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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.