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SURFACE CHEMISTRY INFLUENCE CANCER KILLING EFFECT OF TiO₂ NANOPARTICLES

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Abstract

Photocatalyzed TiO₂ nanoparticles have been shown to eradicate cancer cells. However the required in situ introduction of UV light limits the use of such a therapy in patients. In the present study, the non-photocatalytic anti-cancer effect of surface functionalized TiO₂ was examined. Nanoparticles bearing -OH, -NH₂, or -COOH surface groups, were tested for their effect on in vitro survival of several cancer and control cell lines. The cells tested included B16F10 melanoma, Lewis lung carcinoma (LLC), JHU prostate cancer cells, and 3T3 fibroblasts. Cell viability was observed to depend on particle concentrations, cell types, and surface chemistry. Specifically, -NH₂ and -OH groups exhibited significantly higher toxicity than -COOH. Microscopic and spectrophotometric studies revealed nanoparticle-mediated cell membrane disruption leading to cell death. The results suggest that functionalized TiO₂, and presumably other nanoparticles, may be surface engineered for targeted cancer therapy.

Keywords

Surface functionality; TiO₂ nanoparticles; cancer; toxicity

Background

As an important component in the development of nanotechnology, nanoparticles have been extensively explored for possible medical applications [1–2]. In particular, the potential toxicity of nanoparticles, including titanium dioxide (TiO₂), carbon nanotubes, and fullerenes, has drawn significant attention in recent studies [3–4]. Investigations involving TiO₂ have been particularly prominent, no doubt reflecting the extensive commercial use of TiO₂ nanoparticles at the present time. For example, TiO₂ particles have been shown to trigger reactive oxygen species produced by alveolar macrophages, phagocytes, and microglial cells upon interaction with the cell membranes [5–7]. The potential for tissue specific toxicity also exists depending on mode of delivery; including via implant debris [8], intratracheal delivery [4,9], oral delivery [10], intraperitoneal injection [5], or inhalation [11]. Furthermore, *in vivo* studies of oral uptake of TiO₂ have been shown to enter blood circulation leading to both liver and kidney damage [10]. Intra-tracheal introduction of TiO₂ can also induce severe pulmonary inflammation and

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emphysema [4]. Also, intraperitoneal injection of TiO₂ disseminated in the body was subsequently observed to have been deposited in vital organs, such as spleen, lung, and liver [12]. Recently, hydrophobic functionalized TiO₂ particles were shown to reduce pulmonary inflammatory response [13], though these findings are in disagreement with previously published reports that hydrophobic TiO₂ formulations are acutely toxic and lethal [14]. A more recent study suggests that there is no significant difference in hydrophilic and hydrophobic functionalized TiO₂ after intratracheal instillation [15]. Thus, based on somewhat contradictory, albeit limited, data available it is clear added studies of the interactions between TiO₂ nanoparticles and cell are desirable at this time.

Interestingly, the photocatalytic properties of TiO₂-mediated toxicity have been shown to eradicate cancer cells [16–17]. It is now well established that TiO₂ particles, on exposure to UV light, produce electrons and holes leading subsequently to the formation of reactive oxygen species such as hydrogen peroxide, hydroxyl radicals, and superoxides [18]. These oxygen species are highly reactive with cell membranes and the cell interior, with damaged areas depending on particle location upon excitation. Such oxidative reactions affect cell rigidity and chemical arrangement of surface structures, leading to cell toxicity [19]. Despite promising outcomes in killing cancer cells, such treatments would be difficult to implement in clinical setting for the following reasons. First, UV light cannot penetrate deeply into human tissues, thus limiting this technique to superficial tumors [20]. Second, UV-mediated production of reactive oxygen species has a very short life span and thus would not be able to provide a continuous prolonged cancer killing effect [19].

Overall, there have been relatively few studies involving TiO₂ nanoparticles:cell interactions. As previously noted, the available literature on this subject shows some conflicting results. Since a significant body of recent literature documents the fact that surface functionality affects the interactions between particles and cells [21–22], we have undertaken a comprehensive study to examine the effects of varying surface functional groups on TiO₂:cell interactions under non-photolytic conditions. For this study, TiO₂ nanoparticles having different surface chemistries were produced. Specifically, using a pulsed plasma technique, TiO₂ particles were covered with thin polymer films of di(ethylene glycol) vinyl ether (EO2V), allyamine (AA), and vinyl acetic acid (VAA) providing surface functionalities of -OH, -NH₂, and -COOH, respectively. EO2V [H₂C=CH(OCH₂CH₂)₂OH] is a hydrophilic coating bearing -OH groups. These polymer films have been shown to increase surface hydrophilicity and also to reduce protein adsorption [23]. In an arteriovenous shunt model, EO2V coatings were shown to reduce platelet adhesion but induced a significant inflammatory response [24]. Allyamine (AA) is a hydrophilic coating bearing the positively (cationic) charged -NH₂ functional group at physiological pH. Amine surfaces have been shown to favor protein adsorption [25], peptide binding [26], and cell attachment [25,27]. Amine-rich microparticles have been shown to engage in strong ionic interactions with the negatively charged cell membrane. Such interactions facilitates particle uptake into tight-fitting phagosomes [28]. Vinyl acetic acid (VAA) coatings provide a hydrophilic surface with negatively (anionic) charged -COOH functional groups at physiological pH [29]. In general, these surface chemistries, possessing very similar surface energies but offering neutral (-OH), positive (-NH₂), and negative (-COOH) charged functional groups, were selected to provide an interesting initial study to examine surface functional groups and charges on cellular uptake/membrane binding and subsequent cellular toxicity of these nanoparticles.

The cell toxicity of the surface modified TiO₂ particles, along with unmodified particles as controls, was assessed using various cell lines including NIH 3T3 fibroblasts, B16F10 and B16F1 melanoma lines, JHU prostate tumor line, and Lewis Lung Carcinoma (LLC). Our results clearly show that functionalized TiO₂ particles exert a significant surface chemistry-dependent cytotoxic effect on cancer cells especially JHU and LLC.

Materials and Methods

Materials

Dulbecco's modified Eagle's media (DMEM), Hanks's balanced salt solution (HBSS), sodium pyruvate, and nicotinamide adenine dinucleotide were purchased from Sigma Aldrich (St. Louis, MO). Fetal calf serum (FCS) was purchased from Atlanta Biologicals (Lawrenceville, GA). Live/Dead Viability/Cytotoxicity Kit for mammalian cells and FM 1-43FX membrane probes were obtained from Invitrogen-Molecular Probes (Eugene, OR). Aqueous One Solution Cell Proliferation Kit was obtained from Promega.

TiO₂ Particle Coating

TiO₂ particles (P25 standard 80% anatase, 20% rutile), acquired from the DeGussa Corporation, were of uniform size, with an average diameter of 21 nm. The di(ethylene glycol) vinyl ether (EO2V), allyamine (AA), and vinyl acetic acid (VAA) monomers were purchased from Aldrich (Milwaukee, WI) and were of the highest purity available. They were out-gassed repeatedly before use but were not subjected to any additional purification steps.

Radiofrequency gas discharge (RFGD) plasma polymerization [30] was employed to coat the particles. In an effort to achieve efficient coating of the TiO₂ nanoparticles, a 360° rotatable plasma reactor was employed. The continuous rotational motion, while maintaining vacuum, was achieved via use of Ferrofluidic valves located at each end of the reactor (Figure 1). Transport grooves, located on the inside of the glass reactor, move the particles upward during rotation for subsequent gravitational descent through the plasma discharge. This efficient mass transport and agitation of the particles successfully overcomes the tendency for nanoparticle aggregation providing continuous exposure of fresh TiO₂ surface to the plasma generated reactive molecules and ions. The specific process employed has been previously shown to be extremely effective in coating fine powders [31], including nanoparticles [32]. In a typical run, 3.5 g of TiO₂ particles were loaded inside the borosilicate glass reactor and the reactor was evacuated to 5 mTorr background pressure. After this background pressure was achieved, an oxygen plasma-pretreatment was conducted at 100W average power to remove any carbonaceous contaminants on the surface of TiO₂ particles. Subsequently, the PECVD process was initiated using one of the three monomers. In each case the polymerization was carried out using a monomer pressure between 50 and 100 mTorr. The film thicknesses deposited were limited to approximately 5 to 10 nm. The film deposition rates, and thus thickness, were determined in separate experiments using polished silicon substrates and an Alpha-Step profilometer (Tencor, San Jose, CA)

Polymer Characterizations

The structures of the plasma deposited polymer films produced from each of the three monomers employed were characterized using FT-IR spectroscopy. A Bio-Rad, Model FTS-40, operated at 8 cm⁻¹ resolution and transmission mode, was used for the FT-IR characterizations. Additional characterization of these films included measurement of surface wettability using a Rame-Hart sessile drop goniometer, as described previously [23]. For this purpose, as well as for the FT-IR measurements, these film characterization measurements were made on flat substrates coated in the plasma reactor under identical conditions to those employed with the TiO₂ nanoparticles. Polished Si substrates were employed for the water contact angle measurements and KBr discs for the FT-IR determinations. We have previously shown that the film compositions are independent of substrate composition after deposition of the first few monolayers of polymer [33].

The surfaces of the TiO₂ particles were modified using pulsed plasmas to control the chemical composition of the plasma generated thin polymeric films. Under the pulsed condition, the

extent of retention of the functional group in a given monomer increases as the duty cycle employed during the plasma polymerization is decreased [32]. The average power input is defined as: Average power (W) = (Duty cycle) $\times$ Peak Power (W), where the duty cycle represents the ratio of the plasma on time divided by the plasma (on + off) time. In the present study, the plasma duty cycle employed for each monomer was adjusted to generate films which maximized the surface functionalities of the desired chemical group, consistent with achieving sufficiently strong adhesion of the polymer to the TiO₂ particles to resist dissolution of the films when immersed in aqueous solution. As the average power input increases the extent of polymer cross-linking increases, while the retention of monomer functional groups decreases. In effect, the plasma duty cycle adjustment represents a compromise between retention of monomer structure and achieving the requisite insolubility of the films [34]. The spectroscopic results, shown below, document the success of these endeavors.

Cell Source and Culture

The five cell lines used in this investigation were purchased from ATCC. Four of these five cell lines were derived from tumor tissues. Specifically, Lewis lung carcinoma (LLC) was isolated from C57BL mice and is widely used as a model for cancer metastasis. JHU is a prostate cancer cell line isolated from Copenhagen rats. B16F10 and B16F1 are skin melanoma cell lines isolated from C57BL mice. 3T3 fibroblast cell line was established from Swiss mouse embryos and used here as control cells. All cell types were maintained in DMEM supplemented with 10% FCS. For all particle studies, TiO₂ particles were incubated with either DMEM containing 10% FCS or HBSS with 1% FCS for 24 hours prior cell culture, which has been suggested to reduce photocatalytic effects of TiO₂ by passivation with serum components [3].

Cell Viability Analysis

Cell viability analyses were carried out using both Live/Dead cell staining and cell proliferation assay. For viability staining studies, cells were seeded in 24 well plates at a concentration of 1×10^4 cells/mL at 37°C with 5% CO₂. Cells were allowed to grow to confluence. The particles were then added into the cell-seeded well plates and incubated for 24 hours. At the end of incubation, the media was removed and the adherent cells were subjected to Live/Dead staining. For imaging studies and enzyme release assay, cells were cultured with particle solutions for 3 hours. Following incubation, cells were subjected to membrane staining, and Lactate Dehydrogenase (LDH) measurement.

The viability of particle-incubated cells was conducted using the Viability/Cytotoxicity staining method following manufacture's protocol (Molecular Probes, Eugene, OR). Briefly, 1 μ M calcien AM and 2 μ M ethidium homodimer-1 solutions were prepared in PBS. After verification that the majority of cells were attached, the culture media containing TiO₂ particles was removed. After removal of the culture media, the cells were rinsed once in PBS followed by the addition of 100 μ L 1 μ M calcien AM and 2 μ M ethidium homodimer-1 solution. Cells were then photographed using a Leica fluorescence microscope coupled with a digital camera. The live and dead cells were then counted using NIH Image J software (rsb.info.nih.gov/ij/).

MTS assays were conducted on LLC cells grown to confluence in 96 well plates (Promega, Madison, Wisconsin) to analyze against stain viability results. Upon reaching confluence, media containing 1mg/mL TiO₂ functionalized with VAA, EO2V, AA, or uncoated TiO₂ was added to cultures and allowed to incubate for 3 hours at 37° C and 5% CO₂. After 3 hours, 20 μ L of Aqueous One Solution was added to begin the assay. Manufactures suggested protocol was followed concerning solution incubation time and microplate settings. Sample ODs were read at 490nm with background at 630nm.

Membrane Integrity Analysis

To visualize the effects of various particles on cell membrane integrity, cultured cells were subjected to FM1-43 membrane staining following the manufacturer's protocol (Molecular Probes, Eugene, OR). The cell membrane integrity was then recorded with the fluorescence microscope.

Cell toxicity measurement with lactate dehydrogenase (LDH) assay

LDH is a cytoplasm enzyme and LDH reduction is often associated with cell membrane damage and cell death [35]. The activity of LDH was measured spectrophotometrically by assaying reduced nicotinamide adenine dinucleotide oxidation at 340 nm during LDH-catalyzed reduction of pyruvate to lactate [36]. Briefly, cells were cultured as previously described in 24 well plates. TiO₂ particle solutions and coated TiO₂ particle solutions were prepared in HBSS, supplemented with 1% FCS, and stored overnight. The solutions were then added to the cell culture and cultures were then incubated for 3 hours (the maximum time period before cell death can be observed due to serum deprivation). The supernatant was then removed and centrifuged to eliminate the non-adherent particles and cell debris. Adherent cells were lysed with 0.5% Triton X-100. Samples of both lysate and supernatant for each particle type were then analyzed by spectroscopically. Statistical comparison (achieved on the basis of variable cell number) was carried out according to Student t- test. Differences were considered statistically significant when $p < 0.05$.

Results

Composition of Films

The FT-IR spectroscopic results identify the structural features of the films employed and confirm the presence of the different chemical functional groups introduced on the surface of the TiO₂ nanoparticles. The FT-IR spectra of the specific films employed in this study are shown in (Figure 2A), arranged in the order poly(allylamine), poly(vinylacetic acid) and poly(diethyleneglycol vinyl ether) reading top to bottom. The specific plasma parameters employed in synthesis of each film are provided (Table 1). The polyAA film confirms the presence of amine functional groups, as shown by the relatively broad absorption band $\sim 3400\text{ cm}^{-1}$. The polyVAA film exhibits a very broad absorption band extending above 3500 cm^{-1} to below 3000 cm^{-1} which is characteristic of $-\text{COOH}$ groups present in solid films [37]. Additionally, the absorption at 1700 cm^{-1} is also characteristic of a carbonyl stretch specifically associated with $-\text{COOH}$ groups. The polyEO2V films exhibit the absorption band of the $-\text{OH}$ stretch at 3400 cm^{-1} , as well as that of the strong C-O stretch characteristic of the ether group, both functionalities being present in the starting monomer (Figure 1A). All three films exhibited hydrophilic behavior, as expected, given the nature of the polar groups present in these films. The sessile drop static water contact angles measured ranged from 40° to 48° and they are provided in Table 1.

Additional experiments were carried out to determine if the polymer films deposited on the nanoparticles result in a difference in chemical structures from that observed on flat surfaces. For that reason, X-Ray Photoelectron Spectra (XPS) of films obtained from polymerization of vinylacetic acid on the TiO₂ particles were compared to that observed on the Si substrates, for films deposited under identical plasma conditions. The high resolution C(1s) XPS spectra obtained from polyvinylacetic acid, deposited on the nanoparticles at a plasma on/off ratio of 0.75/20 ms is shown in (Figure 2B). This film is in fact essentially identical to one deposited on a flat Si surface, as previously published [34].

An added important consideration involves the morphology of the films deposited on the nanoparticles. In fact, high resolution TEM studies reveal the films are conformal in nature,

having assumed the same shape as the particle. This fact is illustrated in the HRTEM picture shown in (Figure 3), which shows the ~25 nm particle coated with a 5 nm polymeric film. The film conformality is reasonable, given the gas phase nature of the coating process. The conformal aspect of plasma deposited films has been reported in many studies and has long been promulgated as an advantage of this coating technique [33]. Additionally, it is noted that the TEM studies reveal no significant differences in the extent of particle aggregation of TiO₂ particles in comparing coated and uncoated samples.

TiO₂ Nanoparticle:Cell Interactions

Preliminary experiments were conducted to probe the interaction of functionalized TiO₂ with cells. Particle visualization was achieved do to clumping of the particles after 3 hours of culture (Figure 4). Mouse 3T3 fibroblasts were cultured on coverslips with unmodified TiO₂. After 3 hours culture, cells were imaged using phase contrast. Particles can be seen aggregating on the cell membrane in comparison to control unexposed cells.

TiO₂ Nanoparticle Effects on Cell Viability

The effect of various concentrations of uncoated TiO₂ nanoparticles on cell viability was examined. After 24 hour incubation, little to no effect on 3T3 fibroblast survival was observed up to TiO₂ concentrations of 10 mg/ml. In contrast, the various cancer cell lines exhibited significantly different degrees of toxicity towards the untreated TiO₂ particles. Similar to 3T3 fibroblasts, B16 F1 cells appear to be essentially non-responsive to increasing concentration of TiO₂ nanoparticles. On the other hand, the survival of the LLC cell line drops sharply with the increase of the TiO₂ concentration. Specifically, more than 75% of LLC cell were killed at a concentration of 10 mg/mL. The cytotoxic effects of 10 mg/ml uncoated TiO₂ on B16F10 and JHU were intermediate between that of the B16F1 and LLC cells, being 75% and 50%, respectively (Figure 5).

Effects of Surface Functionalized TiO₂ Nanoparticles on Cell Viability

The dose effects of TiO₂ nanoparticles with various functionalities on cell survival were also evaluated using Live/Dead Viability/Cytotoxicity staining (Figure 6). We first found that there was no significant effect of TiO₂ coating on 3T3 and B16F1 cells following incubation for 24 hours, consistent with the fact that uncoated TiO₂ had little to no effect on the cells at concentrations up to 10mg/mL (Figure 6C & 6D). The effect of surface functional groups on cell survival is highlighted at the concentration of 0.1mg/mL for JHU cells (Figure 6B). At this concentration, the survival rates of cells exposed to -NH₂, -OH and -COOH are approximately 60%, 80%, and 100%, respectively, while the viability of cells incubated with uncoated TiO₂ is around 75%. These results support that -COOH functional group diminishes cell toxicity whereas -NH₂ enhance JHU cell toxicity. As concentration increases, particle load overwhelms effects seen by functional coating. Similar surface functionality-dependent cell toxicity can also be found on LLC cells. When exposed to uncoated TiO₂ nanoparticles at 10 mg/ml, the survival of LLC drops sharply to around 25% viability (Figure 5). Similar to responses of JHU cells, the -COOH functional group substantially reduces particle-mediated toxicity to LLC cells (Figure 6A). In addition, compared with uncoated particles, TiO₂ particles with -OH and -NH₂ functional groups have less cell toxicity (~70%) to LLC cells than uncoated particles (25%).

LDH Assay

Our results indicate that TiO₂ nanoparticles may interrupt the continuity of the cell membrane and subsequently lead to membrane breakdown and cytoplasm leakage. To test this assumption, we measured the release of cytoplasmic enzyme - LDH by adherent cells with or without prior exposure to TiO₂ nanoparticles. Assays of LDH activities were observed to increase with

increasing concentrations of uncoated TiO₂ particles confirming the relationship between cell death and the release of LDH enzymes (Figure 7A).

MTS Assay

For further verification, similar studies were carried out using MTS cell proliferation assay of cells culture with functionalized and control TiO₂ nanoparticles at 1mg/mL. As expected, results of the assay were in agreement with previous observations that –COOH functionality reduced toxicity of TiO₂ to LLC cells (Figure 7B). In comparison to control OD, LLC cultured with VAA functionalized TiO₂ (–COOH) had an OD nearly equal to control cultures. ODs for other functionalities were considerably lower; NH₂ (75%), OH (65%), and uncoated TiO₂ (80%).

FM1-43 Stains of TiO₂ Exposed Cells

Through observing the cell morphology, we have noticed that most of the dead cells appear to have broken membranes. We thus assume that binding of TiO₂ nanoparticles interrupts the integrity of cell membranes. To test this hypothesis, FM1-43 staining was used to monitor the fluidity of the membranes of cells with or without prior exposure to TiO₂ nanoparticles. We observed that, without particle exposure, FM1-43 stains of LLC show a smooth, continuous layer of cell membrane (Figure 8A). On the other hand, the spotty, rough cell membranes were found in cultures with uncoated TiO₂ particles (Figure 8B). These results suggest that TiO₂ particles may affect the membrane integrity and lead to cell death. Similar membrane disruptive effects can be seen when compared to control cells with B16F1 (Figure 8C & 8D) and B16F1 (Figure 8E & 8F). This suggests that cell:particle interactions, other than particle deposition on cell membranes, are responsible for cell death. Observation of the cells under higher magnification (×400), confirmed that LLC cells served as focal point for large numbers of TiO₂ particles (Figure 9). Interestingly, TiO₂ particles often coincided with cell membranes and, perhaps, membrane debris. These results suggest that the accumulation of TiO₂ particles may be responsible for the rupture of cell membrane and subsequent cell death.

Discussion

TiO₂ nanoparticles are widely used for industrial and medical applications [38–39]. Since nanoparticles can interact with cell membranes and intracellular organelles in a manner not totally understood, there are increasingly concerns about the adverse health effects of TiO₂ and other nanoparticles. The first part of this study was to analyze the cell toxicity effects of unmodified TiO₂. Among the five cell lines used in this investigation, we find that TiO₂ nanoparticles have low cytotoxicity to B16F10 and B16F1 melanoma cells as well as 3T3 fibroblasts. These findings are in agreement with many recent published results. Specifically, various sizes and concentrations of TiO₂ particles have been reported to be non-toxic in cell monolayer uptake models *in vitro* [40–41], *in vitro* inhalation models [3], and *in vivo* models [5,10]. However, in the case of the JHU prostate tumor cells and Lewis lung carcinoma cells, we found that there are significant differences in viability levels for uncoated TiO₂ particles at concentrations of 1mg/mL for Lewis lung carcinoma cells and 0.1 mg/mL for JHU prostate tumor cells. Our results have shown that TiO₂ particles possess cell-specific toxicity, depending on the concentrations and surface functionality of the particular particles.

TiO₂ particle-mediated tissue toxicity is potentially via particle:cell interactions, possibly related to the surface properties of the TiO₂ particles [9]. It has been reported in several *in vivo* studies that TiO₂ can travel in the bloodstream via binding to plasma proteins, through the lymphatic system after phagocytosis by macrophages, or to the bone marrow via monocytes [42–43]. The specific interactions between TiO₂ particles and proteins are not well understood. Studies have revealed that TiO₂ particles have a net negative charge (at pH = 7) [44] and also

bind preferentially to amino acids containing $-OH$, $-NH$, and $-NH_2$ in their side chains [45]. These observations indicate that TiO_2 particles may react with cell membrane proteins and contribute to cell:particle interaction.

Surface functionality has been shown to affect cell:particle interactions [22]. Although it has been suggested that surface functionality should be the determining factor concerning cell uptake and subsequent activity inside the cell [46], studies that have varied surface functionality to investigate membrane binding, uptake, and internalization of nanoparticles are limited. We thus hypothesize that, by varying surface functionality, the cell toxicity of TiO_2 particles can be altered. Three functional groups with various surface charges ($-OH$, $-NH_2$, and $-COOH$) were included in this investigation. We find that the effect of particle surface functionality on cell cytotoxicity to be cell dependent. 3T3 fibroblasts and B16F10 melanoma cells showed no significant response to functionalized or un-treated particles up to 1mg/mL. These findings are in agreement with recent findings that TiO_2 nanoparticle surface functionality (hydrophilic verses hydrophobic) had insignificant effects on cell toxicity in an intratracheal rat model [15,47]. These differences may be due to protein composition of the cell membrane and how these proteins interact with the TiO_2 particles. In the case of the melanoma and 3T3 cells, weaker particle:membrane interactions may explain the insignificant influence of surface functionality and higher survival rates of particle-exposed cells (Figure 5). In contrast, surface functionality exerts medium influence on Lewis lung carcinoma toxicity, possibly due to increased interaction between the TiO_2 particle surface and the cell membrane. The most significant variances were seen in the JHU prostate tumor cells.

The basis for the observed differential effects of surface functional groups on cell survival is mostly unclear due to the complex interaction between the cell specific membrane properties and nanoparticle surface chemistry. The JHU prostate tumor cells showed a significant susceptibility to $-NH_2$ coated nanoparticles. JHU cells have a relatively high cell toxicity at low particle concentrations (0.1 and 1 mg/ml) with cell survival around only 60% of that of uncoated particles. At high concentration (10 mg/ml), there is no difference of cell toxicity among $-NH_2$ coated or uncoated nanoparticles. The detailed mechanism of such responses has yet to be determined. Since it is well established that positive charged nanoparticles have high affinity to negative charged cell membrane protein [48], it is likely that JHU cell membranes were saturated with $-NH_2$ functionalized particles at lowest concentration (0.1 mg/ml) employed. In addition, using polypropylene microparticles [49], we find that the density of surface functionality has little influence on cell:particle interactions. Therefore, the increase in the surface NH_2 concentration, or the increased exposure to NH_2 groups in the case of nanoparticles, may not have a significant effect on cell survival.

Perhaps, the positive charged amine group prompts a destructive impact on the negatively charged membrane of the cells. The absence of a further decrease in cell viability may be explained by results from a recent study which shows that the properties of a surface functional group override the effects of increases in density or concentration of the functionality [49]. While the positive charged $-NH_2$ coating shows a significantly lower viability for JHU cells compared to uncoated TiO_2 , negatively charged $-COOH$ coated particles have substantially higher cell viability levels except at potential overload concentrations. It appears that positive surface charge may enhance particle accumulation on cell membrane and subsequently particle uptake by cells [48,50], though it has been suggested that particle:membrane interactions facilitated through particle surface charge may be a cell dependent phenomenon [51]. Additionally, the effects of functionality seem to be skewed at high concentrations, likely due to particle overload conditions or saturation of the cell due to charge interactions, masking the effects of surface modification of the TiO_2 nanoparticles. Also, JHU prostate cancer epithelial cells tend to grow in dense clumps, possibly masking the effects of increased particle concentration due to protection of the cells inside the cell clumps. Taken together, these

observations may suggest why surface functionality effects cell viability via particle:surface interactions, though the effects are not cumulative with increasing concentration.

It is our belief that the decreased viability of particle-exposed LLC cells may be associated with impaired cell membrane function due to TiO₂ particles-induced protein aggregation/denaturation. In fact, our results support this hypothesis. FM1-43 stains of particle-incubated cells have revealed large circular clumps staining positive around the exterior of the cell. Such rough cell membranes are often found on apoptotic or necrotic cells indicating cell death and/or dying cells [52]. It should be noted that FM1-43 stains of JHU cells show similar trend of clumping membrane material around the exterior of cell (data not shown). These observations are in line with many recent results. First, in cancerous cell lines T-24, HeLa, and U937 cells, TiO₂ particles are found to incorporate into the cell membrane and the cytoplasm [19,53]. Second, TiO₂ uptake was decreased in alveolar macrophages in the absence of calcium, preventing influx of calcium into damaged cell membranes [19,54]. Third, results have suggested that particle-mediated production of hydroxyl radical is responsible for the interruption of pulmonary cell and cancer cell membrane [19,55].

When observing uncoated and treated TiO₂ nanoparticles with the LLC cell line, we see that at a concentration of 1mg/mL, cell viability decreases to approximately 75% in the case of untreated, -OH functionalized, and -NH₂ functionalized particles, while viability is unaffected in the case of -COOH functionalized particles. A similar phenomenon is seen at the lower end of concentrations tested in JHU. Though the mechanisms of cell interactions with the negatively charged -COOH group is not well established, many investigations have shown favorable serum protein interactions with -COOH surfaces [56] as well as favorable particle uptake [50]. The -COOH group may allow easy uptake and processing of the particles into intracellular compartments which may prevent aggregation and unfavorable reactions with the cell membrane minimizing damage to the cell membrane. It has also been suggested that a cell's interaction with different surface functionalities affects the charge of the cell membrane [57], which may affect the integrity and function of the cell membrane.

Although our results have shown that surface functionality effect cell viability and particle accumulation on cell membranes, the relationship between particles:membrane interactions and cell death has yet to be determined. Abundant evidence suggests that the disruption of cell membranes often lead to cell death [19,58]. We thus hypothesized that TiO₂ nanoparticle accumulation on cell membranes may lead to cell rupture and then cell death. It is widely established that the release of LDH enzymes can be used to assess and also to quantify the degree of cell rupture [40]. Indeed, there is an excellent relationship between LDH release and cell death (Live/Dead stain), as verified by LDH release. This finding supports our theory that the binding and clumping of TiO₂ with the cell membrane leads to a disruption of the cell membrane, leading to release of intracellular components causing cell death. Further studies are needed to uncover the detailed processes governing particle-associated cell membrane rupture. It should be noted that a proton sponge hypothesis has been suggested to explain the mechanism of rupture [59]. This theory suggests that extensive buffering by the cationic particle surface may lead to unchecked proton transport into the phagosome. As the consequence, excessive water influx may leads to endosome rupture due to the space constraints. Free floating particles within the cytoplasm could then interact with mitochondria and other organelles leading to cell death [59].

This study represents a novel observation on the influence of TiO₂ particles and their surface functionality on cancer cell death. These interesting results suggest that surface functional groups may provide a degree of toxicity based on the interactions between the specific surface chemical group and the properties of a particular cancer cell's membrane. It is our belief that further study may help the development of nanoparticles for targeted cancer therapy.

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Figure 1. Schematic diagram of the 360° rotating RF plasma reactor. 1: gas inlet port; 2: secondary gas inlet port (for low vapor pressure monomers); 3: MKS Baratron pressure transducer; 4: exhaust valve controller; 5: ferrofluidic feedthrough valves inside aluminum housing; 6: ground electrode; 7: hot electrode; 8: butterfly valve; 9: flowmeter; 10: two external ground electrodes

Figure 2. Surface characterizations of functionalized surfaces. (A) FT-IR transmission spectra of the polymer films employed to functionalize the surface of the TiO₂ nanoparticles. The spectra are arranged in order (top to bottom): poly(allylamine); poly(vinylacetic acid); poly (diethyleneglycol vinyl ether) (B) High resolution C(1s) XPS spectrum of a pulsed plasma polymerized polyvinylacetic acid film deposited on TiO₂ nanoparticles, using a plasma on/off ratio of 0.75/20 ms.

Figure 3. High resolution TEM picture of a 5 nm plasma generated film deposited on a 25 nm nanoparticle, illustrating the uniform and highly conformal aspect of the coating.

Figure 4. Visualization of the interaction between TiO₂ particles (0.01mg/mL) and 3T3 cells. Cells were imaged using phase contrast after 3 hours exposure. (A) Non-exposed cells appear normal while (B) exposed cells show collection of particles on the cell membrane.

Figure 5.

The influence of particle concentration on survival rates of cells. TiO_2 particles in various concentrations were added into culture plated with confluent cells. After incubation for 24 hours, the cell viability was then quantified with Live/Dead cytotoxicity Viability stain (Molecular Probes). The cell viabilities were then normalized with cells without treatment. Vertical lines denote $\pm 1\text{SD}$ ($n=4$ for all test samples and cells). Significant of differences between cancer cells vs. 3T3 cells ($\blacktriangle$): **, $P<0.05$).

Figure 6.

Effect of coated particle concentrations on viabilities of different cell types, (A) LLC (B) JHU (C) B16F1 (D) 3T3. Various concentration of functionalized TiO_2 particles were added into culture plated with confluent cells. After incubation for 24 hours, the cell viability was then quantified with Live/Dead cytotoxicity Viability stain (Molecular Probes). The percentages of cell viability were then normalized with same cells without treatment. The vertical lines denote $\pm 1\text{SD}$ ($n=4$ for all test samples and cells). Significant of differences between -NH₂ coated and -OH coated particles vs. -COOH coated particles ($\circ$): **, $P<0.05$).

Figure 7.

Cell toxicity of functionalized particles. (A) LDH release from LLC cells following TiO₂ exposure. Cells with 70–90% confluence were exposed TiO₂ particles for 3 hours. The LDH activities of supernatants and adherent cell lysates were then determined spectrophotometrically. (B) MTT assay on particle-exposed LLC cells. LLC cells were incubated with variously functionalized TiO₂ particles for 3 hours. The activity of adherent cells was then quantified with MTT assay kit. The viable cells were then determined spectrophotometrically. The vertical lines denote $\pm$ 1SD. Significant of differences between particle incubated cells vs. untreated cells: **, $P < 0.01$).

Figure 8.

Effect of particle:cell interactions on cell membrane integrity. Various cancer cells were cultured with TiO_2 particles (1mg/ml) for three hours. The cell membranes were then stained with FM1-43 membrane stain (red) as well as nuclear DAPI staining (blue). (A) Untreated LLC cell showed continuous cell membrane stain. (B) Rough membrane was found on particle exposed LLC cell. (C) Smooth cell membrane on control B16F1 cells. (D) Particle exposed B16F1 cells have prominent disrupted membranes. (E) Complete membranes were found on untreated B16F10 cells, while (F) particle treated B16F10 cells covered with fragmentized membranes.

Figure 9. Particles influence on cell membrane integrity. (A) The disrupted cell membrane of TiO₂ exposed LLC cells were stained with FM1-43 membrane stain (red). (B) TiO₂ particles can be visualized surrounding cells under phase contrast optical microscope. (C) By overlapping membrane stain images and optical image, membranes can be seen to coincide with the particles indicating potential involvement of particles on cell membrane disintegration.

Table 1

Plasma deposition conditions employed and surface wettability of the polymer film generated.

Monomer	RF Peak Power Input, W	Ratio of Plasma on/off Time (in ms)	Monomer Pressure, mTorr	Water Contact Angle
Allylamine	300	10/200	240	40
Vinyl acetic acid	150	0.7/20	284	48
Diethyleneglycol vinyl ether	200	10/160	56	45