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Technical Report on the reduced graphene oxide biosynthesis protocol for biological applications.

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Summary

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Introduction

The interest of scientific community on carbon-based smart materials is growing and, especially focus on graphene oxide (GO) and reduced graphene oxide (rGO). An increasing number of bio-applications such as biological applications as bacterial inhibition [1], drug delivery [2] and photothermal therapy [3] aims the use of GO and rGO. For this reason, the methods used for the synthesis of graphene materials are more important because same of those procedures imply chemical reactions that involve hazardous and toxic reagents. In fact, the biocompatibility and toxicological activity of graphene-related materials is related to the methodologies employed for the synthesis that determine the carbon/oxygen (C/O) ratio of graphene oxide species.

In this technical report, we focused on the synthesis of GO by means of that lead to a biocompatible GO form with a lower oxygen content. Thus, the synthesis of rAsGFP-rGO with the green fluorescent protein allowed us to obtain a biocompatible materials, without using hazardous and toxic reagents. This biocompatibility is the most important prerogative for the use of GO in biological activity assays as reported in several publications [2, 4–7].

Experimental procedures

Experimental procedures were focused on the i) Graphene Oxide chemical synthesis; ii) reduced Grahene Oxide biosynthesis by means of a recombinant Green Fluorescent Protein (rAsGFP) expressed in *Escherichia coli* and produced as a highly purified protein starting from a cDNA of 687 bp isolated from the total RNA of *Anemonia sulcata* tentacles [8]; iii) setting of all parameters for spectroscopic characterization of synthesis products.

Graphene oxide (GO) synthesis

The production of graphene oxide was carried out using a direct oxidation synthesis in protic acid medium, described in our previous work [9]. Briefly, expanded graphite (2.5 g) and $KMnO_4$ (7 g) were introduced into a 500mL round-flask and stirred until homogeneity. The beaker was placed in an water-ice bath and 50 mL of concentrated sulphuric acid (98%) were added slowly (continuous stirring with magnetic stir bars) until a paste (green-petrol colour) was obtained. The beaker was

placed into a water bath at about 30 °C for 30 min to produce a spontaneous volumetric expansion. Next, the hydrolysis of the material was obtained adding 200 mL of distilled water very slowly in order to prevent an uncontrolled temperature increase. Then, the green-brownish liquid was placed into a silicon oil bath at 90 °C for 1 h to obtain a darker dense suspension. The suspension was washed by repeating centrifugation using distilled water up to the pH reaches the neutrality. Eventually, the solid samples were prepared by drying in an oven at 50-60 °C overnight.

Synthesis of reduced Graphene Oxide (rAsGFP-rGO) with rAsGFP

Sample of GO (1 mg mL^{-1}, pH 7) was prepared by dispersing the solid in Milli-Q water and sonicating for 15 min at RT. The dispersion were stirred for 1 h at 300 rpm and centrifugated for 5 min at 10,000 rpm before investigating the soluble fractions (Fig. 1).

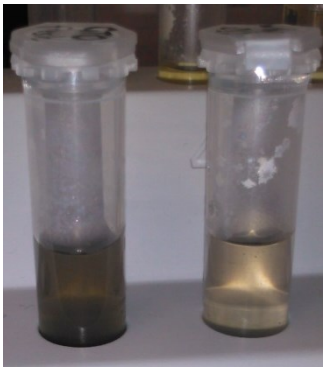

Figure 1 – GO dispersion before (left) and after (right) the sonication and centrifugation.

Using GO as a precursor, our rAsGFP has been used as a reductant and stabilizer. A mixed aqueous solution (pH 7) containing rAsGFP (1 μM) and GO (1 mg mL^{-1}) was sonicated for 15 min, and the mixture was maintained at 40 °C for 1 h. The mixture was then cooled to room temperature for a further 15 min. Subsequently, the mixture was stirred at 90 °C for 1 h at 400 rpm. The resulting black dispersion was centrifuged and then washed with water. Finally, homogenous rAsGFP-rGO suspension was obtained after sonication of the sample and recovery of surpernatant after centrifugation.

Spectroscopic measurements and setting of parameters.

Spectroscopic investigation is used to obtain information about the characteristics of the synthesis products as GO and rAsGFP-rGO. In particular, UV–Vis spectra were recorded in UV2700 spectrometer (Shimadzu) with a quartz cell with a 1 cm path length. Fourier transform infrared spectroscopy (FTIR) measurements were carried out in a Jasco70 FT/IR-620 spectrometer using KBr pellets. Photoluminescence measurements were performed on a RF 5301PC spectrofluorometer (Shimadzu) using 5 nm spectral slit widths for excitation and emission. Wavelength scanning super (about 3000 nm/min) with high sensitivity selection was selected. The S/N ratio of instrument is 150 or higher for the Raman line of distilled water (350 nm excitation wavelength, 5 nm spectral bandwidth, and 2 s response for 98% of the full scale). Wavelength accuracy is ±1.5 nm.

UV–Vis spectra for GO and rAsGFP-rGO were investigated in the range 190–800 nm (Fig. 2). In the region from 400 to 900 nm, we did not identify absorption bands. The maximum absorbance for GO takes place at 236 nm with a shoulder at 300 nm. A redshift of the maximum absorbance peak to 247 nm is observed for rAsGFP-rGO comparing with pristine GO.

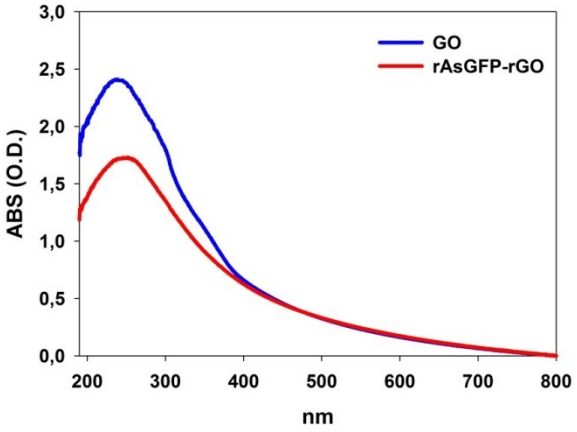

Figure 2 – GO and rAsGFP-rGO UV-Vis spectra.

Figure 3 shows FTIR spectra for the GO and rAsGFP-rGO. The GO spectrum exhibits the following characteristic bands: 3440 cm^{-1} corresponds to the O–H stretching vibrations, tiny doublet peaks at 2920 and 2850 cm^{-1} correspond to the symmetric and antisymmetric CH2 stretching, 1710 cm^{-1} is the C=O stretching, and 1630 cm^{-1} is sometimes assigned to the O–H vibrations due to the presence of adsorbed water [10]. This doublet may correspond to keto–enol

tautomerism . The peak at 1384 cm^{-1} can correspond to the bending vibrations of O–H or C–H. The peak at 1090 cm^{-1} can be related to an alkoxy (R–O) or to the stretching vibration of C–OH in the alcoholic group.

The interaction of GO and rAsGFP produces a different spectrum. Any variation takes place for the O–H stretching frequencies. At the fingerprint region, the relative intensity of keto–enol doublet is modified. The peak at 1710 cm^{-1} is lowered with the presence of rAsGFP, while the peak at 1630 cm^{-1} retains the same position with a shoulder at 1583 cm^{-1}. Below 1300 cm^{-1}, only a broad peak is recorded.

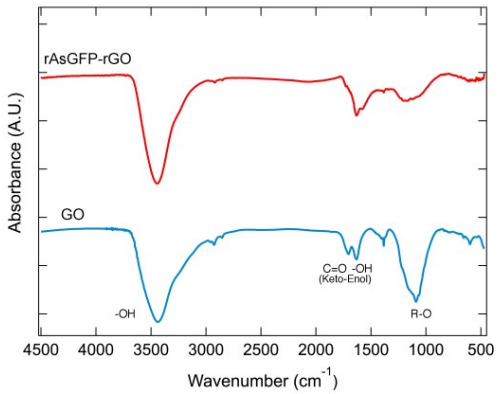

Figure 3 – GO and rAsGFP-rGO FTIR spectra.

The contour plot of GO is reported in Fig. 4 using an excitation wavelength (λ_{ex}) in the range 280-400 nm. The red colour identifies the maximum GO emission (447 nm) at λ_{ex} 330 nm.

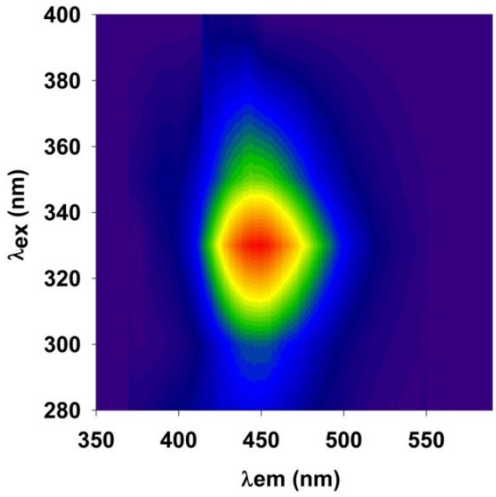

Figure 4 – GO photoluminescence (PL) spectra.

Figure 5 shows the emission spectrum of rAsGFP, GO, and rAsGFP-rGO. Concerning rAsGFP, we observed an intense fluorescence emission band, once excited in the visible range (λ_{ex} 470 nm). It presents a single main peak at 500 nm with a shoulder at about 530 nm. The liquid suspension of GO exhibited an equivalent PL peak centred at around at 446 nm (λ_{ex} 330 nm). After the reduction with rAsGFP, we analysed the products of the reaction. It is evident that the reduced sample lost the luminescent characteristics of GO as the typical fluorescence of rAsGFP

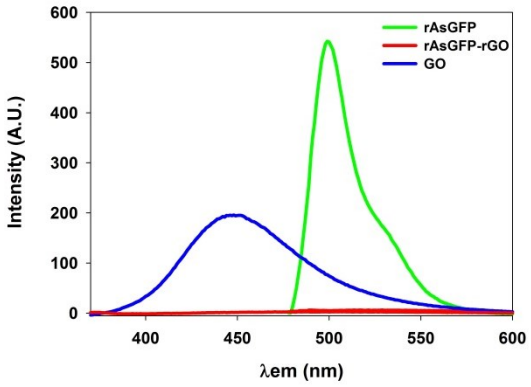

Figure 5 – PL spectra of GO (λ_{ex}330 nm), rAsGFP-rGO (λ_{ex} 330/470 nm), and rAsGFP (λ_{ex}470 nm) .

Conclusions

The biosynthesis procedure used in our work by means of rAsGFP as reductant and stabilizer and GO as precursor, determined the formation of reduced graphene oxide as supported by spectroscopic measurements. In fact, the red shift of the maximum absorption peak of reduced GO and the disappearance of the absorption shoulder at 300 nm indicate the complete reduction and the removal of carbonyl functional groups (11). The interaction of rAsGFP with the alkoxy reduced and broadened the peak at 1090 cm^{-1}, shifting the tautomeric equilibrium toward the enol form and reducing drastically the keto form [12, 13]. The partially reduced GO solution after photothermal reduction is a gradual shift of the PL peaks, but for longer exposure, the quenching of the PL signal was evident [11]. Thus, the quenching in our systems can be attributed to a massive reduction of GO by means of rAsGFP and a possible effect of shielding of the groups responsible for PL.
This reduced GO will use for future biological activity assays.

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