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Enzymatic Oxidative Biodegradation of Nanoparticles: Mechanisms, Significance and Applications

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Abstract

Biopersistence of carbon nanotubes, graphene oxide (GO) and several other types of carbonaceous nanomaterials is an essential determinant of their health effects. Successful biodegradation is one of the major factors defining the life span and biological responses to nanoparticles. Here, we review the role and contribution of different oxidative enzymes of inflammatory cells - myeloperoxidase, eosinophil peroxidase, lactoperoxidase, hemoglobin, and xanthine oxidase - to the reactions of nanoparticle biodegradation. We further focus on interactions of nanomaterials

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with hemoproteins dependent on the specific features of their physico-chemical and structural characteristics. Mechanistically, we highlight the significance of immobilized peroxidase reactive intermediates *vs* diffusible small molecule oxidants (hypochlorous and hypobromous acids) for the overall oxidative biodegradation process in neutrophils and eosinophils. We also accentuate the importance of peroxynitrite-driven pathways realized in macrophages via the engagement of NADPH oxidase- and NO synthase-triggered oxidative mechanisms. We consider possible involvement of oxidative machinery of other professional phagocytes such as microglial cells, myeloid-derived suppressor cells, in the context of biodegradation relevant to targeted drug delivery. We evaluate the importance of genetic factors and their manipulations for the enzymatic biodegradation *in vivo*. Finally, we emphasize a novel type of biodegradation realized via the activation of the "dormant" peroxidase activity of hemoproteins by the nano-surface. This is exemplified by the binding of GO to cyt *c* causing the unfolding and "unmasking" of the peroxidase activity of the latter. We conclude with the strategies leading to safe by design carbonaceous nanoparticles with optimized characteristics for mechanism-based targeted delivery and regulatable life-span of drugs in circulation.

Keywords

carbon nanotubes; graphene oxide; peroxidase activity; oxidants; free radicals; phagocytes

Introduction

Advances in the development, production and applications of nanomaterials inevitably lead to numerous chemical and biochemical interactions at the interfaces of biological systems with nanoparticles that result in a variety of responses by the former as well as modifications of the latter. One can imagine that during the repetitive cycles of these interactions both the materials and the organisms are affected resulting in alterations that may change the consequences and the meaning of the interfacing partners. One of the first important modifications of the nanoparticles in biological systems is the formation of protein-lipid "corona" (Monopoli et al, 2012; Kapralov et al., 2012; Tenzer et al., 2013). The composition and properties of "corona" are dependent on the local microenvironments in biological fluids, tissues and cells, thus determining the specificity of nanoparticles-evoked reactions (Walkey et al., 2012; Sacchetti et al., 2013; Cai et al., 2013; Treuel et al., 2015). The physicochemical characteristics of the corona can also undergo marked changes due to metabolic conversions in the body and also via chemical reactions catalyzed by active ingredients of nanoparticles (Gao et al., 2014; Ma et al., 2015; Docter et al., 2015). This, in turn, triggers strongly modified biological responses (Dutta et al., 2007; Wang et al., 2013). Among the constituents of the protein corona, there may be enzymes contributing to the biodegradation process such as peroxidases, isoforms of CYP450, lysosomal hydrolases, etc.

Overall, nanoparticles tend to either be readily degradable or resistant to degradation in terms of their sensitivity to biodegradation. The first group includes nano-liposomes and polymeric nano-arrangements (such as dendrimers, micelles) (Kamaly et al., 2012; Xie et al., 2014; Chuan et al., 2015). Effective degradation of these nanomaterials is particularly important in the context of drug delivery aimed at the achievement of the prolonged

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circulation of the nano-vehicle with the payload (Owens et al., 2006; Loos et al., 2014; Pérez-Herrero et al., 2015). The second group comprises carbon-based nanoparticles with sp2 hybridization of carbon atoms (i.e. carbon nanotubes, nanohorns and graphene family materials) (Kotchey et al., 2013a; Zhang et al., 2014a). These nanomaterials are more persistent and can either display prolonged life-time at the sites of their entry or migrate to distant locations (Liu et al., 2008; Yang et al., 2008; Shvedova et al., 2012a, 2014). The mechanisms underlying the resistance of the latter type of nanoparticles to biodegradation are important not only for their biomedical applications but also in regards to unintended exposures in occupational and environmental settings.

While studies of nanoparticles degradation have been conducted essentially from the time of their discovery and initial applications (mostly in the field of anticancer therapy (McDevitt et al., 2007; Liu et al., 2009; Burke et al., 2009; Liang and Chen, 2010)), the discovery of *in vivo* biodegradation of carbonaceous nanomaterials by enzymatic machinery of inflammatory cells (Kagan et al., 2010; Shvedova et al., 2012a) and enhancement of the enzymatic degradation of carbon nanotubes by surface modification caused a new wave of interest to this issue (Ali-Boucetta and Kostarelos, 2013; Orecchioni et al., 2014; Sureshbabu et al., 2015). This was mostly driven by exploration of new approaches to regulate the life-time of nanoparticles in desirable ways: increasing the circulation time of drug nano-carriers and enhancing the biodegradation process of nanomaterials causing inflammatory responses and toxicity after inadvertent exposures (Liu et al., 2010; Sacchetti et al., 2013; Shvedova et al., 2012b). Notably, a variety of microbial biodegradation enzymatic mechanisms have been described with the emphasis on their potential role in biodegradation of environmental nanoparticles (Zhang et al., 2013).

Enzymatic oxidative degradation of carbonaceous nanoparticles

The chemical oxidative degradation of pristine carbonaceous materials using strong acids and oxidants (such as mixtures of sulfuric acid and hydrogen peroxide, different chemical generators of hydroxyl radicals) has been known for quite some time (Liu et al., 1998; Zhang et al., 2003; Allen et al., 2009). However, the biological relevance of these oxidative processes remained elusive in spite of the fact that the catabolic pathways for oxidative degradation of different organic molecules in the body (e.g., by different P450 isoforms) have been well characterized (Hrycay and Bandiera, 2015; Olsen et al., 2015). One of the first indications that biologically relevant peroxidase reactions may be responsible for degradation of nanomaterials came from experiments with single-walled carbon nanotubes (SWCNTs) by a plant enzyme, horseradish peroxidase (HRP) (Allen et al., 2008). Subsequent detailed studies of the mechanisms and the reaction products (Allen et al., 2009; Zhao et al., 2011) demonstrated that other bio-peroxidases, particularly those present in inflammatory cells, can also effectively oxidatively “metabolize” carbonaceous nanomaterials (Kagan et al., 2010; Kotchey et al., 2012). Indeed, a number of different oxidative enzymes have been tested and found effective as a mechanism of nanoparticle biodegradation (Kotchey et al., 2013a). The list of enzymes includes myeloperoxidase (MPO), eosinophil peroxidase (EPO), lactoperoxidase, hemoglobin and xanthine oxidase (Table 1). Contrary to HRP, another plant metallo-enzyme Mn peroxidase, was shown to degrade pristine but not carboxylated SWCNTs (Zhang et al., 2014b). These studies also

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established that two types of reactive intermediates – those formed within the protein (particularly oxo-ferryl iron (Fe4+=O) of heme-peroxidases (Compound I)) as well as freely diffusable low molecular weight oxidants such as hypochlorous and hypobromous acids (HOCl and HOBr) – can be responsible for the oxidative modification of carbonaceous nanomaterials, (Table 2) (Sutherland et al., 1993; Kagan et al., 2010; Vlasova et al., 2011). The relative contribution of these two types of oxidants to the overall degradation process may vary dependently on the type of enzyme, conditions (particularly pH), pro-/anti-inflammatory status, etc (Kotchey et al., 2013b; Vlasova et al., 2012). In all cases, however, the presence of catalytic metals is necessary for triggering the degradation process.

Numerous studies emphasized the effects of diversified organic compounds, particularly hydrophobic molecules, with the expression of different isoforms of CYP450 and their activity (reviewed in Zanger and Schwab, 2013; Hrycay and Bandiera, 2015). Moreover, particular carbonaceous materials can also cause robust changes in the functions of CYP450 system (Ji et al., 2009; Fröhlich et al., 2010; Che Abdullah et al, 2014; etc.). This catalytic responsiveness of CYP450 has lead to the development and applications of carbonaceous nanoparticles complexes with these hemoproteins as CNT-conjugated P450-biosensors (Pauwels et al., 2010; Carrara et al., 2011; Baj-Rossi et al., 2012).

Surprisingly, to the best of our knowledge there have been no studies demonstrating the propensity of CYP450 to biodegrade nanoparticles. This may represent an interesting direction of research because highly reactive Compound I is generated during P450 catalyzed metabolism of xenobiotics or direct interaction of these hemoproteins with H_2O_2 or organic hydroperoxides (peroxidase shunt) (Table 1) (Krest et al., 2013; Shoji and Watanabe, 2014).

In contrast to highly likely and straightforward capacities of CYP450 to be involved in the degradation of carbonaceous nanoparticles, the potential role of catalase is less obvious. In contrast to widely opened and solvent exposed active sites, characteristic of most hemoprotein-based peroxidases, the heme of catalases is deeply buried into the protein structure and connected with the surface via a very long and narrow access channel (Vidossich et al., 2012). Catalase compound I is accessible only for very small molecules to effectively fulfill the function of oxoferryl iron to either oxidize H_2O_2 or one of the aromatic residues of the protein thus leading to the formation of tyrosyl of tryptophanyl radicals (Kirkman and Gaetani, 2007; Alfonso-Prieto et al., 2012). The peroxidase activity of native catalase is relatively low. It is possible, however, that catalase monomers formed upon dissociation of multi-meric protein and/or their structural re-arrangements on the surface of nano-materials that will lead to the heme exposure and appearance of peroxidase-like activity with a typical catalytic competence to oxidize phenolic compounds (Horozova et al., 1998). Interestingly, this type of peroxidase activity of catalase has been explored in reactions of controllable degradation of C3N4 to obtain biocompartible fluorescent N-C dots (Liu et al., 2014).

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Pseudo-peroxidase degradation by adventitious transition metals present in nanomaterials

The production of carbonaceous nanomaterials is often associated with the employment of significant amounts of transition metal catalysts, including iron, copper, manganese, etc (Laurent et al., 1998; Panish et al., 2013). The presence of these metals should be inevitably associated with the pseudo-peroxidase function inherent to nano-materials. Moreover, electron donor-acceptor specificity of nano-environments may be conducive to the unusual peroxidase-like activities of metals not traditionally associated with redox catalysis such as gold, silver, etc (Garg et al., 2015; Tao et al., 2013). Indeed, many studies have documented the peroxidase-like activities of a variety of metal-containing nanoparticles inherent to their structure or present as adventitious metals (Gao et al., 2014; Kagan et al., 2006a). Intrinsic catalytic activity of graphene oxide (GO) may be associated with its paramagnetic properties (Song et al., 2010; Su et al., 2012; Garg et al., 2015). As an illustration, we present EPR spectra of GO samples demonstrating the presence of Mn(II) inclusions with paramagnetic propensities and narrow paramagnetic signal of GO structure (Fig. 1A). Notably, the Mn-containing GO samples displayed peroxidase-like activity as revealed by their ability to oxidize typical peroxidase substrates Amplex Red and dichlorofluorescein (DCFH) (Fig. 1B). This type of peroxidase activities associated with integrated or adventitious metals in nanoparticles can act as an important biodegradation factor. It may act as a self-propelled biodegradation mechanism, including one that may be intentionally built-in as a self-biodegradation factor.

Awakening of dormant peroxidase activity during interactions of hemoproteins with nano-surfaces

Specific interactions of nanomaterials with hemoproteins that can trigger the conversion of hexa- to penta-coordinated states of the heme iron in “dormant” peroxidases are of particular interest in the context of biodegradation (Kagan et al., 2004; Kapralov 2007). As a typical example, one can consider interactions of cytochrome *c* (cyt *c*), the hexa-coordinated hemoprotein in the intermembrane space of mitochondria, with GO. The negative charges on GO’s surface favor binding to basic proteins such as cyt *c* that has eight positive charges on its surface (Fig. 2) (Koppenol et al., 1982; Yang et al., 2013). Extensive previous work established a very peculiar behavior of cyt *c* upon its interactions with negatively charged phospholipids, particularly, cardiolipin, a unique doubly-charged phospholipid of mitochondria (Kagan et al. 2005; Kapralov et al., 2011). During this interaction, the protein undergoes structural rearrangements leading to its conversion from the hexa-coordinated to the penta-coordinated electron configuration resulting in “unmasking” of its peroxidase activity (Vlasova et al., 2006). This phenomenon has been extensively studied in mitochondria, cells, and tissues and its role in the execution of apoptotic cell death program and consequences for tissue damage have been well established (Kagan et al., 2006b; Hüttemann et al., 2011). Notably, these specific interactions of hemoproteins with nano-surfaces are meaningful in the context of degradation of nanomaterials. The schema of these interactions and several sets of experimental data illustrate the pathways and significance of GO binding of cyt *c* resulting in unfolding of the protein, weakening of the heme iron/Met80

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sulfur bond and low to high spin transition leading to peroxidase activation (Fig.3). The respective transitions of cyt *c* can be characterized by spectral changes (Figs. 4A, 4B) (Jiang et al., 2014). Accordingly, cyt *c* displays significant peroxidase activity upon its binding to the GO surface (Fig. 5) (Yang et al., 2013; Hua et al., 2012). Most importantly, the peroxidase reactive intermediates of cyt *c* can directly oxidize GO causing its degradation detectable by visible-NIR spectroscopy, TEM and XPS (Fig. 6 and Table 3). Thus, by unfolding and activating cyt *c* into a peroxidase, GO inflicts self-degradation. It is likely that cyt *c*/GO interactions represent a prototypical example of a very interesting new type of biodegradation reactions triggered by interactions of different hemoproteins with charged nano-surfaces. This new type of biodegradation reaction may lead to the design and development of new generations of nano-platforms for drug delivery as well as for modulating the physicochemical characteristics of nanomaterials. Indeed, oxidative modifications of GO have been associated with the changes of its conducting-semiconducting characteristics (Kotchey et al., 2011). In this regard, the entire family of globins, particularly a recently discovered cytoglobin (Kawada et al., 2001; Beckerson et al., 2015), can represent a promising instrument for the controlled and targeted modification/degrade action of carbonaceous nanomaterials (Ascenzi et al., 2013).

Alternatives (non-peroxidase) to enzymatic oxidative degradation of nanomaterials

In addition to peroxidase-based mechanisms, other oxidative metabolic reactions may contribute to the biodegradation process. Among the physiologically relevant mechanisms, peroxynitrite generating reactions have been identified as potent mechanisms of nanoparticle biodegradation (Kagan et al., 2014). Oxidative biodegradation of SWCNTs via superoxide/NO• → peroxynitrite-driven pathways of activated macrophages facilitate clearance of nanoparticles from the lung. This particular pathway includes two enzymatic components producing NO• and superoxide radicals, respectively – iNOS and NADPH oxidase. Interestingly, another generator of superoxide radicals, xanthine oxidase, can also contribute to degradation of oxidized multi-walled carbon nanotubes (ox-MWCNTs) (Table 1) (Sureshbabu et al., 2015).

Oxidative degradation of nanoparticles by inflammatory cells

Among many encounters with the gateway cells of the body, interactions of nanoparticles with immune/inflammatory cells are of particular interest for at least two reasons. Inflammatory cells are “armed” with different types of generators of reactive oxygen and nitrogen species (ROS and RNS, respectively) as well as hypochlorous acid. These chemical agents have strong oxidizing redox potentials enabling their reactivity towards the carbonaceous surface of nanoparticles (Sutherland et al., 1993; Davies et al., 2008) (Table 2). Notably, oxygenation (along with nitration, chlorination) of the lipid-protein “corona” of nanoparticles generates clusters of hydrophilic and/or negatively charged functionalities recognizable by inflammatory cell receptors, thus triggering a vicious cycle of interactions leading to a severe inflammatory response (Konduru et al., 2009; Shvedova et al., 2005, 2012b). In professional phagocytes, the major events include engulfment, uptake, possible intracellular metabolism, and digestion (Nauseef, 2007; Dale et al., 2008; Elgrabli et al.,

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2008). Two major factors – enzymatic machinery generating reactive intermediates and a source of oxidizing equivalents (e.g., H_2O_2, lipid hydroperoxides) – are required for the effective degradation of carbonaceous nanomaterials. While oxidative enzymes are always constitutively expressed in certain types of immune cells, the levels of their expression may be increased many-fold by pro-inflammatory conditions (Brüne et al, 2013; Mangge et al., 2014), including those triggered by the nanoparticles (Lee et al., 2012; Bussy et al., 2012).

Neutrophils are the first line of responders to pro-inflammatory stimulation and their `response reaction includes the activation of MPO-driven pathways. In the context of biodegradation, two mechanisms – immobilized reactive intermediates of the protein itself and highly diffusible small molecule oxidants such as hypochlorous acid HOCl – have been identified as components of the oxidative process (Nauseef et al., 2007, Arnhold and Flemmig, 2010). The role and contribution of these two factors into the overall degradation may depend on the specific conditions (e.g., the presence of sufficient amounts of Cl^- ions) in the microenvironment. *In vitro*, activation of neutrophils (by fMLP and cytochalasin B or by serum opsonized zymosan) is required to achieve significant levels of oxidative biodegradation (Kagan et al., 2010; Lu et al., 2014). Normally occurring opsonization of particles in the body –stimulating their uptake by phagocytes - can be mimicked by their functionalization with immunoglobulins to facilitate the particles' uptake by neutrophils. Under these conditions, neutrophils respond by oxidative burst detectable by the generation of superoxide anion radicals which dismutate to yield H_2O_2. The latter is required to feed the peroxidase reaction of MPO, thus causing oxidative biodegradation of nanoparticles. The efficiency of this pathway has been documented for SWCNTs (including their PEGylated forms) and GO (Kagan et al., 2010; Bhattacharya et al., 2014; Kurapati et al., 2015). Not only active MPO but also sufficiently high activity of NADPH oxidase – generating superoxide radicals - is necessary to maintain the degradation process. The essentiality of this function of NADPH oxidase has been demonstrated both pharmacologically (using its inhibitors) as well as genetically (using NADPH oxidase deficient animals (Shvedova et al., 2008; Kagan et al., 2014)).

Eosinophils another class of immune cells - combat multicellular parasitic organisms engaging a specialized peroxidase, EPO, capable of generating hypobromous acid (HOBr) and low levels of hypochlorous acid at acidic pH (Davies 2008). In murine eosinophils activated by cytochalasin B plus platelet-activating factor (PAF), the oxidizing enzyme, EPO, is released to cause extracellular degradation of SWCNTs (Andón et al., 2013).

Macrophages employ the complex of reactions leading to the production of peroxynitrite, another potent oxidant capable of oxidative degradation of carbonaceous nanomaterials. Two enzymatic systems - NADPH oxidase and NO synthase - produce superoxide radicals and NO•, respectively. Both of these molecules are not reactive enough to oxidatively biodegrade nanoparticles. However, O_2^-• and NO• can effectively react to yield peroxynitrite, whose oxidizing potency is sufficient to cause biodegradation (Ischiropoulos et al., 1992). *In vitro*, effective biodegradation capacity has been demonstrated for several types of macrophages such as RAW 264.7, THP-1, and human monocyte-derived macrophages (Kagan et al., 2014; Elgrabli et al., 2008, 2015; Zhang et al., 2015) (Table 1). Because peroxynitrite-dependent oxidation reactions are independent of the direct binding of

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the reactive protein intermediates with nanoparticles, the degradation process driven by macrophages is independent on the specific positioning of the oxidative machinery on the surface of nanoparticles. As a result, different types of nanomaterials – oxidatively pre-modified as well as pristine - may undergo degradation by macrophages. It is also possible that macrophages "prime" the nano-objects to generate "oxidized" sites where released enzymes of neutrophils and eosinophils can selectively "land" to propagate the process initiated by macrophages.

It is possible that other types of immune cells – specific to particular organs and/or disease conditions – may be involved in biodegradation of carbonaceous nanomaterials. For example, microglial cells in the brain can act similar to macrophages and catalyze the reactions leading to peroxynitrite and ROS formation, hence effectively biodegrading nanoparticles (Goode et al., 2015; Bussy et al., 2016). The evidence for the occurrence and effectiveness of this pathway in the brain is accumulating (Nunes et al., 2012). Another example is myeloid-derived suppressor cells (MDSC) – a heterogenous population of immature cells from the myeloid lineage. As a result of an altered hematopoiesis, amounts of MDSCs can be highly increased in severe disease conditions, particularly chronic infections and cancer (Gabrilovich et al., 2012). These pathological conditions lead to over-expression of NADPH oxidase, iNOS and MPO in MDSC creating a highly pro-oxidant intracellular environment (Youn et al., 2012). These specific features of MDSC may be exploited for the targeted degradation of nanomaterials for optimized delivery of drugs (Zhao et al., 2015). MDSC-derived oxidants can open carbon nano-cups (NCNCs) loaded with antitumor drug paclitaxel and corked with gold nanoparticles (GNPs) and release paclitaxel (Figs. 7, 8).

Biodegradation of nanomaterials by inflammatory cells *in vivo* : role in pulmonary inflammation and fibrosis

There is a common opinion about biopersistence of nanoparticles in the body. While extended circulation of drug nano-carriers with payloads may be desirable, the ineffective elimination of nanoparticles from the organs after unintentional exposure or as a result of therapeutic attempts seems to represent a serious problem. Poorly degradable nanomaterials can accumulate in organs and inside cells where they can cause detrimental effects (Fig. 9). Even with regards to carbonaceous nanomaterials that are readily susceptible to biodegradation, carbon nanotubes may remain inside macrophages in the spleen and liver for prolonged periods of time following parenteral administration (Yang et al., 2008, Clichici et al., 2014; Albini et al., 2015). Moreover, SWCNTs have been observed in the lungs of exposed mice up to one year after pharyngeal administration (Shvedova et al., 2014). Overall, however, high-aspect, bulky carbonaceous nanomaterials tend to have longer retention times within the tissues and are less effectively cleared than short functionalized particles that are readily taken up and degraded by phagocytes (Mercer et al., 2008; Murphy et al., 2011, Bussy et al., 2012). Notoriously, the appearance of nanoparticles in tissues triggers robust inflammatory responses. Given that immune cells can spend some of their pro-oxidant potential on biodegradation of the nanoparticles, and thus display a weakened immune response, studies of nanoparticle biodegradation *in vivo* and possible regulation of

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biodegradation in the context of inflammation *in vivo* became necessary (Shvedova et al., 2007; Kotchey et al., 2013b; Dumortier et al., 2013). These issues have also stimulated the concepts of creating safe-by-design nanoparticles as well as employment of inflammatory cells for targeted drug delivery (Sureshbabu et al., 2015; Zhao et al., 2015).

In line with the ability to take up and biodegrade carbonaceous nanomaterials, professional phagocytes are believed to be mostly accountable for the clearance of the engulfed nanoparticles *in vivo*. Several studies established the association between the clearance of carbonaceous nanoparticles in the lungs and the amounts of neutrophils and macrophages in the respective tissues (Shvedova et al., 2005, 2012a). These correlational relationships imply that, indeed, biodegradation reactions taking place in inflammatory cells are substantial contributors to the overall elimination of nanoparticles from the tissues. In support of this conclusion, the data on the time course of inflammatory responses and SWCNTs elimination in mice with k/o MPO clearly demonstrated the dependence of these biomarkers on the genetic manipulations with the major biodegrading oxidative enzyme of neutrophils, MPO. Quantitative imaging clearly demonstrated the link between MPO-catalyzed degradation of nanoparticles and one of the hallmarks of the inflammation – pulmonary fibrosis – in wild type (WT) versus MPO k/o animals (Shvedova et al., 2012a). The role of NADPH oxidase as a supplier of superoxide for the subsequent reactions of dismutation (to generate H_2O_2 as a fuel for MPO) or with NO• (to produce peroxynitrite in macrophages) has been revealed in experiments with genetically manipulated animals. Clearance of SWCNTs was 10-fold less effective in NADPH oxidase-deficient mice ($gp91^{phox(-/-)}$ mice) *vs* WT animals. Photoacoustic imaging also documented significantly reduced rate of SWCNTs clearance in the lung of NADPH-deficient mice compared to WT control animals (Kagan et al., 2014). There are clear experimental indications that microglial cells - with their highly developed oxidative enzymatic machinery similar to that in macrophages – are primarily responsible for the biodegradative elimination of MWCNTs from the brain (Nunes et al., 2012; Bardi et al., 2013; Bussy et al., 2016).

Employment of enzymatic biodegradation of nano-containers for targeting inflammatory cells in cancer. Peroxidase degradation of payloads vs nano-containers – significance for drug delivery

Design and development of nano-platform based carriers for drug delivery represents one of the active fields for biomedical applications (Battigelli et al., 2013). In this context, effective timely degradation of drug carriers becomes particularly important but must be optimized with regards to nano-carrier vs drug payload degradation (Farokhzad et al., 2006). It has been well documented that oxidative enzymes of inflammatory cells, particularly MPO, can catalytically destroy different types of small organic molecules, including drugs, in circulation (Davies 2008). This wasteful drug metabolism may be exceptionally strong in pro-inflammatory conditions associated with increased amounts and activation of inflammatory cells. This raises the question of possible “protective” role of nano-carriers in preventing unnecessary degradation of payloads and preservation of their therapeutic potential. Notably, experimental assessments of nano-carrier vs drug degradation have not been adequately addressed. A recent study compared degradation of an antitumor drug,

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doxorubicin (DOX), in free form vs its conjugate with SWCNTs in the presence of MPO or ONOO-generating systems (Seo et al., 2015). The evaluations in simple biochemical enzymatic systems clearly demonstrated that the SWCNTs-associated drug molecules (DOX-SWCNTs) degraded more slowly than free DOX. Notably, cytostatic and cytotoxic effects of free DOX, but not nanotube-carried drug, on melanoma and lung carcinoma cell lines were abolished in the presence of tumor-activated MDSC known to express high levels of MPO, NADPH oxidase and iNOS thus providing enhanced myeloperoxidase- and peroxynitrite-induced conditions for biodegradation of organic molecules. Optimizing the balance between the degradation and resistance of the drug carrier and the payload towards the oxidants generated by inflammatory cells is critical to meet the needs for safety and prolonged circulation while orchestrating the stability and therapeutic effect of the drug. This strategy opens opportunities for exploring new parameters in biodegradation and developing controllable degradation properties by chemical modification of the surface of nanotubes. Further studies are necessary to elucidate important details relevant to the degradation characteristics of different drug nano-delivery systems in the context of achieving their optimized therapeutic potential.

Concluding remarks

The currently accelerating progress in nanotechnologies has already accepted the “safe-by-design” principle as a necessary requisite in the development of new nanomaterials. This principle has to include the “safe-by-biodegradation” component, providing for the optimized life-time and clearance of nanoparticles from the body. In this context, the broad applications of nitrogen-doped carbon nanomaterials offer a very important advantage in creating defects in the structure that can serve as “biodegradation” initiation centers. The introduction of principally new nanomaterials with unexpected features of their interactions with the intracellular mechanisms of genetic, epigenetic, and metabolomics regulation calls for new approaches to assessments of their toxic mechanisms. For example, a recently introduced spherical nucleic acids (Service, 2015) – nano-DNA arrays that can readily enter the cells, “seeks and binds” very low quantities of complementary DNA and RNA from pathogenic bacteria and viruses in the blood. As this affects the expression of selected genes, the resourcefulness in evaluating potential side effects, toxicity, and clearance become particularly important. New generations of nano-platforms are designed and used not only as effective delivery vehicles but also for the engagement of their unique nano-characteristics in manipulations of cell metabolism, cell-cell communications, and pathogen-host interactions. Oxidative modifications of these innovative materials by immune cells may result in the emergence of unforeseen propensities and, consequently, unexpected effects on cell functions. Exploration and utilization of targeted, enzymatically-catalyzed, oxidative modification/biodegradation of these new nanomaterials represent an exciting future area of research.

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Highlights

Nanoparticles can be degraded by oxidative enzymatic machinery of inflammatory cells.

Peroxidase-generated oxidants are the reactive species executing the biodegradation.

Unmasked by GO binding peroxidase activity of cyt biodegrades GO.

Professional phagocytes are accountable for the clearance of nanoparticles *in vivo*.

Carbonaceous nano-carriers of drugs protect against degradation of payloads.

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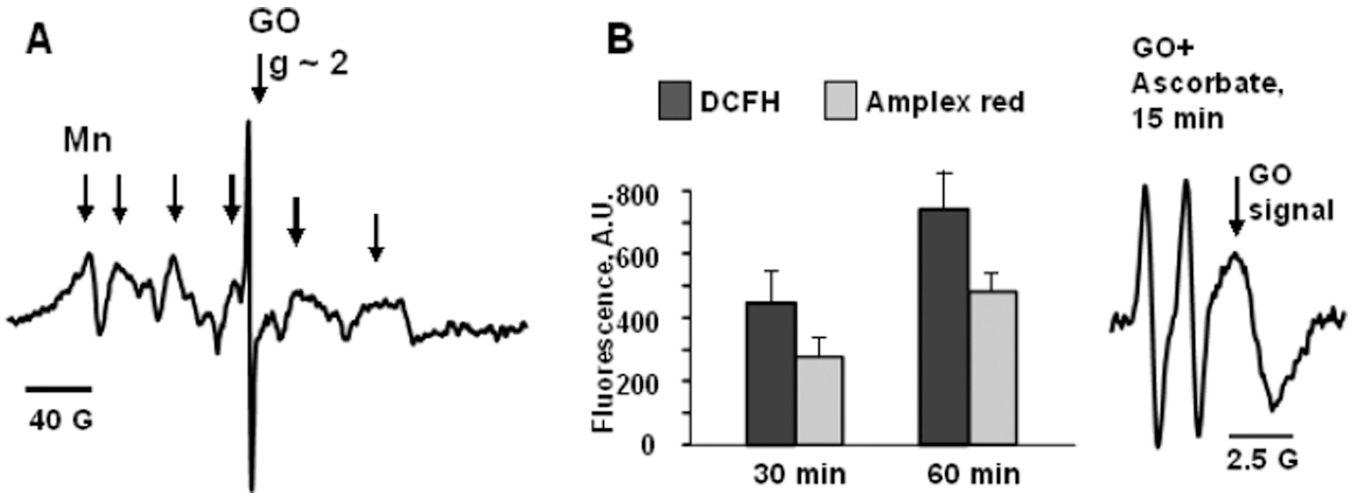

Fig. 1. Paramagnetic properties of GO are accountable for its peroxidase-like activity

A) – EPR spectrum of GO measured at 77K is a composition of a narrow EPR spectrum at g~2.0 which is due to intrinsic paramagnetic properties of GO and hexa-component EPR spectrum of manganese embedded into carbon structure.

B) – GO can oxidize peroxidase substrates characterized by low reduction potentials: Amplex red, dichlorofluorescein (DCFH), and ascorbate (n=3). H_2O_2 (100 μM) was added to the solutions of substrates (50 μM) and GO (0.1 mg/ml) and formation of fluorescent products was detected at 30 or 60 min. In the absence of GO fluorescence responses from Amplex red or DCFH were lower than 10 A.U. EPR signal of ascorbate (1 mM) could be measured in the absence of H_2O_2 but not in the absence of GO (0.5 mg/ml).

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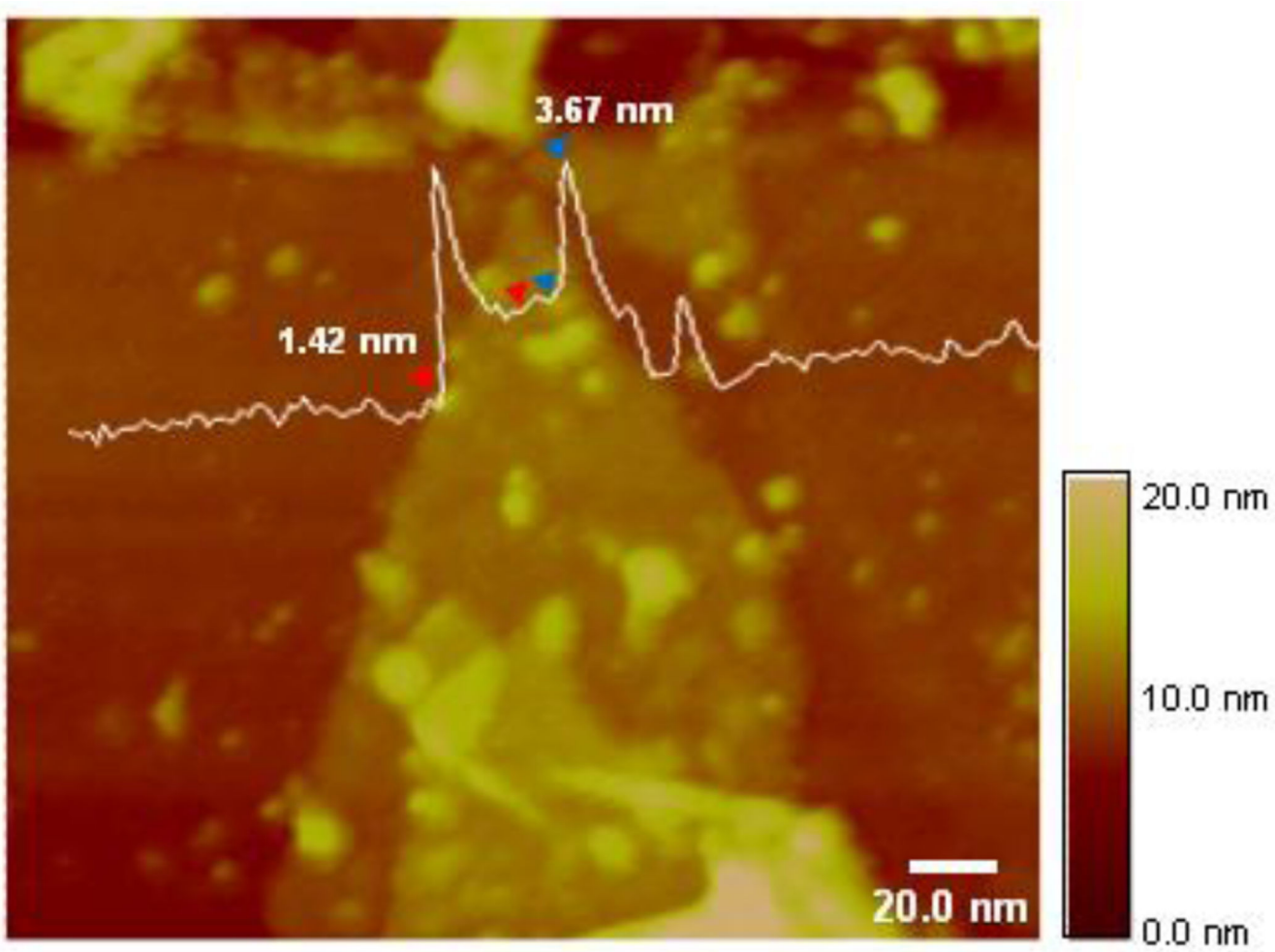

Fig. 2.

AFM evidence for cyt *c* binding with GO. 5 μM cyt *c* were added to a suspension of GO (0.2 mg/ml) in 20 mM Na-phosphate buffer, pH 7.4 and tapping mode AFM height analysis was performed. Average GO height is 1.5 nm, and height of cyt *c* ranges from 3.3 to 3.7 nm.

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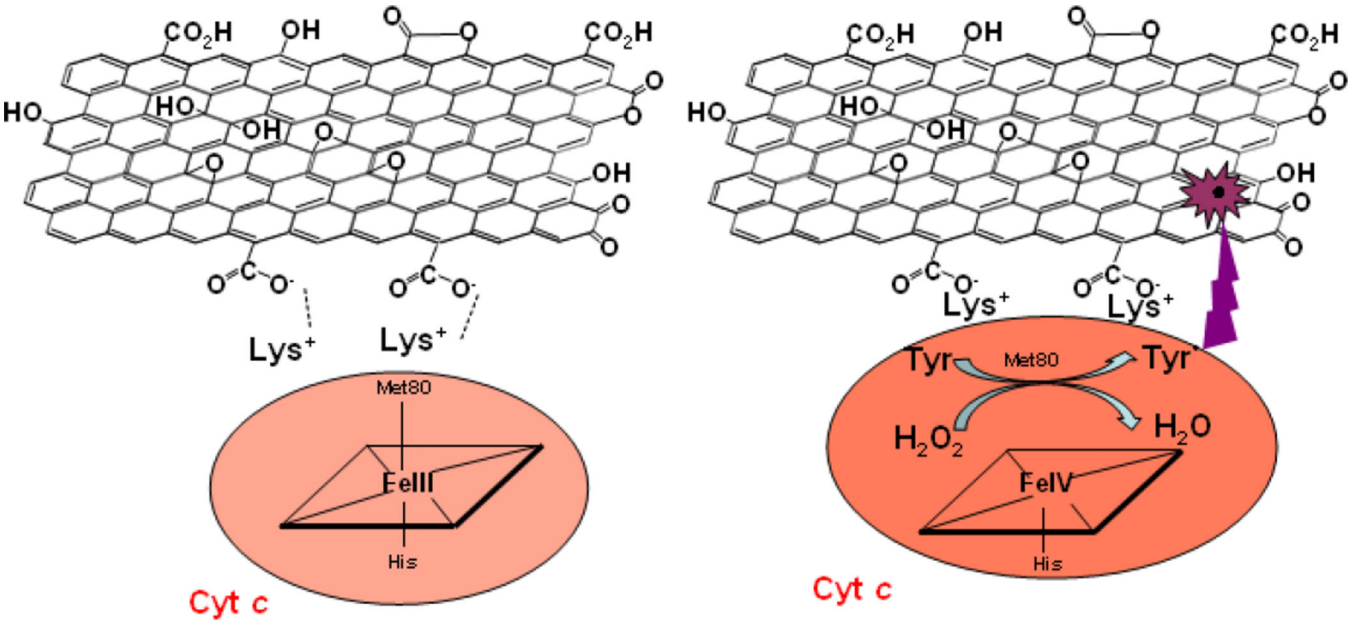

Fig. 3.

Cyt c binding and peroxidase activation upon interaction with GO. Eight positive charges on the surface of cyt c (Lys+) can electrostatically interact with the negatively charged oxygen-containing functional groups of GO. This interaction of cyt c with GO leads to protein unfolding associated with the “awakening” of dormant peroxidase activity. This peroxidase activation of cyt c can trigger – in the presence of oxidizing equivalents (H2O2, organic hydroperoxides) -oxidative modification/degradation of GO.

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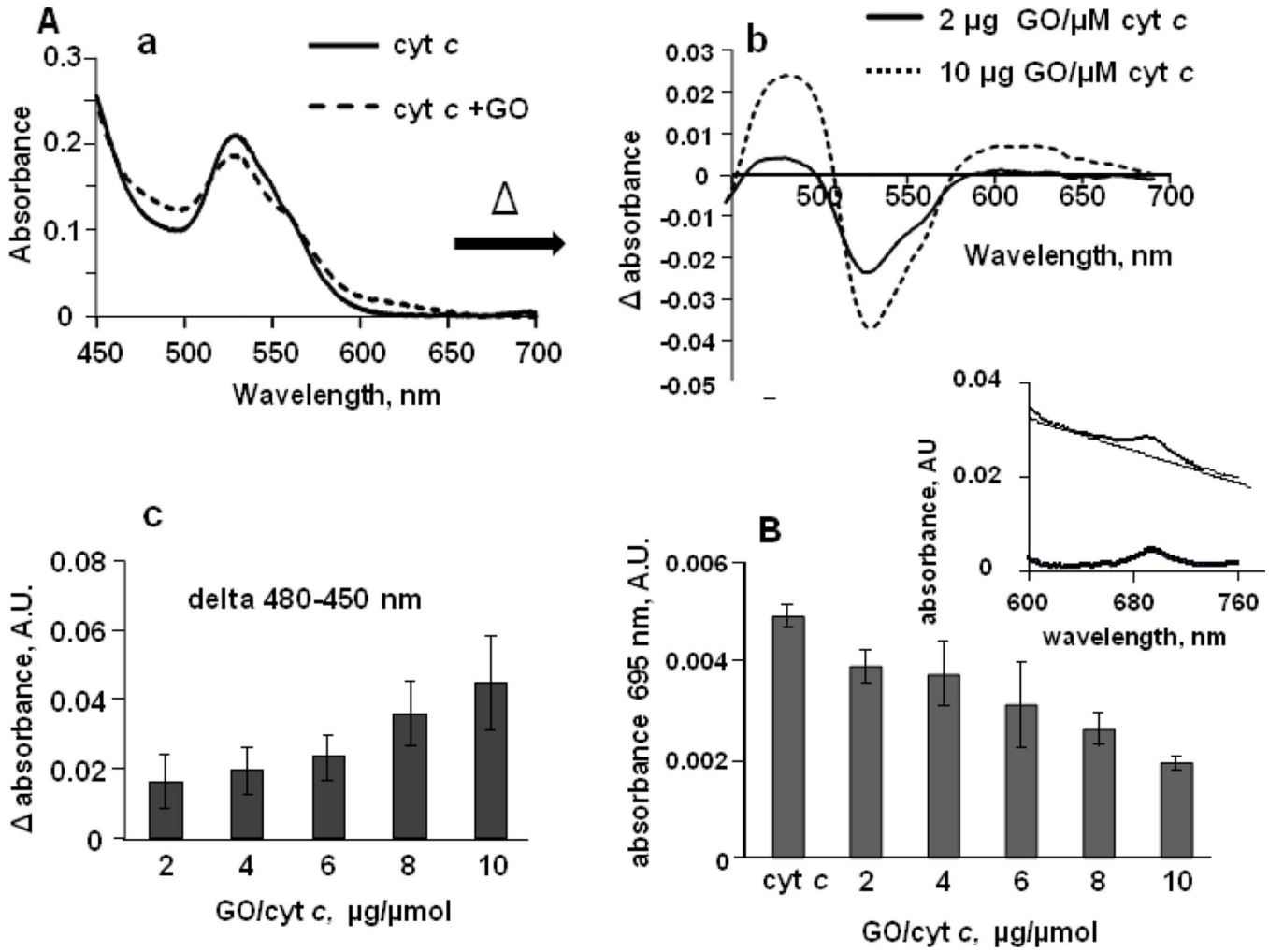

Fig. 4. Absorption spectra of cyt *c* illustrating GO-induced formation of high-spin form of heme-iron and weakening of the heme iron/Met80 sulfur bond

A) - Effect of GO on the formation of high-spin iron state of the hemoprotein. a) - Optical absorption spectra of cyt *c* in the absence (solid line) and in the presence (dashed line) of GO; b) - differential absorption spectrum (obtained by subtracting spectrum of cyt *c* from spectrum of cyt *c* incubated with GO) shows positive features at 480–495 and 610–625 nm accompanied by a clear trough at 700 nm indicative of high-spin ferric heme; c) Effect of GO on the height of a peak at 480 nm (75 μM cyt *c*, n=3).

B) - Dependence of the absorbance of the Fe-S(Met80) bond (λ 695 nm) on GO/cyt *c* ratio. Characteristic absorbance band at 695 nm is associated with an axial coordination of the heme iron by the sulfur atom of Met80 in cyt *c* active site. In the presence of GO, loss of absorbance band at 695 nm was observed indicating the disruption or weakening of the Fe-S(Met80) coordination. Insert - optical absorption spectrum of cyt *c* at region around 695 nm before and after subtracting of baseline. The absorbance was measured in 20 mM HEPES buffer containing 100 μM DTPA (pH 7.4) using 50 μM of cyt *c*, n=7-9.

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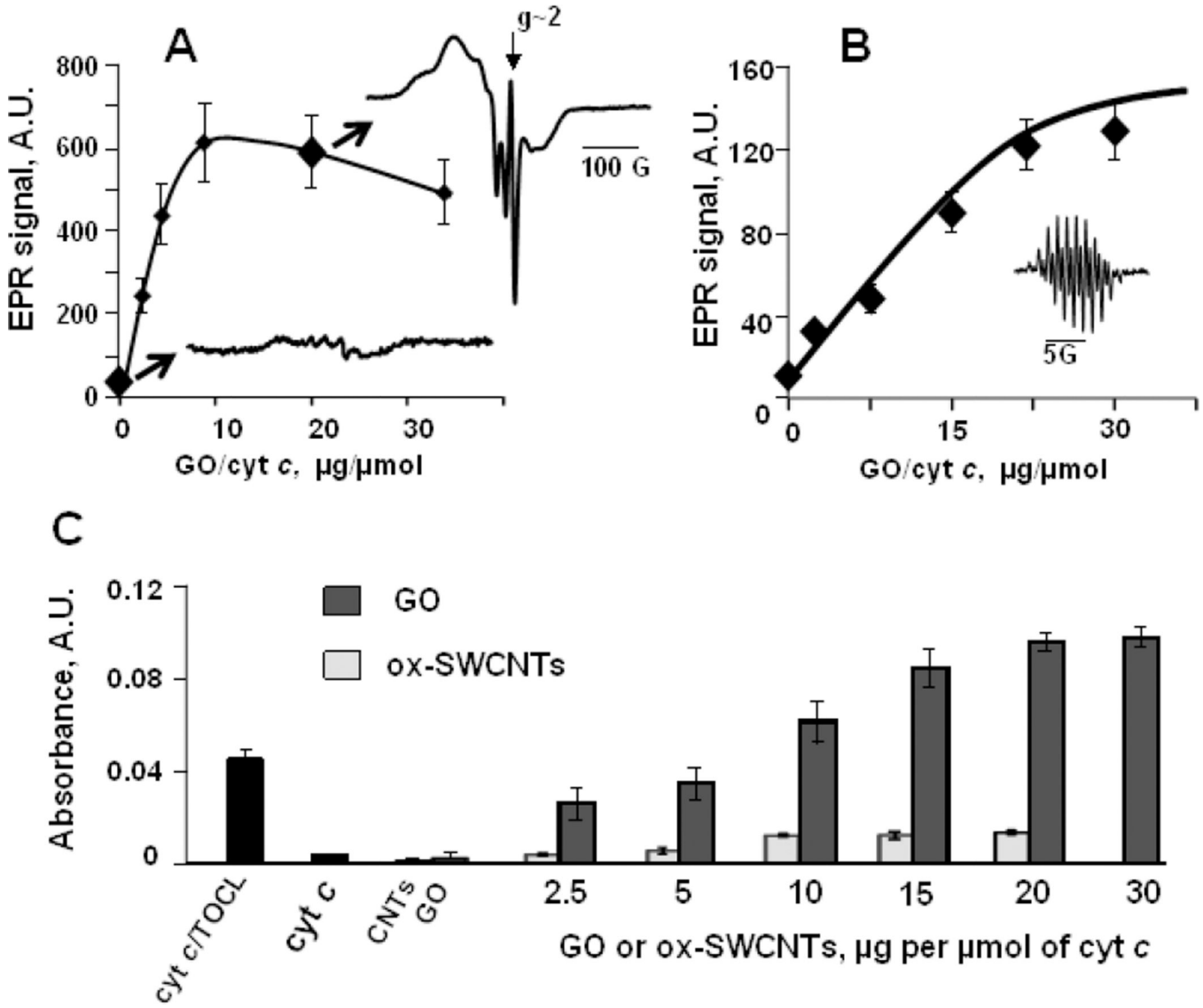

Fig. 5. Binding of GO to cyt *c* causes unfolding of the protein and increases the accessibility of cyt *c* active site

A) - GO facilitates cyt *c* nitrosylation as evidenced by low temperature EPR spectroscopy. Magnitude of the signal of nitrosylated cyt *c* depends on GO/cyt *c* ratio revealing the increase of the accessibility of cyt *c* active site towards nitric oxide upon enzyme binding to GO (Vlasova et al., 2006). Inserts are EPR spectra of cyt *c* nitrosylated in the absence (blue) and in the presence (red) of GO. EPR spectrum of nitrosylated cyt *c*/GO complexes includes pronounced narrow GO spectrum at g~2. Reduced cyt *c* (100 μM, 500 μM ascorbate) was incubated with GO at 37°C for 5 min in 50 mM sodium phosphate buffer, pH 7.4, containing 100 μM DTPA, then 750 μM PAPANONOate was added and incubation was continued for additional 15 min. The reaction was stopped by freezing the samples in liquid nitrogen. EPR spectra of nitrosylated cyt *c* were measured at 77K.

Binding to GO confers peroxidase activity on cyt *c*: B) - Magnitude of EPR spectrum of etoposide-phenoxyl radicals (insert) generated in the peroxidase reaction of cyt *c*/GO

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complexes in 2 min after addition of 100 μM H_2O_2 (7.5 μM cyt *c*) (Kagan et al., 1999); C) - Oxidation of peroxidase substrate ABTS by cyt *c*/GO. Oxidation of ABTS to yield the product with absorbance maximum at 410 nm was performed at reagent concentrations: 5 μM cyt *c*, 1 mM ABTS and 100 μM H_2O_2. The effect of GO on cyt *c* peroxidase activity is compared to the effects of TOCL (blue bar) and ox-SWCNTs.

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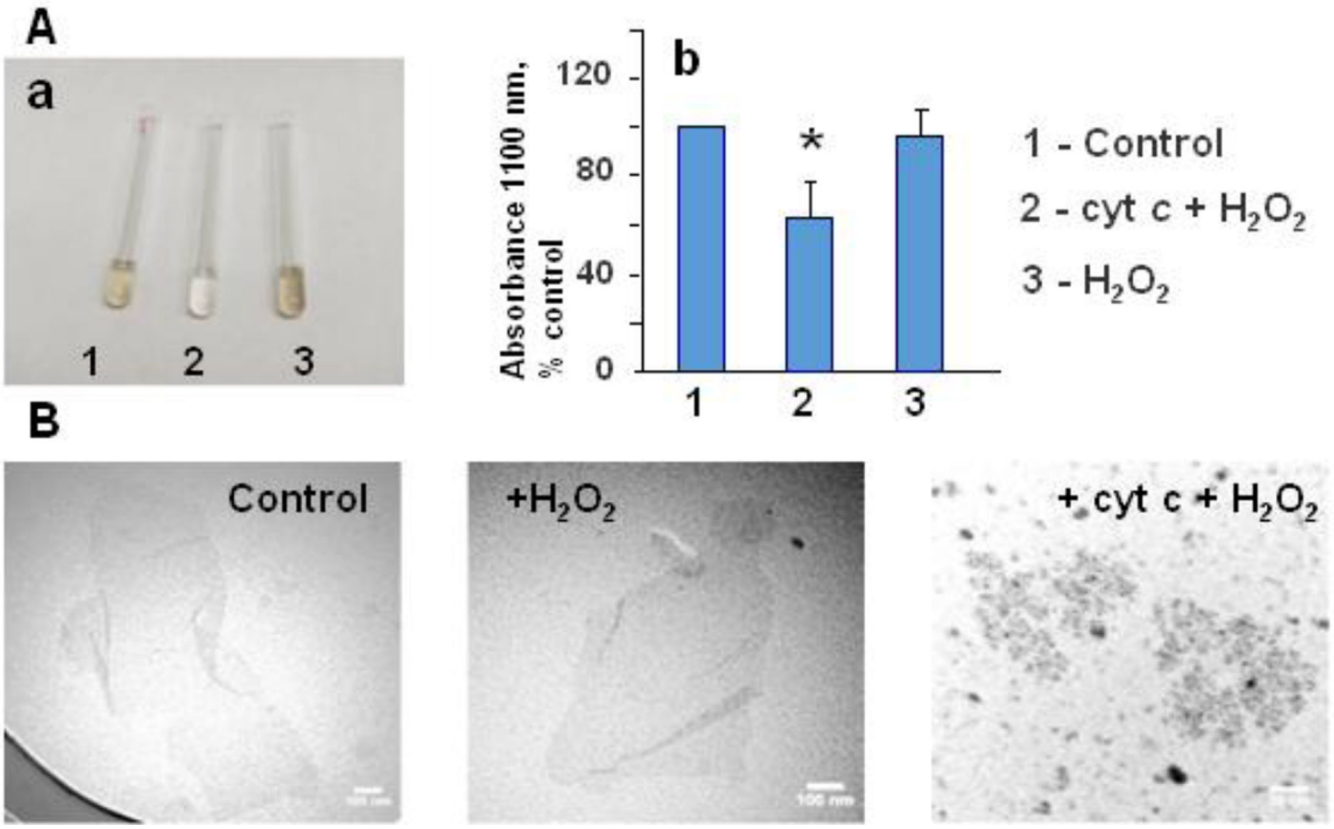

Fig. 6.

Activation of cyt *c* into peroxidase caused biodegradation of GO. GO (100 μg/ml) was suspended in 50 mM Na-phosphate buffer, containing 100 μM DTPA, pH 7.4. Cyt *c* (2.5 μM) was added once a day and micro aliquotes of H_2O_2 (50 μM after each addition) were added 5 times a day during 5 days. a) - Visual evidence of GO degradation as a result of treatment with cyt *c* and H_2O_2 (sample 2). Pictures of GO samples were taken in 6 days of incubation; b) -optical absorbance of the sonicated samples at 1100 nm. For each sample, data are averaged for three independent suspensions, * $p<0.05$ versus control. c) - TEM images of untreated GO (control), GO incubated with H_2O_2, and GO treated with cyt *c* and H_2O_2.

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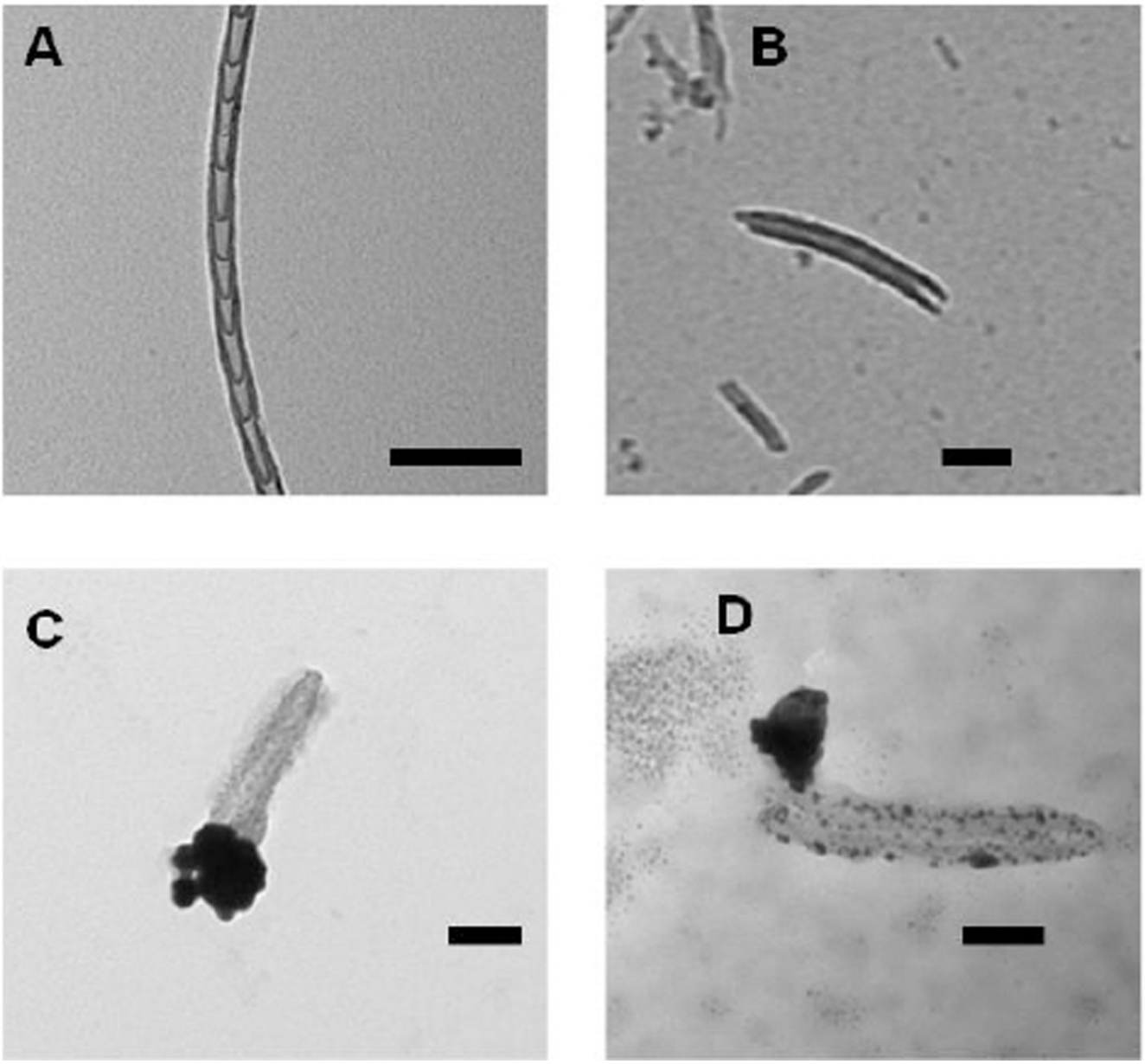

Fig. 7.

Transmission electron microscopy (TEM) images of nitrogen-doped carbon nanotube cups (NCNCs). A) NCNCs synthesized from chemical vapor deposition; B) NCNCs after separation through probe-tip sonication; C) separated NCNCs loaded with paclitaxel and corked with gold nanoparticles (GNPs) through citrate reduction of chloroauric acid; D) GNP corked NCNCs after incubation with myeloid-derived suppressor cells (MDSC), removal of the GNP cork and degradation of nanotube sidewalls is demonstrated in addition to release of loaded cargo. All scale bars are 100 nm.

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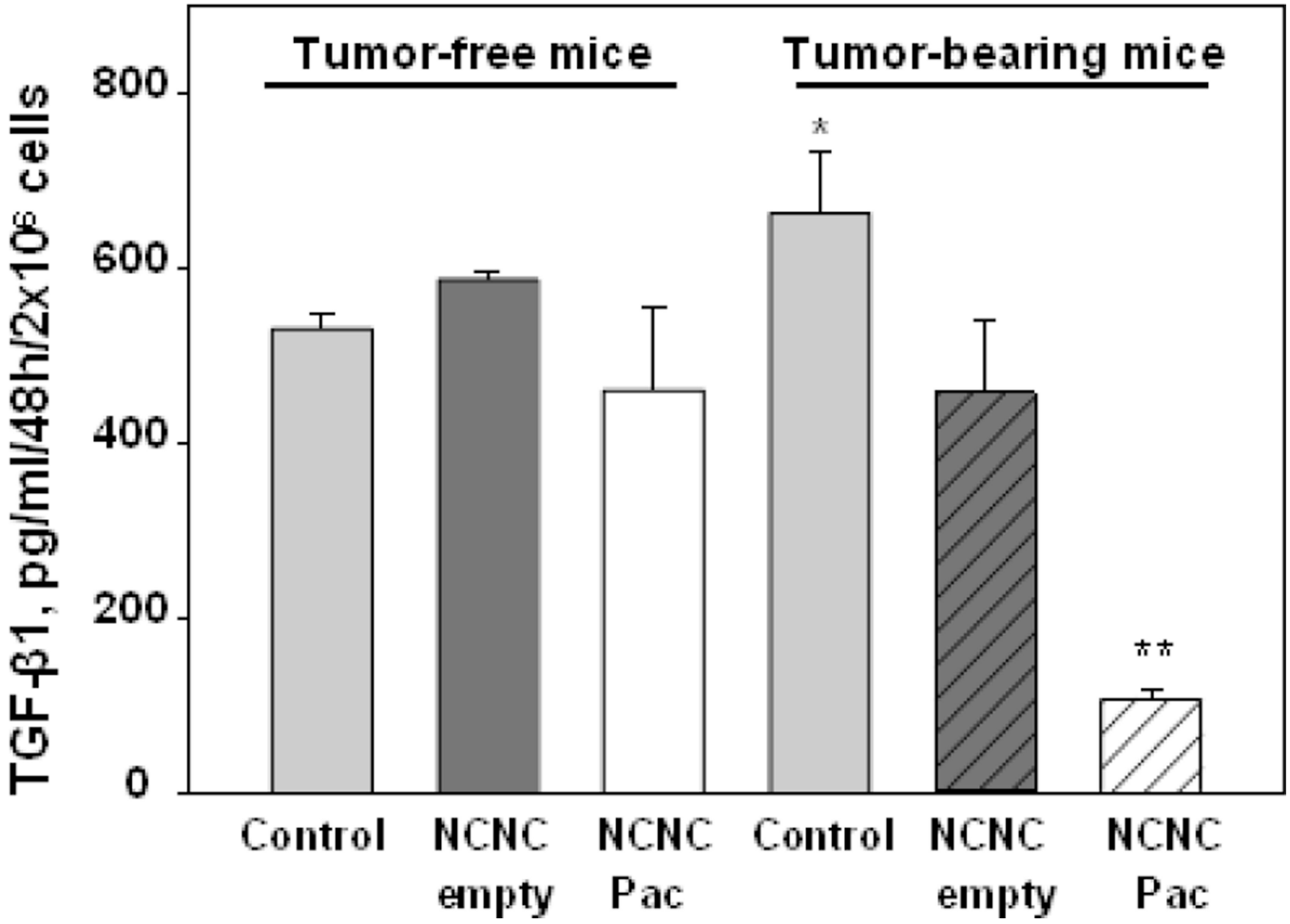

Fig. 8.

Nitrogen-doped carbon nanotube cups (NCNC) loaded with paclitaxel (Pac) block TGF-β production by tumor-associated MDSC. Bone marrow MDSC were sorted from tumor-free and B16-bearing mice (3 weeks), incubated with medium (Control), empty NCNC and NCNC/Pac. TGF-β was measured by ELISA. *, $p<0.05$ (vs controls in tumor-free mice; **, $p<0.05$ (vs NCNC Pac).

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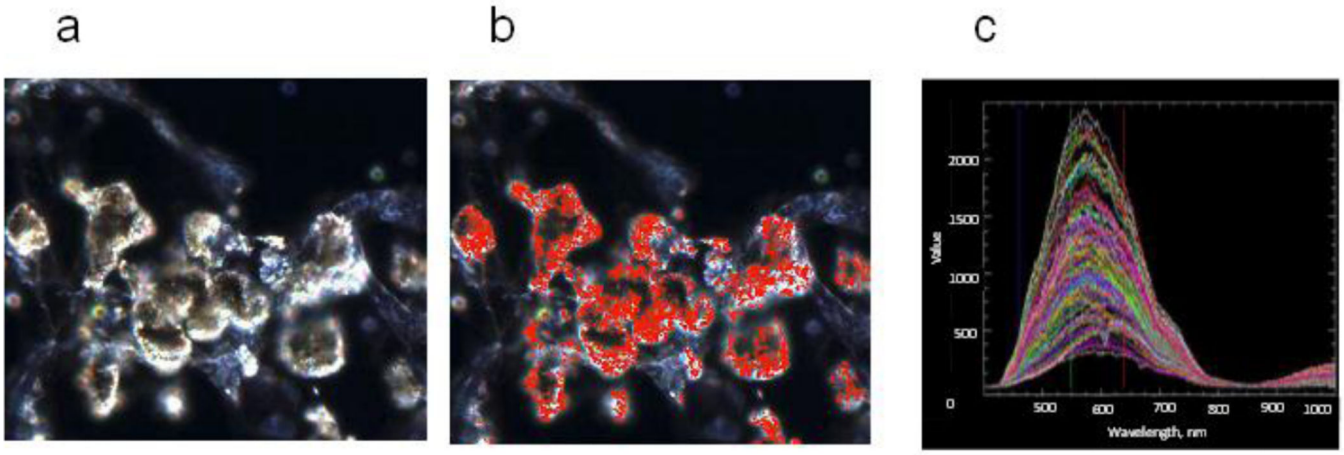

Fig. 9.

Hyperspectral images (CytoViva) of the lung tissue of mice at day 7 after pharyngeal aspiration of SWCNTs. The scanned images a) were collected at 40 × employing an Olympus BX-51 microscope and a 100 W quartz-halogen light source. The pixels that match the SWCNTs spectral profiles were mapped in red b). Spectral data c) was captured with the CytoViva spectrophotometer utilizing an integrated CCD camera.

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Table 1

Enzymatic degradation of carbonaceous nanomaterials with sp^2 hybridization by *in vivo* relevant oxidative systems.

Biological system	Oxidative equivalents	Carbonaceous nanomaterials	References
Enzymes + H_2O_2			
Myeloperoxidase (MPO)	Compound I, Compound II,	c-SWCNTs PEG-SWCNTs	Kagan et al., 2010; Vlasova et al., 2012;
	HOCl, HOBr	PEG-SWCNTs	Bhattacharya et al., 2014
		Nitrogen-doped CNT cups	Zhao et al., 2014
		Graphene oxide	Kurapati et al., 2015
		Carbon nanohorns	Zhang et al., 2015
Eosinophil peroxidase (EPO)	Compound I, Compound II,	c-SWCNTs	Andón et al., 2013
	HOBr; (low HOCl)		
Lactoperoxidase (LPO)	Compound I,	c-SWCNTs, PEG-SWCNTs ox-SWCNTs and	Vlasova et al., 2011, 2012
	HOBr, HOSCN	ox-SWCNTs+lung surfactant	Bhattacharya et al., 2015
Xanthine oxidase + NO			
(without H_2O_2)	ONOO	c-SWCNTs	Kagan et al., 2014
Xanthine oxidase		ox-MWCNTs, f-MWCNTs	
Hemoglobin	$O_2^{\bullet-} + H_2O_2 \rightarrow HO^{\bullet}$		Sureshbabu et al., 2015
	Tyr-O$^{\bullet}$, HOO$^{\bullet}$, HO$^{\bullet}$	c-SWCNTs, PEG-SWCNTs	
Cytochrome *c*	Tyr-O$^{\bullet}$, HO$^{\bullet}$	Graphene oxide	Vlasova et al., 2011, 2012
Phagolysosomal simulant fluid + H_2O_2	n.d.	ox-SWCNTs, ox-MWCNTs	the present paper Russier et al., 2010
Murine bronchoalveolar lavage fluid + H_2O_2 + NaSCN	LPO, MPO, HOSCN	ox-SWCNTs	Bhattacharya et. al., 2015
Neutrophil extracellular traps + H_2O_2 + NaBr	MPO, HOCl, HOBr	ox-SWCNTs	Farrera et al., 2014
Human neutrophils + fMLP and cytochalasin B + fMLP and cytochalasin B + serum opsonized zymosan	MPO, HOCl	c-SWCNTs+IgG PEG-SWCNTs HSA-SWCNTs	Kagan et al., 2010;
			Bhattacharya et al., 2014
Macrophages:	ROS, ONOO-		Lu et al., 2014
Human monocyte-derived		c-SWCNTs+IgG	
THP-1		c-SWCNTs	
THP-1		MWCNTs-NH_2	Kagan et al., 2010
RAW 264.7, THP-1		Carbon nanohorns	Kagan et al., 2014
			Elgrabli et al., 2015
Murine eosnophils + cytochalasin B and PAF	EPO, HOBr	ox-SWCNTs	Zhang et al., 2015

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Biological system	Oxidative equivalents	Carbonaceous nanomaterials	References
Myeloid-derived suppressor cells	MPO, HOCl	Nitrogen-doped CNT cups	Andón et al., 2013
Primary microglia	n.d. (ROS, RNS)	ox-MWCNTs, MWCNT-NH_2,	Zhao et al., 2014
Experimental animals			
-Pharyngeal aspiration		ox-MWCNT-NH_2	Bussy et al., 2016
w/t and MPO k/o mice -Pharyngeal aspiration	MPO, HOCl	c-SWCNTs	
w/t and NADPH-oxidase-deficient mice	ONOO-	c-SWCNTs	Shvedova et al, 2012
-Stereotactic administration			Kagan et al, 2014
C57BL/6 mice	(ROS)	MWCNT-NH_3^+	Nunes et al., 2012

ROS – reactive oxygen species; RNS – reactive nitrogen species; n.d. – not determined.

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Table 2

Standard reduction potentials for redox couples of oxidants which are capable to oxidize and degrade carbonaceous nanomaterials *in vivo* (E° > 0.5 V). pH 6.5-7.0, 25°C.

Redox couple	Reduction potentials (E°, V)	Reference
Myeloperoxidase:		
Compound I/compound II	1.35	Furtmuller et al., 2003
Compound II/ferric enzyme	0.97	Furtmuller et al., 2003
Eosinophyl peroxidase	n.d.	
Lactoperoxidase:		
Compound I/compound II	1.14	Furtmuller et al., 2005
Compound II/ferric enzyme	1.04	Furtmuller et al., 2005
Cytochrome P450		
Compound I/Compound II	1.35	Koppenol, 2007
$HO^{\bullet}$, H^{+}/H_2O	2.31	Buettner, 1993
$RO^{\bullet}$, H^{+}/ROH	1.60	Buettner, 1993
$HOO^{\bullet}$, H^{+}/H_2O_2	1.06	Buettner, 1993
$ROO^{\bullet}$, H^{+}/H_2O_2	1.00	Buettner, 1993
$Trp^{\bullet}$, H^{+}/Trp	1.05	DeFillipis et al., 1989
$Tyr\text{-}O^{\bullet}$, $H^{+}/Tyr\text{-}OH$	0.94	DeFillipis et al., 1989
$ONOOH$, H^{+} /$^{\bullet}NO_2$, H_2O	1.40	Koppenol et al., 1992
$ONOOH$, H^{+} /NO_2^{-}, H_2O	1.20	Koppenol et al., 1992
$HOCl$, H^{+}/Cl^{-}, H_2O	1.48	Vanýsek, 2011
$HOBr$, H^{+}/Br^{-}, H_2O	1.33	Vanýsek, 2011
$HOSCN$, H^{+}/SCN^{-}, H_2O	0.56	Arnhold et al., 2006

E° = 0.5V – reduction potential of the valence band for SWCNTs (Gratzel, 2001; Choi et al., 2002).

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Table 3

X-Ray Photoelectron Spectroscopy (XPS) characterization of GO samples. Carbon/oxygen content (calculated from survey spectra) and relative concentrations of functional groups (calculated from hi-res C1s spectra) in graphene oxide samples

	Control	**+ H_2O_2**	**+ cyt *c* + H_2O_2**
C:O	1.72	2.34	1.52
C−O (%)	18.19	17.20	28.05
C=O (%)	0.23	0	0.01
O−C=O (%)	1.40	1.72	0.15
C−C (%)	80.17	81.07	71.79