Peroxidase-induced degradation of single-walled carbon nanotubes: hypochlorite is a major oxidant capable of in vivo degradation of carbon nanotubes

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Abstract. Due to their extraordinary properties, single-walled carbon nanotubes (SWNTs) have a tremendous potential for medical applications such as clinical diagnostics, targeted drug (or gene) delivery and cancer therapy. Hence, effects of SWNTs on living systems as well as mechanisms for biodegradation of SWTNs are of great importance and must be studied before starting to explore SWNTs for medical use. This study was undertaken to compare the potential of different peroxidases in degrading carboxylated SWNT (c-SWNT) and to elucidate the role of peroxidase-generated reactive products in this process. A detailed study showed that neither reactive intermediate products nor free radicals generated via peroxidase cycle can considerably oxidize c-SWNT. Biodegradation of c-SWNT in model system can be induced by free radicals generated as a result of heme degradation. The latter explains why hemoglobin, which is a pseudo-peroxidase possessing low peroxidase activity, is able to oxidize carbon nanotubes with a higher efficiency than horseradish peroxidase. However, c-SWNT in the presence of blood plasma (15 vol %) demonstrated no degradation even at high concentrations of hemoglobin and H₂O₂. The comparison of the ability of various peroxidases to degrade SWNTs in vitro revealed that MPO, due to its ability to produce hypochlorite, and lactoperoxidase, due to its ability to produce hypobromite, are extremely efficient in degrading carbon nanotubes. Since neutrophils are a main source of human MPO, we tested the effect of SWNTs on these cells. SWNTs were unable to stimulate neutrophils. On the other hand, they dose-dependently enhanced opsonized zymosan-induced cell stimulation as detected by measuring the amount of hypochlorite produced. This finding may be relevant to the in vivo situation, for example, at inflammatory sites. In order to imitate conditions characteristic of phagosomes and inflammatory sites, we titrated the suspension of c-SWNT in the presence of diluted blood plasma at pH 5.8 with high concentrations of (MPO + H₂O₂) or hypochlorite and found significant degradation of nanotubes. Collectively, our results indicate that hypochlorite is a main candidate for oxidative degradation of carbon nanotubes in vivo.

The extraordinary properties of single-walled carbon nanotubes (SWNTs) make them potentially useful in a wide variety of applications not only in hi-tech industry, but also in biotechnologies and medicine, in particular, for clinical diagnostics, targeted drug (or gene) delivery, cancer therapy [1, 2]. Effects of SWNTs on living systems as well as mechanisms for biodegradation of SWTNs and ways for their clearance from the body are of great importance.
and must be studied before starting to explore SWNTs for medical use. It was shown previously that free radicals and oxidants generated in the reactions mediated by horseradish peroxidase (HRP), by myeloperoxidase (MPO), and by free iron ions can oxidize and destruct carboxylated SWNT (c-SWNT) [3, 4]. Moreover, degradation of nanotubes was induced not only by their incubation with MPO [4, 5] but also by the incubation with neutrophils [4], the cells containing MPO and releasing it upon stimulation.

This study was undertaken to compare the potential of different peroxidases in degrading c-SWNT and to elucidate the role of peroxidase-generated reactive products in this process. We employed HRP, hemoglobin, MPO, and lactoperoxidase (LPO). Among them, MPO and LPO exhibit, in addition to peroxidase activity, halogenation activity, oxidizing halide ions (e.g., Cl$^{-}$ and Br$^{-}$) to the corresponding hypohalogenites [6]. SWNT degradation was assessed spectrophotometrically by measuring the absorption near-infrared spectra of nanotubes pretreated by sonication. Decrease in the absorption correlates well with nanotube degradation (Fig. 1A). Such approach was confirmed by other methods [3, 4].

In the absence of halide ions, the addition of the enzymes and H$_2$O$_2$ to nanotube suspension led to c-SWNT degradation, though the extent of degradation was low (Fig. 1B). A more detailed study showed that neither reactive intermediate products nor free radicals generated via peroxidase cycle can considerably oxidize c-SWNT. The destruction of the active site in the presence of H$_2$O$_2$ and further free radical formation mediated by heme, Fe$^{2+}$, Fe$^{3+}$ and H$_2$O$_2$ could account for peroxidase-induced nanotube degradation [3]. So, hemoglobin, which is a pseudo-peroxidase possessing low peroxidase activity, is able to oxidize carbon nanotubes with a high efficiency (Fig. 1B). The hemoglobin heme group is indeed noncovalently inserted in the heme pocket and, hence, is not stable. However, it is unlikely that this pathway for nanotube degradation will be realized in the body, since antioxidant systems, among which the mechanisms preventing the appearance of heme and free iron ions in the bloodstream, impede the accumulation of free radicals in the amount needed to degrade resistant structures such as SWNTs. In line with this assumption, c-SWNT demonstrated no degradation in the presence of blood plasma (15 vol %) even at high concentrations of hemoglobin and H$_2$O$_2$ (Fig. 2A).

The addition of chloride ions to the incubation medium caused an increase in peroxidase-induced degradation of c-SWNT only when the latter were exposed to MPO (Fig. 1B). Since MPO, in the presence of chloride ions, generates hypochlorite, this result provides compelling evidence that enzymatically produced hypochlorite contributes considerably to the degradation of c-SWNT with MPO. It was also shown previously that hypochlorite added as reagent can oxidize carbon nanotubes [4, 5, 7]. Our experimental protocol of repeated sequential additions of the MPO and H$_2$O$_2$ aliquots to nanotube suspension (see legend for Fig. 1) precluded the participation of iron ions in nanotube degradation and proved that hypochlorite generated in MPO-catalysis provides for efficient degradation of c-SWNT. Similarly, when c-SWNT suspension was titrated with LPO and H$_2$O$_2$ in the presence of bromide ions, the nanotube degradation increased twice compared to the Br$^{-}$-free medium (Fig. 1,). This can be again attributed to enzymatically produced hypohalogenite, namely, hypobromite.

Chemical modification of carbon nanotubes is used to increase their efficacy in the medical field. Functionalization of SWNT by polyethylene glycol (PEG) increases nanotube solubility and prevents their interaction with macrophages [2, 8]. In our work, we revealed that functionalization of SWNT by PEG had no effect on the pattern of peroxidase-induced degradation of carbon nanotubes. The best degradation of PEG-functionalized SWNT (PEG-SWNT) was observed with the MPO/Cl$^{-}$/H$_2$O$_2$ system or the LPO/Br$^{-}$/H$_2$O$_2$ system.

Thus, our experiments demonstrated that biodegradation of SWNTs can occur under action of MPO due to its exclusive ability to produce hypochlorite. Can this mechanism be realized in vivo? In blood, carbon nanotubes adsorb plasma proteins. Since the reactions of hypochlorite with protein amino acids are several orders of magnitude faster than with carbon nanotubes,
hypochlorite will react first of all with proteins, in particular with amino groups. Modification of an amino group with hypochlorite produces chloroamine, a strong and stable oxidant, which could oxidize carbon nanotubes. We used a model N-chloramine, chloramine T, and showed that it induces effective degradation of c-SWNT comparable to this obtained with hypochlorite. In an attempt to better simulate the in vivo conditions, we added blood plasma (15 vol %) to c-SWNT suspension. We titrated the mixture with reagent hypochlorite. As a result, the optical absorbance of nanotubes decreased significantly, indicating their degradation (Fig. 2A). This marks a promising possibility for SWNTs to be biodegraded in the body. We hypothesize that nanotube degradation should preferably occur at sites of inflammation and in phagosomes, where high concentrations of MPO (0.5–2 mM) [2], catalytically active NADPH oxidase and the acidic pH levels provide for steady-state high levels of hypochlorite.

Since neutrophils are a main source of human MPO, we tested the effect of SWNTs on these cells. SWNTs were unable to stimulate neutrophils. On the other hand, they do not prevent the interaction of a cell stimulator, opsonized zymosan, with neutrophils but even dose-dependently enhanced stimulator-induced activation of the cells as detected by measuring the amount of hypochlorite produced (Fig. 2B). This finding may be relevant to the in vivo situation, for example, at inflammatory sites. It is difficult to examine the mechanisms for SWNT degradation in neutrophil suspension, since isolated neutrophils are alive only within a few hours. Moreover, loss of cell integrity is accompanied by the appearance of strong antioxidants, taurine and ascorbate, in the medium [6]. For this reason, in order to imitate conditions characteristic of phagosomes and inflammatory sites, we placed c-SWNT in the diluted blood plasma at pH 5.8. Titration of this mixture with MPO and H₂O₂ resulted in the effective degradation of nanotubes (Fig. 2A). On the contrary, at pH 7.4, we failed to observe c-SWNT degradation. This can be explained by the higher pH value that, along with certain experimental conditions, is less favorable for chlorinating activity of MPO.

Collectively, our results indicate that hypochlorite is a main candidate for oxidative degradation of carbon nanotubes in vivo. Potentially, structural and chemical modification resulting from degradation should decrease cytotoxicity and other negative effects of nanotubes on the body. The work was supported by the grant No. 09-04-01043 from the Russian Foundation for Fundamental Research.

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Figure 1. (A) Suspensions of c-SWNT and their spectra after repeated additions of: (control) - water; (LPO) - 400 nM LPO and 200 μM H₂O₂ twice (15 min interval); (LPO/Br⁻) - the same but plus 6 mM Br⁻, (NaOCl) - 1 mM hypochlorite. The additions were made twice a day; 6 additions totally. 100 mM Na-phosphate buffer, 140 mM NaCl, 100 μM DTPA, pH 7.4. M1 and S2- characteristic absorption bands in the spectra of SWNTs.

(B) Degradation of c-SWNT by peroxidase/H₂O₂ and by hypochlorite. The percent degradation was measured by the change in peak absorbance at 1080 nm. The total number of additions – 4. Final concentrations after each addition: 400 nM horseradish peroxidase (HRP); 100 nM hemoglobin (Hb); 200 nM MPO; 400 nM LPO and 2 × 200 μM H₂O₂; 1 μM NaOCl. *p < 0.05 versus Cl⁻-free buffer; **p < 0.05 versus Br⁻-free buffer.
Figure 2. (A) Degradation of c-SWNT in the presence of blood plasma. (a) Absorption of c-SWNT (% control) after incubation with hemoglobin/H$_2$O$_2$, MPO/H$_2$O$_2$ or hypochlorite at different pH values. Plasma 15 % by volume. The total number of additions - 6. Final concentrations after each addition: 2 $\mu$M Hb, 2 $\mu$M MPO and 5x(500) $\mu$M hydrogen peroxide; 10 mM hypochlorite. *$p < 0.05$ versus control. (b) Nanotube suspensions after incubation.

(B) Concentration dependence of the effect of SWNTs on hypochlorite generation by neutrophils. (a) Hypochlorite generation by neutrophils (1 million cells per ml) in the presence of c-SWNT alone and in the presence of both c-SWNT and opsonized zymosan after incubation for 30 minutes at 37. (b) The effect of c-SWNT and PEG-SWNT on hypochlorite generation by neutrophils stimulated with opsonized zymosan. Hypochlorite was measured with the taurine chloramine assay.