found to be a function of the sheet size. Smaller GO sheets possess higher so called “edge-to-centre” ratios which result in more stable colloidal dispersions due to higher hydrophilicity [19]. However, the interfacial functional groups in the interstitial spaces between GO layers are less accessible for chemical interactions due to steric inhibition [20]. As a result, graphene oxide single sheets with thicknesses of ~1 nm are more reactive to compounds such as polyethylene glycol (PEG), a FDA-approved vehicle, which functionalises GO into a material that is potentially suitable candidate for clinical applications [21–23].

Different methods have been introduced to fractionate GO single sheets such as sonication for mechanical exfoliation [24], dispersion in organic solvents as a liquid exfoliation, organic solvent or surfactant assisted sonication [25], thermal exfoliation [26], and exfoliation by polymer/dye functionalization [27]. These methods are based on mechanically separating the sheets by overcoming the van der Waals interactions and inter-sheet π-π

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stacking to enable solubilisation [28]. Mechanical methods have been shown to have detrimental and varied effects on the sizes of the GO sheets and furthermore on the physical and chemical characteristics of the sheets produced [29,30]. These effects change the interaction of GO and its functional derivatives in important size-dependent applications, such as the interactions with cells in clinical studies [31,32].

The strong inter-sheet π-π stacking of GO sheets also decreases the solubility leading to aggregation in solution. Therefore, the proper selection of solvent with suitable repulsive solvation force is required, as many solvents are difficult to remove in the post-processing stage due to their high boiling points (>150 °C) [33]. Association of GO sheets with surfactants, polymers or organic molecules has been used as strategies for the dispersion of GO sheets. These methods rely on induced repulsive electric double layers and/or repulsive steric forces between exfoliated GO sheets to render the sheets soluble [25]. However, removing the associated molecules of surfactants/solvent from GO sheets for biological applications remains problematic.

The first density gradient separation of functionalized GO nano sheets using sucrose density gradient and an centrifugal (50 k rpm) was reported in 2008 [34]. This method was later applied to the separation of pure GO [35]. The gradient density separation method uses a solution containing solvents of different density over a range of compositions to generate the density gradient [36]. The sample solution is placed on the top of the tube and then centrifuged in order to separate the nano-particles by density once the height is equilibrated. The gradient media in this method must be a solvent for the particles to be separated [37]. As a result, the fractionated particles are immersed in a solution in which the gradient molecules are adsorbed to the GO sheets and must be removed prior to further modification using another solvent. This clean-up stage is crucial for GO preparation, as the presence of adsorbed molecules on the separated sheets can render a number of sites inactive for further chemical modification. In the previously reported separation method however, there has not been a clear strategy of purification to remove the adsorbed solvent from the fractionated GO sheets [38].

Another size separation method was introduced in 2011 by Xiluan Wang et al. based on the pH-dependent amphiphilicity of GO molecule [39]. GO has the capacity to buffer the solution due to the structural acid and base groups rendering the pH adjustment difficult [40]. Recently, a polar solvent-selective sedimentation method has been reported [41]. This technique is challenging due to the narrow size distribution, the use of large volumes of organic solvent, and a long standing fractionation time [40]. More recently, Wang et al. presented an alternative size-fractionation method based on filtration [42]. This technique avoids some of the complexities of the previous methods. However, it is both time consuming and not readily applicable in the nanometre size range.

In the current work, a simple and robust gradient centrifugation method is proposed for the separation and fractionization of GO single nano-sheets using water-glycerol media. This procedure eliminates the use of sonication and ultracentrifugation making the separation more controlled and simple. Furthermore, the adsorbed gradient molecules are more readily removed from the separated fractions using dimethylformamide (DMF). Here we report the chemical, optical, auto fluorescence and cytotoxic properties of the fractionized GO nano-sheets using the water-glycerol gradient method.

2. Results and discussion

2.1. Fractionization and separation of GO nano-sheets

In aqueous solution the state of aggregation of GO is determined by the concentration [43]. Hydrogenated functional groups present on the GO sheet surface give the GO charge in solution rendering it soluble in aqueous media. In the presence of charge, the water molecules can cover both sides of the GO sheets. The reason for the concentration-dependent aggregation of the nano-sheets is not fully understood [44]. The measured sedimentation rates of graphene and GO flakes depend on their thicknesses and 2D sizes [45]. Thin flakes are found to have lower densities than the aggregated stacks of sheets [46]. We found that the GO sheets were entirely exfoliated in water at concentrations below ~10^{-2} mg/ml (Supplementary Information, Fig. S1). For low concentrations single sheets are the dominant form and the gradient separation is governed by the 2 dimensional sizes of the dispersed nano-sheets [47]. In the present study, water-glycerol mixtures (5–50%) were used to fractionate the GO sheets in a centrifugation process using a fixed-angle rotor.

Sucrose is a common medium for fractionation with ultracentrifugation, and is generally used for isopycnic (uniform sample and density gradient) mixtures [48]. Glycerol however, is widely used for the separation of components by rate-zonal sedimentation which is the main focus in this research [49,50].

Fig. 1A shows the GO sheets mixed in a water-glycerol media in a centrifuge falcon tube before the separation process. The dark brown layer on top of the tube shows the GO solution (10^{-2} mg/ml) to be separated. By using a fixed angle rotor system (Fig. 1B), a few light brown layers of GO nano-sheets (diluted solution compares to the primitive sample) migrated to their respective buoyant densities (Fig. 1C).

In this figure, GOF_1–GOF_4 represents different fraction zones from smaller to larger sizes of GO nano-sheets. These fractions (GOF_1–GOF_4) were then washed with DMF to remove any adsorbed glycerol molecules attached to the GO sheets via the hydrogenated functional groups. The washed fractions were dialyzed against distilled water for 72 h (water was changed each 12 h) to remove DMF and glycerol (Supplementary Information, Fig. S2). To determine the concentration of the separated fractions, the GO standard curve was used with concentration being determined by reference to absorbance of GO solutions at 230 nm (Supplementary Information, Fig. S3).

2.2. Size distribution analysis of the fractionated GO sheets

The separated GO sheets were then imaged using Atomic Force Microscopy (AFM). A sample of each fraction was placed on a freshly cleaved mica surface and dried under laminar flow to minimize contamination. The average thickness of the single sheets was measured to be ~1.1 ± 0.2 nm on average (See Fig. 2).

Dynamic Light Scattering (DLS) measures the hydrodynamic diameter of GO sheets in solution. The lateral dimension of the sheets absorbed on mica measured by AFM would be expected to be larger than the hydrodynamic size. The measured hydrodynamic radii (DLS) and AFM lateral dimensions are found to be the similar (as seen in Fig. 3 below).

Lotya et al. reported that the hydrodynamic radius in non-spherical particles is often approximated as the radius of a sphere with the same volume as the particle [51]. Other researchers have also measured a correlation between the hydrodynamic DLS radius and measured AFM sizes [52]. Models for interpreting the correlation between actual size and hydrodynamic diameter in irregular and platelet-like particles have been developed [53,54]. Our experimental results show a linear correlation between the measured hydrodynamic radii measured using DLS and the AFM flat sheet sizes as shown in Fig. 3 in which the reported hydrodynamic diameter represents the average size of GO sheets [55] in the range of 150–850 nm diameter.