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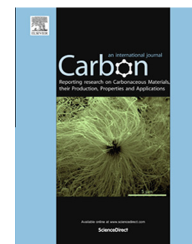

Lactoperoxidase-mediated degradation of single-walled carbon nanotubes in the presence of pulmonary surfactant

Kunal Bhattacharya [a], Ramy El-Sayed [a], Fernando T. Andón [a], Sourav P. Mukherjee [a], Joshua Gregory [b,c], Hu Li [d], Yinchen Zhao [e], Wanji Seo [f], Andrea Fornara [g], Birgit Brandner [g], Muhammet S. Toprak [e], Klaus Leifer [d], Alexander Star [f], Bengt Fadeel [a,*]

[a] Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet, 171 77 Stockholm, Sweden
[b] Centre for Allergy Research, Karolinska Institutet, 171 77 Stockholm, Sweden
[c] Division of Physiology, Institute of Environmental Medicine, Karolinska Institutet, 171 77 Stockholm, Sweden
[d] Department of Engineering Sciences, Uppsala University, 751 21 Uppsala, Sweden
[e] Functional Materials Division, Department of Materials and Nanophysics, Royal Institute of Technology, 164 40 Stockholm, Sweden
[f] Department of Chemistry, University of Pittsburgh, Pittsburgh, PA 15260, United States
[g] Unit for Chemistry, Materials and Surfaces, SP Technical Research Institute of Sweden, 114 86 Stockholm, Sweden

ARTICLE INFO

Article history:
Received 9 December 2014
Accepted 7 May 2015
Available online 13 May 2015

ABSTRACT

Carbon nanotubes (CNTs) may elicit inflammatory responses following pulmonary exposure. Conversely, enzymatic biodegradation of CNTs by inflammatory cells has also been reported. The aim of this study was to study the degradation of oxidized single-walled CNTs (ox-SWCNTs) by lactoperoxidase (LPO), a secreted peroxidase present in the airways, and whether pulmonary surfactant affects this biodegradation. To this end, ox-SWCNTs were incubated *in vitro* with recombinant bovine LPO + H_2O_2 + NaSCN in the presence and absence of porcine lung surfactant (Curosurf®) and biodegradation was monitored using UV–Vis–NIR spectroscopy, Raman spectroscopy, and scanning electron microscopy. The interaction of recombinant LPO with bundles of ox-SWCNTs was confirmed by atomic force microscopy. Cell-free biodegradation of ox-SWCNTs was also observed *ex vivo* in murine bronchoalveolar lavage fluid in the presence of H_2O_2 + NaSCN. Our study provides evidence for biodegradation of ox-SWCNTs with a lung surfactant 'bio-corona' and expands the repertoire of mammalian peroxidases capable of biodegradation of ox-SWCNTs. These findings are relevant to inhalation exposure to these materials, as LPO serves as an important component of the airway defense system.

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* *Corresponding author at:* Institute of Environmental Medicine, Nobels väg 13, Karolinska Institutet, 171 77 Stockholm, Sweden. Fax: +46 8 34 38 49.
E-mail address: bengt.fadeel@ki.se (B. Fadeel).

Abbreviations: AFM, atomic force microscopy; ELISA, enzyme-linked immunosorbent assay; bLPO, lactoperoxidase (bovine); HOCl, hypochlorous acid; HOSCN, hypothiocyanous acid; mBALF, bronchoalveolar lavage fluid (murine); MPO, myeloperoxidase; NIR, near infrared; SCN, thiocyanate; SEM, scanning electron microscope; SWCNTs, single-walled carbon nanotubes; UV–Vis–NIR, ultraviolet–visible–near infrared

http://dx.doi.org/10.1016/j.carbon.2015.05.022
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1. Introduction

Carbon nanotubes (CNTs) are promising candidates in the field of nanomedicine as drug carriers, and for imaging and biosensing due to their unique electrical, mechanical and optical properties [1]. However, the fiber-like dimensions of these materials and their potential 'asbestos-like' pathogenicity due to their physico-chemical properties (i.e., high aspect ratio) are still a major cause of concern for intentional or occupational exposure [2,3]. Therefore, effective removal of CNTs from the body following exposure is of paramount importance [4]. Pristine CNTs are insoluble and may cause significant toxicity *in vitro* and *in vivo* [5]. Therefore, surface modifications (such as, carboxylation) of CNTs are required to make these materials more biocompatible [6,7].

Surface functionalization (such as, carboxylation) of single-walled carbon nanotubes (SWCNTs) has been found to greatly influence their biodurability in the presence of physiologically relevant and mildly oxidizing phagolysosomal simulant fluids [8]. Indeed, oxidation with introduction of carboxyl groups is a prerequisite for enzymatic biodegradation of CNTs by plant and animal peroxidases [9–12]. A few studies have also investigated the biodegradation of multiwalled CNTs using horseradish peroxidase (HRP) [13,14]. The enzymes, myeloperoxidase (MPO) and eosinophil peroxidase (EPO) are released from innate immune cells – neutrophils and eosinophils, respectively – upon activation, and we and others have shown that these enzymes effectively 'digest' oxidized single-walled carbon nanotubes (ox-SWCNTs) [12,15–17]. The third major member of the peroxidase family, lactoperoxidase (LPO) is a secreted peroxidase, which unlike MPO and EPO is constitutively expressed by goblet cells present in the epithelial lining of the respiratory tract as well as in many human exocrine secretions including tears, milk, saliva, vaginal fluids, and lung fluidic lining [18]. Thiocyanate (SCN^-), and iodine (I^-), but not chlorine (Cl^-) have been found to be suitable substrates for the LPO catalyzed oxidation [19]. However, since the ratio of I^- to SCN^- in biological fluids is 10:100, and SCN^- competes effectively with I^- for LPO catalyzed oxidation the influence of I^- is negligible [20] and the hypothiocyanous acid (HOSCN) that is produced may be responsible for the destruction of the organic backbone of the ox-SWCNTs (Supplementary Fig. S1). Vlasova et al. [21] have demonstrated *in vitro* biodegradation of PEGylated SWCNTs using LPO and I^- as a substrate, which provides evidence of an effective system for the degradation of SWCNTs, but is not physiologically relevant. Furthermore, following inhalation into the airways, CNTs are coated with pulmonary surfactant biomolecules [22] and it has been argued that pulmonary surfactant is indispensible when performing *in vitro* studies in order to simulate the *in vivo* situation [23]. Pulmonary surfactant produced by type II epithelial cells located around the air–liquid interface and consisting of 85–90% of phospholipids and ~10% specific surfactant proteins (SP)-A, -B, -C, and -D surround the CNTs immediately upon exposure forming a corona [24,25]. Surfactant coating has been found to affect the attachment of other molecules to the surface of CNTs and to modulate uptake by macrophages and other biological responses to CNTs [22,26,27]. However, the impact of pulmonary surfactant on the biodegradability of CNTs has not been assessed previously.

Therefore, in the present study, we determined the biodegradation of ox-SWCNTs in an *in vitro* system using recombinant LPO supplemented with physiologically relevant concentrations of SCN^- and H_2O_2. We also pre-coated the ox-SWCNTs with pulmonary surfactant in order to study its impact on biodegradation. We further assessed whether any biodegradation of the ox-SWCNTs occurred in bronchoalveolar lavage fluid (BALF) obtained from C57BL/6 mice. Our results showed that LPO is capable of performing oxidative biodegradation of ox-SWCNT bundles both in the presence and absence of pulmonary surfactant, and that biodegradation also occurs in cell-free BALF thus demonstrating that biodegradation of CNTs can occur in complex biological fluids.

2. Experimental

2.1. *Oxidation of SWCNTs*

Pristine single-walled carbon nanotubes (P2-SWCNTs) were bought from Carbon Solutions, Inc. (Riverside, CA). Approximately 10 mg of P2-SWCNTs were sonicated (Branson 1510, frequency 40 kHz) in 20 mL of concentrated H_2SO_4:HNO_3 at a ratio of 3:1 at 70 °C for 40 min. Detailed description of the methodology is reported elsewhere [15]. For transmission electron microscope (TEM) analysis, 7 μL the sample (0.05 mg/mL) was placed on a lacey carbon grid (Pacific-Grid Tech, San Francisco, CA) and allowed to dry in ambient conditions for 2 h prior to TEM imaging (FEI Morgagni, 80 keV, Hillsboro, OR). The average length for the 40 min-oxidized (ox-)SWCNTs was 1254 ± 479 nm (n = 110) as measured from TEM images [15], and the average diameter of the ox-SWCNT bundles was 90.3 ± 41.9 nm (n = 150) based on SEM images (Supplementary Fig. S2).

2.2. *X-ray photoelectron spectroscopy*

To quantify oxygen content and formation of oxygen-containing functional groups in P2 or ox-SWCNTs, we performed X-ray photoelectron spectroscopy (XPS) using a Thermo Scientific ESCALAB 250xi photoelectron spectrometer using monochromated Al K Alpha X-rays as the source. The spot size of the sample prepared on a Si substrate was 400 μm. Charge compensation was provided by a low energy electron source and Ar^+ ions. Survey scans were collected using a pass energy of 150 eV, and high resolution scans were collected using a pass energy of 50 eV. The average percentages (Supplementary Table S1) indicate means representing three different sample spots.

2.3. *Collection of murine BALF*

Murine BALF was extracted following the protocol of Duguet et al. [28]. In brief, C57BL/6 mice (Charles River Laboratories, Sulzfeld, Germany) were deeply anesthetized with intraperitoneal injections of 50 mg/kg of sodium pentobarbital (MTC Pharmaceuticals, Joliet, IL). The upper trachea was then

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