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ROS evaluation for a series of CNTs and their derivatives using an ESR method with DMPO

S Tsuruoka[1], K Takeuchi[2], K Koyama[2], T Noguchi[1], M Endo[1], F Tristan[1], M Terrones[1], H Matsumoto[3], N Saito[4], Y Usui[2], D W Porter[5] and V Castranova[5]

[1]Shinshu University, Research Center for Exotic Nanocarbons, 4-17-1 Wakasato, Nagano, Japan
[2]Shinshu University, Faculty of Engineering, *ditto*
[3]Department of Organic and Polymeric Materials Tokyo Institute of Technology, 2-12-1 Ookayama, Meguro-ku, Tokyo, JAPAN
[4]Department of Applied Physical Therapy, Shinshu University, School of Health Sciences, 3-1-1 Asahi, Matsumoto, Nagano, Japan
[5]National Institute for Occupational Safety and Health, Morgantown, WV, USA

s_tsuruoka@shinshu-u.ac.jp

Abstract. Carbon nanotubes (CNTs) are important materials in advanced industries. It is a concern that pulmonary exposure to CNTs may induce carcinogenic responses. It has been recently reported that CNTs scavenge ROS though non-carbon fibers generate ROS. A comprehensive evaluation of ROS scavenging using various kinds of CNTs has not been demonstrated well. The present work specifically investigates ROS scavenging capabilities with a series of CNTs and their derivatives that were physically treated, and with the number of commercially available CNTs. CNT concentrations were controlled at 0.2 through 0.6 wt%. The ROS scavenging rate was measured by ESR with DMPO. Interestingly, the ROS scavenging rate was not only influenced by physical treatments, but was also dependent on individual manufacturing methods. Ratio of CNTs to DMPO/ hydrogen peroxide is a key parameter to obtain appropriate ROS quenching results for comparison of CNTs. The present results suggest that dangling bonds are not a sole factor for scavenging, and electron transfer on the CNT surface is not clearly determined to be the sole mechanism to explain ROS scavenging.

1. Introduction

Carbon nanotubes (CNTs) are expected to have broad potential to materialize new and prominent technologies over the next decades [1-3], and CNT hybrid materials with the other carbon groups and/or doped elements are also under development [4]. For instance, a specific rubber composite of CNTs is being successfully commercialized, which significantly increases recovery from oil wells [5] and may significantly impact the global energy market. For another instance, particular types of CNTs exhibit super conductivity without addition of other elements [6]. These results will accelerate the product development with CNTs further.

It is critically important to evaluate the material safety and develop appropriate in-use regulations for CNTs and nano carbon materials prior to the major industrialization. There are many reports on the evaluation of CNT safety which discuss the similarity to asbestos in shape and suggest that CNTs may exhibit asbestos-like carcinogenicity [7]. It has been proposed that conventional approaches be employed using physicochemical characteristics and in vitro or in vivo assays to evaluate toxicity of nanoparticles [8]. Maynard et al. [9] summarized the early stage studies on nanotechnology and occupational health following intensive discussions of possible adverse effects of CNTs [10-15]. The first systematic evaluation on CNT toxicity was reported by Shvedova et al. [16] followed by an inhalation [17] and bolus instillation [18, 19, 20] studies. CNTs were demonstrated to induce fibrosis and granulomatous lesions at relatively low exposure doses. In addition, CNT-induced mesothelioma was reported at higher doses after installation into the abdomen or scrotum of rodents [21, 22]. Results suggest that inflammation of the mesothelial lining is dependent on the length of CNTs [23].

It is essentially required to utilize physicochemical properties of materials to reinforce the conventional toxicological evaluations. However, it has been recently argued whether those conventional methods are applicable for fibril materials such as CNTs because bolus instillation and inhalation evaluation results do not correspond with *in vitro* tests (as discussed at the annual conference of Society of Toxicology Mar. 2012 in San Francisco [24]). Results of reactive oxygen species (ROS) measurement appear to correspond with CNT *in vitro* results. Shvedova et al. [25] conducted examination of free radical generation after exposure of HaCaT cells to Single-Walled CNTs (SWCNTs) using electron spin resonance (ESR) spin trapping. Fenoglio et al. [26] discussed the relationship between ROS and biological evaluations. The current spin trapping method is pursued using indirect measurement with a spin adduct that forms the stable amine-oxide radical and relatively-long-lived free radicals. The adduct gives a measurable hyperfine coupling constant of spin, and the concentration of free radical is obtained analyzing area of the ESR signal spectrum. Measurement of HO and HO_2 free radicals with nitrones (the N-oxide of an imine) was proposed by Harbor et al. [27]. Among various spin trapping compounds, 5, 5-dimethyl-1-pyrroline-N-oxide (DMPO) is often used for biological radical spin trapping because it dissolves into aqueous solution well with selectivity of radical molecules captured [28]. The author, nevertheless, addressed the limitations of DMPO. Shvedova et al. [25] likewise discussed the issue that ferrous irons associated with SWCNTs can cause the decomposition of hydrogen peroxide or alkoxyl radicals from lipid peroxide, since the unpurified SWCNTs used in that study contained up to 30% iron residue. They concluded that raw SWCNTs have a relatively high risk potential to induce inflammatory lesion at their manufacturing places. Murray et al. [29] reported thereafter that the OH radical generation was mostly caused by the iron residue via the Fenton reaction. Furthermore, Porter et al. [18] demonstrated that purified Multi-Walled CNTs (MWCNTs) scavenge OH radicals generated by the Fenton reaction and do not generate ROS in an acellular system containing hydrogen peroxide. Fenoglio et al. [26, 30, 31] conducted ROS-ESR measurement of CNTs by several methods and concluded that purified MWCNTs quench OH radicals.

The present study aims to evaluate ROS generation by number of CNTs and verifies the assumption that surface morphology of CNTs affects ROS scavenging potential. In addition, we evaluated the importance of the CNT to DMPO/hydrogen peroxide ratio to obtain appropriate scavenging rates.

2 Materials and Methods

2.1. Materials

Seventeen kinds of CNTs were prepared and titanium dioxide was used as a reference. As shown in Table 1, Group A consists of a cup-stack type of MWCNTs (cs-CNTs) and their derivatives. Particular CNTs identified as CS1 of Group A were the pivotal ones in the present study and were prepared and characterized intensively. CNTs identified as CS2 through CS5 were prepared to investigate effects of surface morphological changes. CS2 was prepared by annealing at 2800°C in inert gas atmosphere.

CS3 and CS4 were chopped by machines to shorten the length. CS5 was rubbed to de-bulk the agglomerated CS1 mechanically so that the outer surfaces were damaged significantly. CS4-PT and CS5-PT were deposited with platinum (20 wt%) after annealing at 2800 °C. Figure 1 shows a transmission electron micrograph of CS1 and CS5-PT. As CS1 has a lot of dangling bonds on the surface in comparison with usual CNTs, it was assumed that physical treatments might give quantitative differences by significantly causing morphological changes as suggested by Fubuni et al. [32]. Group B consists of commercially available MWCNTs. This group was designed to compare the dependence on manufacturing processes of CNTs. CT1 and CT1-N were synthesized to evaluate nitrogen-doping effects only.

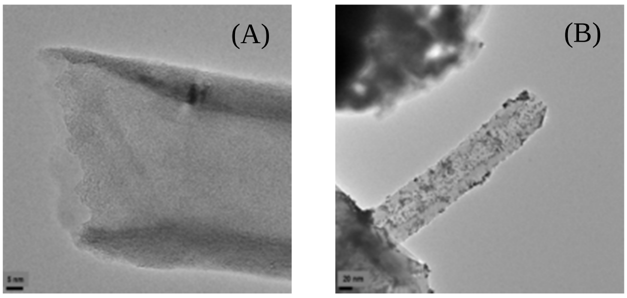

Figure 1. Transmission electron micrograph: (A) cs-MWCNT (CS1), (B) platinum deposited cs-MWCNT (CS5-PT); dots are Pt particles deposited on the CNT surface.

2.2 Measuring method

First of all, dispersibility of the prepared CNTs was tested to avoid agglomeration during the ESR measurements. All CNTs were reasonably dispersed in a DMPO and hydrogen peroxide solution with surfactant after sonication, with no clumping by CNT agglomeration in the pipet or the measuring cell of ESR equipment observed.

The generation and scavenging of radical oxygen species by CNTs were measured at room temperature by ESR (JES-FA200, JEOL) using DMPO as the spin trapping agent. ESR settings were: frequency 9415.404 MHz, power 0.998 mW, field center 335 mT, sweep time 2 min., width +/- 5 mT, and modulation frequency 100 kHz. Fresh frozen DMPO (Dojindo Laboratories, Kumamoto Japan) was thawed at room temperature and diluted to 100 mM with ultrapure water. Hydrogen peroxide (hydrogen peroxide 30%, Wako Pure Chemical Industries, Ltd. Japan) was diluted to 1 mM. Ferrous chloride (Iron (II) Chloride Tetrahydrate, Wako Pure Chemical Industries, Ltd. Japan) was dissolved into ultrapure water at 0.1 mM. Titanium dioxide (Evonik Degussa P25) and CNTs listed in Tables 1-A and 1-B were dispersed into aqueous solution made of 16 mg surfactant (Sodium Dodecyl Benzensulfonate, Kanto Chemical Co., Inc. Japan) and 10 ml ultrapure water. ROS generation by CNTs was measured with the mixed solution (Mixture A) consisting of 0.1 ml of each solution of DMPO, surfactant, hydrogen peroxide and CNTs and the addition of ultrapure water for a total volume of 5 ml. Scavenging ROS generated by Fenton reaction was measured with the mixed solution (Mixture B) consisting of 0.1 ml of each solution of DMPO, ferrous chloride, hydrogen peroxide and CNTs, and ultrapure water for a total volume of 5 ml. CNT concentration was determined by weight in the total volume of 5ml. The blank solution was made with the ultrapure water instead the CNT solution. Each suspension was dispersed by sonication. All of measuring samples were prepared just before ESR measurement to avoid any change over time. Each sample was measured five or more times repeatedly and averaged after elimination of the highest and lowest values.

3. Results and Discussion

The effect of surfactant on the ESR signal is shown in Figure 2. The results show OH radical quenching was proportional to the concentration of surfactant present. Thus, the surfactant concentration was kept constant throughout further experiments. Figure 3 shows a typical ESR spectrum of ROS generation by the Fenton reaction and quenching by CNTs. CNTs and TiO_2 in the absence of ferrous chloride did not produce ROS (Figure 3(A)). Figure 3(B) shows the spectra that indicate radical quenching by CNTs. OH radicals generated by the Fenton reaction between $FeCl_2$ and hydrogen peroxide were apparently quenched by CNTs in a concentration dependent manner.

Table 1-A. CNT properties used for ROS evaluations (Group A)

CNTs	Description	SSA[a] (m^2/g)
CS1	Cup-Stack type MWCNT, diameters: outer = 80 ~100 nm, inner = 50 ~ 70 nm, length = 5 μm ave., purity > 95wt%	49.0
CS2	Graphitized CS1 annealed at 2800 °C	49.5
CS3	Mechanically chopped CS1, average length = 1.5 μm	52.5
CS4	Mechanically chopped CS1, average length = 1.0 μm	74.2
CS5	Mechanically de-bulked CS1, slightly shorter length than CS1	45.8
CS4-PT	Graphitized at 2800 °C of CS4 and deposited with Pt (20wt%)	57.1
CS5-PT	Graphitized at 2800 °C of CS5 and deposited with Pt (20wt%)	39.4

[a] SSA stands for specific surface area measured by BET isotherm adsorption method.

Table 1-B. CNT properties used for ROS evaluations (Group B)

CNTs	Properties[a]			Making methods
	Ave. outer diameter (nm)	Ave. Length (μm)	Purity (wt%)	
DA1	< 1.5	N/A	> 95	CVD[b]
CA1	13 ~ 16	>1	> 95	CVD[b]
CA2	13 ~ 16	>1	> 95	CVD[b]
CA3	~ 13	1.5	> 90	CVD[b]
CA4	10 ~ 15	0.1 ~ 10	> 90	CVD[b]
CA5	0.7 ~ 70	10	> 95	CVD[b]
CA6	15	3	> 95	CVD[b]
CB1	40 ~ 90	4.9[d]	99	FCM[c]
CB1-T	40 ~ 90	4.9[d]	99	FCM[c]
CB2	80	N/A	> 99	FCM[c]

[a] Properties of manufactured CNTs are from manufacturers' specifications.
[b] Catalytic chemical vapor deposition
[c] Floating catalyst method
[d] The measured length by scanning electron micrograph

Figure 4(A) and 4(B) show the scavenging rates for various CNTs at concentrations of 0.2, 0.4 and 0.6 wt%, and those normalized figures by CNT weight concentrations, respectively. The scavenging rate was calculated in comparison to the blank. Figure 4(A) clearly shows that radical quench ability depends on the particular CNT tested. CA1 and CA2 were obtained from the same manufacturer but exhibited different dispersibilities in media. They did not quench OH radicals, but enhanced OH radical generation when compared to the blank. CA3 behaves as an OH radical scavenger at low concentrations, but generates radicals at the highest CNT concentration. As CA4 through CA6 were produced from the similar process but did not generate OH radicals, it suggests that CA1 through CA3 included something reacting with $FeCl_2$. CB1-T, according to the manufacturer, was a reduced bulk volume product of CB1 using glue. However, as CB1-T behaved differently, the glue or the gluing process lowered OH radical quenching at the low concentration. CT1-N was the same as CT1 but nitrogen-doped. Nitrogen doping reduced quenching. These results suggest that different CNTs exhibit different surface activity. Thus, each product must be evaluated individually in terms of occupational health and environmental safety. Figure 4(B) specifically tells us that balance between CNTs and

DMPO/hydrogen peroxide has to be considered carefully when conducting ROS evaluations. Scavenging rate may reach almost 100% at the lowest concentration of CNTs but quenching per weight decreases at the higher CNT concentration

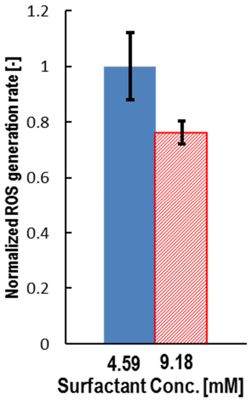

Figure 2. Interaction of surfactant to OH radical quench using Mixture B without CNTs; The vertical axis indicates that the normalized average intensity of the ESR signal was set at 1.00. The deviation bars indicates standard deviations.

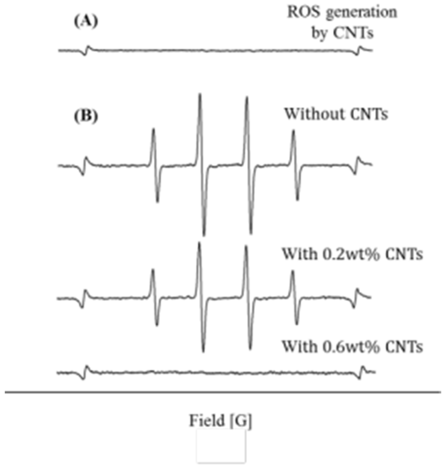

Figure 3. Typical ESR spectrum intensity of free radical adducts with DMPO using CS1; (A) OH radicals generated by CNTs without $FeCl_2$. (B) OH radicals generated by the Fenton reaction with $FeCl_2$. The more radicals were scavenged with the higher CNTs concentration.

The relationship between CNT surface morphology and OH radical quenching was evaluated using CS1 and its derivatives. Figure 5 shows results normalized by the individual specific surface area. It demonstrates that surface morphology has a small effect on OH radical quenching as does addition of Pt, i.e., augmentation of quenching. Since platinum catalyzes hydrogen peroxide, synergetic decomposition of OH radicals may occur. Figure 5 suggests that the shortest CNT (CS4) exhibited the lowest scavenging rate, and that radical quenching corresponds to length in addition to the surface properties. Meanwhile the graphitization that increases crystallinity and decreases the number of dangling bonds apparently affected quenching. DA1, double-walled CNTs, were also strong ROS quenchers (Figure 4). Further investigation is necessary to determine the role of electron charge transfer on the CNT surface with and without dangling bonds.

The present study demonstrates two major points. First, commercial CNTs have different characteristics, depending on processing, which affect ROS quenching. Second, CNT scavenging rate depends on the surface morphology, length and particles deposited on the surface. In addition, since CNTs have high OH radical quench ability, concentration ratio between CNTs and DMPO/ hydrogen peroxide has to be considered carefully in order to obtain appropriate ROS results. The present ROS measuring method by ESR is simple and reproducible but chemical reactions of CNTs with DMPO have to be reviewed.

4. Conclusion

Seventeen CNTs at three concentrations were evaluated to determine their ROS generation or scavenging rate. The majority of CNTs tested quenched OH radicals. The scavenging rate depended on individual characteristics determined by the CNT synthesis process and surface morphology. CNTs from Group A demonstrate a dependence on surface morphology, which supports the assumption discussed by Fenoglio et al. [30] and Fubini et al. [32]. On the other hand, the OH radical degeneration reaction is far more complicated than predicted. Parameters to describe CNT scavenging characteristics have to include not only metal impurities but also surface morphology, such as the number of dangling bonds, length, and deposition of particles. The results suggest necessity of the further investigation of the role of impurities and CNT surface features in hydroxyl radical generation or scavenging.

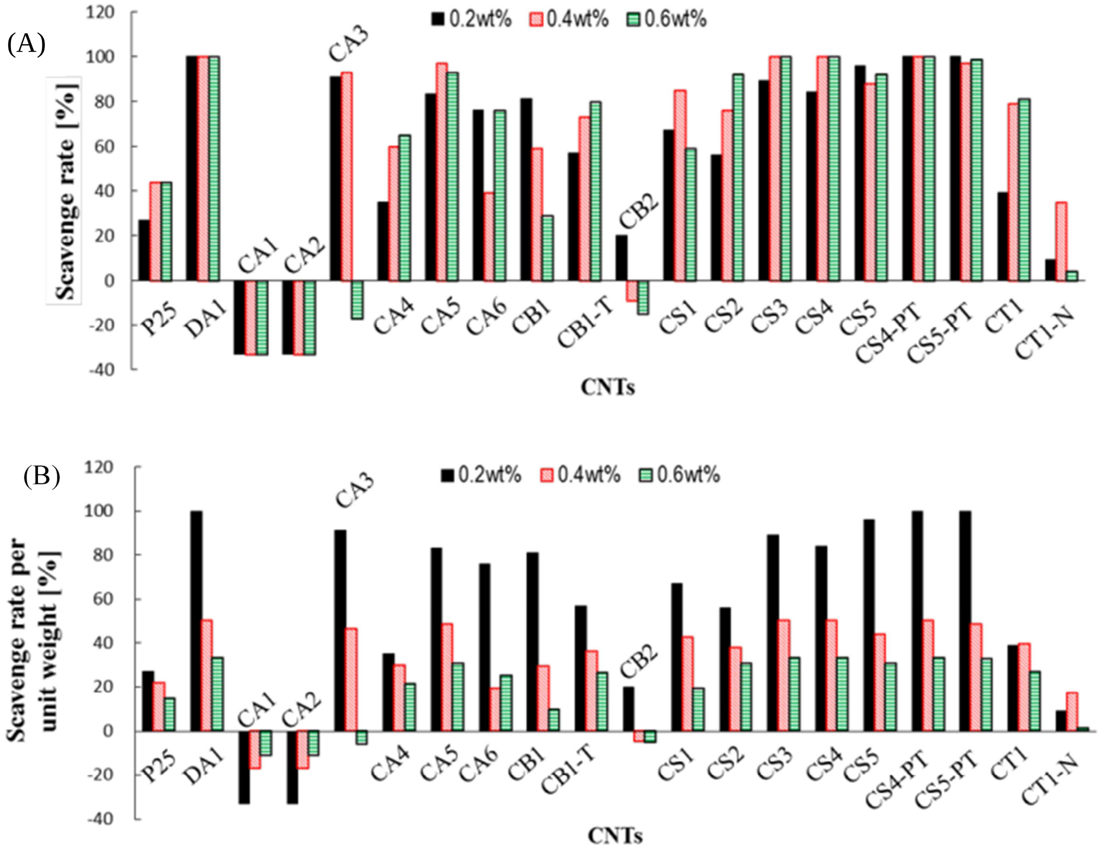

Figure 4. Scavenge rate of OH radicals by various CNTs with concentration change: (A) Scavenge rate at 0.2, 0.4 and 0.6 wt% of CNTs. (B) Scavenge rate per unit CNT weight (0.2 wt%) at 0.2, 0.4 and 0.6 wt % of CNTs; OH radicals were generated by the Fenton reaction.

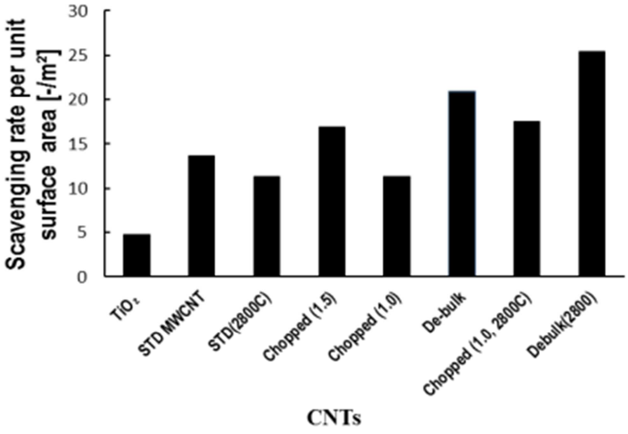

Figure 5. Scavenging rate of cs-CNT derivatives; scavenging rates at 0.2 wt% of CNT were normalized by specific surface area measured by BET isotherm adsorption.

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