

Easy and versatile synthesis of bulk quantities of highly enriched ^{13}C -graphene materials for biological and safety applications

Viviana Jehová González[#], Javier Frontiñan-Rubio^{#,§}, M. Victoria Gomez^{#,}, Tiziano Montini^{†,§}, Mario Durán-Prado[§], Paolo Fornasiero^{†,§}, Maurizio Prato^{*,†,‡} and Ester Vázquez^{*,#,‡}*

[#] Universidad de Castilla-La Mancha, Instituto Regional de Investigación Científica Aplicada (IRICA), 13071, Ciudad Real, Spain.

[§] Universidad de Castilla-La Mancha, Cell Biology Area, Department of Medical Sciences, Faculty of Medicine, 13071, Ciudad Real Spain.

^{*} Universidad de Castilla-La Mancha, Faculty of Chemical Science and Technology, 13071, Ciudad Real, Spain.

[†] Department of Chemical and Pharmaceutical Sciences, INSTM, UdR Trieste, Via Giorgeri 1, University of Trieste, 34127 Trieste, Italy.

[§] ICCOM-CNR, University of Trieste, Via L. Giorgieri 1, 34127 Trieste, Italy.

^{||} Center for Cooperative Research in Biomaterials (CIC biomaGUNE), Basque Research and Technology Alliance (BRTA), Paseo de Miramon 194, 20014 Donostia San Sebastián, Spain.

[‡] Basque Foundation for Science (IKERBASQUE), Plaza Euskadi 5, 48013 Bilbao, Spain.

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ABSTRACT

The preparation of bulk quantities of ^{13}C -labelled graphene materials is relevant for basic investigations and for practical applications. In addition, ^{13}C -labelled graphene materials can be very useful in biological and environmental studies, as it may allow the detection of graphene or its derivatives in cells or organs. In this paper, we describe the synthesis of tens of mg scale of ^{13}C -labelled graphene materials (few-layer graphene, FLG, and graphene oxide, GO), starting from ^{13}C -labelled methane to afford carbon fibers, followed by liquid phase exfoliation (FLG) or oxidation (GO). The materials have been characterized by several analytical and microscopic techniques, including Raman and nuclear magnetic resonance spectroscopies, thermogravimetric analysis, X-ray photoelectron spectroscopy and X-ray powder diffraction. As a proof of concept, the distribution of the title compounds in cells has been investigated. In fact, the analysis of the

$^{13}\text{C}/^{12}\text{C}$ ratio with isotope ratio mass spectrometry (IRMS) allows the detection and quantification of very small amounts of material in cells or biological compartments, with high selectivity, even when the material has been degraded. During the treatment of ^{13}C -labelled FLG with HepG2 cells, 4,1 % of the applied dose was found in the mitochondrial fraction, while 4.9 % ended up in the nuclear fraction. The rest of the dose did not enter into the cell and remained in the plasma membrane or in the culture media.

2D Nanomaterials have opened a new world with pathbreaking applications in nearly every field of development¹. In biological applications, the possibility of these materials to interact, at the nanoscale level, with biological entities gives rise to innovative approaches such as the creation of innovative therapeutic agents or the development of alternative theranostic approaches². Nevertheless, real-life advances in this area rely on the investigations of the mechanisms with which these materials interact with cells, organs or other biological compartments and their environment. Another important issue is that state-of-the-art nanomaterials still generate toxicological concerns³: their end-use and the commercialization of final devices for any particular purpose depend on safety assessments to exclude possible hazards and risks for human health or for the environment, including the determination of exposure limits⁴.

A particular challenge for nanosafety studies is to detect minimal amounts of 2D nanomaterials in complex biological and environmental contexts. For metal containing materials, the quantification can be achieved relatively easily by standard analytical techniques, such as inductively coupled plasma-mass spectrometry (ICP-MS)⁵. However, the quantitative determination of graphene materials is much more difficult, as these materials are mostly composed of carbon atoms and, to a lesser amount, of oxygen and hydrogen atoms, which are the same elements found in the environment or in living systems⁶.

Graphene materials are usually detected by microscopy techniques and Raman spectroscopy. Black, semi-transparent shapes in the transmission electron microscopy (TEM) or confocal microscopy images of biological cross sections are associated to graphene flakes⁷, while the detection of D- and G- bands in Raman spectra of natural samples is correlated to the presence of the 2D material⁸. However, even considering only detection without quantification, these techniques are insufficient, mainly because: i) once inside the biological tissues or the natural backgrounds, graphene materials can be degraded⁹ and transformed to structurally and/or chemically different amorphous species which further complicate the recognition; ii) using these techniques, it is very difficult to locate the real position of the material within the different biological compartments. For these reasons, some authors have tried to label graphene materials with fluorescence organic labels or radioactive atoms³, but this modification changes the nature/surface of the nanomaterial and can affect their properties and their behavior. Moreover, quenching phenomena and/or detachment of these added moieties from the surface of the 2D nanomaterial can occur, thus making biological and environmental tracing unreliable¹⁰.

A different approach, very robust and reliable for nanosafety studies and for environmental tracing, is the use of isotope labelling of the graphene material itself¹¹. Some studies have already

reported the use of ^{14}C radioisotope to describe bioaccumulation and biotransformation of graphene in complex biological matrices^{12, 13}. However, a simpler concept is the employment of stable isotopes such as ^{13}C . ^{13}C and ^{12}C graphene materials synthesized by the same protocol have the same physicochemical properties and intrinsic behavior¹⁴. The analysis of the $^{13}\text{C}/^{12}\text{C}$ ratio with isotope ratio mass spectrometry (IRMS) allows the detection and quantification of very small amounts of material in a biological system or compartment, with high selectivity, even when this material has been degraded¹⁵. Moreover, although one of the main advantages of the use of radioactive isotopes as ^{14}C is its quantification through imaging techniques, the use of ^{13}C magnetic resonance spectroscopic imaging (MRSI) is becoming progressively established¹⁶⁻¹⁸. Finally, stable isotope labelling avoids issues associated with the production of radioactive waste and approval of special experimental conditions when using radioactive materials for tracing¹⁹.

Some authors have already reported the preparation of ^{13}C graphene materials, mostly describing the physicochemical characteristics of graphene prepared by chemical vapor deposition (CVD) process²⁰⁻²⁸. The cost of the ^{13}C starting materials and the low yield of the synthetic approaches usually prevent the preparation of bulk quantities of ^{13}C -graphene materials. However, the enrichment of bulk quantities of GO has also been described with around 7.1 atom % of ^{13}C atoms in the final GO, which has allowed to investigate the bioaccumulation and toxicity of this 2D material in wheat.²⁹

In this work, we detail a completely unprecedented approach for the production of readily available bulk quantities of ^{13}C graphene materials, both graphene and GO, with different C/O ratios and lateral sizes, suitable for biological studies. The high ^{13}C % in the graphene materials (from 95 to 65%) has made it possible to trace, for the first time, graphene materials in individual cell compartments, such as the nucleus and the mitochondria. The protocol described herein will stimulate further investigations to quantify the biodistribution of graphene in different organisms and ecosystems and will permit to study their biological degradation.

RESULTS AND DISCUSSION

The process leading to ^{13}C graphene starts with the preparation of ^{13}C carbon fibers via a CVD process, which are then exfoliated by a standardized ball milling procedure^{30, 31}. Ball milling treatments show significant lower environmental impact for the production of graphene³² and can be easily scaled up in order to produce various graphene related materials^{30, 31, 33, 34}. Our group has previously described that, following these treatments, the preparation of graphene can be achieved by exfoliation of graphite or carbon fibers³⁵. In the present work, ^{12}C and ^{13}C nanomaterials have been prepared using carbon nanofibers obtained by CVD process (see experimental section). ^{12}C nanomaterials have been prepared as controls and their physicochemical characterization have served to prove that ^{13}C labelling does not change the intrinsic structure nor the properties of the materials.

Synthesis of Carbon Nanofibers

Carbon nanofibers were grown by methane decomposition over Ni nanoparticles in a tubular furnace. **Figure 1** presents, in a schematic way, the principal steps of the procedure: parameters such as amount of Ni catalyst, concentration of CH₄ in the reaction mixture, gas flow and reaction time for nanofiber growth, were optimized to maximize the amount of carbon nanofibers obtained from each preparation. In a typical experiment^{36, 37}, carbon nanofibers were grown from a H₂/CH₄, 30/70 mixture (200 mL min⁻¹) over a Ni catalyst (obtained by reduction of NiSO₄ sprayed over Al₂O₃ plates) at 800 °C for 8h. After cooling under Ar flow, the carbon nanofibers were washed in diluted HCl (to remove accessible Ni), water and ethanol, finally yielding ¹²C-CNF nanofibers. ¹³C-CNF nanofibers were prepared following the same protocol but replacing CH₄ with ¹³CH₄. Fibers prepared in this way showed high quality by TEM. However, to eliminate any trace of nickel metal, the nanofibers were further graphitized at 2700 °C under helium atmosphere during 2 hours with 0.5 bar overpressure.³⁵ The ¹²C and ¹³C graphitized carbon nanofibers were labelled as ¹²C-GCNF and ¹³C-GCNF, respectively. All samples were characterized using Raman spectroscopy, thermogravimetric analysis (TGA), elemental analysis, transmission electron microscopy (TEM) and X-ray powder diffraction (XRD).

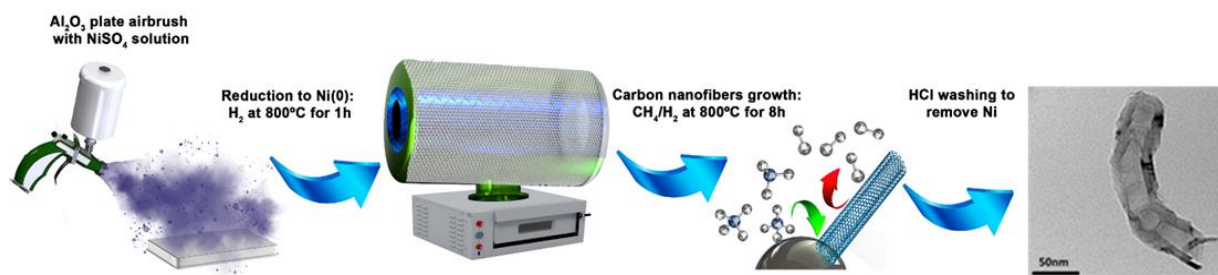


Figure 1. Schematic representation of the synthesis of carbon nanofibers. On the right: typical TEM image of the produced fibers.

Figure 2 shows the XRD spectra for ¹³CNF and ¹³GCNF in which the two peaks, at 2θ=26.39° and 42.44°, can be assigned as typical graphitic 002 and 101 planes, respectively. Through the 002 peak intensity plane, it is possible to determine the degree of crystallinity in carbon samples, using the following equation 1:³⁸

eq. 1.

$$G = \frac{3.461 - d_{002}}{3.461 - 3.352}$$

where 3.461 °Å and 3.352 °Å correspond to the d spacing for a fully turbostratic disordered and highly oriented pyrolytic graphite, respectively.

In general, graphitized nanofibers show a higher degree of crystallinity determined by the d002 spacing peak (**Table 1**). Moreover, carbon nanofibers showed a high quantity of residual metal related to their synthesis. However, after graphitization no metal residue was observed in the XRD spectra (**Figure 2** and **S1**). The corresponding values for all the parameters obtained through XRD are showed in Table 1 using the peak (002) of carbon in all the different samples.

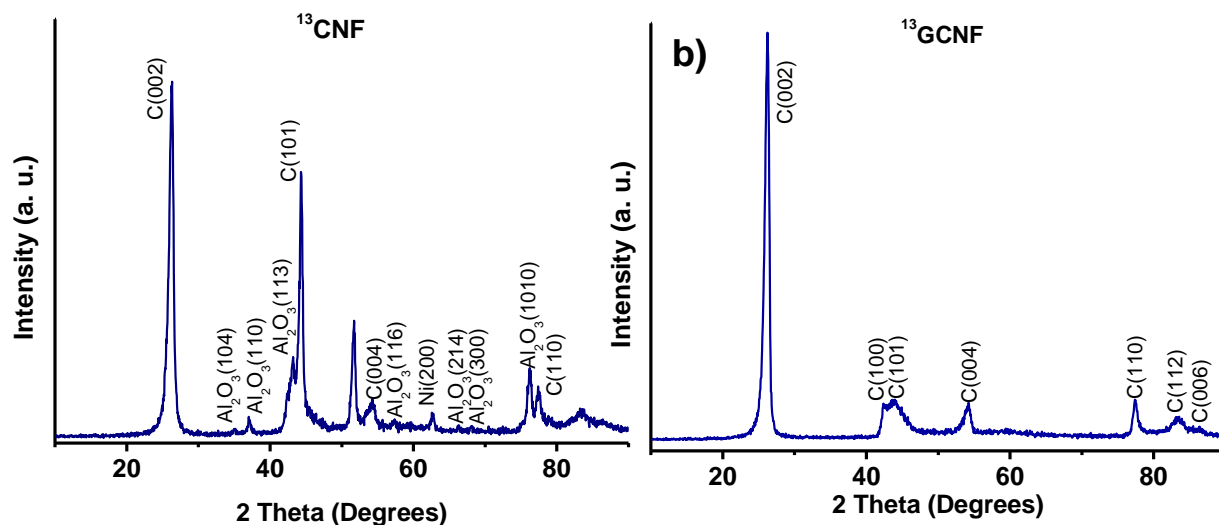


Figure 2. XRD of a) ¹³CNF and b) ¹³GCNF.

Sample	2theta (°)	d (Å)	G(%)
¹² CNF	26.40940	3.37082	82.73
¹² GCNF	26.42480	3.36889	84.50
¹³ CNF	26.29798	3.38485	69.86
¹³ GCNF	26.35370	3.37782	76.31

Table 1. Different parameters obtained from XRD for ¹²CNF, ¹²GCNF, ¹³CNF, ¹³GCNF.

TGAs performed in air atmosphere are shown in **Figure 3** and **Figure S2** for ^{13}C and ^{12}C fibers, respectively. As expected, higher thermal stability is observed for graphitized samples (779.93°C for ^{12}C -GCNF, 773.21°C for ^{13}C -GCNF) in comparison to pristine nanofibers (683.95°C for ^{12}C -CNF, 683.52°C for ^{13}C -CNF), corroborating the higher crystallinity in graphitized samples. Moreover, a total weight loss is observed for graphitized samples at 800°C, while a residual (metallic) mass remains in the non-graphitized fibers (14.12% for ^{12}C -CNF, 9.31% for ^{13}C -CNF), which indicates the complete removal of all the catalytic nanoparticles in the graphitized nanofibers.

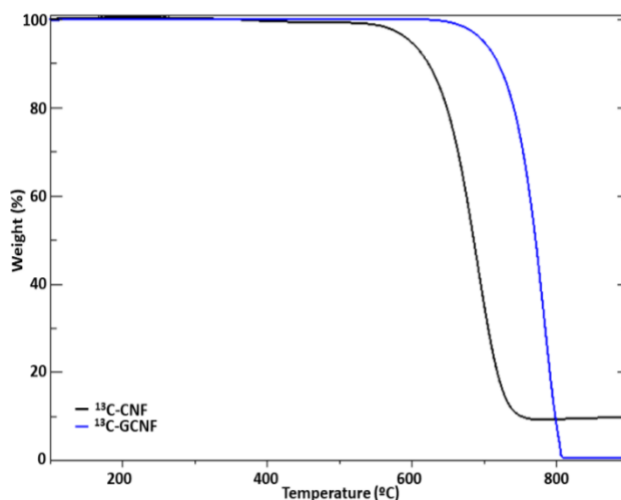


Figure 3. TGA of ^{13}C non-graphitized and graphitized carbon nanofiber.

XPS was also used to evaluate the type and abundance of oxygen groups in the samples (**Figure S3a** for ^{12}C and **Figure S3b** for ^{13}C). The C1s and O1s core-level spectra were fitted with Gaussian–Lorentzian (90G/10L) peaks to give separate main components.

The components of C1s at different binding energies are: 284.5 eV (sp^2 carbon bonds), 286.4 eV (sp^3 , C-O-C bond), 287.8 eV (C=O bonds), 289.3 eV (C(O)O bonds)³⁹ and, 291.4 eV (π - π^* satellite)⁴⁰ this last one observed in graphitized nanofibers.

On the other hand, the deconvolution of O1s spectra produces three main peaks in non-graphitized fibers. These peaks at around 531.08, 532.03, are generally assigned to C=O (either in carbonyl or carboxyl groups)⁴¹, and 533.43 eV C-O (singly bonded oxygen)⁴². The graphitization produces in general a reduction in the oxygen content mainly visible in the C=O peaks.

The increase in intensity of the $\pi \rightarrow \pi^*$ shake-up satellite band of graphitic carbon band (**Figure S4**) observed between non-graphitized and graphitized ^{12}C and ^{13}C carbon nanofibers is due the reduction of defects in the material after removal of oxygen atoms.⁴³

^{12}C -CNF and ^{13}C -CNF were also analyzed by Raman spectroscopy (**Figure 4**) where three bands are observed corresponding to D, G and 2D bands. The introduction of heavier ^{13}C atoms into the structure leads to downshifts in all the Raman modes. The magnitude of these downshifts

depends on the frequency of the mode: those at higher frequencies shift more than modes at lower frequencies, as described by the simple harmonic oscillator model.^{27, 44, 45}

For this reason it is possible to quantify the percentage of enrichment of ¹³C of our sample, following the next equation^{46, 47}:

eq. 2.
$$\frac{(\omega_0 - \omega)}{\omega_0} = 1 - \sqrt{\frac{12 + c_0}{12 + c}}$$

Where:

ω_0 is the frequency of ¹²C sample,

ω corresponds to the frequency of ¹³C sample,

c corresponds to the concentration of ¹³C in the enriched sample,

and $c_0=0.0107$ is the natural abundance of ¹³C.

After applying this equation, we find that before graphitization, carbon nanofibers ¹³CNF have an enrichment of 95.48 wt % (1519.89 cm⁻¹). After graphitization, this value decreases to 65.10 wt% (1538.56 cm⁻¹) for ¹³C-GCNF. These data agree with the results of elemental analysis quantifying ¹²C with 2.82 wt% and 33.12 wt% for ¹³C-CNF and ¹³C-GCNF respectively. The reason for this exchange in the ¹³C/¹²C ratio is not clear yet and it is currently under investigation in our groups. Different graphitization conditions might also be employed to avoid the dilution in ¹³C content⁴⁸.

Raman technique allows to study the degree of crystallization and graphitization in carbon structures, through the relation between the intensities of D (I_D), G (I_G) and 2D (I_{2D}) bands^{49, 50}. The lower value in the ratio between I_D/I_G intensities after the graphitization process (around 0.10-0.14 for both samples, **Table 1**) indicates the loss of defects and the increase of the crystallization of the carbon nanofibers.

The percentage of ¹²C in the ¹²C samples observed by elemental analysis was 87.20 wt% for the pristine fibers (¹²C-CNF) and 98.79 wt% for the graphitized ones (¹²C-GCNF), which confirms once more the high quality of the graphitized fibers. Meanwhile, the percentage of H, N and S is similar in both non-graphitized (0.14 wt% of H, 0.03 wt% for N and, 0.03 wt% for S) and graphitized carbon nanofibers (0.01 wt% of H, 0.02 wt% for N and, 0.02 wt% for S). For ¹³C samples similar results and percentages of the elements in the samples were found in ¹³C-CNF (0.12%wt of H, 0.04 wt% for N and, 0.03 wt% for S) and ¹³C-GCNF (0.01 wt% of H, 0.03 wt% for N and, 0.02 wt% for S).

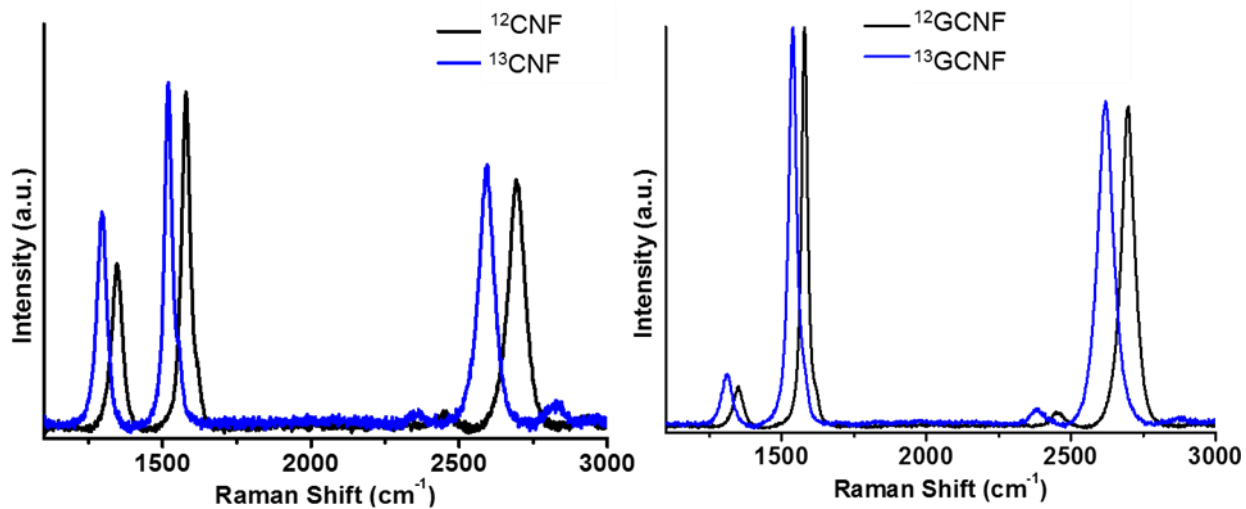


Figure 4. Raman of a) ^{12}C and ^{13}C of non-graphitized and b) graphitized carbon nanofibers.

Finally, **Figure 5** and **Figure S5** show typical TEM images for graphitized and non-graphitized carbon nanofibers, which have homogeneous structure and uniform diameter of around 180 nm with lengths about 800 nm.

All the characterization techniques led us to conclude that both ^{12}C and ^{13}C fibers show similar structures. Pure graphitized samples exhibit an improvement in their thermal properties, due to a higher degree of crystallinity and to the absence of catalytic nanoparticles.

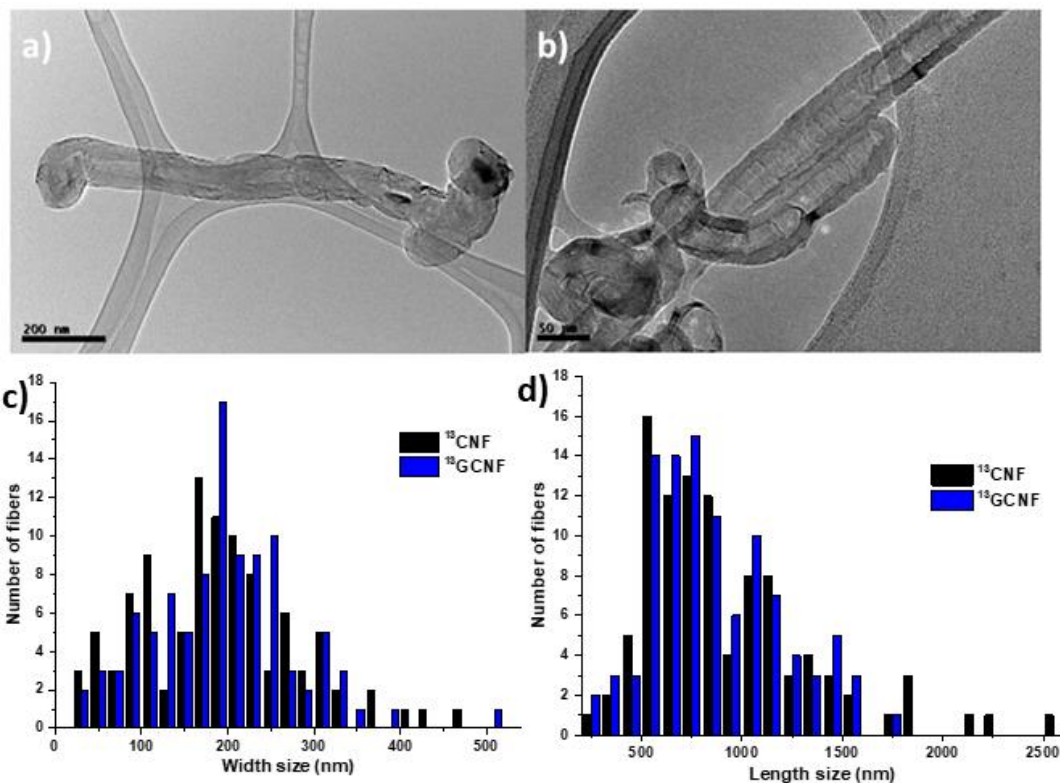


Figure 5. TEM image of a) ^{13}C non-graphitized and b) graphitized nanofiber, c) distribution of diameters and d) length size of ^{13}C carbon nanofibers.

Synthesis of Few-Layer Graphene

The exfoliation of carbon nanofibers was performed by using two exfoliating agents, melamine and glucose, through a ball milling treatment for the synthesis of FLG^{30, 51, 52}. The advantage of using a ball milling treatment is that by changing the exfoliating agent and the milling parameters there is the possibility of obtaining graphene flakes with different sizes and of tuning the C/O ratios^{30, 33}. Moreover, the process can be considered a green protocol because the exfoliating agent can be recycled and reused in different treatments.

In a typical experiment, the carbon nanofibers and the exfoliating agent are introduced in a stainless-steel grinding bowl with ten stainless-steel balls (1 cm diameter) and the mechanochemical treatment is performed in a Resch ball mill at room temperature and pressure conditions. After the treatment, the solid is suspended in 20 mL of deionized water, sonicated for 1 min and then dialyzed to remove the exfoliating agent. The final dispersion is stored for 5 days to allow the remaining carbon fibers to separate from the graphene sample. Finally, the resulting supernatant is separated and lyophilized at $-80\text{ }^{\circ}\text{C}$ with a pressure of 0.005 bar to obtain FLG powder.

All the results of the exfoliation of the carbon nanofibers were analyzed mainly by Raman spectroscopy to characterize the quality of the flakes, although the final yield of the process was also considered (**Figure S6, Table S2**).

In the Raman spectra, there are three principal bands useful to characterize graphene materials (D, G and 2D bands). The D band is related to the presence of defects, whereas the G band represents the degree of graphitization, therefore the intensity ratio between these two bands (I_D/I_G) can serve to quantify the density of defects in the graphene material⁵³. Finally, the 2D band can be used to determine the number of layers through its full width at half maximum (FWHM⁵⁴), a narrow 2D bands indicates a low number of layers⁵⁵.

Considering the information obtained from the Raman spectra and the final yield of graphene obtained, we established a time of milling of 2 h at 100 rpm and 5 h at 250 rpm when using melamine and glucose, respectively, as exfoliating agents.

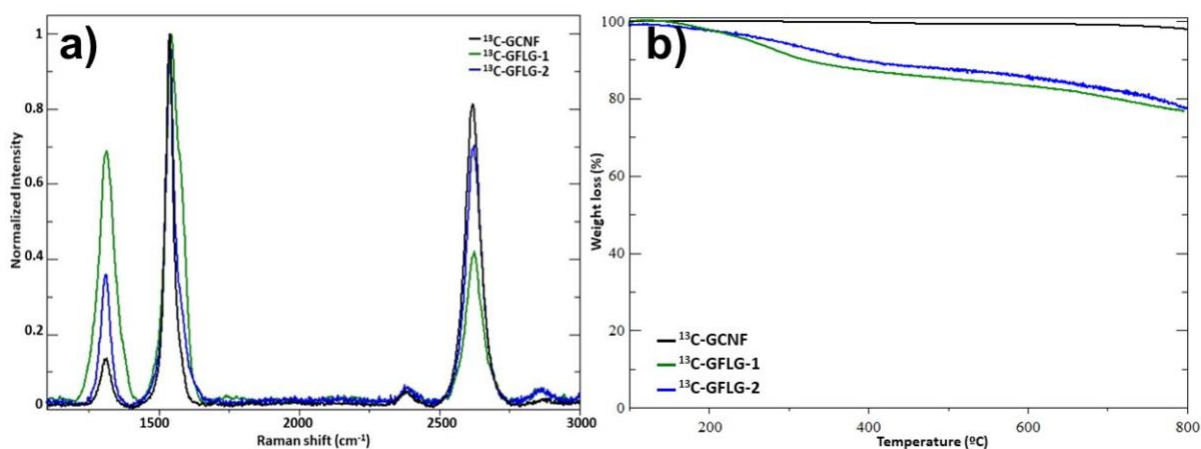


Figure 6. a) Raman spectra; b) Thermogravimetric analysis of carbon nanofibers using graphitized carbon nanofibers and melamine or glucose as exfoliants (¹³C-GFLG-1 and ¹³C-GFLG-2 respectively).

Figure 6 shows the Raman spectra and the TGA of the graphene materials prepared under the optimized conditions, starting with the ¹³C-GCNF (**Figure S7** for ¹²C materials). As already described elsewhere^{30, 52}, the ratio I_D/I_G in the spectra of the graphene materials is lower (**Table 1**) when using melamine as exfoliating agent in comparison to glucose, which indicates the presence of fewer defects in the former sample. This agrees with the TGA results.

Figure 7 shows a comparison between the Raman spectra of the different fibers and FLG prepared using melamine with graphitized (¹³C-GFLG-1) and non-graphitized (¹³C-FLG-1) carbon nanofibers, where the shifts in the Raman bands depend on the percentage of ¹³C as previously described (**Figure 7a** and data in **Table 1**). Also, in **Table 1** we observe the relationship between the different bands I_{2D}/I_G , I_D/I_G and the FWHM for the different ¹³C nanomaterials. It is important to mention that the percentage of ¹³C in FLG is 95.48 wt% for ¹³C-FLG and 65.10 wt% for ¹³C-GFLG (the same value as the starting fibers). Only CVD graphene grown on a substrate has been described in the literature with such a high ¹³C- percentage.^{20, 22-}

24, 26-28, 44, 56 Other papers describe the synthesis of GO in which the amount of ^{13}C is only around 7%.²⁹ Our values are much higher in comparison to the data found in the literature for ^{13}C graphene materials prepared in mg scale and it is also important to note the high yield in which the material is obtained along with the possibility of recycling the non-exfoliated fibers to produce other batches of ^{13}C -FLG.

Sample	$I_{\text{D}}/I_{\text{G}}$	$I_{2\text{D}}/I_{\text{G}}$	FWHM (cm^{-1})	2D Position (cm^{-1})	G Position (cm^{-1})
^{13}C -CNF	0.66	0.72	69.08	2593.78	1519.89
^{13}C -FLG-1	1.48	0.54	61.60	2586.60	1519.26
^{13}C -GCNF	0.14	0.80	68.22	2619.20	1538.56
^{13}C -GFLG-1	0.70	0.42	63.77	2622.70	1545.98
^{13}C -GFLG-2	0.36	0.81	63.76	2619.02	1539.80
^{13}C -GGO	0.86	0.09	-	2634.67	1565.34
^{12}C -CNF	0.50	0.76	71.10	2693.80	1578.50
^{12}C -GCNF	0.10	0.78	62.91	2696.88	1579.04
^{12}C -GFLG-1	1.33	0.57	69.43	2697.74	1583.88
^{12}C -GFLG-2	0.55	0.67	62.49	2699.91	1581.54
^{12}C -GGO	0.82	0.12	-	2677.62	1584.85

Table 1. Raman spectroscopy parameters ($I_{2\text{D}}/I_{\text{G}}$, $I_{\text{D}}/I_{\text{G}}$ bands, FWHM, 2D and G position band) of ^{12}C and ^{13}C nanomaterials: carbon nanofiber, graphitized carbon nanofibers (^{12}C -CNF, ^{12}C -GCNF, ^{13}C -CNF and ^{13}C -GCNF), FLG prepared by exfoliation of carbon nanofibers using glucose as exfoliating agent (^{13}C -FLG-1, ^{12}C -GFLG-1, ^{13}C -GFLG-1) and melamine (^{12}C -FLG-2 and ^{13}C -GFLG-2) and graphene oxide prepared from graphitized carbon nanofibers (^{12}C -GGO and ^{13}C -GGO).

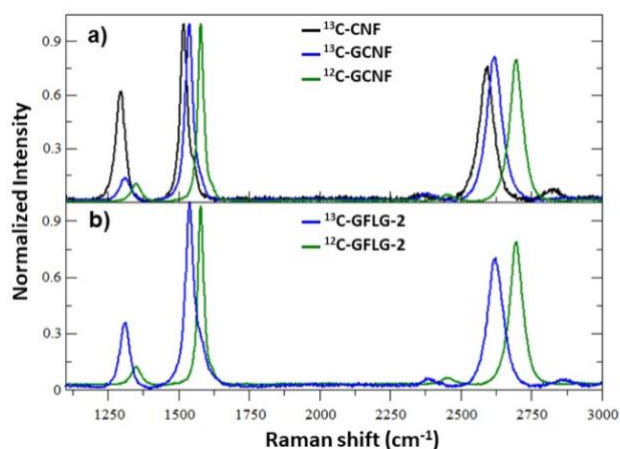


Figure 7. Comparison of Raman spectra of ^{13}C and ^{12}C nanomaterials: (a) carbon nanofiber and graphitized carbon nanofibers (^{13}C -CNF and ^{13}C -GCNF, ^{12}C -GCNF) and, (b) FLG prepared by exfoliation of carbon nanofibers and graphitized carbon nanofibers using melamine (^{13}C -GFLG-2 and ^{12}C -GFLG-2).

Raman spectra were used to analyze the different characteristics of the ^{13}C graphene materials (**Figure S7a** for ^{12}C and **Table 1**). In general terms, all the experiments related to the exfoliation

of graphitized carbon nanofibers have shown a small width of FWHM in 2D band and a I_{2D}/I_G ratio lower than 1, which corresponds to few-layer graphene. As already commented, lower values of I_D/I_G are observed when using melamine as exfoliating agent, indicating a less defective graphene in the case of melamine^{30, 33}. However, it should be emphasized that the exfoliation with glucose offers the advantage of using a non-toxic and environmentally friendly agent.

TGA was used to check the thermal stability of all samples of graphene (**Figure 6** and **Figure S7b** for ^{12}C) under nitrogen atmosphere. ^{12}C -GFLG and ^{13}C -GFLG show a similar behavior with a residual loss of 1.8 wt% and 1.9 wt%, respectively. However, the graphene obtained by melamine has a higher thermal stability in comparison with the graphene obtained using glucose as exfoliant agent, ^{13}C -GFLG-2 (11.9 wt%), ^{12}C -GFLG-2 (14.4 wt%) in comparison with glucose samples ^{13}C -GFLG-1 (21.9 wt%) and ^{12}C -GFLG-1 (17.2 wt%). This confirms the less defective nature of FLG prepared using melamine as exfoliating agent. All these values are due to pyrolysis of the residual oxygen groups on the graphene surface⁵⁷.

TEM Image and size distribution of graphene obtained by the two methods of exfoliation are shown in **Figure 8** for ^{13}C and **Figure S8** for ^{12}C . The size distribution of ^{12}C and ^{13}C materials is very similar and mainly depends on the exfoliating conditions, observing larger flakes when using melamine as exfoliating agent.

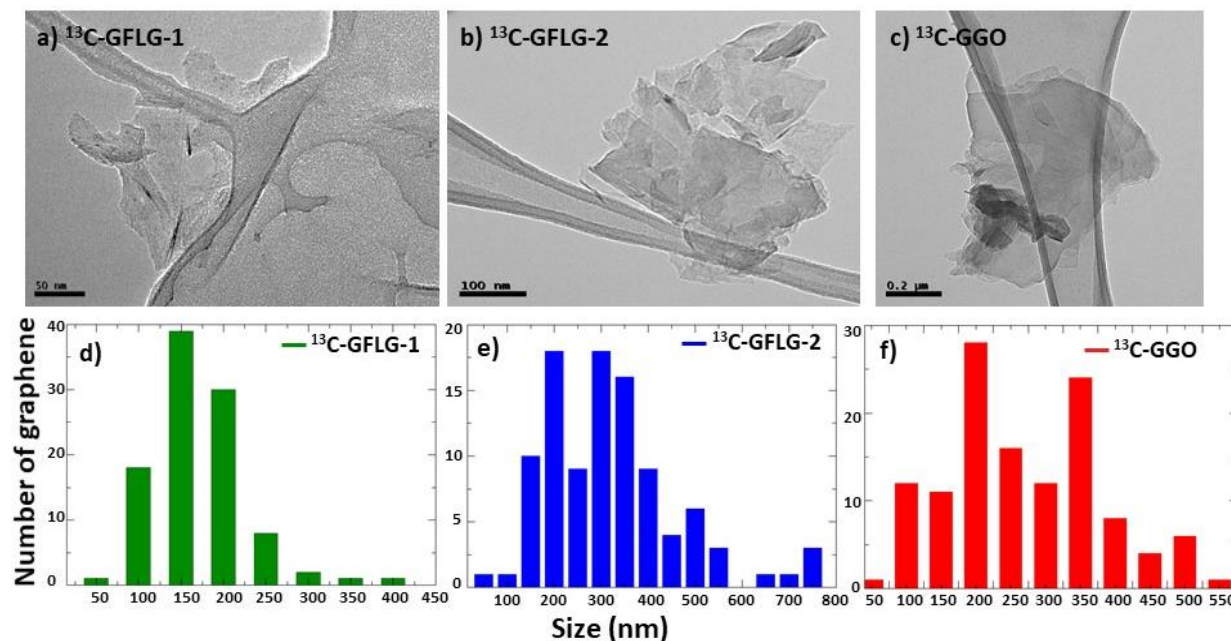


Figure 8. TEM Image and size distribution of graphene obtained by the exfoliation of the ^{13}C graphitized carbon nanofibers: a, d) ^{13}C -GFLG-1, b, e) ^{13}C -GFLG-2, c, f) ^{13}C -GGO.

Although the yield of the preparation of FLG, considering the recovery of the non-exfoliated fibers, is quite high (around 44 %), we have also exploited the possibility of resubmitting these fibers to a new exfoliation process. **Figure 9** shows the characterization of the FLG materials when using glucose and melamine as exfoliating agents, in a second exfoliation procedure, ^{13}C -GFLG-1-remain and ^{13}C -GFLG-2-remain respectively. In general, we found smaller flakes, but the TGA shows a similar thermal stability compared to the previous results of FLG, which

corroborates the absence of oxidation during the treatment. Moreover, in Raman spectra, it is possible to observe an increase in the I_D/I_G ratio that is related to the decrease in the flake sizes.

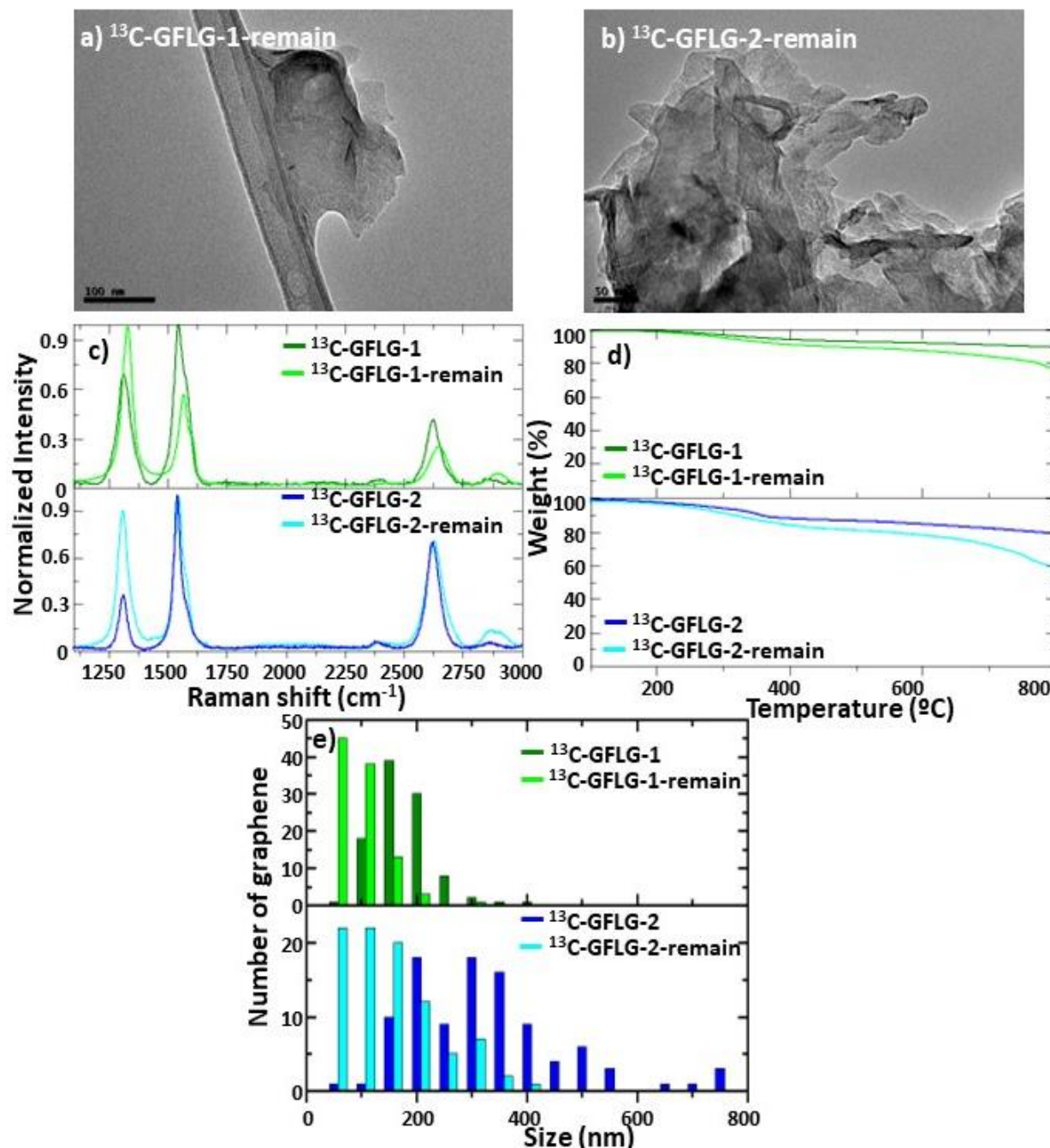


Figure 9. Comparison of characterization results obtained for FLG materials prepared from pristine graphitized carbon fibers (^{13}C -GFLG-1, ^{13}C -GFLG-2) and recovered graphitized carbon fibers (^{13}C -GFLG-1-remain, ^{13}C -GFLG-2-remain), a) and b) TEM Images, c) Raman spectra, d) TGA and e) size distribution of FLG flakes.

Synthesis of Graphene Oxide

GO was prepared using a modified Hummers method starting from ^{12}C GCNF or ^{13}C GCNF as starting material⁵⁸ (see experimental section).

Raman spectroscopy was used to follow the process from graphitized carbon nanofibers to graphene oxide, where it is possible to observe the disappearance of the 2D band in the GGO for both samples (**Figure 10a** for ^{13}C , **Figure S9a** for ^{12}C and **Table 1**), due to loss of interlayer bonding of graphene layers⁵⁹. It is also possible to observe, for both samples ^{12}C and ^{13}C , an increase in the D band correlated with the appearance of defects in the structure.

Figure 10b for ^{13}C and **Figure S9b** for ^{12}C were used to observe the thermal stability of the GO nanomaterial in nitrogen atmosphere. In general, TGA analysis shows a low percentage of oxygen groups on the surface compared to other typical GO synthesis, observing a 31.2% of weight loss for ^{13}C -GGO.

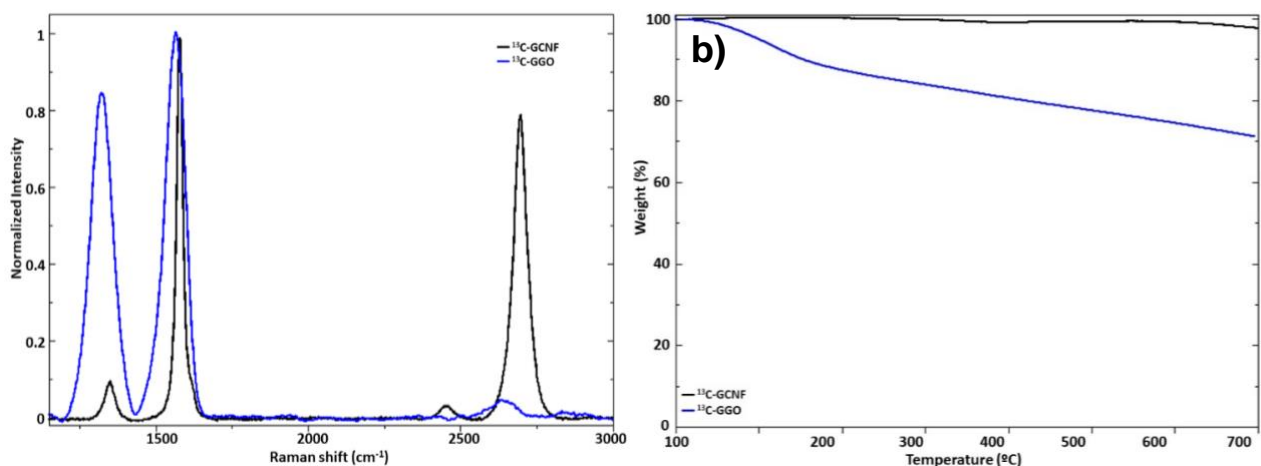


Figure 10. a) Raman spectroscopy and b) Thermogravimetric Analysis of ^{13}C graphene oxide (^{13}C -GGO).

TEM Image and size distribution of graphene oxide is showed in **Figure 10** and **Figure S8**. For ^{12}C -GGO the medium lateral size is around 220 nm (**Figure S8**) and about 240 nm for ^{13}C -GGO (**Figure 8**), again corroborating similar materials from ^{12}C and ^{13}C fibers.

Finally, the colloidal stability of the ^{13}C and ^{12}C materials in deionized water was evaluated by UV-Vis absorption spectroscopy (**Figure S10**) at 660 nm for FLG and 386 nm for GO³⁴. Nanomaterial powders were re-dispersed at three different concentrations (0.2, 0.1 and 0.05 mg/ml). The stability of FLG (^{12}C -GFLG-1, ^{13}C -GFLG-1) increases significantly with respect to that of graphitized carbon nanofibers (^{12}C -GCNF, ^{13}C -GCNF). Moreover, the stability of graphene oxide (^{13}C -GGO, ^{12}C -GGO) was compared to that of a commercial source (GO-Antolin). In all the experiments, it is possible to observe a high stability of our ^{13}C nanomaterials, showing a minor sedimentation after 48h (around 25% and 22.5 %, for ^{13}C -GGO and ^{13}C -GCNF-1 respectively). Another great advantage of this methodology is the use of glucose as exfoliating agent, which makes the material prepared with the present protocol ideal for biological experiments and applications.

Solid state C-13 Magic-Angle-Spinning, Nuclear Magnetic Resonance Spectroscopy (C-13 MAS NMR)

Finally, high-resolution solid state NMR spectroscopy combined with magic angle spinning (MAS) has been used to characterize ^{13}C -GGO and ^{13}C GFLG-1, based on the work from Bianco & col.^{60, 61} where MAS NMR showed a key role for a comprehensive characterization of functionalized graphene derivatives. **Figure 11**, left, shows the MAS NMR spectroscopy for ^{13}C -GGO where two peaks are observed. The signal at 168.4 ppm is attributed to the carbonyl $^{13}\text{C}=\text{O}$ species existing in GO, in agreement with theory and previously reported data^{62, 63}. The peak centered at 130.0 ppm can be attributed to the aromatic carbon atoms of GO and possibly aromatic C-OH (phenol and/or aromatic diol species) according to previous peak assignment.⁶²⁻⁶⁴ The absence of peaks at around 60-70 ppm indicates the non-existence or the low presence of aliphatic C-OH and epoxy groups attached to aliphatic carbons⁶⁴. The lack of these aliphatic peaks in the MAS NMR spectrum is in good agreement with the TGA spectrum which shows the low oxidation degree of this sample (**Figure 10b**). On the other hand, CP-MAS NMR spectroscopy was also carried out in the ^{13}C GFLG-1 sample. The sample could be tuned properly because it is a graphitized sample (absence of metals) in contrast to other works where electric conductivity is causing tuning problems⁶¹. The spectrum for ^{13}C GFLG-1 shows a single and broad peak at 102.0 ppm that corresponds to sp^2 - ^{13}C from aromatic entities and conjugated double bonds as observed for ^{13}C graphene oxide (**Figure 11**, right). Due to the lack of functionalization, no other peaks are expected.

The shift to lower chemical shift (101.5.0 ppm) compared to GO (130.0 ppm) can be explained due to the absence of oxygen in the ^{13}C - sp^2 surroundings or to magnetic susceptibility effects, knight shift effects and/or to others, as previously reported by Bianco & col⁶¹. It is consistent with the lower chemical shift values for ^{13}C - sp^2 carbon signals found for graphite nanofibers (^{13}C - sp^2 chemical shift of 80 ppm⁶⁵) and graphite powder (^{13}C - sp^2 chemical shift of 97 ppm⁶⁶) in comparison to oxidized graphene samples.

The bandwidth of the ^{13}C - sp^2 peaks for ^{13}C -GGO (10400 Hz (26 ppm)) and for ^{13}C -GFLG-1 (26000 Hz (65 ppm)) are common values observed for solid-state NMR due to chemical shift anisotropy and other factors^{61, 65}. As observed for graphite nanofibers (bandwidth of 90 ppm)⁶⁵ or graphite⁶⁶, it can be related to the presence of different chemical species with different magnetic susceptibility (heterogeneity in the types of sp^2 carbons or many non-equivalent ^{13}C sites) or even due to structural heterogeneity which could include sheet stacking that could modulate chemical shifts, or even due to the presence of conduction electrons.

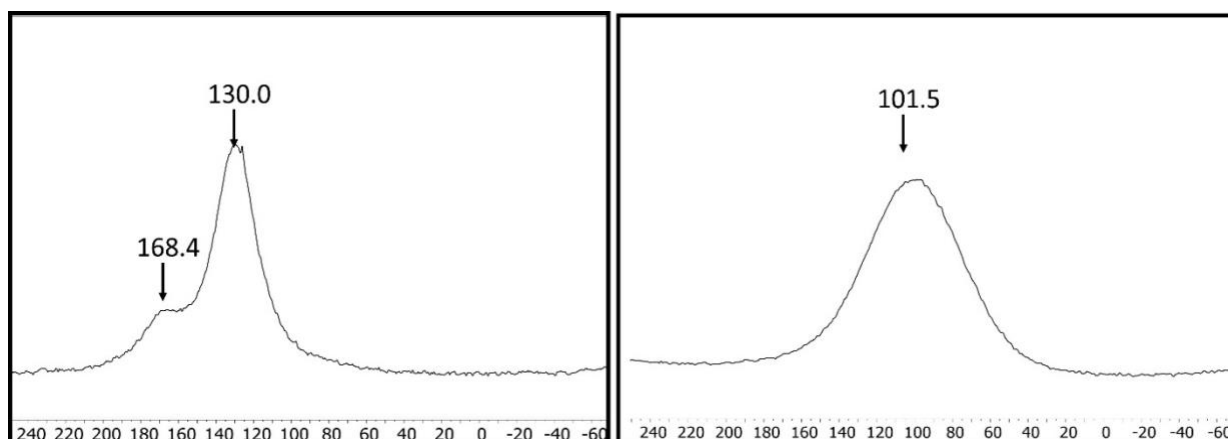


Figure 11. MAS ^{13}C NMR spectra for a) ^{13}C -GGO and, b) ^{13}C -GFLG-1.

Proof of concept. Detection of graphene in subcellular organelles

Stable isotope labeling of materials is a relevant method to evaluate their environmental impact or their degradation (biostimulation, bioaugmentation, etc.⁶⁷). However, as far as we know, no study has described the distribution, the detection and quantification of graphene materials inside the different cellular compartments. In the present work, our ^{13}C -graphene material is suitable to trace graphene derivatives assimilated by cells, even at very low concentrations. For this purpose, HepG2 cells were exposed to a dispersion of ^{13}C and ^{12}C FLG derivatives (10 $\mu\text{g}/\text{mL}$). In order to avoid any effect produced by metal or melamine traces, graphitized FLG samples prepared using glucose as exfoliating agent were used (GFLG-1), even though these materials present a lower percentage of ^{13}C (63 vs 95%).

After 7 days of incubation, a subcellular fractionation was performed by means of serial centrifugation steps (**Figure 12a**). We were able to separate fractions including the nucleus (NF), cytoplasm (CF), membranes (MmF) and mitochondria (MF). To ensure that the FLG present in the medium does not contaminate the different fractions, the cells were washed twice, trypsinized and seeded for 24 hours before starting the fractionation process. Moreover, and to completely exclude the possible contamination of the subcellular fractions with graphene materials, for the control experiment, ^{13}C GFLG-1 was added to untreated cells immediately before the fractionation process.

The extracted fractions were lyophilized, analyzed by IRMS analysis and the results were given in the form of stable carbon isotope ratios ($\delta^{13}\text{C}$). $\delta^{13}\text{C}$ values of the different subcellular fractions can be found in **Table 2** and they are plotted in **Figure 12b** for comparison. $\delta^{13}\text{C}$ values of cytoplasm and membrane fractions were similar in all cases, while nuclear and mitochondrial fractions of cells treated with ^{13}C -GFLG-1, were enriched in ^{13}C by around 1 ‰ relative to both control and ^{12}C -GFLG-1 treated cells. It is important to note that values found in the buffer obtained from the washing steps of nuclear and mitochondrial fractions, (wash 1 and wash 2, respectively) were similar in all the cases, what excludes contamination. Additionally, plasma membrane fractions, extracted in the trypsin part, present an enormous increase in ^{13}C , (**Table 2**) corroborating the fact that graphene materials in the culture media and those attached to the plasma membrane were removed from the cellular environment.

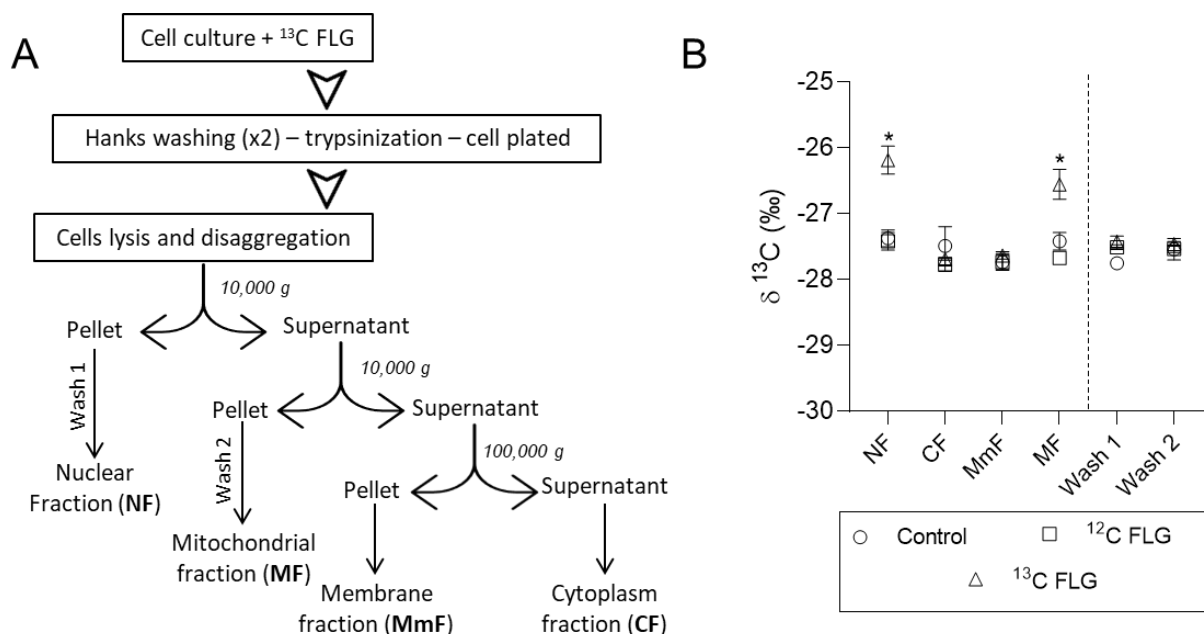


Figure 12. a) Subcellular fractionation at serial centrifugation steps; **b)** and their comparison.

Samples	(SD)						
	Trypsin	NF	Wash 1	MF	Wash 2	CF	MmF
Control $\delta^{13}\text{C}$ (‰)	-22,75	-27,37	-27,75	-27,42	-27,54	-27,49	-27,73
	(0,033)	(0,090)	(0,02)	(0,095)	(0,115)	(0,205)	(0,080)
%C	17,45	38,43	38,63	38,37	39,48	38,48	38,75
C-GFLG-1 $\delta^{13}\text{C}$ (‰)	-22,77	-27,42	-27,50	-27,67	-27,53	-27,77	-27,73
	(0,175)	(0,095)	(0,025)	(0,05)	(0,040)	(0,075)	(0,09)
%C	17,80	38,14	40,76	38,56	38,40	38,07	38,75
C-GFLG-1 $\delta^{13}\text{C}$ (‰)	-13,39	-26,18	-27,42	-26,55	-27,45	-27,69	-27,63
	(0,057)	(0,150)	(0,06)	(0,16)	(0,025)	(0,01)	(0,040)
%C	16,04	38,87	39,08	38,41	38,09	38,81	38,37

Table 2. $\delta^{13}\text{C}$ values of the different subcellular fractions.

To quantify ^{13}C -FLG in nuclei and in mitochondrial fractions, $\delta^{13}\text{C}$ values were transformed into the amount of ^{13}C -FLG in the different organelle fractions ($m_{13\text{C-FLG}}$) following equations 1-3. The relation $m_{\text{dry}}/m_{\text{wet}}$ was 0.1 for nuclei fractions and 0.09 for mitochondria ones, being the weight of the lyophilized pellets of around 3 and 3.5 mg for nuclei and mitochondria fractions, respectively. Moreover, cells were treated with a concentration of 10 mg/mL with a total dose of 50 mg. With these data and using equation 4, it is possible to calculate that 4.1 % of the applied dose can be found in the mitochondrial fraction, while a 4.9 % of the dose ends up in the nuclear fraction. The rest of the dose cannot enter the cell and remains in the plasma membrane or in the culture media. These studies and the possibility of detecting graphene derivatives within cellular compartments may help study the mechanisms of interaction of these materials at the cellular level.

Conclusion

The synthesis of bulk quantities of ^{13}C -labelled graphene materials is important for basic studies. But especially, the use of graphene in real-world applications makes it increasingly necessary to use methods for the detection of this material and his derivatives in natural matrices, which will favor its traceability and the development of safe and environmentally friendly systems. The present work contributes significantly to this target by describing the synthesis of graphene materials, with varying degrees of oxidation and sizes, all enriched with high amounts of ^{13}C that have not been described to date for bulk materials.

The approach is simple and easily scalable, enabling high yields so that materials can be prepared in high enough quantities to be used in a wide range of applications.

^{13}C labelling also enables graphene to be detected even after it has been modified or degraded, without the need for D- or G-band Raman detection or microscopy imaging analysis, so it could be used for degradation studies or even for developing occupational exposure limits.

The results suggest that the use of ^{13}C materials is a suitable method to assess the biodistribution of graphene in different models and organisms. To date, ^{14}C -graphene has been used for such studies but, although it is a valid approach, it generates a number of technical and practical issues due to radioactivity. With the use of ^{13}C -graphene, these problems would be avoided, generating an approach that is simpler and applicable in all types of environments. One of the main advantages of using ^{14}C is its quantification by imaging techniques. However, the use of MRSI is progressively gaining ground [25-27]. Combining this technique with the use of ^{13}C -graphene would enable real-time biodistribution studies in animal models.

^{13}C materials could also be used for bioaccumulation studies in different ecosystems. The use of ^{13}C -graphene allows for much simpler and less environmentally invasive experiments than the use of ^{14}C . Previous studies with ^{13}C -labelled toxic compounds demonstrate the feasibility and applicability of our proposal [61].

EXPERIMENTAL SECTION

MATERIALS

Unless otherwise noted, materials were purchased from Fluka, Aldrich, Acros, ABCR, Merck, and other commercial suppliers and used as received. $^{13}\text{CH}_4$ (99% ^{13}C) was purchased from SIAD (Società Italiana Acetilene Derivati S.p.A.)

EQUIPMENT

XRD spectra were recorded on a Philips (Panalytical) model using Cu KAlpha1 (1.54056 Angstroms) at 40 kV and 40 mA. Diffraction patterns were collected in a range of 10–90° 2 θ .

Raman spectra were recorded on an InVia Renishaw using powder samples with a 532 nm point-based laser with a power density below of 1 mW μm^{-2} to avoid laser heating effects. The resulting

spectra (with around 30–40 random locations on each sample) were fitted with Lorentzian-shaped bands in each characteristic band of graphene (D, G and 2D).

TGA were performed on a TGA Q50 (TA Instruments) at $10\text{ }^{\circ}\text{C min}^{-1}$ under nitrogen or air flow depending on the sample, from $100\text{ }^{\circ}\text{C}$ to $800\text{ }^{\circ}\text{C}$.

TEM were performed on a High-Resolution Transmission Electron Microscope (HRTEM) JEOL 2100 at an accelerating voltage of 100 kV using stable dispersions of graphene and dip-cast on Lacey copper grids (3.00 mm, 200 mesh), coated with carbon film, for further drying under vacuum.

UV-Vis spectra were recorded in a Cary 5000 UV-vis-NIR spectrophotometer with 1 cm quartz cuvettes. Dual beam mode and baseline correction were used throughout the measurements of FLG (660nm) and GO (386nm), for 2 h to 48 h at different time intervals. The determination of the concentration of graphene was determined from the optical absorption coefficient at maximum absorbance, using:

$$\text{eq. 3.} \quad A = \alpha \cdot l \cdot c,$$

Where:

l (m) is the light path length,

c (g L^{-1}) is the concentration of our material of interest, and

α ($\text{L g}^{-1} \text{m}^{-1}$) is the absorption coefficient, with $\alpha = 690\text{ L g}^{-1} \text{m}^{-1}$ at 660 nm FLG and $\alpha = 1130\text{ L g}^{-1} \text{m}^{-1}$ at 386 nm for GO. The optical absorbance divided by cell length against the concentration exhibited Lambert–Beer behaviour.

Magic Angle Spin Nuclear Magnetic Resonance (MAS-NMR) Spectroscopy was performed in a Bruker AV400 wide bore spectrometer operating in a 9.4 T magnetic field (^{13}C , 100.62 MHz) and equipped with a 2.5 mm diameter solid-state probe head. A multinuclear double channel probe (BL2.5 170-31P/19F-1H) with a spinning speed up to 35 kHz was employed. An amount of around 10 mg of sample was used for each material, ^{13}C -GGO and ^{13}C -GFLG-1. A pulse sequence of direct excitation on ^{13}C via ^1H decoupling was used. ^{13}C MAS NMR spectra of both materials were recorded with a spectral width of 496.9 ppm, a fid size of 2048, with 256 scans, an acquisition time of 0.02 s, a pulse width of 6.2 μs , and a long recycle delay of 180 s based on the previous work reported by Cai et al.⁶³ The spin rate for the ^{13}C MAS NMR experiment of graphene oxide ^{13}C was 10 KHz and for FLG ^{13}C was 20 kHz. MestReNova vs 12.0 was used for data processing.

^{13}C quantification by elemental-analysis coupled to isotope ratio mass spectrometry (EA-IRMS)

Lyophilized subcellular fractions were used to detect ^{13}C -contents IRMS. Each sample was weighed with precision balance MX5 (Metler, Toledo), and then encapsulated in tin capsules for isotope analysis. EA-IRMS analysis were performed using a Flash EA1112-ConFloIV Analyzer with a MAS 200R carousel autosampler and an IRMS Delta V Advantage (Thermo Scientific, Bremen, Germany) was used as the detection system. The elemental analyser also has a thermal conductivity detector (TCD) for elemental analysis. In the elemental analyser, the samples pass

through a combustion and reduction reactor at 1020°C, transforming them into CO₂ and N₂ gases, which are separated in a chromatographic column at 45°C. These gases are then transferred to the TCD and IRMS. The carrier gas (Helium) was maintained during the analysis at 100 ml min⁻¹ and the reference gases (CO₂ and N₂) at 250 ml/min. The reference gases were calibrated against internationally certified reference materials supplied by the International Atomic Energy Agency (IAEA). Certified reference materials (IAEA-N1, IAEA-N2, IAEA-CH6, NBS-22, USGS-40 and USGS-41) and laboratory standards were also introduced in each sample sequence.

METHODS

Synthesis of carbon nanofibers

According to the optimized synthesis conditions, 10 mL of an aqueous solution containing 36.6 mg of NiSO₄ were slowly sprayed over the four alumina plates (75 x 15 mm) heated at 200 °C, in order to homogeneously cover their surface. NiSO₄ were sprayed over both sides of alumina plates. For each batch of carbon nanofibers, twelve sprayed alumina plates were placed inside the tubular reactor (inner diameter 45 mm) within a quartz holder properly designed to maximize the exposed area. The tubular reactor is placed within a furnace to homogeneously heat the materials. Prior each treatment, the reactor was purged from air by an Ar flow (200 mL min⁻¹) for 30 minutes. Subsequently, the furnace was heated at 800 °C (10 °C min⁻¹) maintaining the Ar flow in the reactor. After stabilizing the final temperature for 15 minutes, the gas flow was switched to H₂ (140 mL min⁻¹) to completely reduce the Ni salt forming Ni nanoparticles on the surface of the Al₂O₃ support. After reduction for 1 hour, CH₄ (60 mL min⁻¹) was added to the H₂ flow reaching a total flow of 200 mL min⁻¹ and linear velocity of ~ 12.6 cm min⁻¹. The H₂/CH₄ mixture was flowed within the reactor for 8 hours in order to grow the carbon nanofibers. Then, the furnace was purged by an Ar flow (200 mL min⁻¹) for 15 minutes and cooled. To avoid oxidation and/or partial degradation of the products by accidental exposure to air, the reactor was left to stabilize at room temperature overnight before being opened to remove the Al₂O₃ plates with the carbon nanofibers grown on them. The carbon nanofibers were detached from the alumina plates by sonication in EtOH 96% for 1 hour and subsequently collected by centrifugation at 4500 rpm for 30 minutes. Then, the fibers were suspended in aqueous HCl 5% for 24h, in order to remove the accessible Ni nanoparticles, and washed twice with EtOH 96%. Finally, the material was dried at 60 °C overnight.

The preparation of ¹³C-based carbon nanofibers has been performed following the same procedure but replacing CH₄ with ¹³CH₄.

Synthesis of Few-Layer Graphene

Different milling parameters (time and rpm) were used to determine the best conditions of exfoliation of the carbon fibers, using ¹²C-CNF as a model for further implementation with ¹³C nanofibers (See supporting info, **Table S1**).

Synthesis of Graphene Oxide

Graphene oxide was prepared using graphitized carbon nanofibers as starting material using 1 mg of ^{13}C -GCNF or ^{12}C -GCNF, 1 ml of H_2SO_4 and 1 mg KMnO_4 , stirred for 1 h at room temperature and 6 hr at 70°C . After this time, the material was washed with water until getting a neutral pH.

Subcellular fractionation

HepG2 cells were plated into T25 flasks and incubated for 7d with $10\ \mu\text{g mL}^{-1}$ of ^{13}C or ^{12}C -Few-layer graphene materials (^{12}C -GFLG-1 and ^{13}C -GFLG-1). Then, to ensure the full removal of adsorbed graphene materials, cells were washed with Hanks solution, detached with trypsin and plated again into T25 flasks for another 24h. After this time, cells were scraped and lysed using $500\ \mu\text{L}$ pH 7.4 fractionation buffer (250 mM Sucrose, 20 mM Hepes 10 mM KCl, 1.5 mM MgCl_2 , 1mM EDTA, 1mM EGTA, 1mM dithiothreitol) supplemented with 1% protease inhibitor cocktail. Lysate were passed through a 25G needle 10 times using 1 mL syringe and incubated on ice for 20 minutes. Lysates were centrifuged at 720 g for 5 minutes, obtaining the nuclear and cytoplasm/membrane fractions (pellet and supernatant, respectively). The nuclear fraction (NF; pellet) was washed by adding $500\ \mu\text{L}$ of fractionation buffer, resuspended with a pipette, passed through a 25 G needle 10x and then centrifuged again at 720 g for 10 minutes. Buffer was removed (wash 1) and NF pellet were resuspended in fractionation buffer. Supernatants were centrifuged at 10.000 g, obtaining the mitochondrial (MF) and cytoplasm/membrane fraction (pellet and supernatant, respectively). MF was washed by adding $500\ \mu\text{L}$ of fractionation buffer, resuspended with a pipette, passed through a 25 G needle 10x and then centrifuged again at 10.000 g for 10 minutes. Buffer was removed (wash 2) and mitochondrial pellet were resuspended in fractionation buffer. Finally, cytoplasm/membrane fraction was centrifuge at 100,000 g for 1 hour. Supernatant corresponds to cytoplasm fraction. For membrane fraction, the pellet was washed by adding $400\ \mu\text{L}$ of the fractionation buffer, and then re-centrifuge for 45 min. All the fractions were freeze-dried using a Telstar Lyoquest.

As a control, to completely exclude the possible contamination of graphene materials between the subcellular fractions, ^{13}C GFLG-1 was added to untreated cells immediately before the fractionation process (avoiding the incubation step).

Quantification of ^{13}C - graphene materials in subcellular organelles

To quantify the amount of graphene materials entering inside the different organelles, the extracted pellets were lyophilized to obtain a dry powder. The samples were analyzed by IRMS and the results were provided as δ values (**Table 2**). The δ value was converted into $^{13}\text{C}/^{12}\text{C}$ ratio (r) following equation 4, where the $(^{13}\text{C}/^{12}\text{C})_{\text{standard}}$ was 0.0111802, the ratio of the Vienna Pee Dee Belemnite (VPDB) standard sample. $\omega_{^{13}\text{C}}$ (equation 5) is the percentage of ^{13}C in mass (total weight of ^{13}C atoms/ total weight of carbon atoms), thus $\omega_{^{13}\text{C}}$ (organelle fraction) is the percentage of ^{13}C in mass for the organelle fraction from cells exposed to ^{13}C -FLG; $\omega_{^{13}\text{C}}$ (control) is the percentage of ^{13}C in mass for the organelle fraction from control cells. $\omega_{^{13}\text{C}}$ -FLG (the

percentage of ^{13}C in mass in ^{13}C -FLG) was calculated from Raman data, obtaining a value of 68,5% for ^{13}C -GFLG-1.

The amount of ^{13}C -FLG in the different organelle fractions ($m^{13}\text{C-FLG}$) can be calculated from equation 6, where ω_{carbon} is the content of carbon in each fraction obtained by IRMS (**Table 2**), m_{wet} and m_{dry} are the weights of the organelle fractions before and after lyophilising.

The content of ^{13}C -FLG in the different organelles were also expressed as percentage of applied dose (%ID, equation 7).

$$\text{eq. 4.} \quad r = \left(\frac{\delta}{1000} + 1 \right) \times \left(\frac{^{13}\text{C}}{^{12}\text{C}} \right)_{\text{standard}}$$

$$\text{eq. 5.} \quad \omega_{13\text{C}} = \frac{r \times 13}{r \times 13 + 12} \times 100\%$$

$$\text{eq. 6.} \quad m^{13}\text{C-FLG} = \frac{[\omega_{^{13}\text{C}}(\text{organelle fraction}) - \omega_{^{13}\text{C}}(\text{control})] \times (\omega_{\text{carbon}} \times m_{\text{organelle}} \times \frac{m_{\text{dry}}}{m_{\text{wet}}})}{\omega_{^{13}\text{C-FLG}}}$$

$$\text{eq. 7.} \quad \%ID = \frac{m_{^{13}\text{C-FLG}}}{\text{dose}} \times 100\%$$

SUPPORTING INFORMATION

Additional experimental details of the synthesis of graphene, including characterization XRD, TGA, XPS, TEM of carbon nanofibers; Raman, TGA, TEM characterization of ^{12}C graphene and, colloidal stability of the synthesized nanomaterials are available from the authors.

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ABBREVIATIONS

FLG	Few-layer graphene
GO	Graphene oxide
NMR	Nuclear magnetic resonance
TGA	Thermogravimetric analysis

XPS	X-ray photoelectron spectroscopy
XRD	X-ray powder diffraction
IRMS	Isotope ratio mass spectrometry
ICP-MS	Inductively coupled plasma-mass spectrometry
TEM	Transmission electron microscopy
MRSI	Magnetic resonance spectroscopic imaging
CVD	Chemical vapor deposition
FWHM	Full width at half maximum
NF	Nucleus
CF	Cytoplasm
MmF	Membranes
MF	Mitochondria
MAS-NMR	Magic Angle Spin Nuclear Magnetic Resonance
TCD	Thermal conductivity detector
VPDB	Vienna Pee Dee Belemnite

AUTHOR CONTRIBUTION

V.J.G. synthesized and characterized FLG and GO. J.F.R. performed the biological studies. T.M. synthesized carbon nanofibers. M.V.G. performed C-13 MAS NMR studies. E. V. designed the methodology and conceptualized the paper. V.J.G. E.V. J.F.R. and M.P. wrote the paper. E.V. M.P. P.F. M.V.G. and M.D.P. participated in analysis of the experimental data, interpretation and correction of the paper. E.V. and M.P. conceived this work. All authors discussed the results, revised the final manuscript and gave the final approval.

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