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Degradation-by-design: Surface modification with functional substrates that enhance the enzymatic degradation of carbon nanotubes

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ABSTRACT

Biodegradation of carbon-based nanomaterials has been pursued intensively in the last few years, as one of the most crucial issues for the design of safe, clinically relevant conjugates for biomedical applications. In this paper it is demonstrated that specific functional molecules can enhance the catalytic activity of horseradish peroxidase (HRP) and xanthine oxidase (XO) for the degradation of carbon nanotubes. Two different azido coumarins and one cathecol derivative are linked to multi-walled carbon nanotubes (MWCNTs). These molecules are good reducing substrates and strong redox mediators to enhance the catalytic activity of HRP. XO, known to metabolize various molecules mainly in the mammalian liver, including human, was instead used to test the biodegradability of MWCNTs modified with an azido purine. The products of the biodegradation process are characterized by transmission electron microscopy and Raman spectroscopy. The results indicate that coumarin and catechol moieties have enhanced the biodegradation of MWCNTs compared to oxidized nanotubes, likely due to the capacity of these substrates to better interact with and activate HRP. Although azido purine-MWCNTs are degraded less effectively by XO than oxidized nanotubes, the data uncover the importance of XO in the biodegradation of carbon-nanomaterials leading to their better surface engineering for biomedical applications.

1. Introduction

The assessment of the biodegradation of carbon-based nanomaterials, including carbon nanotubes and graphene, is becoming one of the key issues in the development of these materials in the biomedical domain. Only recently, carbon nanotubes were believed to be biopersistent and resistant to almost any type of enzyme [1]. The discovery of the degradation of carbon nanomaterials by oxidative enzymes has opened new possibilities in their use and in their fate after *in vivo* administration [2–4]. In a seminal work, the group of Star illustrated that oxidized single-walled carbon nanotubes (SWCNTs) could be degraded by the plant enzyme horseradish peroxidase (HRP) [5]. Since then, various research groups started to explore the possibilities of degradation by other enzymes using different conditions and different types of carbon nanotubes [6–8]. It has been demonstrated that intracellular peroxidases, such as myeloperoxidase (MPO) [9,10] and eosinophil peroxidase (EPO) [11], are capable to degrade oxidized SWCNTs. Biodegradation of CNTs has been also demonstrated using microbial cultures including bacteria and fungi [12,13]. Biodegradation by MPO and EPO was assessed *ex vivo* using primary murine neutrophils and eosinophils [9,11,14,15]. Other types of immunocompetent cells, for example, macrophages or microglia, follow instead a different route to degrade oxidized SWCNTs [16,17]. Activated lung macrophages are able to oxidatively digest nanotubes using the superoxide/peroxynitrite oxidative pathway, leading to the generation of potent peroxonitrites and subsequent clearance of the nanomaterials from the organs [16].

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Recently, alternative enzymes capable of degrading pristine SWCNTs but not oxidized nanotubes have also been discovered [18,19]. Manganese peroxidase for example was shown to degrade non-functionalized nanotubes [19]. This finding expands the panel of oxidative enzymes and proves that pristine nanotubes can also undergo enzymatic transformation. It has also been repeatedly reported that SWCNTs can be degraded *in vitro* and *in vivo* by the neutrophil MPO [9,14,15]. The elucidation of such catalyzed enzymatic degradation is still not fully understood. For this purpose antioxidants have been used to evaluate their effect on the pathway of oxidized SWCNT degradation by MPO [20]. Antioxidants like ascorbic acid and glutathione suppress the capacity of the peroxidase to degrade the nanotubes, therefore it is important to take into consideration their use if the oxidative stress induced by the nanotubes needs to be regulated as this can counterbalance the biodegradation potential [20]. In an attempt to elucidate the mechanism of degradation, a recent report has shown that the degree of biodegradation is enhanced by the interaction with human serum albumin [21]. This protein, highly abundant in blood, can form a complex with the carboxylic groups of oxidized SWCNTs and trigger the damage process of the tubes into neutrophils via an ameliorated cellular uptake, the stimulation of MPO release and the production of hypochlorous acid [21]. More recently, SWCNTs coated or functionalized with different PEG chains were demonstrated to undergo a combined process of de-functionalization (called stripping) and biodegradation *in vitro* using MPO or *ex vivo* using freshly isolated primary human neutrophils, reinforcing the concept of CNTs as degradable nanostructures [22]. Surface coating and functionalization are also aimed to reduce the risks of toxicity of carbon nanotubes [23,24]. We have demonstrated that the pathogenic effects of long nanotubes, that hold high similarity with asbestos fibers, can be attenuated by an appropriate functionalization [24]. The degree of functional groups plays also an important role in the biodistribution of CNTs [25]. Along the same direction, biocompatible molecules like polyethylene glycol or bovine serum albumin greatly reduced the toxicity not only of carbon nanotubes but also of graphene, the 2D form of carbon nanomaterials [26,27]. Functionalization with these types of molecules, however, seems to provoke a reduction of graphene biodegradability that can be re-enhanced if the bonds between the macromolecules and the nanomaterial contain a cleavable intracellular sensitive linker [28]. More recently, Mata et al. highlighted the importance of surface functionalization of CNTs, preparing CNTs modified via Diels–Alder cycloaddition reaction with 1,3-butadiene and further oxidized to generate carboxylic acid groups. The functionalized CNTs showed better biocompatibility as well as faster biodegradability over pristine CNTs when they were implanted into mice [29]. Thus, these studies encourages the design of suitable functionalizations to modulate the toxic effects and to ensure at the same time the biodegradability of the carbon nanomaterials. Inspired by these works, we were interested to explore the degradation potential of oxidized multi-walled carbon nanotubes (MWCNTs), as they represent our benchmark material for a wide range of biomedical applications including therapy, imaging and diagnosis [30–33]. Until now, no work has been reported aimed to enhance the peroxidase activity by functionalizing the surface of CNTs with specific molecules that are known to interact well with the enzymes, and augment their catalytic activity. Thus, we have selected coumarin and catechol derivatives that were reported to interact with HRP, acting as good reducing substrates for HRP [34,35]. One challenging approach to enhance the process of CNT degradation is to introduce functional molecules on the material surface capable to stimulate the activity of peroxidases or other oxidative enzymes. To prove this concept, we have conceived and designed new carbon nanotubes functionalized with selected moieties that stimulate the activity of HRP and xanthine oxidase (XO), another oxidative enzyme very little explored in the biodegradation of CNTs [16].

2. Experimental section

2.1. Synthesis of MWCNT **1**

First, MWCNT-Alkyne (7 mg) and 3-azido-7-hydroxycoumarin (14 mg) were dispersed in a mixture of THF/H_2O (3:1, 4 mL) and the suspension was sonicated in a water bath for a few minutes. The mixture was flushed under argon for 15 min and then catalytic amounts of $CuSO_4 \times 5H_2O$ and sodium ascorbate previously dispersed in 1 mL each of water were added to the dispersion. The reaction was left under stirring for 48 h. Then, the reaction mixture was filtered using Omnipore® membrane filtration from Millipore (0.1 μm). The solid was dispersed in 5 mL of DMF, sonicated for 5 min in a water bath and the suspension was filtered. This procedure was repeated once using DMF, and twice with methanol, DCM, and deionized water. Finally, the solid was dried under vacuum.

2.2. Synthesis of MWCNT **2**

MWCNT-Alkyne (7 mg) and 4-methyl-7-(azidopropyl)-coumarin (14 mg) were dispersed in a mixture of THF/H_2O (3:1, 4 mL) and the suspension was sonicated in a water bath for a few minutes. The mixture was flushed under argon for 15 min and then catalytic amounts of $CuSO_4 \times 5H_2O$ and sodium ascorbate previously dispersed in 1 mL each of water were added to the dispersion. The reaction was left under stirring for 48 h. Then, the reaction mixture was filtered using 0.1 μm membrane. The solid was dispersed in 5 mL of DMF, sonicated for 5 min in a water bath and the suspension was filtered. This procedure was repeated using THF, deionized water, DMF, methanol and DCM. Finally, the solid was dried under vacuum.

2.3. Synthesis of MWCNT **3**

MWCNT-Alkyne (12 mg) and 9-(3-azidopropyl)-purine (24 mg) were dispersed in a mixture of THF/H_2O (9:1, 10 mL) and the suspension was sonicated in a water bath for a few minutes. The mixture was flushed under argon for 15 min and then catalytic amounts of $CuSO_4 \times 5H_2O$ and sodium ascorbate previously dispersed in 1 mL each of water were added to the dispersion. The reaction was left under stirring for 48 h. Then, the reaction mixture was filtered using 0.1 μm membrane. The solid was dispersed in 30 mL of DMF, sonicated for 5 min in a water bath and the suspension was filtered. This procedure was repeated twice using methanol and once using deionized water and DCM. Finally, the solid was dried under vacuum.

2.4. Synthesis of MWCNT **4**

A solution of 3,4-dihydroxybenzoic acid (0.100 g) and thionyl chloride (0.277 g, 3.6 eq.) was heated at reflux for 24 h under argon atmosphere. Then, excess $SOCl_2$ was removed under reduced pressure and a suspension of amino-MWCNT (12 mg) dispersed in dry THF (25 mL) was added. The mixture was heated at reflux for 48 h under argon atmosphere. The reaction mixture was filtered using 0.1 μm membrane. The solid was dispersed in DMF (10 mL), sonicated for 5 min in a water bath and filtered. This procedure was repeated once again with DMF, twice with methanol and once with DCM. Finally, the solid was dried under vacuum.

2.5. Enzymatic degradation of oxMWCNT, MWCNT **1**, **2** and **4** by HRP

A suspension of oxMWCNT, MWCNT **1**, **2** or **4** (10 or 20 μg/mL) in 2.5 mL of phosphate-buffered saline (PBS) was sonicated in a water bath for 1 min, followed by the addition of HRP (4 mg, corresponding to 208.8 U/mL enzyme activity, Sigma–Aldrich) solubilized in 2.5 mL of PBS. To enable the enzymatic activity, 15.5 μL of H_2O_2 (550 μM) were added six times per day from day 1 to day 10 and 13.3 μL (six times per day) from day 11 to day 20. The suspensions were kept in the dark and stirred for the entire duration of the experiment. Aliquots (500 μL) were taken at time 0, 10 and 20 days and stored at −20 °C in the dark until characterization by TEM and Raman spectroscopy.

2.6. Degradation of oxMWCNT and MWCNT **3** by XO

A suspension of oxMWCNT or MWCNT **3** (20 μg/mL) in 2.5 mL of PBS was sonicated in a water bath for 1 min followed by the addition of microbial XO (6.25 mg, corresponding to 10 U/mL enzyme activity, Sigma–Aldrich) solubilized in 2.5 mL of PBS. A solution (15.5 μL) of H_2O_2 (550 μM) was added six times per day from day 1 to day 10 and 13.3 μL (six times per day) from day 11 to day 20. The dispersions were kept in the dark and stirred for the entire duration of the experiment. Aliquots (500 μL) were taken at time 0, 10 and 20 days and stored at −20 °C in the dark until characterization by TEM and Raman spectroscopy.

2.7. Transmission electron microscopy

In Fig. S1, the observations were performed using TEM grids onto which few microliters of a suspension of the MWCNT samples in MeOH/H_2O (1:1) were previously deposited and left for drying. The TEM images of Fig. 2 were taken using grids onto which a few microliters of aliquots of the degradation reactions were previously deposited and left for drying. In some cases, the observation of the MWCNTs was difficult due to the presence of salts and the enzyme. Therefore, the TEM grids were washed by adding few microliters of deionized water and by absorbing water with a paper tissue. When this procedure was not efficient enough to purify the grids, the samples were diluted in a mixture of methanol/diethyl ether (1:3) and precipitated by centrifugation. This sequence was repeated three times. Then, the precipitate was resuspended in deionized water (500 μL) by sonication.

2.8. Raman spectroscopy

The samples for Raman analysis were prepared by drop-casting 50 μL of respective samples on Si window (Thorlabs) and left for drying at room temperature.

3. Results and discussion

3.1. Design, synthesis and characterization

Different reports on the enzymatic degradation of multi-walled carbon nanotubes have already indicated the key role of functional groups (i.e. carboxylic acids generated during the oxidation process), defects or heteroatoms (i.e. nitrogen-doped CNTs) in this process [5,6]. Indeed, their presence renders nanotubes highly biocompatible and biodegradable, thus reducing the risks of accumulation into specific organs for prolonged periods of time that could lead to inflammatory events, eventually evolving into more adverse effects [17,25,36]. Another possibility to enhance the capacity of the oxidative enzymes (i.e. peroxidases) to degrade faster the nanotubes and in particular MWCNTs is their functionalization with substrates for these specific enzymes. This is a new approach that has not been previously explored. Such substrates should allow a better interaction between the protein and its ligand, increasing the rate of the degradation process.

We have used MWCNTs from Nanostructured & Amorphous Materials Inc. with an outer and inner average diameter of 20–30 nm and 5–10 nm, respectively. The number of walls (~20–30) was estimated from the sheet interlayer distance (0.34 nm). We have selected a series of substrates for HRP and XO, the latter recently used in combination with xanthine [16]. Coumarin derivatives and catechols (i.e. 3,4-dihydroxybenzoic acid) are good substrates for oxidative enzymes [34,35,37–39]. Catechols act also as redox mediators of this type of enzymes by favoring electron transfer and preventing the enzyme inactivation [35]. Coumarins and catechols have been already described as efficient substrates for HRP, while purines are able to stimulate XO [34,35,37–42]. We have designed and synthesized oxidized multi-walled CNTs and subsequently modified the COOH groups with two coumarin derivatives, with 3,4-dihydroxybenzoic acid and with an azido purine. Scheme 1 illustrates the synthetic strategy based on the functionalization of the nanotubes using click chemistry and amidation reactions. The two coumarins were prepared to evaluate the effect on biodegradation of the chemical modification of the

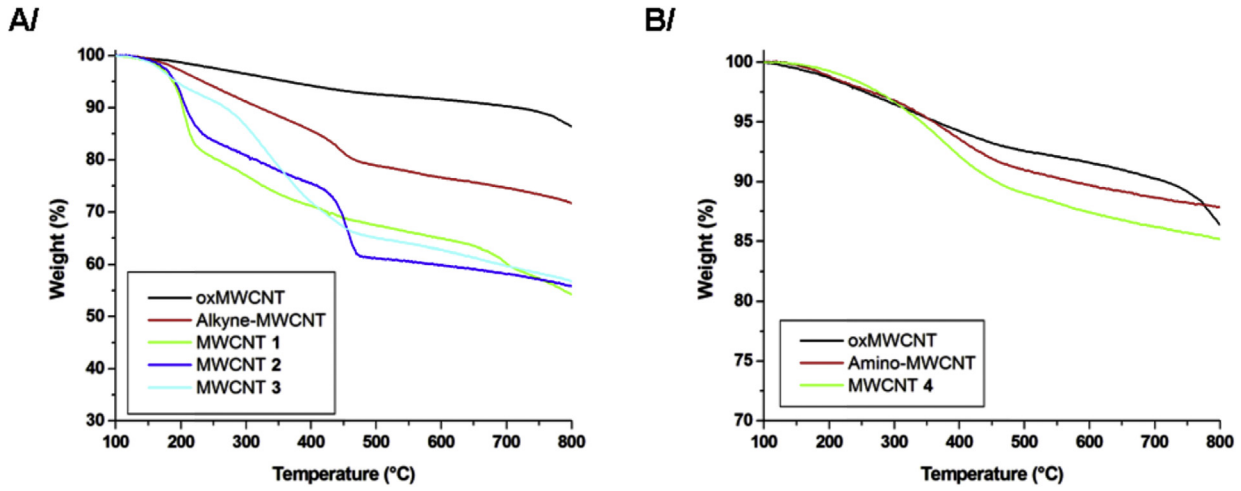


Fig. 1. TGA curves of the different intermediate and final MWCNTs under N_2. A) oxMWCNT, Alkyne-MWCNT and MWCNT **1**–**3**. B) oxMWCNT, Amino-MWCNT and MWCNT **4**.

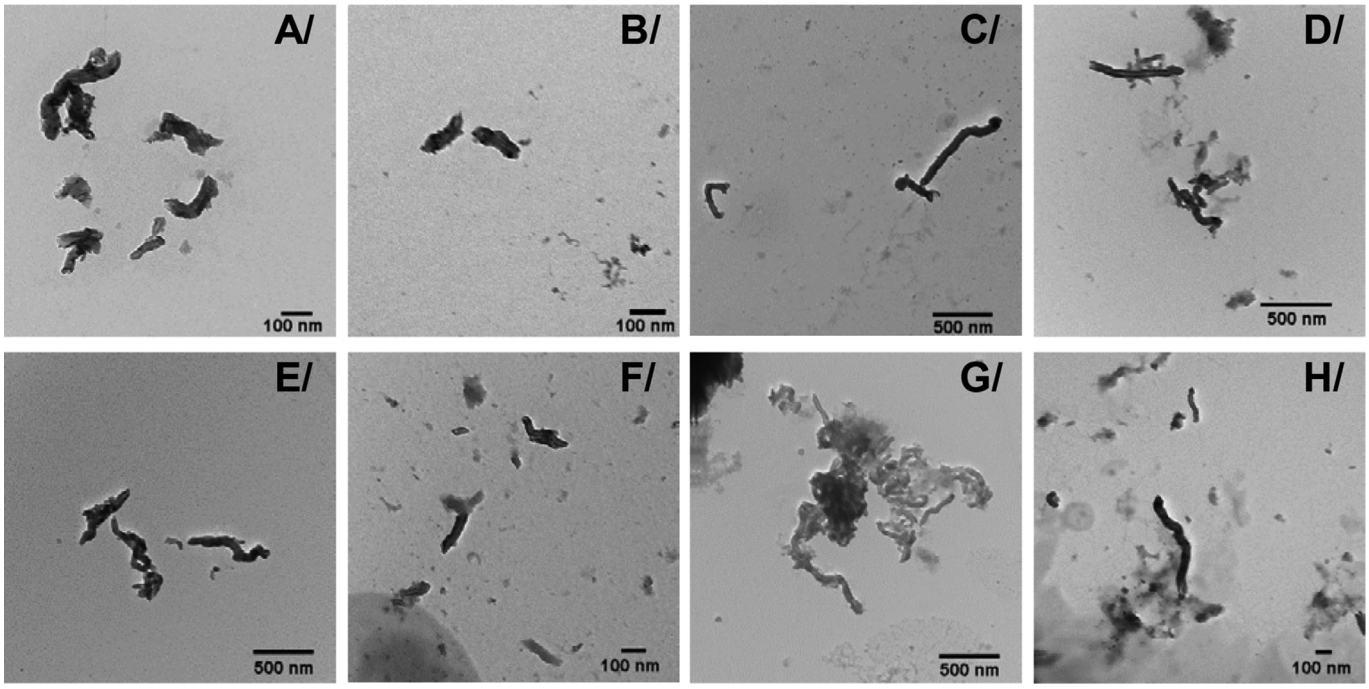
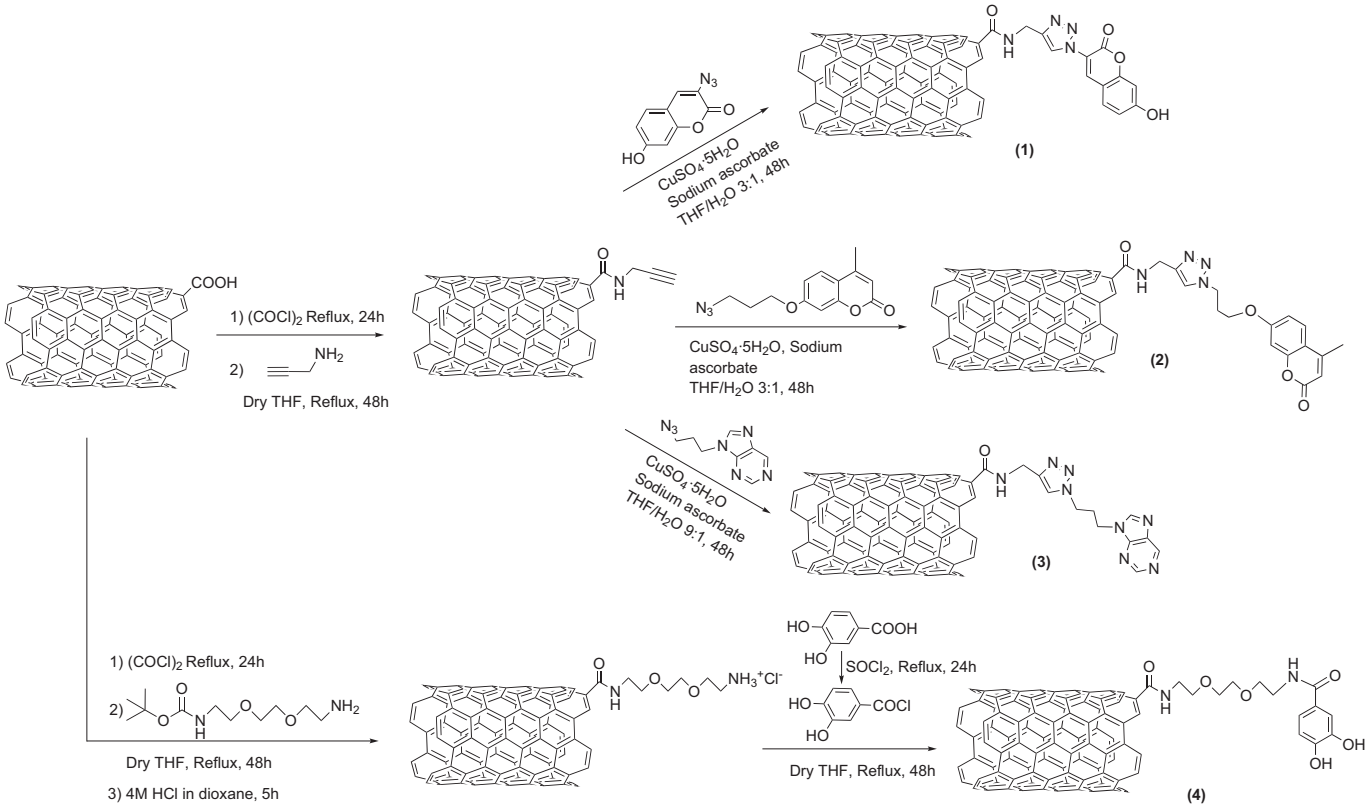


Fig. 2. TEM images of degrading nanotubes at different time points. (A) MWCNT **1** at Day 0; (B) MWCNT **1** at Day 20; (C) MWCNT **2** at Day 0; (D) MWCNT **2** at Day 20; (E) MWCNT **4** at Day 0; (F) MWCNT **4** at Day 20; (G) MWCNT **3** at Day 0; (H) MWCNT **3** at Day 20.

functional groups on the benzopyran core. Commercially available 7-hydroxycoumarin was functionalized with an azide in position 3, while a spacer was introduced on the hydroxyl group in position 7 of 7-hydroxy-4-methyl coumarin (see SI for details). Both molecules were then "clicked" on oxidized MWCNTs modified with an alkyne group (MWCNT **1** and **2**). Dihydroxybenzoic acid was



Scheme 1. Synthesis of the different functionalized MWCNTs.

instead bound to the tubes tethered with a diaminotriethylene glycol chain via an amidation reaction (MWCNT **4**). The substrate for XO was prepared from purine by alkylating the nitrogen in position 3 of the imidazole ring with a 3-azido propyl linker. Azido purine was then "clicked" on alkyne derivatized nanotubes (MWCNT **3**). All functionalized carbon nanotubes and precursors were characterized by complementary analytical techniques. The degree of functional groups was calculated using thermogravimeric analysis (TGA) (Fig. 1). The weight loss for each functionalized CNT was determined at 600 °C.

The amount of coumarin moieties corresponded to 576 and 644 μmol/g for MWCNTs **1** and **2**, respectively. MWCNT **4** were functionalized with 149 μmol/g of catechol derivative, while the amount of purine resulted 679 μmol/g on MWCNT **3**. The morphology of the different functionalized MWCNTs was observed by transmission electron microscopy (TEM) (see SI Fig. S1) and Raman spectroscopy (at 514 nm) was used to characterize all the functionalized MWCNTs. As already demonstrated previously [43], the chemical functionalization did not affect the structure of the nanotubes. The oxidative process resulted in short MWCNTs containing carboxylic groups with an average length of 451 nm (starting length in the range of 0.5–2 μm) containing carboxylic. After oxidation and further functionalization, the MWCNTs were less aggregated in comparison with the pristine MWCNTs. The strong acid treatment did not modify the nanotube diameter (between 20 and 30 nm). The oxidized MWCNTs (oxMWCNT) and the four functionalized nanotube derivatives (MWCNTs **1**–**4**) were subsequently used for the degradation studies.

3.2. Enzymatic degradation

Coumarin and catechol functionalized MWCNTs (**1**, **2** & **4**) were treated with HRP, while purine modified MWCNT **3** was treated with XO to verify if the different substrates were able to enhance the degradability of the tubes. The enzymatic oxidative activity on functionalized nanotubes was compared to that on simply oxidized MWCNTs, previously shown using HRP [6,7]. We then selected XO as an alternative enzyme. XO is widely distributed in the mammalian tissues including humans [44]. In general, XO is known to play a major role in the purine metabolism by oxidizing for example hypoxanthine to uric acid, through generation of reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet -}$) [44]. In addition, XO plays also a crucial role in the innate immune system, especially in the pathogenesis of ischemia-reperfusion. It was demonstrated that the concentration of XO increased in the vascular endothelial cells, subsequently leading to rise in the production of ROS that initiated neutrophil activation and antimicrobial response [45]. The generated different species could interact to give stronger reactive radicals like hydroxy radicals ($HO^{\bullet}$) by the combination of $O_2^{\bullet -}$ with H_2O_2 or with NO to generate peroxynitrite ($ONOO^{-\bullet}$) [46]. These highly potent radicals can damage the tissues by lipid peroxidation or kill the pathogens more effectively [44,47].

We were interested to test the biodegradation of MWCNTs with and without functional purine moieties via oxidative catalysis by XO in a test tube. Control oxMWCNT and functionalized MWCNTs **1**–**4** were treated in the same conditions and the progress of the degradation and the generated by-products were analyzed using TEM and Raman spectroscopy. TEM allowed determination of the average length of the remaining nanotubes and provided evidence for the presence of material debris along with partially exfoliated tubes. MWCNT **1** and **2** functionalized with coumarin and MWCNT **4** with catechol were treated with HRP for 20 days in the presence of H_2O_2. The same conditions were used to assess the degradation of MWCNT **3** functionalized with purine by XO. In a typical experiment, the nanotubes (10 μg/mL) were dispersed in PBS (phosphate buffer saline) followed by regular addition of the enzyme and aliquots of hydrogen peroxide (see Experimental Section for details). The dispersions were kept at room temperature in the dark for the duration of the experiment. Fig. 2 shows the TEM images of MWCNTs **1**, **2** and **4** at day zero (Panel A, C & E, respectively) and the remaining short tubes after 20 days (Panel B, D & F, respectively) in the presence of HRP/H_2O_2. Highly damaged nanotubes, graphitic debris and short tubes can be clearly observed in the TEM images.

TEM micrographs were used to calculate the length of the residual tubes present after 20 days. The dimensions of more than 100 tubes were measured for the different HRP treated dispersions at day 0 and 20 for MWCNT **1**–**4**. Under the action of HRP, after 20 days the average length of oxMWCNT decreased from 451 nm to 328 nm, with 10% of nanotubes shorter than 150 nm (Figs. S2 and S3), whereas for MWCNT **1**, average length was reduced from 451 nm to 226 nm, with 27% of nanotubes shorter than 150 nm (Fig. 3A). These data allowed us to conclude that the hydroxyl coumarin moiety had accelerated the degradation process by enhancing the HRP enzyme activity.

For MWCNT **2**, a higher concentration of nanotubes (20 μg/mL) was used to provide more substrate available for HRP. However, this did not show a clear effect in enhancing the degradative action of HRP in the presence of H_2O_2, when compared to HRP-treated oxMWCNT, as evidence from the TEM analysis. After 20 days the average length of MWCNT **2** decreased from 451 nm to 324 nm with only 9% of nanotubes shorter than 150 nm. From this result we hypothesize that the 7-hydroxyl group on the benzopyran ring of the coumarin is important to enhance the activity of HRP [48]. Indeed, for the derivative covalently bound to MWCNT **1** the hydroxyl group remained free, while for the second type of nanotubes, the hydroxyl function was used to insert the linker for the coupling to the tubes. The −OH group at position 7 of the benzopyran ring seems to play an important role in the enhanced degrading action of HRP, likely acting as an effective donor of radical species [5,6,18]. Our results are in a good agreement with a previously reported work, where 7-hydroxycoumarins were shown to enhance the oxidation of ascorbic acid in the presence of HRP/H_2O_2, while coumarins devoid of 7-hydroxyl group or 4-acetoxylated coumarins lost this activity [48]. Thus, MWCNT **2** which contained a methyl group in the position 4 and lacked the hydroxyl group in the position 7 did not undergo a degradation similar to MWCNT **1**, likely due to the inhibition of the catalytic action of HRP. These 7-hydroxycoumarins were also described as bioactive molecules [48,49], especially in the combination chemotherapy along with other drugs [48], and shown to be oxidized more efficiently by MPO in the presence of H_2O_2, while 4-acetoxy coumarins again were not affected by this enzyme. Based on these observations, we believe that the 7-hydroxy coumarin-conjugated MWCNTs may be used in the combination therapeutic strategies for treating cancers [49]. Indeed, Star and co-workers recently reported that nitrogen doped CNTs could be useful as biodegradable drug delivery chemotherapeutic vehicles, since the nanotubes are degraded by MPO [8]. Alternatively, treatment with HRP of the nanotubes functionalized with the catechol derivative (MWCNT **4**) led to an average length of 238 nm, with 26% of tubes shorter than 150 nm. Similar to coumarin conjugated to MWCNT **1**, the 3,4-dihydroxybenzamide moiety seems to accelerate the degradation process by enhancing the HRP oxidative activity. Taking into account the difference in the amount of substrate on MWCNT **4** (149 μmol/g) versus MWCNT **1** (576 μmol/g), it seems that the catechol is more efficient in augmenting the enzymatic activity of HRP. Catechol is known as a very good reducing co-substrate for HRP [37–38]. The degradation of the MWCNT **4** is likely carried out by starting with the oxidation of the catechol moieties, leading to further oxidation of the nanotubes by

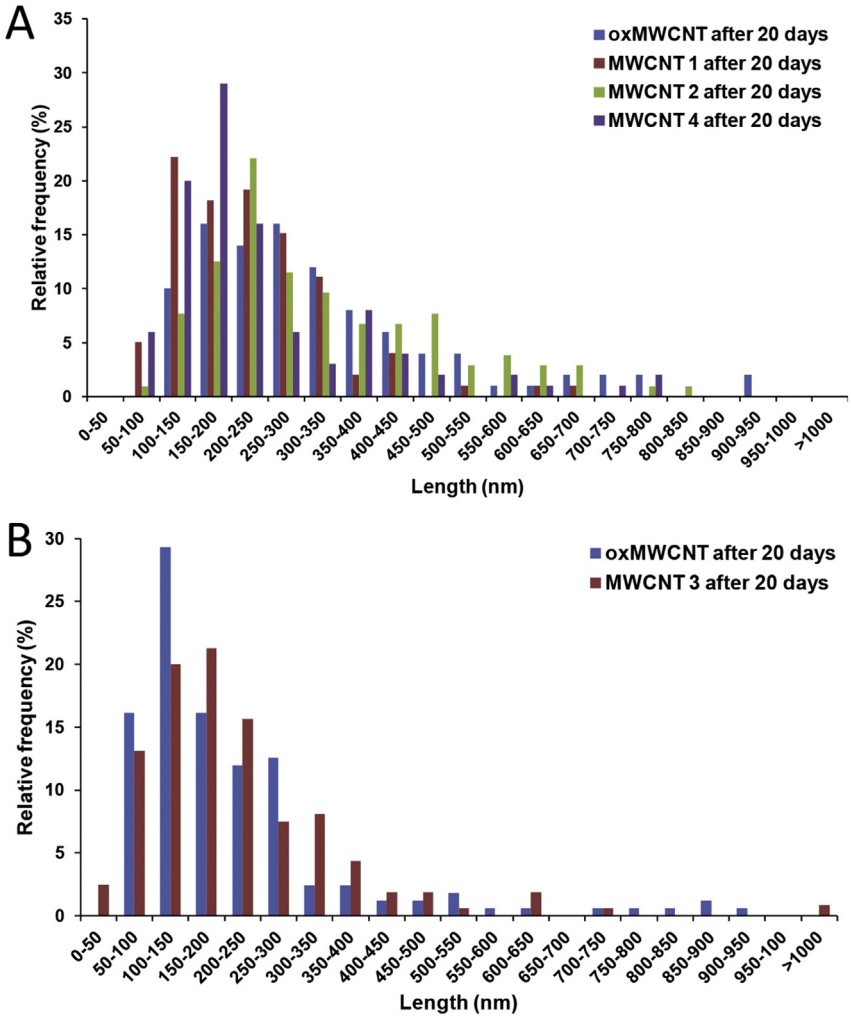


Fig. 3. Distribution of length of degrading nanotubes. (A) oxMWCNT, MWCNT **1**, **2** and **4** after 20 days, (B) comparison between oxMWCNT and MWCNT **3** after 20 days.

generation of HRP reactive radical intermediates. The catechol derivatives also proved as good substrates for myeloperoxidase and other leukocyte peroxidases [37], thus these catechol conjugated MWCNTs could be interesting in potential therapeutic applications.

In order to expand the scope of the oxidative enzymes that can degrade functionalized carbon nanotubes developed for biomedical applications, we also selected XO. This enzyme is present mainly in the liver, kidney and intestine, and in endothelial and epithelial cells of mammalian species [44,46]. XO is known to produce several ROS species, mainly superoxide ($O_2^{\bullet -}$) during the oxidation of purine molecules (e.g. hypoxanthine) to uric acid. To interrogate the activity of this enzyme, we have used nanotubes surface-modified with a purine derivative, a well-known substrate of XO. MWCNT **3** were dispersed in PBS followed by the addition of XO, along with regular additions of low concentration of H_2O_2. Fig. 2 shows TEM images of MWCNT **3** at day zero and the remaining tubes after 20 days (Panels G & H, respectively). After 20 days, XO was able to induce a decrease of the length of MWCNT **3** from 451 to 281 nm, with 36% of remaining nanotubes shorter than 150 nm (Fig. 3B). However, we surprisingly discovered that XO was also able to degrade oxidized MWCNTs, even more efficiently. In fact, these nanotubes were shortened from 451 to 215 nm, with 46% of remaining nanotubes below 150 nm (Fig. S2 and S3). Comparing these results, we concluded that the purine moiety was not really capable to accelerate the degradation process by improving the enzymatic activity of XO. The degradation or oxidation of the MWCNTs could be possible through the attack of the highly active hydroxy ($HO^{\bullet}$) radicals produced by the combination of generated superoxides and the externally added H_2O_2. The $HO^{\bullet}$ radicals have very high reduction potential (2.33 V) when compared to superoxide radicals (−0.33 V), which is adequate to oxidize the CNTs, while the latter cannot oxidize CNTs (~0.5 V) [16]. We believe that the formation of hydroxy radicals by the combination of externally added hydrogen peroxide with the *in situ* generated superoxide occurred during this treatment [44]. A similar mechanism has been shown under specific inflammatory conditions, mainly during ischemia-reperfusion, where hydroxy species are used to damage the endothelial cells [46]. The reduced degradation of MWCNT **3** compared to oxMWCNT, may be due to the production of the antioxidant uric acid molecules as the end products of the purine metabolism catalyzed by XO [50]. Uric acid was shown to act as a scavenger of the generated ROS or hydroxy radicals. Thus, in the case of purine-modified CNTs, we observed reduced degradation of the nanotubes compared to the CNTs that were only oxidized. Based on the lower efficient oxidation of MWCNT **3** by XO catalysis, we hypothesize that the simply oxMWCNT were acting as a better reducing substrate for XO than purine modified nanotubes in our tested conditions. This is likely due to the fact that purine moieties

conjugated onto CNTs can be transformed into uric acid (a radical scavenger) [50]. This enzyme was previously used only on single-walled carbon nanotubes in the presence of xanthine along with inducible nitric oxide synthase (iNOS) to produce highly reactive radicals, such as peroxynitrite ($ONOO^-$) with the oxidation potential measured to be around 1.4 V, that could oxidize effectively the SWCNTs [16]. The results obtained here and the previously reported data suggested that XO can be complimentary to other mammalian enzymes, including myeloperoxidase and eosinophil peroxidase that have been recently used in different degradation studies [9,11]. More investigations and the use of other substrates (i.e. xanthine) are necessary to clarify the role of purine on the degradation, which seems not to be crucial when the tubes are simply treated with XO in the presence of hydrogen peroxide.

We then used Raman spectroscopy to confirm the changes on the ratio between the G and D bands of the nanotubes. The I_D/I_G ratio is one of the parameters indicative of the transformation in the structural morphology of the nanotubes [5,7]. It does not provide conclusive information of a complete degradation of the tubes, especially for multi-walled CNTs [7], but it allows observation of the structural evolution of the graphitic walls during the enzymatic digestive process. Fig. 4 shows the D and G bands for the different degraded nanotubes between zero and 20 days. Samples were collected and studied after 0, 10 and 20 days. In the case of MWCNT **1**, we can observe a significant increase in the D band intensity within the timeframe of the experiment (Fig. 4A, 5A and Table S1), which is higher compared to oxMWCNT (Fig. S4A). Normalization of the Raman signal associated to the G band revealed that, the differences between the samples at 0 and 20 days were remarkably different, confirming that the nanotubes grafted with the hydroxycoumarin derivative were undergoing major changes in their graphitic structures. It seemed that the degradative action of HRP in the presence of hydrogen peroxide was creating more defects on the nanotubes rather than exfoliating their external surface, liberating "more graphitically pristine" nanotubes [7]. The results from the Raman spectroscopy studies and the images obtained by TEM together indicated that the nanotubes were much shorter and therefore they might have a higher amount of defects. A similar analysis was performed on MWCNT **4**. Again there was evident increase in the D band intensity at the end of experiment. We can assert that the nanotubes grafted with the 3,4-dihydroxybenzamide derivative were degrading faster than the starting oxidized nanotubes (Fig. 4C, 5A and Table S1). We would like to highlight that our previous study on the degradation of the oxidized and shortened nanotubes was conducted for 60 days, while the current data collected after 20 days are much earlier and in full agreement with a higher average length [7]. As control experiment, all functionalized MWCNTs **1**, **2** and **4** including oxMWCNT were incubated with HRP without addition of hydrogen peroxide, and the Raman analysis was performed on the treated samples (Fig. S5, data shown only for oxMWCNT). The D/G ratio at day 0 and after 20 days remained the same, confirming the role of hydrogen peroxide for the degradation of nanotubes as reported previously [5,6,51]. In addition, we also performed another control experiment only with addition of H_2O_2 to the respective MWCNTs without the enzyme. We added 550 μM of hydrogen peroxide 6 times per day for 20 days similar to the degradation experiments in the presence of the enzyme. The Raman analysis of control samples after 20 day treatment are shown in Figs. S6–S8 for oxMWCNT and MWCNTs **1** and **4**, respectively. For all the samples, the I_D/I_G ratio was slightly increased or remained the same compared to their respective I_D/I_G values measured for HRP control samples at day 0 (Fig. 5). This slight increase was expected since it is known that the addition of hydrogen peroxide to CNTs can induce a few defects on the surface of CNTs [6,52].

To further understand the morphological changes of MWCNT **3** during the oxidation by XO, samples were also collected after 0, 10 and 20 days and analyzed by Raman spectroscopy. After 20 days, there was a significant decrease of the D band (Fig. 4B, 5B and Table S1). This is still indicative of the changes in the morphology and physico-chemical characteristics of the CNT walls. The control experiment only with addition of H_2O_2 to MWCNT **3** (550 μM for 6 times in a day) was performed without XO for 20 days and the Raman analysis revealed that the D/G intensity ratio was slightly increased (Fig. S9). This result confirmed how H_2O_2 plays an important role for the degradation of CNTs in the presence of XO, since there was a significant decrease in the D/G ratio after treating with XO/H_2O_2 for 20 days. In contrast to HRP, the degradation action of XO is likely reducing the number of defects on the nanotubes, likely enhancing the exfoliation process of the external surface, leading to more pristine nanotubes, present within the concentric layers of the multi-walled CNTs. On the other hand, this mechanism seems not to happen on oxidized MWCNTs that are devoid of the purine functional groups that exhibited an almost unchanged I_D/I_G ratio (Fig. S4B and 5B, Table S1). The interaction between horseradish peroxidase and oxidized CNTs has been described by theoretical models and molecular dynamics simulations [52], while for xanthine oxidase the possible binding to the

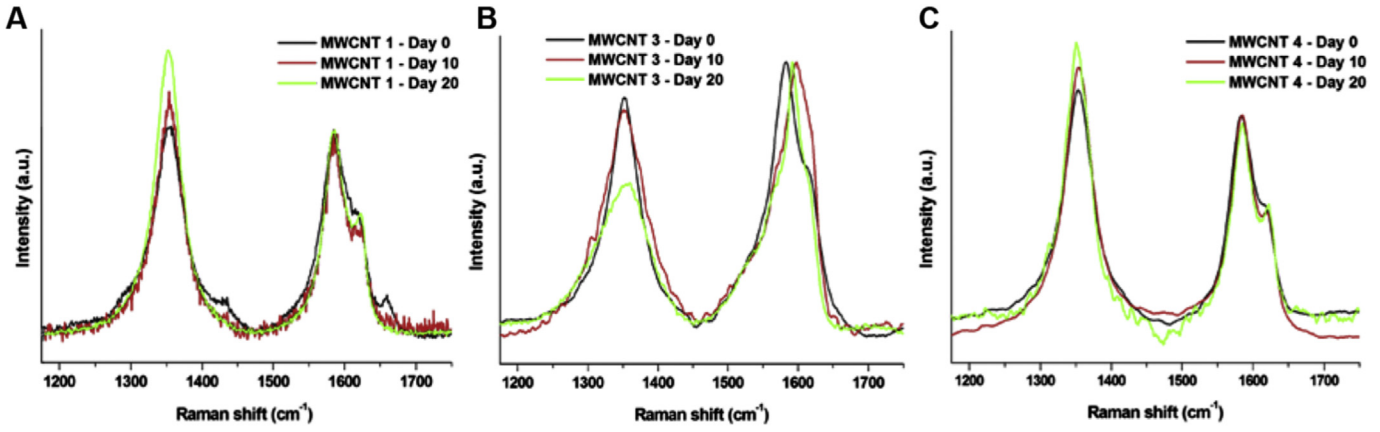


Fig. 4. Raman spectra of MWCNT **1** A), MWCNT **3** (B), and MWCNT **4** (C) during the degradation process. MWCNT **1** and **4** were treated with HRP, while MWCNT **3** was treated with XO. The samples were observed at day zero (black) and after 10 (red) and 20 days (green). All spectra have been normalized on the G band. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

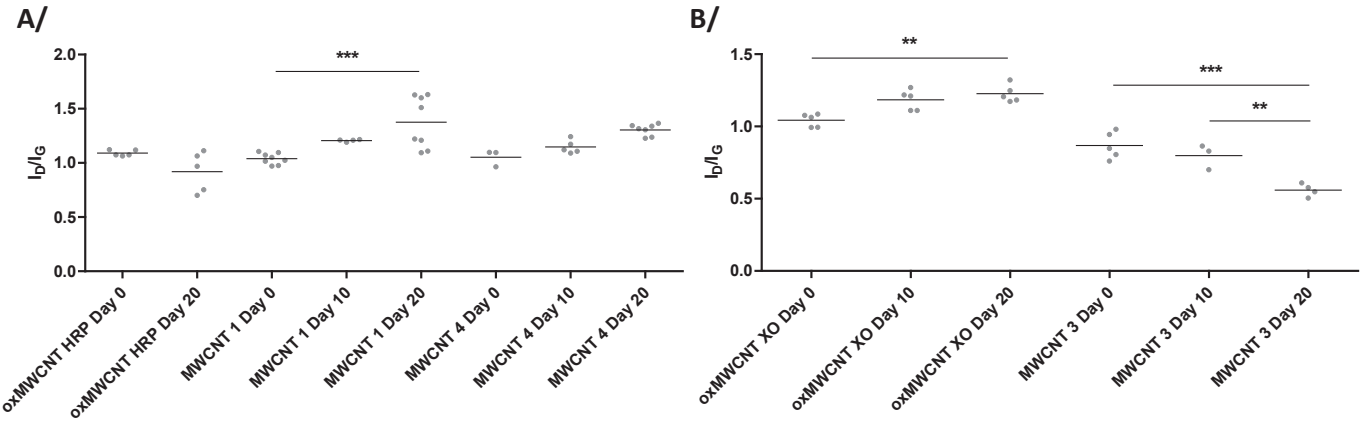


Fig. 5. I_D/I_G band ratio calculated from Raman spectra. An overall increase in I_D/I_G ratio was observed with time for MWCNT **1** and **4** treated with HRP. In the case of MWCNT **3** treated with XO we observed a decrease in I_D/I_G value, while in the same conditions I_D/I_G ratio increased for the control oxMWCNT.

tubes and in particular to the functional groups remains unidentified. As the active site and the catalytic cycle of HRP and XO are different, this might explain the changes in the evolution of the D band in the two studied cases. More experiments are necessary to assess the mechanism of degradation by XO, although based on the data generated, this enzyme has the ability to degrade the carbon nanotubes provided that an appropriate substrate is presented on the nanotube surface.

4. Conclusions

In this work, we have demonstrated that the covalent functionalization of MWCNTs with specific functional molecules such as potential reducing substrates (e.g. coumarin derivatives) and redox mediators (e.g. catechols) can lead to accelerated degradation of the nanotubes in comparison to simply oxidized CNTs. In particular, we have evidenced the importance of the hydroxyl group in the position 7 of the benzopyran ring of the coumarin, as when this function was blocked the enhanced catalytic action of HRP was abolished. Similarly, catechol-functionalized MWCNTs have been more effectively degraded than simply oxidized MWCNTs, again confirming the catalytic active role of the hydroxyl groups. Both coumarin and catechol are known as good reducing substrates and catalytic enhancers for HRP, accounting for the particular efficacy on the degradation of the nanotubes. To assess the importance of surface functionalization of CNTs for their potential biomedical applications, we demonstrated that purine modified MWCNTs could be effectively degraded by the oxidative enzyme, xanthine oxidase, in the presence of hydrogen peroxide. Though oxidized MWCNTs were degraded more efficiently than purine conjugates, this work offers alternative possibilities for the biodegradation of the CNTs *in vivo* in addition to the reported peroxidase enzymes, like MPO and EPO. Overall, this work has illustrated the crucial importance of the type of surface functionalities onto CNTs as a strategy to modulate their enzymatic biodegradability.

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Appendix A. Supplementary information

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.08.046.

References

[1] A. Bianco, K. Kostarelos, M. Prato, Making carbon nanotubes biocompatible and biodegradable, Chem. Commun. 47 (2011) 10182–10188.
[2] G.P. Kotchey, S.A. Hasan, A.A. Kapralov, S.H. Ha, K. Kim, A.A. Shvedova, et al., A natural vanishing act: the enzyme-catalyzed degradation of carbon nanomaterials, Acc. Chem. Res. 45 (2012) 1770–1781.
[3] G.P. Kotchey, Y. Zhao, V.E. Kagan, A. Star, Peroxidase-mediated biodegradation of carbon nanotubes in vitro and in vivo, Adv. Drug Deliv. Rev. 65 (2013) 1921–1932.
[4] S. Marchesan, K. Kostarelos, A. Bianco, M. Prato, The winding road for carbon nanotubes in nanomedicine, Mater. Today 18 (2015) 12–19.
[5] B.L. Allen, P.D. Kichambare, P. Gou, I.I. Vlasova, A.A. Kapralov, N. Konduru, et al., Biodegradation of single-walled carbon nanotubes through enzymatic catalysis, Nano Lett. 8 (2008) 3899–3903.
[6] Y. Zhao, B.L. Allen, A. Star, Enzymatic degradation of multiwalled carbon nanotubes, J. Phys. Chem. A 115 (2011) 9536–9544.
[7] J. Russier, C. Ménard-Moyon, E. Venturelli, E. Gravel, G. Marcolongo, M. Meneghetti, et al., Oxidative biodegradation of single- and multi-walled carbon nanotubes, Nanoscale 3 (2011) 893–896.
[8] Y. Zhao, S.C. Burkert, Y. Tang, D.C. Sorescu, A.A. Kapralov, G.V. Shurin, et al., Nano-gold corking and enzymatic uncorking of carbon nanotube cups, J. Am. Chem. Soc. 137 (2015) 675–684.
[9] V.E. Kagan, N.V. Konduru, W. Feng, B.L. Allen, J. Conroy, Y. Volkov, et al., Carbon nanotubes degraded by neutrophil myeloperoxidase induce less pulmonary inflammation, Nat. Nanotechnol. 5 (2010) 354–359.
[10] W. Seo, A.A. Kapralov, G.V. Shurin, M.R. Shurin, V.E. Kagan, A. Star, Payload drug vs. nanocarrier biodegradation by myeloperoxidase- and peroxynitrite-mediated oxidations: pharmacokinetic implications, Nanoscale 7 (2015) 8689–8694.
[11] F.T. Andõn, A.A. Kapralov, N. Yanamala, W. Feng, A. Baygan, B.J. Chambers, et al., Biodegradation of single-walled carbon nanotubes by eosinophil peroxidase, Small 9 (2013) 2721–2729.
[12] G. Chandrasekaran, S.K. Choi, Y.C. Lee, G.J. Kim, H.J. Shin, Oxidative biodegradation of single-walled carbon nanotubes by partially purified lignin peroxidase from Sparassis latifolia mushroom, J. Ind. Eng. Chem. 20 (2014) 3367–3374.
[13] L. Zhang, E.J. Petersen, M.Y. Habteselassie, L. Mao, Q. Huang, Degradation of multiwall carbon nanotubes by bacteria, Environ. Pollut. 181 (2013) 335–339.
[14] A.A. Shvedova, A.A. Kapralov, W.H. Feng, E.R. Kisin, A.R. Murray, R.R. Mercer, et al., Impaired clearance and enhanced pulmonary inflammatory/fibrotic response to carbon nanotubes in myeloperoxidase-deficient mice, PLoS One 7 (2012) e30923.
[15] C. Farrera, K. Bhattacharya, B. Lazzaretto, F.T. Andón, K. Hultenby, G.P. Kotchey, et al., Extracellular entrapment and degradation of single-walled

carbon nanotubes, Nanoscale 6 (2014) 6974–6983.
[16] V.E. Kagan, A.A. Kapralov, C.M. St Croix, S.C. Watkins, E.R. Kisin, G.P. Kotchey, et al., Lung macrophages digest carbon nanotubes using a superoxide/peroxynitrite oxidative pathway, ACS Nano 8 (2014) 5610–5621.
[17] A. Nunes, C. Bussy, L. Gherardini, M. Meneghetti, M.A. Herrero, A. Bianco, et al., In vivo degradation of functionalized carbon nanotubes after stereotactic administration in the brain cortex, Nanomedicine 7 (2012) 1485–1494.
[18] D.X. Flores-Cervantes, H.M. Maes, A. Schäffer, J. Hollender, H.-P.E. Kohler, Slow biotransformation of carbon nanotubes by horseradish peroxidase, Environ. Sci. Technol. 48 (2014) 4826–4834.
[19] C. Zhang, W. Chen, P.J.J. Alvarez, Manganese peroxidase degrades pristine but not surface-oxidized (carboxylated) single-walled carbon nanotubes, Environ. Sci. Technol. 48 (2014) 7918–7923.
[20] G.P. Kotchey, J.A. Gaugler, A.A. Kapralov, V.E. Kagan, A. Star, Effect of antioxidants on enzyme-catalysed biodegradation of carbon nanotubes, J. Mater. Chem. B 1 (2013) 302–309.
[21] N. Lu, J. Li, R. Tian, Y.Y. Peng, Binding of human serum albumin to single-walled carbon nanotubes activated neutrophils to increase production of hypochlorous acid, the oxidant capable of degrading nanotubes, Chem. Res. Toxicol. 27 (2014) 1070–1077.
[22] K. Bhattacharya, C. Sacchetti, R. El-Sayed, A. Fornara, G.P. Kotchey, J.A. Gaugler, et al., Enzymatic 'stripping' and degradation of PEGylated carbon nanotubes, Nanoscale 6 (2014) 14686–14690.
[23] G. Prencipe, S.M. Tabakman, K. Welsher, Z. Liu, A.P. Goodwin, L. Zhang, et al., PEG branched polymer for functionalization of nanomaterials with ultralong blood circulation, J. Am. Chem. Soc. 131 (2009) 4783–4787.
[24] H. Ali-Boucetta, R. Sainz, M. Herrero, B. Tian, M. Prato, A. Bianco, et al., Asbestos-like pathogenicity of long carbon nanotubes alleviated by chemical functionalization, Angew. Chem. Int. Ed. 52 (2013) 2274–2278.
[25] K.T. Al-Jamal, A. Nunes, L. Methven, H. Ali-Boucetta, S. Li, F.M. Toma, et al., Degree of chemical functionalization of carbon nanotubes determines tissue distribution and excretion profile, Angew. Chem. Int. Ed. 51 (2012) 6389–6393.
[26] C. Bussy, H. Ali-Boucetta, K. Kostarelos, Safety considerations for graphene: lessons learnt from carbon nanotubes, Acc. Chem. Res. 46 (2012) 692–701.
[27] A. Bianco, Graphene: safe or toxic? The two faces of the medal, Angew. Chem. Int. Ed. 52 (2013) 4986–4997.
[28] Y. Li, L. Feng, X. Shi, X. Wang, Y. Yang, K. Yang, et al., Surface coating-dependent cytotoxicity and degradation of graphene derivatives: towards the design of non-toxic, degradable nano-graphene, Small 10 (2014) 1544–1554.
[29] D. Mata, M. Amaral, A.J.S. Fernandes, B. Colaco, A. Gama, M.C. Paiva, et al., Diels-Alder functionalized carbon nanotubes for bone tissue engineering: in vitro/in vivo biocompatibility and biodegradability, Nanoscale 7 (2015) 9238–9251.
[30] K.T. Al-Jamal, L. Gherardini, G. Bardi, A. Nunes, C. Guo, C. Bussy, et al., Functional motor recovery from brain ischemic insult by carbon nanotube-mediated siRNA silencing, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 10952–10957.
[31] L. Delogu, G. Vidili, E. Venturelli, C. Ménard-Moyon, M. Zoroddu, G. Pilo, et al., Functionalized multiwalled carbon nanotubes as ultrasound contrast agents, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 16612–16617.
[32] G. Lamanna, A. Garofalo, G. Popa, C. Wilhelm, S. Bégin-Colin, D. Felder-Flesch, et al., Endowing carbon nanotubes with superparamagnetic properties: applications for cell labeling, MRI cell tracking and magnetic manipulations, Nanoscale 5 (2013) 4412–4421.
[33] X. Liu, I. Marangon, G. Melinte, C. Wilhelm, C. Ménard-Moyon, B.P. Pichon, et al., Design of covalently functionalized carbon nanotubes filled with metal oxide nanoparticles for imaging, therapy, and magnetic manipulation, ACS Nano 8 (2014) 11290–11304.
[34] R. Prodanovic, R. Ostafe, M. Blanusa, U. Schwaneberg, FACS based high throughput screening systems for gene libraries in double emulsions, Progr. Colloid Polym. Sci. 139 (2012) 51–57.
[35] D.C. Goodwin, T.A. Grover, S.D. Aust, Roles of efficient substrates in enhancement of peroxidase-catalyzed oxidations, Biochemistry 36 (1997) 139–147.
[36] S. Lanone, P. Andujar, A. Kermanizadeh, J. Boczkowski, Determinants of carbon nanotube toxicity, Adv. Drug Deliv. Rev. 65 (2013) 2063–2069.
[37] A. Sadler, V.V. Subrahmanyam, D. Ross, Oxidation of catechol by horseradish peroxidase and human leukocyte peroxidase: reactions of o-benzoquinone and o-benzosemiquinone, Toxicol. Appl. Pharmacol 93 (1988) 62–71.
[38] E. Ghibaudi, E. Laurenti, C. Pacchiardo, G. Suriano, N. Moguilevsky, R. Pia Ferrari, Organic and inorganic substrates as probes for comparing native bovine lactoperoxidase and recombinant human myeloperoxidase, J. Inorg. Biochem. 94 (2003) 146–154.
[39] R.W. Miller, J.-C. Sirois, H. Morita, The reaction of coumarins with horseradish peroxidase, Plant Physiol. 55 (1975) 35–41.
[40] R.D. Morrison, A.L. Blobaum, F.W. Byers, T.S. Santomango, T.M. Bridges, D. Stec, et al., The role of aldehyde oxidase and xanthine oxidase in the biotransformation of a novel negative allosteric modulator of metabotropic glutamate receptor subtype 5, Drug Metab. Dispos. 40 (2012) 1834–1845.
[41] K.G. Paul, Y. Avi-Dor, The oxidation of uric acid with horse radish peroxidase, Acta Chem. Scand. 8 (1954) 637–684.
[42] A.M. Azevedo, V.C. Martins, D.M.F. Prazeres, V. Vojinović, J.M.S. Cabral, L.P. Fonseca, Horseradish peroxidase: a valuable tool in biotechnology, Biotechnol. Annu. Rev. 9 (2003) 199–247.
[43] A. Battigelli, J.T.W. Wang, J. Russier, T. Da Ros, K. Kostarelos, K.T. Al-Jamal, et al., Ammonium and guanidinium dendron-carbon nanotubes by amidation and click chemistry and their use for siRNA delivery, Small 9 (2013) 3610–3619.
[44] R. Harrison, Physiological roles of xanthine oxidoreductase, Drug Metab. Rev. 36 (2004) 363–375.
[45] J. Kaszaki, A. Wolfárd, L. Szalay, M. Boros, Pathophysiology of ischemia-reperfusion injury, Transpl. Proc. 38 (2006) 826–828.
[46] R. Harrison, Structure and function of xanthine oxidoreductase: where are we now? Free Radic. Biol. Med. 33 (2002) 774–797.
[47] A.W. Girotti, J.P. Thomas, Superoxide and hydrogen peroxide-dependent lipid peroxidation in intact and Triton-dispersed erythrocyte membranes, Biochem. Biophys. Res. Commun. 118 (1984) 474–480.
[48] L.M. Kabeya, C.N. Fuzissaki, S.H. Taleb-Contini, Ferreira A.M.da C., Z. Naal, E.O.L. Santos, et al., 7-Hydroxycoumarin modulates the oxidative metabolism, degranulation and microbial killing of human neutrophils, Chem. Biol. Interact. 206 (2013) 63–75.
[49] B.G. Lake, Coumarin metabolism, toxicity and carcinogenicity: relevance for human risk assessment, Food Chem. Toxicol. 37 (1999) 423–453.
[50] S. Tan, R. Radi, F. Gaudier, R.A. Evans, A. Rivera, K.A. Kirk, et al., Physiologic levels of uric acid inhibit xanthine oxidase in human plasma, Pediatr. Res. 34 (1993) 303–307.
[51] B. Czech, P. Oleszczuk, A. Wiącek, Advanced oxidation (H2O2 and/or UV) of functionalized carbon nanotubes (CNT-OH and CNT-COOH) and its influence on the stabilization of CNTs in water and tannic acid solution, Environ. Pollut. 200 (2015) 161–167.
[52] B.L. Allen, G.P. Kotchey, Y. Chen, N.V.K. Yanamala, J. Klein-Seetharaman, V.E. Kagan, et al., Mechanistic investigations of horseradish peroxidase-catalyzed degradation of single-walled carbon nanotubes, J. Am. Chem. Soc. 131 (2009) 17194–17205.