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Nanotoxicology

Surfactant effects on carbon nanotube interactions with human keratinocytes

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Abstract Interactions of multiwalled carbon nanotubes (MWCNTs) with human epidermal keratinocytes (HEKs) were studied with respect to the effect of surfactant on dispersion of MWCNT aggregates and cytotoxicity. Our earlier studies had shown that the unmodified MWCNTs were localized within the cytoplasmic vacuoles of HEKs and elicited an inflammatory response. However, MWCNTs in solution tend to aggregate and, therefore, cells are exposed to large MWCNT aggregates. The purpose of this study was to find a surfactant that prevents the formation of large aggregates of MWCNTs without being toxic to the HEKs. HEKs were exposed to serial dilutions (10% to 0.1%) of L61, L92, and F127 Pluronic and 20 or 60 Tween for 24 hours. HEK viability, proportional to surfactant concentration, ranged from 27.1% to 98.5% with Pluronic F127; viability with the other surfactants was less than 10%. Surfactants dispersed and reduced MWCNT aggregation in medium. MWCNTs at 0.4 mg/mL in 5% or 1% Pluronic F127 were incubated with HEKs and assayed for interleukin 8 (IL-8). MWCNTs were cytotoxic to HEKs independent of surfactant exposure. In contrast, MWCNT-induced IL-8 release was reduced when exposed to 1% or 5% Pluronic F127 ($P < .05$). However, both MWCNTs and surfactant, alone or in combination, increased IL-8 release compared with control exposures at 12 and 24 hours. These results suggest that the surfactant-MWCNT interaction is more complex than simple dispersion alone and should be investigated to determine the mode of interaction.

Key words: Surfactant; Multiwalled carbon nanotubes; Human epidermal keratinocytes

Nanomaterials are structures with dimensions characteristically between 1 and 100 nm; when engineered appropriately, these materials exhibit a variety of unique and tunable chemical and physical properties. These unique physical properties (eg, conductivity, reactivity) have placed these materials in the forefront of emerging technologies and may result in useful applications in biology and medicine. The field of nanoscience has undergone unprecedented growth during the last few years and has received a great deal of attention. Industrial production of nanomaterials is of public concern with respect to their impact on the environment and on human health. Many challenges must be overcome before we can apply nanotechnology to the field of nanomedicine or conduct science-based occupational or environmental exposure risk assessments. There are limited data on the toxicology of chemically modified or unmodified manufactured nanomaterials both in vivo and in vitro. Pulmonary toxicity studies via

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intratracheal instillation of 5 mg/kg of single-walled carbon nanotubes (SWCNTs) in rats for 24 hours detected multifocal granulomas. A mortality rate of 15% resulted from the mechanical blockage of the upper airways by the instillate due to a foreign body reaction and not to the SWCNT particulate [1]. When mice were exposed to nanotubes manufactured by three different methods and catalysts, epithelioid granulomas were formed [2]. Both instillation studies showed the presence of multifocal granulomas and inflammation depending on the type of particle used. Once carbon nanotubes reached the lung they were more toxic than carbon black or quartz dust, two known pulmonary toxicants [2]. This may be secondary to the particles' tendencies to self-aggregate when removed from controlled conditions.

When immortalized nontumorigenic human epidermal (HaCaT) cells were exposed to unrefined SWCNTs, significant cellular toxicity occurred after 18 hours [3]. Previously, we have reported the presence of chemically unmodified multiwalled carbon nanotubes (MWCNTs) within the cytoplasmic vacuoles of human epidermal keratinocytes (HEKs) [4]. These MWCNTs induced the release of the proinflammatory cytokine interleukin 8 (IL-8) from HEKs in a time-dependent manner. These data clearly show that MWCNTs that have been neither derivatized nor optimized for biological applications are capable of both localizing within and initiating an irritation response in a target epithelial cell; this composes a primary route of occupational exposure for manufactured nanotubes. It must be stressed that respiratory exposure of particulate matter such as nanoparticles is fundamentally different from dermal or oral exposure, because the lung is designed to trap particulate matter. In dermal exposure, the stratum corneum is the barrier to particulate absorption.

Cell culture techniques remain a powerful tool to study chemical or particle interaction with most cell types. Uptake of nanotubes into the such cells as keratinocytes may be limited by the substantial van der Waals attractions that cause them to readily agglomerate in aqueous culture medium. To obtain the optimum effect as in drug delivery studies, a permeation enhancer such as a surfactant could be used to disperse the MWCNTs and to alter the membrane permeability, thus increasing the effective concentration of the MWCNTs. There are several classes of surfactants (ie, anionic, cationic, amphoteric, and nonionic) that are categorized by their polar functional groups. Surfactants are primarily found in detergents, preservatives, foam boosters, and emulsifying agents [5]. The nonionic surfactants are known to be less irritating and can affect the permeability of biological membranes by solubilizing the lipid membrane; moreover, some have been shown also to function as penetration enhancers in transdermal delivery systems because of their nonirritating nature and low toxicity. Occupational exposure to both manufactured nanomaterials and surfactants can occur during the manufacturing process. In contrast, the surfactants used to prevent aggregation in cell culture safety protocols cannot be toxic or irritating to the HEKs, otherwise false positive indicators of toxicity may occur. Suspension of individual nanoparticles in aqueous solution is necessary to understand the potential biomedical applications and to understand the mechanisms of toxicity. These issues suggest that knowledge of surfactant effects on nanomaterial behavior in cell culture is important from both occupational and experimental design perspectives.

Our purpose here was to evaluate the effects of five commonly used nonionic surfactants (Pluronic L61, Pluronic L92, Pluronic F127, Tween 20, and Tween 60) on the dispersal of MWCNTs and their toxicity to HEKs. Specifically, the objective was to identify the optimal surfactant that causes minimal toxicity to HEKs, while preventing nanotube aggregation without affecting cell viability or causing inflammation as determined by IL-8 release.

Methods

Surfactants and MWCNT

Pluronic L61, Pluronic L92, and Pluronic F127 were a gift from BASF Corp (BASF Corp, New Milford, CT). Tween 20 and Tween 60 were purchased from Sigma Chemical Co (Sigma Chemical Co, St. Louis, MO). Pluronic L61 (polyoxyethylene-polyoxypropylene glycol) is a liquid having a molecular weight (MW) of 2000 and is insoluble in water. Pluronic L92 (polyoxypropylene-polyoxyethylene block copolymer) is a liquid with MW 3650 and is considered to be moderately soluble in water (>1%). Pluronic F127 (polyoxyethylene-polyoxypropylene block copolymer) is a solid with MW 12,600 and is soluble in water (>10%). Tween 20 (polyethylene glycol sorbitan monolaurate) is a viscous liquid nonionic detergent with MW approximately 1228 that is water soluble. Tween 60 (polyethylene glycol sorbitan monostearate) is an amber paste at room temperature that is soluble in water.

The vertically aligned carbon nanotube films were grown using a microwave plasma–enhanced chemical vapor deposition system. The alignment of the MWCNTs in this system has been attributed to the electric field at the edge of the plasma [6]. This growth chamber has been used to grow diamond films [7]. The catalyst was an 80-nm iron film deposited on 2.5-cm silicon wafers by direct current magnetron sputtering. The iron-coated silicon substrates were introduced into the reactor, which was pumped to a base pressure of less than 1×10^{-3} torr, with the working pressure maintained at 20 torr. The growth temperature (900°C) was achieved through heating from induction and microwave plasma. The ammonia and acetylene precursors were introduced into the chamber at a flow rate of 70 and 18 standard cubic centimeters/min and the plasma ignited (600 W) for 30 minutes. Two and three-dimensional atomic force microscope images of the iron film surface were obtained with the Thermo Microscope M5 model in contact

mode, and the sputtered iron surface (with no pretreatment) had island-like structures approximately 100 nm in diameter with a surface height of approximately 3 nm. These structures serve as carbon nanotube nucleation centers in the growth phase. The nanotube films were characterized using scanning electron microscopy and high-resolution transmission electron microscopy (HRTEM). The film was grown perpendicular to the substrate with an approximate thickness of 50 μm and an approximate average diameter of 100 nm. The yield was relatively high, with a density of 1×10^{10} nanotubes/cm^2. The HRTEM images depict multiwalled structures that resemble a "bamboo shoot" [4]. The intergraphitic layers between two compartments comprise as many as 30 sheets, with a thickness of about 12 nm. These nanotubes are characterized as multiwalled because their internal structure approaches that of carbon fibers, they exhibit a base mode growth, they have very little disordered carbon, and they are well ordered and aligned. Auger electron spectroscopy detected no iron at the film surface. The technique is sensitive to a depth of approximately 2 nm from the exposed surface and has a sensitivity of less than 1%. The results support an earlier study that indicated base growth mode for these MWCNTs [8]. The MWCNTs were removed from the growth surface by manual scraping with a razor blade and subjected to elemental analysis to exclude the iron catalyst as a contaminant that could be potentially responsible for toxicity in the cell cultures. Carbon, not iron, was present above background.

HEK treatment with surfactants and cytotoxicity assays

To determine which surfactant and what surfactant concentration would be optimal, studies were conducted with HEKs. Cryopreserved pooled neonatal HEKs (BioWhittaker Inc, Walkersville, MD) were stored under liquid nitrogen until passage and plating. HEKs (~170,000) were diluted in 15 mL of keratinocyte growth medium (KGM-2) in each 75-cm^2 culture flask and grown at 37°C in a humidified 95% O_2/5% CO_2 environment. HEKs were harvested at approximately 60% confluency, passed again, and plated in 96-well culture plates (0.32 cm^2 growth area) at a density of approximately 7000 cells per well. Upon reaching approximately 80% confluency, the HEKs in 96-well plates were treated with serial dilutions of 10%, 5%, 1%, 0.5%, and 0.1% (n = 8 wells/treatment) of each surfactant in KGM-2 for 24 hours in a humidified 5% CO_2 atmosphere at 37°C. After treatment of HEKs with Pluronic L61, Pluronic L92, Pluronic F127, Tween 20, and Tween 60, their viability was compared to that of the media controls using the neutral red (NR) uptake method [9,10]. The culture medium in each of the wells were replaced with NR medium (0.05 mg/mL in KGM-2) and then incubated for 3 hour. The medium was aspirated from each well; then the cells were washed and fixed, and the dye extracted with 50% ethanol–1% acetic acid (v/v). Color formation after 20 minutes was evaluated by measuring absorption at 550 nm in an absorbance microplate reader (Multiskan RC, Labsystems, Helsinki, Finland). Absorbance values (n = 8 wells/treatment) were then normalized against the control wells (n = 8) and expressed as a percentage of viability relative to the controls (100% viability), which is proportional to the cytotoxicity.

HEK treatment with MWCNTs + Pluronic F127 surfactant

Pluronic F127 at 1% and 5% (w/v) dilution proved to be the optimal surfactant, resulting in less lethality to the HEKs as determined by the NR viability assay (Figure 1). Therefore, Pluronic F127 was also tested to determine its effectiveness in breaking up MWCNT aggregates. The concentrations of the solutions were monitored under the inverted microscope to view the dispersion of the MWCNTs. HEKs were seeded in two 96-well plates as summarized above. MWCNTs at 0.4 mg/mL were added to KGM-2, with and without Pluronic F127 at 5% and 1% (w/v) dilution (n = 8 wells/treatment). The solutions were vortexed for 3 minutes, followed by a 20-minute ultrasonification step. Then HEKs were treated with the MWCNT solutions along with appropriate controls. Medium was harvested at 1, 2, 4, 8, 12, and 24 hour after exposure and stored at –80°C until assayed for IL-8. The 24-hour viability of HEKs exposed to both 5% (w/v) Pluronic F127 + MWCNTs and 1% (w/v) Pluronic F127 + MWCNTs was determined. The treatments with Pluronic F127 + MWCNTs were monitored over 24 hours to determine whether the surfactant reduced nanotube aggregates by viewing under a microscope relative to MWCNTs in medium alone.

Analysis of IL-8

Cell culture medium was thawed and assayed in triplicate for IL-8 concentration using an enzyme-linked immunosorbent assay cytoset kit (Biosource International, Camarillo, CA). Briefly, human anti-IL-8 monoclonal antibody was coated overnight onto 96-well immunoassay plates (Greiner, Frichenhausen, Germany) at 4°C. Nonspecific binding was blocked with bovine serum albumin, and samples or standards were incubated in each well with a biotinylated IL-8 detection antibody. Streptavidin–horseradish peroxidase conjugate was added to each well for 30 minutes and the wells developed in the dark with *o*-phenylenediamine dihydrochloride (Sigma Fast, Sigma Chemical Co, St. Louis, MO). Sample absorbance was quantitated at 450 nm using a Multiskan RC plate reader (Labsystems, Helsinki, Finland). The final IL-8 concentration (pg/mL) for all treatments and time points were calculated using Genesis Lite Version 3 for Windows software (Labsystems, Helsinki, Finland), with data normalized by corresponding 24-hour HEK viability.

Statistics

The mean values for percentage viability and IL-8 concentration for each treatment were calculated and the

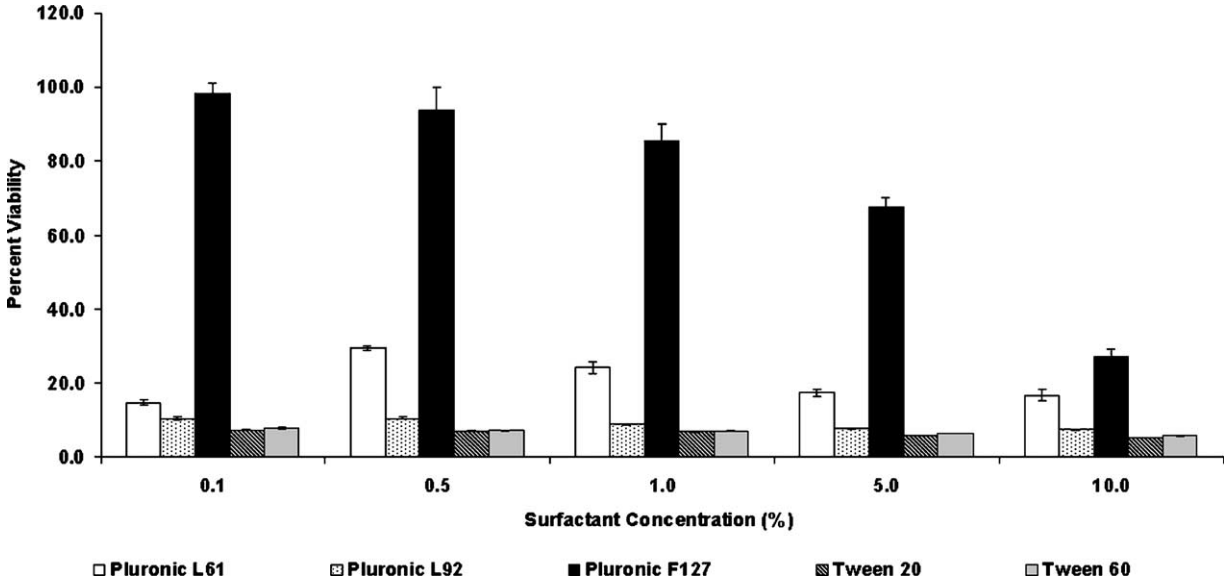

Fig 1. NR viability of HEKs treated for 24 hours with 0.1%, 0.5%, 1%, 5%, and 10% (w/v) concentrations of Pluronic L61, L92, F127, Tween 20 and 60 surfactants. Data represent the means ± SEM.

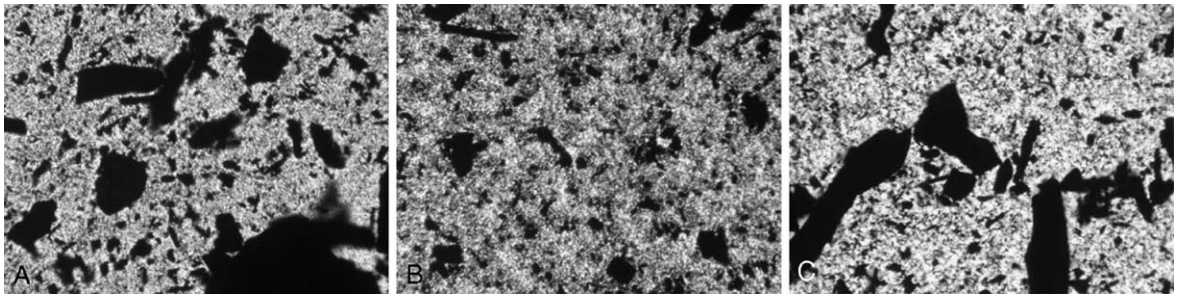

Fig 2. Light micrograph of 0.4 mg/mL of MWCNTs. **A,** MWCNTs alone. **B,** MWCNTs in 1% (w/v) Pluronic F127. **C,** MWCNTs in 5% (w/v) Pluronic F127. Note the dispersion of the MWCNTs in 1% (w/v) Pluronic F127. Original magnification ×50.

significant differences ($P < .05$) determined using the least significance differences in the analysis of variance procedure of SAS (SAS 9.1 for Windows; SAS Institute, Cary, NC). Multiple comparisons among different treatments were conducted within each exposure length and sampling time using the least significance difference at the $P < .05$ level of significance.

Results

HEK treatment with surfactants

Cytotoxicity was expressed as HEK percentage viability normalized to media controls. The percentage viability of HEKs exposed to 0.1%, 0.5%, 1.0%, 5.0% and 10.0% (w/v) dilutions of Pluronic L61, Pluronic L92, Pluronic F127, Tween 20, and Tween 60 showed that the surfactants, with the exception of Pluronic F127, decreased HEK viability at all concentrations (Figure 1). Pluronic F127 also decreased viability in a concentration-dependent manner. The values ranged from 27.1% viable cells at the highest concentration (10% w/v) of the surfactant to 98.5% viable cells at the lowest concentration (0.1% w/v).

HEK treatment with MWCNTs + Pluronic F127

Because Pluronic F127 was found to be the least toxic surfactant and the most promising upon examination of both viability and morphology, we then conducted additional experiments to evaluate its effects on the MWCNTs. Immediately after the treatment solutions were prepared, the nanotubes dispersed within the KGM-2 formed large aggregates within the medium and in contact with the wall of the vial. In contrast, Pluronic F127 dispersed the nanotubes within the medium but formed fewer aggregates. After 3 days, MWCNTs dispersed in 1% and 5% (w/v) Pluronic F127 were still partly suspended, while the nanotubes dispersed in KGM-2 alone were almost completely settled. Suspensions of 0.4 mg/mL in KGM-2 in cell culture wells without any surfactants developed very large

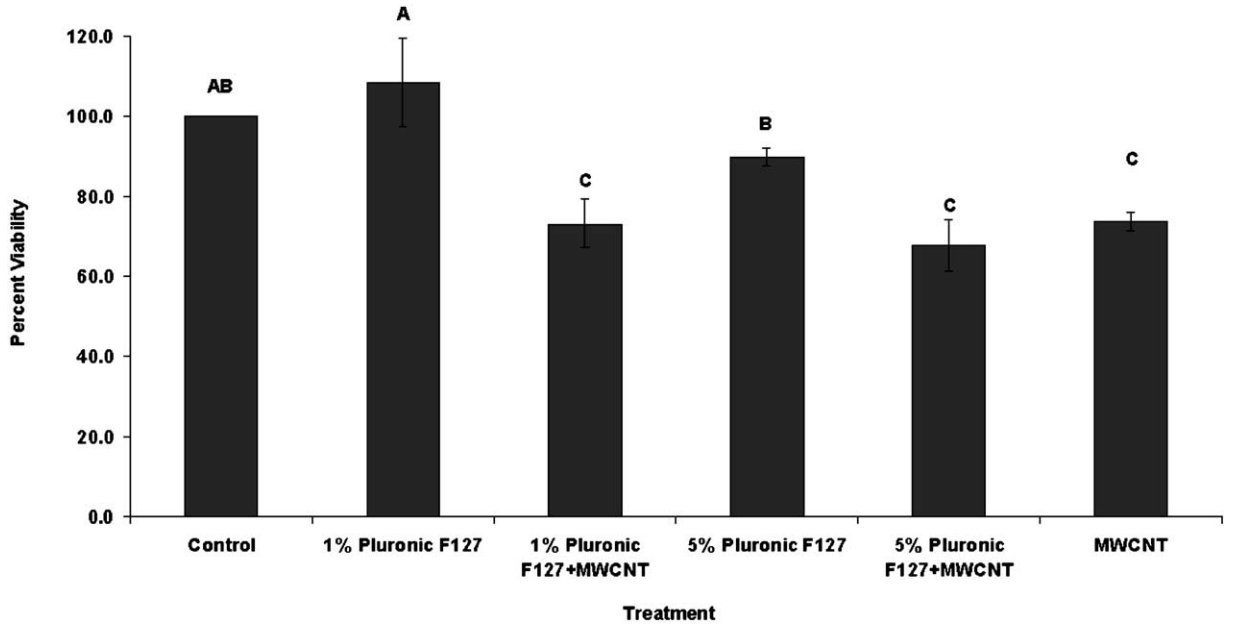

Fig 3. NR viability of HEKs treated with MWCNTs alone, with 1% (w/v) Pluronic F127 + MWCNTs, and with 5% (w/v) Pluronic F127 + MWCNTs at 24 hours. Histograms with different letters (A, B, C) denote mean values that are statistically different ($P < .05$). Data represent the means ± SEM.

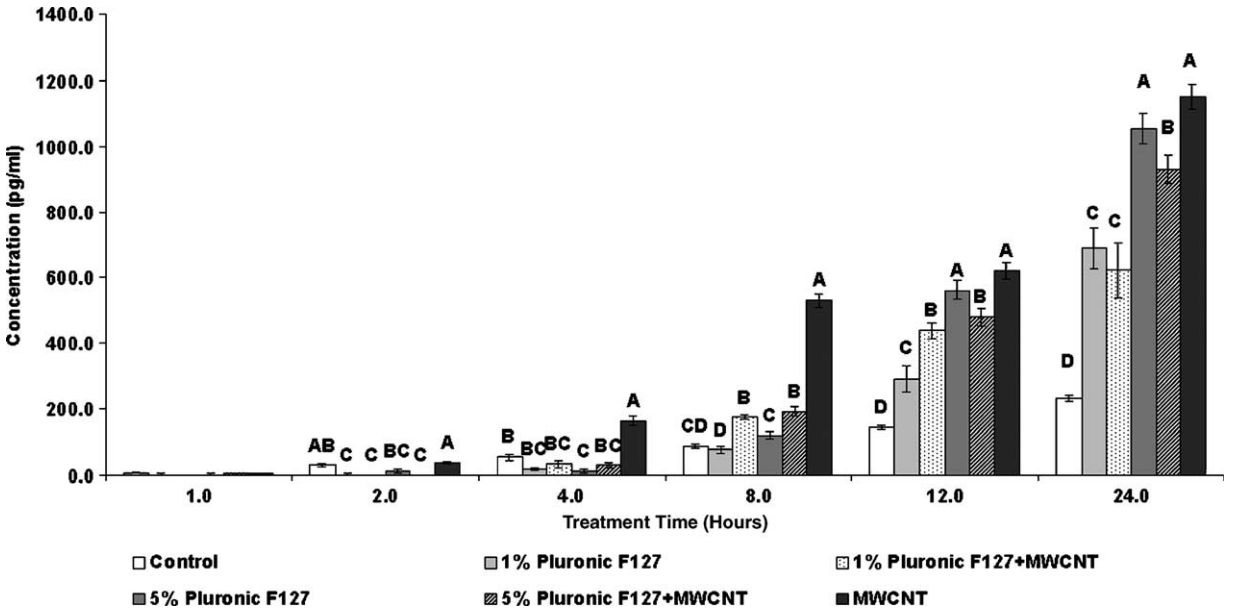

Fig 4. IL-8 release by HEKs treated with MWCNTs alone, with 1% (w/v) Pluronic F127 + MWCNTs, and with 5% (w/v) Pluronic F127 + MWCNTs for 24 hours. Histograms with different letters (A, B, C, D, E) denote mean values that are statistically different ($P < .05$). Data represent the means ± SEM.

agglomerates. With the addition of 1% (w/v) Pluronic F127 to the MWCNTs, fewer agglomerates were present; however, when 5% (w/v) Pluronic F127 was added to the MWCNTs, larger agglomerates were present that often resembled the nonsurfactant MWCNT control (Figure 2).

At 24 hours the viability of HEKs exposed to 1% and 5% (w/v) Pluronic F127 did not differ significantly ($P < .05$) from the HEKs exposed to media controls. However, MWCNTs alone and with both 1% (w/v) Pluronic F127 + MWCNTs and 5% (w/v) Pluronic F127 + MWCNTs significantly reduced HEK viability ($P < .05$) compared with the corresponding media and surfactant controls (Figure 3). The viability of HEKs exposed to the 1% (w/v) Pluronic F127 + MWCNTs did not differ significantly from that of the HEKs exposed to 5% (w/v) Pluronic F127 + MWCNTs. The viability of the MWCNTs alone (in media control with no surfactant) did not differ statistically from the 1% and 5% (w/v) Pluronic F127 + MWCNTs.

By 8 hours after treatment, normalized IL-8 release (pg/mL) was significantly greater ($P < .05$) in the HEKs

treated with 5% (w/v) Pluronic F127 than in those treated with 1% (w/v) Pluronic F127. Normalized IL-8 release from HEKs exposed to the 1% Pluronic F127 + MWCNTs and the 5% (w/v) Pluronic F127 + MWCNTs treatments was significantly greater ($P < .05$) than the corresponding media controls from 8 to 24 hours after treatment. The two surfactant concentrations were only significantly different at 24 hours (Figure 4). MWCNTs dispersed in medium without surfactant caused a significantly greater ($P < .05$) release of IL-8 by HEKs at 4 hours and 8 hours, compared with all treatments and controls. By 24 hours, MWCNTs in medium caused a significantly greater ($P < .05$) release of IL-8 than the nanotube treatments in Pluronic F127.

Discussion

Earlier we had shown that MWCNTs that have neither been derivatized nor optimized for biological applications are capable of intracellular localization and irritation, as detected by IL-8 release, in a target cell (keratinocyte) that constitutes a primary route of occupational exposure for engineered MWCNTs [4]. However, nanotube agglomeration caused by substantial van der Waals attractions potentially limits the concentration of MWCNTs available to the cell, thus reducing the potential of cytotoxicity. To this end we have investigated the use of five nonionic surfactants to disperse nanotubes and potentially increase the effective dose to HEKs. Nonionic surfactants are less irritating to viable cells and thus are used in many topical pharmaceutical and cosmetic formulations. Although the primary role of the surfactants in these formulations is physical stability or cosmetic appearance, they may also increase the permeability of the cell membrane.

Addition of surfactant to MWCNTs in medium resulted in an increase in dispersion. MWCNTs, with or without surfactant, were equally cytotoxic to HEKs as determined by the NR viability assay. MWCNTs exposed in surfactant were less irritating to HEKs (as assessed by IL-8 release) than when exposed in medium alone, suggesting that simple dispersion is not the sole mechanism involved in the effects of surfactant on cellular interactions, because cytotoxicity (NR assay) and irritation (IL-8 release) were not similar.

Other workers have stated that nonionic surfactants, such as Pluronic F127, cause suspension of nanotubes by coating them [11]. This could lead to complex micelle formation in aqueous systems such as cell culture medium [12]. Such micelle formation would be expected to decrease nanotube availability to cells. Although surfactants may decrease aggregation as seen in this study, the altered surface properties may also modulate how the particles interact with cells. It should also be noted that the higher concentration of Pluronic F127 resulted in less dispersion, further suggesting a complex interaction between surfactant and MWCNTs. In our study MWCNTs in surfactant were equal in toxicity to MWCNT without surfactant as assessed by NR viability, and statistically less irritating as determined by IL-8 release at 8 hours. This suggests that, although 1% (w/v) Pluronic F127 dispersed MWCNTs, this did not increase the available concentration for interaction with cells as originally predicted. Similar losses in viability, but less irritation at early time points assessed by IL-8 release, were seen when MWCNTs were dosed with the surfactant.

Most of the studies thus far with nanoparticles suspended in surfactants have been concerned with the dispersal of nanoparticles outside living systems [11,13,14]. The current work involves using Pluronic F127, a bifunctional block copolymer surfactant that has been shown to cause little toxicity to a living cell (eg, HEKs) to break up nanotube aggregates. Pluronic copolymers, consisting of polyethylene oxide and polypropylene oxide, have been used to modify surfaces to improve the stability of hydrophobic latex particles in aqueous media [15]. Cell culture studies with Pluronic F127 have focused on tissue engineering, drug delivery, and wound healing with a gelled surfactant [12,16,17]. Recent studies have shown that the high-MW Pluronic surfactants enhance dispersal of SWCNTs [11]. It is possible that such surfactant-nanotube interactions alter the nanotubes' surface properties, thus modulating their interaction with viable cells, much as derivatized particles have altered cytotoxicity [18]. Our studies have shown that the ability to disperse nanotubes in aqueous solutions is not a sufficient criterion for predicting cell interactions, since MWCNTs showing different degrees of aggregation (Figure 2) showed the same degree of cytotoxicity as indicated by the loss of viability of HEKs.

Here we confirm the low relative cytotoxicity of this surfactant through an assay of HEK viability. We have conducted viability studies that show the Pluronic F127 surfactant alone is relatively nontoxic to HEKs (Figure 1). HEK viability after treatment with Tween 20, Tween 60, and Pluronic L92 was less than 11%. Therefore, these surfactants would not be appropriate for use in live cell culture studies because they are toxic to HEKs.

In conclusion, these studies demonstrate that Pluronic F127 is relatively nontoxic to HEKs in culture but does not increase the cytotoxicity of MWCNTs. In contrast, MWCNTs dosed alone in the aqueous cell culture medium caused significantly more IL-8 release than when MWCNTs were dosed in Pluronic F127. MWCNTs were equally cytotoxic to HEKs with or without the presence of a surfactant. Studies that model nanotube behavior in nonbiological systems suggest that increased dispersion would result in an enhanced ability to interact with cells. Our studies suggest that, although dispersion occurred with surfactant, biological interactions did not correlate with this property.

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