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Effect of surface functionalizations of multi-walled carbon nanotubes on neoplastic transformation potential in primary human lung epithelial cells

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Abstract

Functionalized multi-walled carbon nanotube (fMWCNT) development has been intensified to improve their surface activity for numerous applications, and potentially reduce toxic effects. Although MWCNT exposures are associated with lung tumorigenesis *in vivo*, adverse responses associated with exposure to different fMWCNTs in human lung epithelium are presently unknown. This study hypothesized that different plasma-coating functional groups determine MWCNT neoplastic transformation potential. Using our established model, human primary small airway epithelial cells (pSAECs) were continuously exposed for 8 and 12 weeks at 0.06 $\mu\text{g}/\text{cm}^2$ to three-month aged as-prepared-(pMWCNT), carboxylated-(MW-COOH), and aminated-MWCNTs (MW-NH_x). Ultrafine carbon black (UFCB) and crocidolite asbestos (ASB) served as particle controls. fMWCNTs were characterized during storage, and exposed cells were assessed for several established cancer cell hallmarks. Characterization analyses conducted at 0 and 2 months of aging detected a loss of surface functional groups over time due to atmospheric oxidation, with MW-NH_x possessing less oxygen and greater lung surfactant binding affinity. Following 8 weeks of exposure, all fMWCNT-exposed cells exhibited significant increased proliferation compared to controls at 7 d post-treatment, while UFCB- and ASB-exposed cells did not differ significantly from controls. UFCB, pMWCNT, and MW-COOH exposure stimulated significant transient invasion behavior. Conversely, aged MW-NH_x-exposed cells displayed moderate increases in soft agar colony formation and morphological transformation potential, while UFCB cells showed a minimal effect compared to all other treatments. In summary, surface properties of aged fMWCNTs can impact cell transformation events *in vitro* following continuous, occupationally relevant exposures.

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Supplemental data for this article can be accessed [here](#).

Keywords

Surface properties; aging effects; functionalization; multi-walled carbon nanotubes; cell transformation

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Introduction

Past multi-walled carbon nanotube (MWCNT) toxicity studies have primarily focused on assessing risk for pulmonary inflammation, genotoxicity, fibrosis, and tumorigenesis due to aerosolization of MWCNTs during synthesis or subsequent handling. MWCNTs cause aneuploidy via centromere abnormalities in exposed human epithelial cells and are considered a possible human carcinogen (Grosse et al., 2014; Sargent et al., 2014; Siegrist et al., 2014). Furthermore, recent *in vivo* evidence suggests that MWCNTs directly induce lung tumors and may be a complete carcinogen (Kasai et al., 2016). MWCNTs differ in their physicochemical properties, such as length, diameter, surface area, and surface chemistry, which can drive adverse pulmonary health effects (Donaldson et al., 2013). Expansion of the MWCNT market for a wide array of purposes has resulted in surface functionalization of MWCNTs, either directly during synthesis (i.e., doping) or post-synthesis, with small organic molecules (e.g., carboxylation) or oxidized metals (e.g., aluminum oxide). Functionalization allows for improved dispersion characteristics for incorporation of hydrophobic MWCNTs into hydrophilic polymers or plastics, or provides enhanced electrostatic properties (Kim, 2011). Advances in plasma-grafting technology have given rise to plasma polymer coatings on numerous surfaces, including carbon nanotubes (CNTs; Khelifa et al., 2016). Recent comparisons of fMWCNTs to pristine MWCNTs suggest that carboxyl fMWCNT induce less lung inflammation, toxicity, and fibrosis following pulmonary exposure compared to pMWCNT (Hamilton et al., 2013a,b; Poulsen et al., 2016; Sager et al., 2014). However, other studies report that fMWCNT exhibit equal or greater potency compared to pMWCNT (Dandley et al., 2016; Patlolla et al., 2010; Ursini et al., 2016).

Estimates for the number of U.S. workers who would come in contact with MWCNTs soon after synthesis is small compared to the potential number of downstream users across the MWCNT life cycle (Mackevica & Hansen, 2016). In most instances, MWCNT exposures are expected to occur over long time frames at low levels (Kuempel et al., 2017). Once MWCNTs move downstream from their manufacturing site into additive manufacturing, modifications to their surface, structural integrity, and other physicochemical properties are expected due to their wide spread use in numerous applications, each with their own physical and chemical requirements. Changes or elimination of surface functionalization will not only change the technological abilities, but possibly the transport, transformation, and toxicological effect of MWCNTs if released into the environment (Petersen et al., 2011). To date, few studies have examined the changes in physicochemical properties of MWCNTs across their life cycle (Dahm et al., 2012; Hedmer et al., 2014), and how these changes potentially impact worker pulmonary health (Bishop et al., 2016; Kuempel et al., 2017). No studies have evaluated the life cycle impacts on MWCNT tumorigenic potential.

Aging and transformation of engineered nanomaterials (ENMs) across their life cycle can directly alter unique physicochemical properties (Mitrano et al., 2015), thus affecting not only their nanotechnological application but also toxicological responses. At present, a majority of both exposure and effect toxicological research has evaluated primarily as-produced, pristine ENMs with little consideration of how life cycle transformations affect hazard, release, fate, exposure, and effect. Given ENMs' unique physicochemical properties that are typically designed for unique applications, it is highly likely that transformation processes across the ENM life cycle will change these physicochemical properties. Transformation of various pristine and functionalized ENMs may increase similarity or result in greater diversity of their physicochemical properties. Since a majority of current ENM risk assessment relies on linking as-produced physicochemical properties of pristine ENM with toxicological effect, it is likely that these predictive estimates may over- or underestimate toxicological hazard over an ENM's life cycle (Lowry et al., 2012). Given the large data gaps and uncertainties associated with ENM transformation, release, and potential long-term exposures in the workplace, it is important that research efforts begin to evaluate potential long-term health effects, including carcinogenesis (Becker et al., 2011).

Consistent with this notion, the main objective of this study was to screen and assess different aged fMWCNTs for potential neoplastic transformation ability using primary human small airway epithelial cells (pSAECs). Based on previous fMWCNT literature, we hypothesized that differences in surface properties would impact aged MWCNT neoplastic transformation ability. We monitored the aging of post-synthesis fMWCNTs in laboratory storage conditions and subsequently conducted long-term fMWCNT exposures of human primary small airway epithelial cells. Cancer cell hallmark assays were employed to determine neoplastic transformation potential. We show that three-month aged MW-NH_x, with low-percent surface oxygen content and enhanced protein-binding ability, caused a greater cell transformation ability than all other fMWCNTs, suggesting that differences in surface properties of aged fMWCNTs impact their cell transformation potential.

Methods

Additional details on methodology can be found in Supplementary materials.

Nanoparticle characterization and dispersion

'As-prepared' (pMWCNT), carboxylated- (MW-COOH), and aminated- (MW-NH_x) multi-walled carbon nanotubes, functionalized by plasma gas, were purchased from Perpetuus Carbon Technologies (Ammanford, UK) with equal lengths and surface area containing less than 1% wt. of contaminants. pMWCNT served as the parent particle which was then functionalized in proprietary plasma gas atmospheres to acquire both MW-COOH and MW-NH_x. Ultrafine carbon black (UFCB; Elftex 12), Mitsui #7 MWCNT (MWCNT-7), and crocidolite asbestos (ASB; NIEHS standard) were acquired as previously described (Table 1; Wang et al., 2014).

Upon arrival, all MWCNT were immediately assessed for functionalization presence via Fourier transform infrared spectroscopy (FTIR) analysis using previously described methods (Hamilton et al., 2013a,b). FTIR was chosen as a primary characterization method to

monitor time-based changes in graphite surface chemistry (Chang et al., 2016). All MWCNT samples were stored at laboratory room temperature (20–22 °C) and 40% humidity in darkness. FTIR and X-ray photoelectron spectroscopy (XPS) analyses were conducted again at 2 and 12 months post-storage to examine the fMWCNT characteristics over time. MW-COOH were previously reported to possess a significant amount of –COOH functional groups amounting between 7.4 and 7.7% oxygen (NCL NIEHS, 2012). In addition, UFCB and Mitsui #7 MWCNT, our laboratory's benchmark carbon nanomaterials, were also characterized by FTIR. Dry samples of aged fMWCNTs were shipped to Perpetuus Carbon Technologies for independent validation using scanning electron microscopy (SEM), FTIR, and XPS analyses.

All carbon nanomaterials and ASB were prepared for small airway epithelial cell exposure as previously described (Wang et al., 2014). Briefly, MWCNTs, UFCB, and ASB were weighed and placed into 1 mg/ml suspension in sterile MilliQ water. UFCB and MWCNT particles were diluted to 0.1 mg/ml using water containing 150 µg/ml of Survanta® (Abbott Laboratories, Columbus, OH) or 1% bovine serum albumin (BSA) in water to obtain stock solutions. To disperse each material, UFCB and MWCNT stock solutions containing Survanta® and ASB in water were lightly sonicated. All particle suspensions were serially diluted with SAGM to acquire final exposure concentrations ($0.288 \mu\text{g/ml} = 0.06 \mu\text{g/cm}^2$) for each particle with < 0.3% water. These techniques were previously shown to result in characteristic *in vitro* neoplastic phenotypes, fibrogenesis, and genomic signatures that correlated well to *in vivo* models for both MWCNT and ASB (Mishra et al., 2012; Wang et al., 2014). Next, zeta potential was determined for all MWCNT suspensions in sterile MilliQ water and SAGM using a Nanoseries Zetasizer ZS (Malvern). To measure dispersion potential, length/width, and elemental content of particles, freshly sonicated stock solutions were prepared and visualized under field emission SEM (FESEM/EDX) as previously described (Mishra et al., 2012). More than 100 individual MWCNTs per particle type were measured using ImageJ (National Institutes of Health, Bethesda, MD) for dispersed length and width.

To discern differences in the ability of various carbon ENMs ability to bind lung surfactant and protein that may impact toxicological response, each carbon ENM was incubated with Survanta lung surfactant in SAGM medium, analyzed by SDS Page gels, and bound protein quantified using previously described methods (Allegri et al., 2016). Stained protein bands on gels were imaged and quantified using FluoroChem SP.

To identify acellular oxidative potential differences among the particles, dispersed particles were assayed via cell-free dithiothreitol (DTT) depletion reaction as previously described (Roberts et al., 2016). Phosphate-buffered saline and Survanta-only water served as controls. Both DTT consumption vs. particle mass (nM/µg) and surface area (nM/m²) were calculated based on absorbance values at 412 nm.

Long-term pSAEC exposure

pSAECs (Lonza) at 2nd passage were seeded overnight in triplicate into 6-well plates holding SAGM (Lonza) and cultured at 37 °C in a humid atmosphere containing 5% CO₂ air throughout the experiment. Following seeding, cells were continuously exposed to 0.06

$\mu\text{g}/\text{cm}^2$ of Survanta-dispersed UFCB, pMWCNT, MW-COOH, MW-NH_x, or water-suspended ASB for 8 and 12 weeks. fMWCNT particles were aged for 3 months under laboratory conditions prior to the start of the exposure. The chosen dose modeled an expected MWCNT deposition of 30 μg in mouse lung which approximates an estimated human occupational exposure of 10–40 $\mu\text{g}/\text{m}^3$ over 27–103 months of light work (Porter et al., 2013). Likewise, it closely matches the deposited dose after inhalation exposure that resulted in MWCNT promotion of lung cancer (Sargent et al., 2014). In addition, this dose gave greater chance for transformation given the shorter life span of pSAECs (<12 weeks; Stueckle et al., 2017) than immortalized SAECs in our previous 0.02 $\mu\text{g}/\text{cm}^2$ six month exposure study (Wang et al., 2014). Water and dispersant exposed cells were used as controls. Every three days old medium was removed, adherent cells rinsed in HEPES buffered saline, and fresh SAGM medium with or without particles was administered. All treatments were passaged every 8–10 days following manufacturer's protocol by washing liberally with saline, incubating with 0.25% trypsin/EDTA, and inactivating trypsin with 2× v/v neutralization buffer. Rinsing attached cells prior to culture medium changes and cell passages, and reseeding at 25% confluence aided in reducing potential bioaccumulation of MWCNT over the exposure time course.

Darkfield enhanced microscopy

Uptake and localization of UFCB, MWCNTs, and ASB in pSAECs was performed and imaged using enhanced darkfield imaging (CytoViva, Auburn, AL) following acute 24 h exposure in low passage pSAECs (Wang et al., 2014).

Cancer hallmarks assessment

To evaluate cancer cell behavioral hallmarks, cells from each exposure replicate were assessed at both 8 and 12 week time points using well-established methods (Creton et al., 2012; OECD 2007; Wang et al., 2014) with minor modifications as described in the Supplementary material. Cell proliferation, transformation and aggressive cell mobility were measured using (a) Trypan blue, WST-1, and colony forming unit (CFU) assays, (b) soft agar colony, and morphological transformation assays, and (c) Transwell migration and invasion chemotaxis assays, respectively.

Statistical analyses

All experimental data sets were evaluated for normal distribution of the residuals and variance homoscedasticity to satisfy ANOVA assumptions. Dispersant-only cells acted as negative passage control in all statistical comparisons. Treatments were compared using one-way ANOVA analyses followed by Dunnett's or Tukey–Kramer honestly significant difference *post-hoc* tests ($\alpha = 0.05$).

Results

Aged MWCNT characterization

Following receipt of shipment, MWCNTs were assessed for differences in surface functionalization using FTIR. All fMWCNTs possessed peaks at 2915 and 2848 cm^{-1} associated with C–H stretch, and a strong peak at 1385 cm^{-1} corresponding to the

deformation vibration (Figure 1(A)). Peaks at 1627 cm^{-1} and 1569 cm^{-1} were associated with MWCNT aromatic ring skeletal vibrations. In addition, a strong peak at 3436 cm^{-1} was associated with O–H stretch. For the MW-COOH particles, peaks at 1720 cm^{-1} associated with C = O, 2511 cm^{-1} was due to carboxylic acid, and the broad $950\text{--}1230\text{ cm}^{-1}$ peak was ascribed to the C–O vibration, respectively, indicated the presence of carboxyl surface functionalization. A peak ranging $1410\text{--}1460\text{ cm}^{-1}$ was assigned to C–O stretching typical of carboxylic groups (Li et al., 2012). For MW-NH_x, weak peaks at 1617 and 1643 cm^{-1} clustered near the 1627 cm^{-1} peak, along with a strong 1539 cm^{-1} and weak 1560 cm^{-1} peak (Supplementary Figure 1A), indicated presence of N–H scissoring and stretching, respectively (Chidawanyika & Nyokong, 2010), which was distinct from all other MWCNTs. A weak 1652 cm^{-1} peak suggested the presence of saturated amide bond. These particles exhibited a noticeable similarity to the MW-COOH, primarily due to C–O peaks. However, the C–O vibration peak was reduced compared to the other two MWCNT particles, indicating potential reduced presence of oxygen during the nitrogen functionalization process. In addition, a peak at 870 cm^{-1} associated with a carboxylic or ether group was observed, but relatively absent from pMWCNTs. Negative zeta potentials reflected the nature of the negatively charged plasma gas during synthesis. These characteristics generally supported those reported by the manufacturer (Table 1).

Prior to exposure, all 2 month aged FTIR spectra were dramatically different compared to freshly shipped material (Figure 1(B)) by experiencing the loss of C–O ($950\text{--}1230\text{ cm}^{-1}$), which coincided with the absence of the $1410\text{--}1460\text{ cm}^{-1}$ peak in MW-COOH and MW-NH_x samples. MW-COOH retained C = O (1720 , and 2511 cm^{-1}) and a weak 1160 cm^{-1} associated with C–O, respectively, indicating residual carboxyl surface functionalization. Both pMWCNT and MW-COOH, but not MW-NH_x, possessed small peaks at 1465 (C–H bend), 1541 , and 1570 cm^{-1} (C aromatic), respectively (Supplemental Figure 1B). The absence of aromatic ring peaks in MW-NH_x may have been due to oxidative damage. For MW-NH_x, all previously observed nitrogen-associated peaks were absent. In addition, the C–H 1382 and 870 cm^{-1} peaks were noticeably reduced compared to the other two MWCNT particles. The main peak of C1s from XPS was deconvoluted by fitting into multiple sub-peaks at 284.8 eV (C–C), 285.7 eV (C–O–C), and 286.8 eV (C–OH). XPS analysis (Figure 1(C)) indicated the presence of C–O–C and C = O. The O 1s peak at 530.7 and 533.0 eV indicated the presence of carboxylic and ether groups (Datsyuk et al., 2008). Percent oxygen content was low compared to previous published studies with similar material, with MW-NH_x possessing the lowest content (Table 2). Repeated analysis following 12 months aging showed surface property convergence of all three MWCNTs with C–O–C and C–OH functional groups present and continued reduction in percent oxygen content for all fMWCNTs (Supplementary Figure 2; Table S1). For comparison, FTIR and XPS analyses were also performed on MWCNT-7 which showed O–H and C–O functional groups (Supplementary Figure 3). These findings indicate that the fMWCNT experienced autoxidation during laboratory storage with MW-NH_x possessing less percent oxygen than the other MWCNTs.

SEM analysis of the MWCNT samples showed that all samples comprised particles with CNT morphology with little presence of amorphous carbon (Figure 2). No differences were observed among the different MWCNT particle morphologies once dispersed in Survanta.

Polydisperse MWCNT morphologies comprised single MWCNT, nanoropes (~55–60 nm wide), and agglomerates measuring several microns in size (Figure 2). Length and width measurements indicated equivalent short lengths and narrow widths for the various dispersed MWCNT samples tested (Table 1; $p > .05$). Particle uptake analysis showed that UFCB and all the MWCNT particles co-localized with either the cytoplasm or nucleus of the cells (Figure 3(A)) with minimal evidence that internalized MWCNTs retained a well-dispersed fiber morphology. All MWCNTs appeared as rough spherical particles or loose agglomerates while fiber-shaped MWCNTs were occasionally found.

Protein corona and surface oxidant potential

Next, UFCB and MWCNTs were characterized for their ability to bind lung surfactant and SAGM protein to assess differences in agglomeration and ability to interact with biomolecules. All carbon ENMs bound significantly more protein than lung dispersant only following 1 h incubation in SAGM medium (Figure 3(B)). Moreover, MW-NH_x bound significantly more total protein (13-fold) than UFCB and the other MWCNTs (5.8-fold) compared to control. Separate quantification of the high molecular weight, low molecular weight, and strong band 75–90 kD proteins resulted in no significant differences among the carbon ENMs (data not shown). Lastly, all three polydisperse MWCNTs showed equivalent moderate surface oxidant potential (nM/μg), which was significantly lower on a surface area basis, but not a mass basis, than UFCB (Supplementary Figure 4), possibly indicating that gas absorption of dry powder does not adequately reflect structure surface area in suspension (Table 1 and Figure 2).

Cell transformation assessment

Cell proliferation was monitored every two weeks as a marker for both stimulation of cell growth signaling and an early marker for cell transformation. After 8 weeks of particle exposure, all MWCNT-exposed cells showed a significant increase in live cells at Day 7 post-seeding in particle-free medium compared to unexposed and UFCB-exposed cells (Figure 4(A)). In contrast, ASB exposure reduced live cell number. Next, UFCB and pMWCNT at both time points, and MW-NH_x 48 h after an 8 week exposure, displayed subtle, but significantly increased mitochondrial metabolism compared to passage control cells (Figure 4(B)), while ASB-exposed cells showed no difference from control. In the CFU assay, none of the exposed cells showed decreased viability compared to dispersant-only controls (Supplementary Figure 5). pMWCNT and MW-COOH exposed cells showed significant moderate increase in attached CFUs. Passaging of pSAECs over 8 weeks appeared to strengthen CFU ability since saline-exposed cells showed reduced ability. Continuation of the exposure to 12 weeks resulted in the loss of cell proliferation ability for all cells compared to 8-week data. Passage control and UFCB displayed a significant 50–60% reduction in proliferation ability while all fiber particle-exposed cells showed a 75–90% loss (Figure 4(C)). All passaged cells lost proliferation ability and exhibited senescence by 14 weeks in culture.

Neoplastic transformation of epithelial cells requires escape from single layer growth morphology, avoiding cell death, and gaining the ability to form both attached, multilayered foci and unattached soft agar colonies (Creton et al., 2012; OECD, 2007). At 8 weeks, only

UFCB and MW-NH_x cells exhibited significantly increased number of soft agar colonies (Figure 5(A,B)). This effect was modest since colony count per well was low compared to our previous data involving pSAEC and SAEC-hTERT cells exposed to MWCNT-7 (Stueckle et al., 2017; Wang et al., 2014). Attempts to collect individual colonies and reestablish clones in culture (Stueckle et al., 2017) resulted in low survival or cell senescence. Mild morphological transformation was observed only in fiber-exposed cells with multiple Type I foci and occasional Type II foci (Figure 5(C)). MW-NH_x cells showed the highest number of Type II foci while passage control and UFCB-exposed cells displayed minimal evidence of Type I foci (Table S2). These data suggested that MW-NH_x-exposed cells underwent cell transformation.

Transformed cells undergoing carcinogenesis towards an aggressive phenotype typically exhibit retention of the ability to migrate and invade neighboring tissue. To assess carbon ENM-exposed cells for this phenotype, cells after 8 and 12 weeks exposure were assayed for enhanced mobility. At 8 weeks, UFCB-, pMWCNT-, and MW-COOH-exposed cells displayed a significant 2-to-4-fold increase in invasion ability (Figure 6). A significant proportion of the UFCB-exposed cell invasion ability was due to enhanced cell mobility. MW-NH_x and ASB-exposed cells showed no difference compared to passage control cells. To ascertain retention of this ability, a sub-passage of cells were cultured in particle-free medium for one week and re-assayed. All cells showed an increase in migration ability and a decrease in invasion ability compared to 8-week-exposed cells. MW-NH_x and ASB cells showed significantly lower migration ability. UFCB-exposed cells still showed a modest, yet significant, elevated invasion ability. At 12 weeks, all cells, except UFCB cells, showed a drastic reduction in both migration and invasion ability compared to saline-exposed pSAEC cells in a sister study done in parallel (Stueckle et al., 2017). Unexpectedly, UFCB-exposed cells showed a significant enhanced invasion ability. These data suggested that observed increases in migration and invasion ability were particle presence-dependent and that the MW-NH_x cell transformation was early in neoplastic development.

Discussion

Surface functionalization of MWCNTs is known to enhance aqueous dispersion for incorporation into nano-enabled products and change its toxicological response following pulmonary exposures. The inherent surface redox potential is thought to influence CNT interactions with cellular components, thus impacting toxicity and eventual risk for disease. Changes to an ENM's surface reactivity due to aging, either during storage or release into the environment, are expected during its life cycle. However, little research has evaluated how these changes may impact disease development (Mitrano et al., 2015). The aim of the present study was to evaluate how differences in surface properties of aged fMWCNTs impacted neoplastic transformation potential in a long-term exposure primary epithelial cell screening model. We found that three-month aged MW-NH_x, with relatively low amounts of surface oxygen and enhanced protein-binding ability harbored a greater cell transformation ability compared to pMWCNT, MW-COOH, and UFCB. These data highlight that plasma fMWCNTs experience atmospheric aging over relatively short time frames during their life cycle and that resulting surface properties can influence measurable toxicological effects.

Numerous techniques for surface modification of CNTs allow a wide variety of enhanced dispersion, improved biocompatibility, technological flexibility, and application for CNTs. However, such advances have induced additional uncertainty into toxicological effects and relative health risks associated with workplace exposure. Different surface functionalization strategies, storage environments, product incorporations, and potential release scenarios represent factors in the 'toxicological evolution' (Liu et al., 2015) of CNTs over their life cycle. Although chemical oxidation of MWCNTs allows control over amount and maintenance of surface functionalization, acid treatment can shorten and damage MWCNTs, which can interfere with technological application (Datsyuk et al., 2008), and has complicated efforts to correlate physicochemical properties with pulmonary toxicological effects. For example, recent *in vivo* studies reported that short-term MW-COOH exposure resulted in reduced inflammation, cytotoxicity, rapid clearance, and fibrosis compared to parent MWCNTs (Hamilton et al., 2013a,b; Jain et al., 2011; Sager et al., 2014). Anionic –COOH functionalization reduced epithelial, fibroblast, and macrophage cytotoxicity and inflammasome activation (Sayes et al., 2006), while strong cationic surfaces increased surface reactivity, resulting in strong inflammasome activation and fibrogenic potential (Li et al., 2013b). Other studies, however, found no difference between –COOH and –NH₂ functionalized MWCNT toxicity, since functional group presence caused increased protein binding and agglomeration (Allegri et al., 2016; Coccini et al., 2013).

Conversely, other studies suggest that functionalization allows for better MWCNT dispersion in biological fluids, thus increasing their toxicity potential. For example, aspiration of well-dispersed –COOH functionalized single-walled CNTs (fSWCNTs) caused greater inflammation than parent particle in CD1 mice (Saxena et al., 2007). Intraperitoneal injection of MW-COOH caused greater clastogenic potential in collected bone marrow cells than poorly dispersed parent MWCNTs (Patlolla et al., 2010). Although no differences between functionalized –COOH, –NH₂ vs. parent MWCNTs to induce inflammation and airway damage were found, a follow-up study identified –NH₂ causing more pronounced PCNA and IL-6 expression in exposed rat alveolar tissue than other MWCNTs (Coccini et al., 2013; Roda et al., 2011). *In vitro* administered fSWCNTs and fMWCNTs with strong negative charge also increased genotoxicity, hampered natural killer cell function, and inhibited cell division. Of note, an increasing number of recent studies show that fCNTs exhibit enhanced genotoxicity and apoptosis induction compared to parent CNTs, but the opposite for cellular damage and cytotoxicity assays, possibly due to uptake route (Alam et al., 2016; Mrakovcic et al., 2015; Ursini et al., 2012).

Alternatively, plasma gas functionalization allows for a rapid and clean method for surface modification for carbon nanomaterials and provides a highly reactive surface with chemical resistance and coating properties for MWCNT incorporation into multiple applications (Vohrer et al., 2007). Plasma coating of surfaces relies on using free radicals to bind unique polymers onto surfaces. Inductive coupled plasma effectively grafts several different functional group types to CNT surfaces with scaling up potential to industrial scales (Felten et al., 2005). Many negative side effects, such as carbon matrix damage, lengthy process, and average shorter tube lengths are avoided (Naseh et al., 2010). The highly reactive surface without stable covalent bonds, however, also renders the surface highly susceptible to atmospheric oxidation (Khelifa et al., 2016).

Here, our FTIR time course study of plasma surface functionalization found that both MW-COOH and MW-NH_x experienced significant atmospheric oxidation in laboratory storage conditions. Studies investigating aging of plasma-deposited polymers reported radical-induced autoxidation, which can occur over several weeks to months resulting in substantial addition of random carbon-oxygen functionalities. Whittle et al. (2000) reported that the loss of oxygen-associated peaks in different spectra analyses was due to the loss of low molecular weight oligomers. In addition, carbon–nitrogen bonds were highly susceptible to these reactions resulting in amides and/or rapid loss of nitrogen altogether (Gengenbach et al., 1996; Whittle et al., 2000). Autoxidation during the first few weeks results in degradation or transformation of the plasma-coated surface to hydroperoxides and, over long time scales, random and uncontrolled oxidation (Khelifa et al., 2016; Whittle et al., 2000). These authors suggested that small quantities of surface radicals on plasma treated surfaces may persist over longer time frames (months). Similar to our observation, Chang et al. (2016) showed that drastically different forms of sp² carbon allotropes with unique surface properties converged over time with atmospheric aging resulting in similar surface properties. Thus, based on the inherent instability of the side wall functional groups, time associated with material characterization, and the long-term exposure experimental design, this study was not able to compare and assess neoplastic potential of freshly synthesized plasma fMWCNTs. We did observe a loss of C–H signatures within the FTIR spectra over time. This also coincided with an initial loss (0 vs. 2 month) and then a gain of C–O signature indicating that MWCNTs experienced biphasic autoxidation as the plasma functionalization became unstable. pMWCNTs also exhibited this phenomenon suggesting that argon plasma-synthesized MWCNTs are susceptible to oxidation over time (Vohrer et al., 2007).

Limited studies have evaluated the effects of atmospheric aging on toxicity of fCNTs. Liu et al. (2015) conducted controlled O₃ and OH radical exposure studies over 12 days to evaluate atmospheric aging impacts on SWCNT cytotoxicity. Aged SWCNTs were found to experience random oxidation/reduction reactions, causing increase in surface carboxylic acids and esters with little change in redox or cytotoxic potential. Surprisingly, ambient urban air reduced toxicity through binding of organic vapors. Other recent atmospheric aging studies on CNTs, including MWCNTs indicated that differences in MWCNT diameter and surface morphology influence oxidation reaction kinetics during aging and may influence redox potential (Tsuruoka et al., 2015a,b).

Here, aged fMWCNTs did not vastly differ from aged pMWCNT in SAEC transformation ability since both fMWCNT lost a substantial amount of their functionalization during aging. All aged MWCNT exhibited similar short, thin, poly-dispersed fibers that coincided with a less potent transformation, which largely differed from our findings with long, thick, and more dispersed MWCNT-7 (Stueckle et al., 2017; Wang et al., 2014). This finding matches the established paradigm that increased length and dispersion of high aspect ratio (HAR) fibers increases their toxicity, cell transformation, and tumorigenic potential (Donaldson et al., 2013). Zeta potential differences among MWCNT particles with intrinsic negatively charged surfaces were minimized once in SAGM, suggesting that residual functionalization was minimized by protein corona effect and probably did not contribute to observed transformation differences. Similar dispersion characteristics for all tested

fMWCNTs matched those previously reported (Jackson et al., 2015; Poulsen et al., 2016). In addition, both publications reported that their –OH and –COOH fMWCNTs possessed less oxygen than the pristine MWCNTs, indicating loss of surface functionalization. Of note, even though our particle stock solutions contained water instead of saline (Wang et al., 2014), the small difference in salt concentration (<0.3%) would be negligible over the course of an 8-week exposure given the presence of Survanta lung surfactant in both particle-free and exposed treatment groups. All fMWCNT stimulated cell proliferation rate, which partially correlated with increased mitochondrial metabolism and matches numerous reports that a low MWCNT dose stimulates epithelial cell proliferation associated with either wound healing or hyperplasia, an early indicator of neoplasia (Donaldson et al., 2013). UFCB- and pMWCNT-exposed cells exhibited increased mitochondrial activity, while all fMWCNTs possessed elevated proliferation rates. Similarly, these fMWCNTs, except MW-NH_x, caused transient, particle- dependent invasion ability. These results coincide with published literature concerning low dose carbonaceous ENM stimulation of cell proliferation and invasion processes associated with wound healing via epithelial-mesenchymal transition. UFCB at low doses is known to stimulate cell signaling pathways, epithelial hyperplasia, ROS damage, inflammation, and is a suspected lung carcinogen (IARC, 2010; Kolling et al., 2011). Ozone oxidation is known to increase the redox activity of black soot and may increase its reactivity once exposed to biological systems (Li Q et al., 2013). Black carbon possesses higher amount of disorganized carbon, quinones, and metals, which possess high reactivity compared to graphene carbon (Li R et al., 2013). ASB-exposed cells exhibited reduced cell proliferation and no observable changes in cell mobility or soft agar colony formation ability. These results are consistent given that low-dose asbestos exposures result in DNA damage, cell cycle delay, and low soft agar colony formation in human SAECs over short exposure time frames (Kisin et al., 2011; Wang et al., 2014). Based on these studies, *in vitro* SAEC transformation does not predict asbestos-induced carcinogenesis. The decline in cell proliferation for all HAR fibers after 12 weeks exposure suggested potential accumulated genetic damage, primary cell senescence, and matched our previous findings with MWCNT-7s (Stueckle et al., 2017).

Conversely, MW-NH_x-exposed cells showed significantly greater attachment-independent growth ability in soft agar, greater number of Type II foci, but reduced cell mobility, indicating a moderate cell transformation effect over short exposure time frame. Early *in vivo* lung neoplasia studies exhibit hyperplasia, defined borders, altered cell survival/death signaling, but minimal ability to invade neighboring tissue. Invasion ability is not a defining trait for neoplastic transformation, but an indicator of malignant transformation (Hanahan & Weinberg, 2011). A sister study conducted in parallel (Stueckle et al., 2017) found that long-term MWCNT-7 exposure on pSAEC resulted ~4-fold increase in soft agar colonies (~120 colonies per well) compared to MW-NH_x-exposed cells. This indicates that MWCNT-7 are much more potent at cell transformation than all MWCNTs tested here. Since MWCNT-7 are well-dispersed in our dispersion preparation (Wang et al., 2014), MW-NH_x agglomeration and reduced length may potentially reduce the ability to induce a strong neoplastic-like phenotype. Regardless, the ability of polydisperse MW-NH_x to elevate cell proliferation, mitochondrial metabolism, Type II foci, and soft agar colony formation

compared to UFCB, but similar to MWCNT-7, indicates that aged MW-NH_x with low oxygen content possess the ability to induce human pSAEC transformation.

The mechanism by which aged MW-NH_x caused increased cell transformation is currently unknown. It is possible that reduced oxygen content, compared to aged pMWCNT and MW-COOH, resulted in a more exposed carbon surface resulting in increased surface reactivity with biomolecules. This hypothesis is supported by UFCB and MWCNT-7 possessing relatively low oxygen content and induction of soft agar colony in pSAEC (Stueckle et al., 2017). Secondly, amine plasma coated surfaces are known to covalently bind and immobilize numerous biological molecules, including DNA (Khelifa et al., 2016), however similar fMWCNTs previously showed relatively little DNA breakage (Jackson et al., 2015). Enhanced lung surfactant and protein-binding in SAGM indicates that MW-NH_x harbored greater ability to interact with biological macromolecules. Although a significant amount of aging occurred during the first two months of storage, our analysis indicates that each fMWCNT was still susceptible to atmospheric aging during the long term exposure. This suggests that aged MW-NH_x harbored some residual surface reactivity that caused increased protein and biomolecule binding resulting in moderate cell transformation. Since no detectable differences were observed with dispersion/agglomeration or co-localization with pSAECs, enhanced proteinbinding ability may have had an impact once MW-NH_x were internalized. *In vivo* pulmonary exposure to similar fMWCNT found that autoxidized amine-fMWCNTs elicited a stronger and persistent neutrophil response (Poulsen et al., 2016). These results suggest that aged MW-NH_x harbor elevated pulmonary damage potential.

Conclusion

Laboratory aging of three different plasma fMWCNT resulted in autoxidation of surface functional groups resulting in a convergence of physicochemical properties over time. Eight week long term *in vitro* exposure of pSAECs to aged MW-NH_x, with less surface oxygen resulted in a moderate cell transformation compared to pMWCNT and MW-COOH. Based on these results, further studies into how ENMs with similar surface modifications transform during their life cycle and how they impact biological response following exposure is important and warranted. As carbon ENMs move through their life cycle, incorporation of *in vitro* screening studies using primary human cells paired with physicochemical characterization can assist in identifying those materials with neoplastic transformation potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Disclosure statement

The findings and conclusions in this article are those of the authors and do not necessarily represent the view of the National Institute for Occupational Safety and Health.

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Figure 1. FTIR characterization of the MWCNT samples. (A) Fresh 0month; (B) 2-month following laboratory aging ($n = 3$). (C) C1s (left) and O1s (right) XPS spectra for 2 month old MW-COOH.

Figure 2. FESEM characterization of UFCB and aged MWCNT samples in dry powder (left) and lung surfactant suspension (right). All MWCNT samples exhibited poly-disperse fibers consisting of singlets (white arrows), nanoropes, and loose agglomerates.

Figure 3.

Particle localization comparison of MWCNTs compared to UFCB and crocidolite asbestos in primary small airway epithelial cells at 24 h. (A) White arrows in enhanced dark field images indicate particle co-localization with nucleus. Dashed arrow and inset (lower left panel) = suspended CNT singlet. (B) Protein-binding comparison of carbon ENMs incubated in lung surfactant and culture medium. Lane order (left) matches sample order (right). Bars represent mean $\pm$ SE. * and ** indicate significant differences among treatments ($n = 3$; $p < .05$).

Figure 4.

Cell proliferation of human pSAECs following long term MWCNT exposure compared to UFCB and asbestos. (A) Trypan blue live cell count after 8 week exposure. All MWCNT-exposed cells exhibited significantly greater proliferation than all other treatments ($n = 9$; $p < .05$), while ASB-treated cells exhibited significantly diminished proliferation at 7 d, respectively. (B) 8-week and (C) 12-week WST-1 mitochondrial metabolism ($n = 9$). Data were standardized to 8-week passaged SURV controls in Panel B. Points and bars represent mean $\pm$ SE. * and # indicate significant differences from SURV control and other fiber-exposed cells, respectively ($p < .05$).

Figure 5.

Attachment-independent growth and morphological transformation assessment of MWCNTs. (A) and (B) UFCB and MW-NH_x at 8 weeks displayed significant increase in number of soft agar colonies compared to controls ($n = 9$). Bar = 100 μm . Bars represent mean $\pm$ SE. (C) Foci in a morphological transformation assay ($n = 9$). White arrow = Type II focus; Bar = 500 μm . * indicate significant difference vs. SURV control ($p < .05$).

Figure 6.

Transient invasion and migration ability of pSAECs following 8 and 12 week long term exposure ($0.06 \mu\text{g}/\text{cm}^2$) to MWCNT, UFCB, and asbestos. (A) Representative invasion micrographs at 8 weeks of exposure. Quantification of migration and invasion (B) immediately after exposure for 8 weeks, (C) one-week post-8 week exposure, and (D) migration and invasion at 12-week exposure. Bars represent mean $\pm$ SE. * and # indicates treatments significantly increased or decreased, respectively, from SURV control ($n = 9$; $p .05$).

Table 1

Physicochemical characterization of functionalized MWCNT.

Particle	UFCB	pMWCNT	MW-COOH	MW-NH ₂	Crocidolite Asbestos
Name/Source	Elfex 12, Cabot	Perpetuus	Perpetuus	Perpetuus	NIEHS, Kalahari Desert
Synthesis ^a	Vapor-phase pyrolysis	Argon Plasma	Proprietary Oxygen Blend Plasma	Nitrogen Plasma	Natural
Primary Functionality ^a	non-functionalized	non-functionalized	COOH	N=H	n/a
Other Functionalities ^a	n/a	atmospheric gas	COH, C=O, other oxygen groups	N-H, O=C-N-H ₂ , C≡N	n/a
Dry Mean Length (μm) ^a	n/a	1 to 12	1 to 12	1 to 12	10
Dry Mean Width (nm) ^a	37	13 to 18	13 to 18	13 to 18	210
Dispersed Mean Length (μm)	n/a	1.51 ± 0.001	1.86 ± 0.18	1.50 ± 0.078	ND
Dispersed Mean Width (nm)	316.6 ± 25.8	26.0 ± 5.4	26.5 ± 1.0	21.6 ± 0.6	ND
BET Surface Area	43 m ² /g ^b	214 m ² /g ^a	214 m ² /g ^a	214 m ² /g ^a	9.8 m ² /g ^b
Zeta Potential (water)	-7.02	-24.2	-30.1	-32.5	-35.2
Zeta Potential (medium)	-9.28	-9.58	-8.66	-10.12	-8.04
% carbon (w/w)	>99%	>99%, 0% ash	>99%, 0% ash	>99%, 0% ash	<1%

Data represent mean ±SE. ND = not determined.

^aSupplied by the manufacturer.^bPreviously reported (Wang et al. 2014).

Table 2

MWCNT surface elemental analysis from XPS electron signature at 2 months.

Particle	Atomic Element (%)				
	Oxygen 1s	Carbon 1s	Nitrogen 1s	Fluorine 1s	Chlorine 2p
pMWCNT	1.82	98.09	0	0.09	0
MW-COOH	2.6	97.4	0	0	0
MW-NH _x	1.1	98.9	0	0	0