Oxidative biodegradation of single- and multi-walled carbon nanotubes†

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In this study we compare the biodegradation of both single-walled (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) using two different oxidative conditions. In particular, we demonstrate that oxidized multi-walled carbon nanotubes are highly degraded, although not to completeness when treated with horseradish peroxidase (HRP) in the presence of hydrogen peroxide.

The applications of carbon nanotubes (CNTs), covering many different domains including materials science and biomedicine, are rapidly expanding.[1–7] Health impact, bio-persistence and environmental accumulation are currently considered as key issues for the development of this class of nanomaterial.[8–10] The increasing industrial production of CNTs, and particularly of multi-walled carbon nanotubes, is raising many concerns about their fate. Indeed, it is difficult at the moment to anticipate the amount of nanotubes that will come in contact with air, soil, water and living species in the near future. As a consequence, it is of fundamental importance to explore the possibility that CNTs could be degraded in some conditions, ideally by the action of microorganisms or at cellular level as regards biomedical applications.[11] It is noteworthy that, although an active field of research is focussed on the biocompatibility of these nanomaterials, little has been done to explore the possibility of their biodegradation. In particular, low toxicity of CNTs has been correlated to the presence of functional groups on the external surface of the tubes.[12,13] Moreover, experimental data on the accumulation in different organs and the elimination of CNTs through urine and bile partly support their bioavailability.[14–16]

Although studies involving living organisms potentially able to degrade CNTs are still missing, evidence that oxidative conditions can induce degradation of CNTs has recently appeared as proof-of-concept.[17–20] It has been demonstrated that single-walled carbon nanotubes can be biodegraded by exogenous and endogenous enzymes or by fluids mimicking phagolysosome content.[17,20] Star and colleagues have recently devised a method for the degradation of carboxylated SWCNTs. Their approach is based on the catalytic activity of horseradish peroxidase (HRP) in the presence of low concentration of hydrogen peroxide.[17,18] In 10 days nearly all the nanotubes were degraded. This finding was further supported by a detailed analysis of the enzymatic degradation. Oxidized aromatic fragments were detected in the course of the nanotube degradation which eventually evolved to carbon dioxide. SWCNTs can be also degraded inside cells such as neutrophils and macrophages by action of myeloperoxidase.[19] This result has important implications on the inflammatory responses induced by inhaled CNTs and to a wider extent on their use as vehicle for drug delivery and targeted therapies. Cellular degradation of double-walled CNTs (DWCNTs) was envisaged in another recent study.[21] Evidence of the intracellular modifications of DWCNTs has been obtained by mapping the Raman signature of this type of tubes. More precisely, defects seemed to accumulate on the external graphene sheet of the nanotubes after internalization by human prostate adenocarcinoma (PS3) cells. Alternatively, bio-persistence of SWCNTs was evaluated *in vitro* by simulating the phagolysosomal conditions (corresponding to a mild physiological oxidizing environment).[20] In the latter study, a clear dependence on the type of CNT functionalization was observed. In fact, the presence of carboxylic functions and defects on the graphitic surface likely offers the site of interaction with the oxidative agents that are responsible for the degradation. The process of shortening CNTs often used to improve their dispersibility by introducing carboxylic functions at the tips and the defect sites clearly enhances the biodegradation rate under the oxidative conditions generated by the treatment with hydrogen peroxide.[17,20] Another class of carbon-based nanomaterials comprises graphene oxide (GO), which is entering large-scale production for a wide range of applications. The recent assessment of GO biodegradation by environmental bacteria is of great importance and might pave the way to new bioremediation processes of carbon nanotubes.[22]

To assess the capacity of the different conditions described in the literature to biodegrade a wider variety of nanotubes, we have expanded the study to both single- and multi-walled nanotubes. SWCNTs were obtained from Unidym (SWCNTs **1**) while MWCNTs were purchased from the companies NanoAmor (MWCNTs **2**) and Nanocyl (MWCNTs **3**), respectively. As biodegradation increased with the introduction of functional groups, we initially submitted the nanotubes to a chemical oxidation treatment.[23–25]

Table 1 displays the characteristics of the three types of nanotubes used in this study. These CNTs were oxidized under strong acidic conditions as previously reported.[23–25] Following this treatment, the tubes contained between 1.7 and 2.5 mmol g^{-1} of COOH as assessed by thermogravimetric analysis. *ox*-SWCNTs **1** and *ox*-MWCNTs **2** and **3** were then dispersed in two different buffers enriched by the addition of H_2O_2. The nanotubes were observed by transmission electron microscopy (TEM) at different time points up to two months. Their characteristics were further analyzed by Raman spectroscopy and dynamic light scattering (DLS). Although it was

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Table 1 Characteristics of the CNTs before biodegradation

	Starting nanotubes		Oxidized nanotubes[a]		
Entry	Length/μm	Diameter/nm	Average length/nm	Functional groups/mmol g^{-1}	Weight loss (%)[b]
1, *ox*-SWCNTs	0.1–1	0.8–1.2	ND[c]	2.5	11
2, *ox*-MWCNTs	0.5–2	20–30	403	1.7	7.3
3, *ox*-MWCNTs	1.5	9.5	372	1.9	8.3

[a] SWCNTs (**1**) and MWCNTs (**2** and **3**) were oxidized for 73 h and 24 h, respectively. [b] Calculated by TGA at 350 °C. [c] ND not determined.

previously shown that changes in the colour of the solutions were indicative of the disappearance of the nanotubes, we could not rely on this observation, as the solutions never became colourless, independently of the type of nanotubes and conditions.

In this comparative experiment, we evaluated the effects of two different conditions on CNT morphological features. First, *ox*-SWCNTs **1**, *ox*-MWCNTs **2** and **3** have been subjected to the degrading action of an enzyme-free reaction buffer corresponding to the phagolysosomal simulant fluid (PSF). This buffer has been shown to mimic the chemical environment of phagolysosomes,[26] where nanomaterials are located after phagocytosis.[27] To fully simulate the physiological oxidizing environment of phagolysosomes, H_2O_2 was added weekly.

A second experimental protocol has been carried out by incubating the nanotubes with HRP.[17] To enable the enzymatic activity, H_2O_2 was added daily. All the solutions were kept in the dark and stirred for the entire duration of the experiment. Samples (500 μL) were drawn at time 0, 1, 7, 15, 30 and 60 days, washed three times with a mixture of methanol and diethyl ether (1 : 3), resuspended at the initial volume (500 μL) in distilled water and stored at 4 °C in the dark until characterization.

During the entire period of incubation, the highly entangled *ox*-SWCNTs, as initially evidenced by the TEM observation, started to disaggregate and degrade. The degree of degradation of these tubes followed different evolutions in PSF or in the presence of HRP. TEM images support the changes in the morphology of SWCNTs. In addition, little debris, pieces of nanotubes, and carbonaceous nanoparticles associated to the graphitic tubular structure were detected by TEM (Fig. 1). After 30 days SWCNTs are highly deteriorated in PSF and completely destroyed by the enzymatic treatment, similarly to the data reported in the literature.[17,20] To follow the evolution of the length of the nanotubes dispersed in oxidizing solutions, we used DLS (see ESI, Fig. S1†). Although the theoretical model applied to measure the hydrodynamic radius is only adapted to spherical objects, the physical modifications on the degrading tubes allow to qualitatively indicate a variation in their size and aggregation state. It is obvious from the DLS curves that nanoobjects or globular material of smaller dimensions are present in solution after 60 days. However,

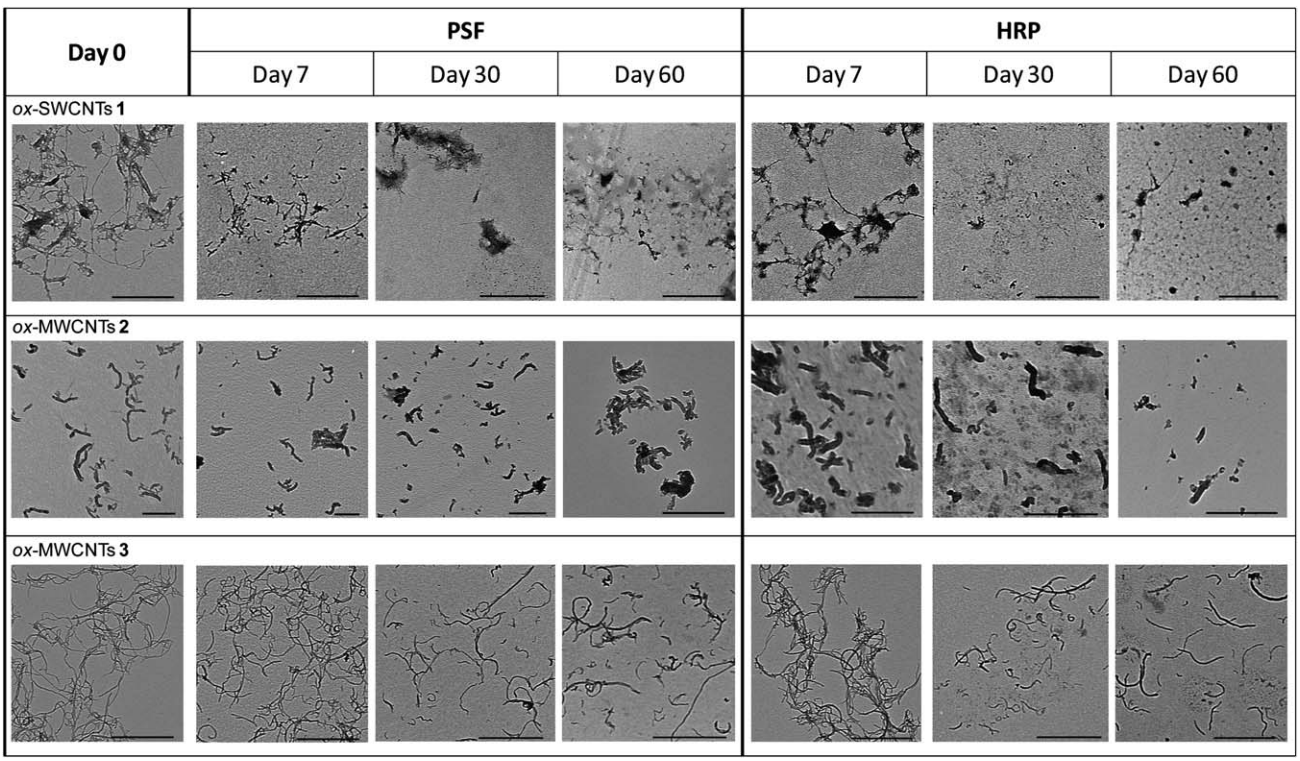

Fig. 1 TEM images of *ox*-SWCNTs **1** (top), *ox*-MWCNTs **2** (middle) and **3** (bottom) during the degradation process. The samples were observed after 7, 30 and 60 days in both oxidizing conditions and compared to the starting material. Scale bars correspond to 500 nm.

from the DLS data we could not correlate these entities to the presence or the absence of short nanotubes.

To further verify that SWCNTs followed a degradation process, Raman spectroscopy provided clear evidence that SWCNTs underwent strong modifications. We have only reported Raman spectra normalized to the G band because absolute values should be normalized on the volume of homogenous samples, which are very difficult to obtain. Therefore, the spectra displayed in Fig. 2 allow the monitoring of the qualitative evolution of the oxidation of the nanotubes rather than the quantitative determination of their presence. The starting *ox*-SWCNTs **1** already exhibit an intense D band (~1335 cm^{-1}) associated to the functional groups and defects generated by the initial acidic treatment (Fig. 2, top). A progressive decrease of the D band during the process indicates that the nanotubes with the highest amount of defects are degraded first. The complete disappearance of both G and D bands after 60 days points out that PSF and HRP conditions are efficient in destroying carboxylated SWCNTs (Fig. 2, top). The fact that phagolysosomal simulant fluid destroys almost all oxidized SWCNTs after 30 days in comparison to the data reported by Liu *et al.* is certainly due to the high amount of carboxylic functions already present in our starting material.[20] This further supports the conclusions of Liu *et al.* that functionalization affects the degradation process of SWCNTs. Raman spectra indicated also a fastest degradation kinetics for the nanotubes treated with HRP (less than one month).

To extend the study to other types of CNTs, we explored the behavior of two different batches of oxidized multi-walled nanotubes

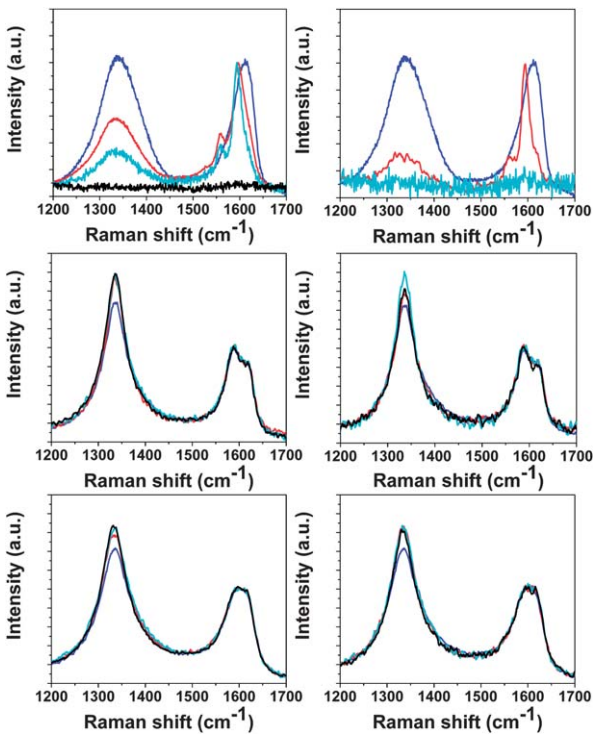

Fig. 2 Raman spectra of *ox*-SWCNTs **1** (top), *ox*-MWCNTs **2** (middle) and **3** (bottom) during the degradation process. Left column corresponds to the CNTs treated with PSF, while right column corresponds to the CNTs treated with HRP. The samples were observed after 7 (red), 30 (cyan) and 60 days (black) using both oxidizing conditions. Blue lines correspond to spectra of the starting materials. All spectra have been normalized on the G band.

which were again incubated in PSF and HRP solutions. These nanotubes, obtained from NanoAmor and Nanocyl, differ mainly by their diameter. While NanoAmor's tubes have a diameter between 20 and 30 nm, Nanocyl's material has a smaller diameter (9.5 nm).[23,25] Their morphology is also different as MWCNTs **3** seem more regular and less distorted. A preliminary observation of the reacted tubes using DLS provided evidence of changes in the aggregation and eventually morphological state of both *ox*-MWCNTs **2** and **3** (see ESI, Fig. S2 and S3†). The characterization by TEM clearly showed that *ox*-MWCNTs are changing (Fig. 1). In particular, the treatment with HRP is the most effective in degrading the nanotubes. Comparing the activity of PSF and HRP on both types of tubes, it is clear that *ox*-MWCNTs **2** (from NanoAmor) are more affected (Fig. 1, middle). This is not surprising as these tubes are more defective than those obtained from Nanocyl. To further understand the morphological changes of *ox*-MWCNTs, the samples collected after 7, 30 and 60 days were analyzed by Raman spectroscopy. This technique is generally useful for the characterization of multi-walled nanotubes to put in evidence the variation of the intensity of the D to the G bands and therefore the defect density on the nanotubes (Fig. 2, middle and bottom). Although the D band is already very intense on both *ox*-MWCNT samples, there is a significant increase of this band in the timeframe of the experiment. The differences between the samples are subtle, nevertheless we can assert that the nanotubes are undergoing major changes in their structures. It seems that the biodegrading action in the presence of hydrogen peroxide is creating more defects on the nanotubes rather than exfoliating their external surface. If we compare the results of the Raman spectra and the images obtained by TEM, it is clear that the nanotubes are much shorter and therefore they might have a higher amount of defects. Curiously, some TEM images seem to support also exfoliation although it was not observed thoroughly (see ESI, Fig. S4†). As this type of nanotubes is not completely degraded after two months, we have compared the length of the tubes at the end of the experiments with the starting material. Fig. 3 displays the statistical length distribution, as measured by TEM, of both types of *ox*-MWCNTs treated either with PSF or HRP. *ox*-MWCNTs **2** became very short by action of HRP. Indeed the average length decreased from 403 nm to 148 nm with more than 65% of the nanotubes shorter than 150 nm. PSF seems instead less effective on these tubes with an average length around 310 nm. In the case of the *ox*-MWCNTs **3** from Nanocyl, the PSF resulted more effective (average length 188 nm) than HRP (average 280 nm). Although the mean length of MWCNTs **3** decreased compared to the starting material (372 nm), they remain on average longer than the tubes from NanoAmor. The higher efficiency of our *in vitro* degradation conditions on the NanoAmor tubes is probably correlated to their more defective structure than Nanocyl tubes and their higher amount of carboxylic functions at the surface. Finally, it is important to note that the degrading action is less efficient on both *ox*-MWCNTs which are not completely degraded after two months in comparison to the *ox*-SWCNTs. This is not surprising as the number of concentric layers in MWCNTs is more important.

The mechanism of degradation seems also different between SWCNTs and MWCNTs, as the latter appear to be extensively shortened rather than unzipped, although we observed a few examples in which the nanotubes are peeled off (see ESI, Fig. S4†). In the TEM images of MWCNTs we also observed many small pieces and debris of nanotubes that we did not take into account in the calculation of the length distribution. These carbonaceous fragments are,

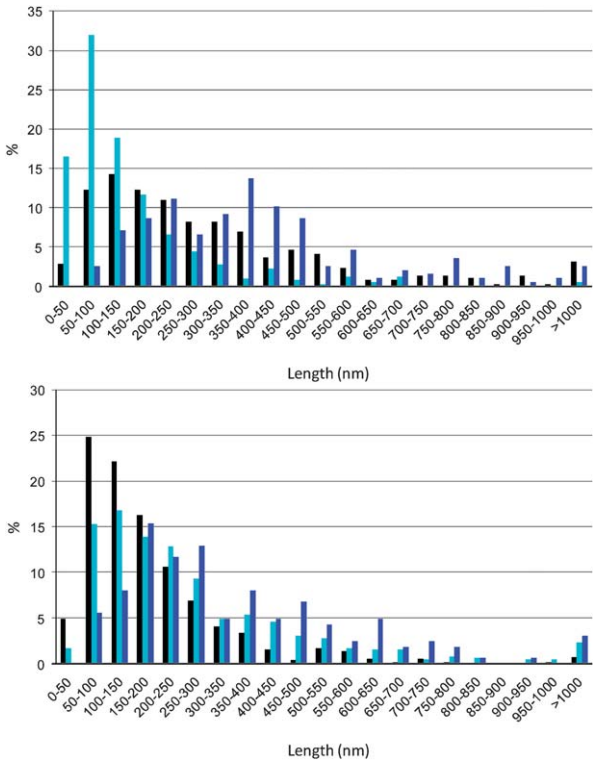

Fig. 3 Statistical distribution of the length of the *ox*-MWCNTs **2** (top) and **3** (bottom) at the end of the degradation process (60 days). Blue bars correspond to the starting material. Black and cyan bars correspond to the PSF and HRP treatment, respectively.

however, indicative of an efficient oxidative and enzymatic action. The morphology of the starting nanotubes influences the rate of degradation as the "more perfect" Nanocyl's tubes are less destroyed. To further explore this behaviour, we are also currently analyzing the degradation of other types of functionalized carbon nanotubes not only bearing simple carboxylic functions. This is of fundamental importance in view of the development of this type of material for biomedical applications.[11]

Conclusions

The contribution of the present work consists in the assessment of the degrading action on carboxylated/shortened multi-walled carbon nanotubes in a comparative analysis with single-walled nanotubes. We have used two types of oxidative conditions, namely PSF and HRP in the presence of hydrogen peroxide. In both conditions, *ox*-SWCNTs are rapidly degraded, especially when using HRP. After two months *ox*-MWCNTs are highly modified on their morphology and highly degraded although not completely. The possibility that CNTs undergo a degradation process is not only unique to SWCNTs but it is also demonstrated for MWCNTs. This finding supports and encourages future developments of biodegradable functionalized MWCNTs towards applications in different fields.

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