

FUNCTIONALIZED NANOPARTICLES FOR THE SELECTIVE KILLING OF
CANCER CELLS

by

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ABSTRACT

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The use of nanoparticles for cancer imaging and drug delivery has produced some success in improving the efficacy and safety of cancer chemotherapy. It is generally believed that the efficiency of nanoparticle-based cancer therapy would be greatly improved if such particles could be designed to directly interact with and eradicate tumor. Many recent studies have revealed that surface functionality plays an important role in particle:cell interactions. Using titanium dioxide (TiO₂) nanoparticles (21 nm diameter) as test subjects, nanoparticles with different functional groups, hydroxyl (-OH), amine (-NH₂), and carboxyl (-COOH) were produced and used in this investigation. An in vitro cell culture system was used to evaluate the interaction of a series of surface functionalized TiO₂ nanoparticles with a number of normal animal and

cancer cell lines, including B16F10 melanoma, Lewis lung carcinoma (LLC), JHU prostate cancer cells, and 3T3 fibroblasts. It was observed that cell viability following exposure to TiO₂ nanoparticles was dependent on particle concentration, cell line, and TiO₂ surface chemistry. Overall, the TiO₂ nanoparticles, both functionalized and uncoated, exerted the strongest toxicity effects on LLC cells and least toxicity on B16F10 cells and 3T3 fibroblasts. Cell viability was observed to depend on the specific surface chemistry of the particles. In general, particles with –NH₂ or –OH functional groups exhibited significantly higher toxicity than those functionalized with –COOH groups. Microscopy analysis of cell viability as well as spectrophotometric analysis of lactate dehydrogenase (LDH) content and arrays of cell proliferation indicated that the TiO₂ nanoparticles disrupt the cell membrane integrity leading to cell death. Overall, the present results suggest that functionalized TiO₂ nanoparticles, and presumably other nanoparticles similarly surface modified, may be engineered specifically for targeted cancer therapy.

The ability to fully understand how a precisely chemically defined surface interacts with both cell monolayers and body tissues will help scientists and engineers in the design of materials that will perform their desired function while also minimizing unfavorable interactions within the body.

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LIST OF ABBREVIATIONS

| | |
|---|--|
| TiO₂ – Titanium Dioxide | FCS – Fetal Calf Serum |
| LLC – Lewis Lung Carcinoma | ATCC – American Type Cell Culture |
| LDH – Lactate Dehydrogenase | EthD-1 - Ethidium homodimer-1 |
| OH – Hydroxyl | RFGD – Radiofrequency Glow Discharge |
| NH₂ – Amine | EO2V - Di(Ethylene Glycol) Vinyl Ether |
| COOH – Carboxyl | AA – Allyamine |
| ROS – Reactive Oxygen Species | VAA – Vinyl Acetic Acid |
| CH₃ – Methyl | MTS - [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (Aqueous One Cell Proliferation Assay) |
| IgG – Immunoglobulin G | HBSS – Hank’s Balanced Salt Solution |
| PVA – Poly(Vinyl Alcohol) | |
| PLGA – Poly((Lactic-co-Glycolic Acid) | |
| FN – Fibronectin | |
| DMEM – Dulbecco’s Modified Eagle’s Media | |

CHAPTER 1

INTRODUCTION

Cancer is one of the principle causes of death in the Western world (Schulenburg et al. 2006) and has passed heart disease as the leading cause of death for persons younger than 85 years of age (Jemal et al., 2007). Tumors are cause by abnormalities in cellular genetics causing unregulated cell growth, and possibly metastasis of the cells to other locations within the body, though the mechanisms of events such as metastasis and malignant growth are largely unknown (Schulenburg et al., 2006).

Methods and strategies for the treatment of different types of cancers are continually developing and rapidly expanding into the realm of nanotechnological treatments (Kim, 2007). Nanoparticles are at the forefront of modern research in the treatment of cancer (Yezhelyev et al., 2006; Kim, 2007). Treatment strategies are shifting from intrusive and invasive surgical approaches, for both detection and treatment, to the use of nanoparticles followed by chemotherapy with targeted therapeutics such as controlled drug delivery from nanoparticles (Santra et al., 2005; O'Neal et al., 2005). Concurrent with developments in medicinal biology and

chemistry, nanoparticles have emerged as a powerful weapon in the fight against cancer.

1.1 Nanoparticles in Cancer Therapy

Nanoparticles have been increasingly employed as vehicles with which to deliver anti-cancer agents to tumors as well as tumor imaging. Nanoparticles have shown potential as intravascular as well as cellular probes which may help lead to early diagnosis of certain types of cancers. They also have been shown to provide targeted delivery, allowing the delivery of analyte probes and therapeutic agents not only to cells, but to specific compartments within the cell (Liu et al., 2007). The ability to conjugate cell-penetrating peptides as well as load nanoparticles with therapeutic gene and DNA therapies has advanced both cancer research and medicinal chemistry. One such example is the delivery of suicide genes to cancer cells via nanoparticles (Czupryna and Tsourkas, 2006).

Cancer research using nanoparticles has seen application in many different scenarios, including cancers of the breast (reviewed by Yezhelyev et al., 2006), prostate (Johannsen et al., 2007), skin (Ito et al., 2004), and lung (Zhou et al., 2006). Each type of cancer provides unique challenges related to delivery, uptake, and successful eradication of cancerous cells, which can be met by the quickly expanding arsenal of nanoparticles. Quantum dots, nano-shells, magnetic nanoparticles, and carbon

nanotubes are just a few of the many newly developed nanoparticles that each have distinct advantages for particular facets of cancer therapy including imaging, drug delivery, magnetic field targeting, radiation sensitizing, and photothermal ablation (Cuenca et al., 2006).

Recently, several chemotherapeutic agents have become available in nanoparticle formulations with equivalent efficacy and fewer side effects (Yezhelyev et al., 2006). Though many methods have been developed to characterize nanoparticles *in vitro*, the nature of how nanoparticles interact with tissue and cells *in vivo* is only vaguely understood.

1.2 Surface Interactions of Biomaterials after Implantation

Implanted biomaterials, including nanoparticles, are mostly hydrophobic. As a result, upon implantation, biomaterials are usually covered with a layer of plasma proteins. It is the protein composition and the degree to which these proteins denature which ultimately affects the functionality of the implant or drug release device (Dadsetan et al., 2004, Wang et al., 2004). In the case of implants, it is believed that the conformation changes of the adsorbed proteins are responsible for initiating adverse reactions such as inflammation, coagulation, and foreign body response (Shen et al., 2004; Nath et al., 2004). For nanoparticles, this protein adsorption assists macrophages and the immune system in identifying and eliminating the particles (Moghimi and

Hunter, 2001). The content and conformation of the adsorbed protein layer will ultimately determine how the nanoparticles will interact with cells and tissues.

Surface functionality has been shown to affect the extent of protein adsorption and denaturation (Keselowsky et al., 2004). Surface engineering strategies for biomaterial implants has shifted to focus on controlling protein adherence and denaturation to decrease end products of inflammation, most importantly fibrous capsule formation around the implant (Barbosa et al., 2006; Keselowsky et al., 2007). In the case of the much smaller nanoparticles, especially those which come into contact with blood after intravenous injection, surface chemistry has also been shown to affect the degree of opsonization (Peracchia et al., 1997). This reduction, when combined with the nanoscale size of the particles can decrease recognition of the particles by macrophages and the immune system (Vonarbourg et al., 2006). Surface modification can also help to tailor particle solubility/dispersion and stability (Choi et al., 2003), enhance cell uptake (Win and Feng, 2005), and provide chemical reactivity on the particle surface for further conjugation of bioactive ligands (Nobs et al., 2003).

We have chosen to focus this project on titanium dioxide (TiO₂) nanoparticles. TiO₂ has many unique properties, most notably the ability to photocatalyze. There are however many discrepancies concerning the biocompatibility of TiO₂. Very little has been reported on the effects of altering the surface functionality of TiO₂. The goal of

this study is understand how TiO₂ interacts with cancer cells and if altering the surface properties of TiO₂ can alter the cellular response.

1.3 TiO₂ Nanoparticles

Titanium has been widely used as an implant material. Overall, the material has been established as a biocompatible material, with some investigations showing anti-inflammatory properties due to the ability to degrade reactive oxygen species. However, there have been conflicting results concerned with the toxicity of TiO₂ nanoparticles. When exposed to water, titanium forms an oxide layer resulting in a chemical structure TiO₂ (Sahlin et al., 2006). Titanium implants have been shown to release TiO₂, which can accumulate locally or disseminate systemically (Olmedo et al., 2002). TiO₂ is used commercially and industrially as a white pigment and is generally developed in particulate form less than 1µm. TiO₂ particles can be classified as poorly soluble in water and have been classified as relatively inert and non-toxic (Bermudez et al., 2002).

In biological applications, TiO₂ is most often used as a disinfectant due to its photocatalytic properties, mainly to purify water and industrial waste. In the medical industry, TiO₂ has been applied to increase biocompatibility of surfaces (Abou Neel et al., 2007), as a biosensor coating for glucose monitoring (Nakamura et al., 2007), as a functional surface for living-cell microarrays in drug development (Carbone et al.,

2007), and as an additive to materials to both mimic natural bone and enhance apatite formation (Hashimoto et al., 2007).

CHAPTER 2

TiO₂ NANOPARTICLES BACKGROUND

TiO₂ nanoparticles in medicine have been investigated mainly in the fields to toxicology (Jeng and Swanson, 2006), sterilization (Sekiguchi et al., 2007), and cancer treatment (Seo et al., 2007). As previously mentioned, TiO₂ has many industrial and medical applications which, due to their small size and unique properties require an in depth understanding of any potential toxicity associated with exposure.

The photocatalytic properties of TiO₂ have been well documented in recent years (Blake et al., 1999). Researchers have sought to harness these properties to both sterilize surfaces which may become exposed to pathogens as well as apply particles to cancerous cells with the hopes of disrupting cellular functions and thus viability, through photocatalysis. Though these photocatalytic applications have been thoroughly investigated, the mechanisms are still only generally understood (Blake et al., 1999).

2.1 Formulations of TiO₂ and Their Properties

Two types of crystalline structure have been investigated related to medicine, rutile and anatase. The properties of these two crystalline structures have been shown to differ regarding both chemical reactivity and photocatalytic capabilities (Brunella et al., 2007; Warheit et al., 2007). The most commonly investigated nanoparticle variant of TiO₂ is the P25 formulation, consisting of 80% anatase 20% rutile, commonly used as a white pigment (Blake et al., 1999).

The photocatalytic properties of TiO₂ and their applications have been thoroughly studied for the past few decades (Blake et al., 1999). It has been demonstrated that the anatase structural form of TiO₂ has superior photocatalytic capabilities (Brunella et al., 2007), as well as increased toxicity *in vivo* compared to the rutile phase (Warheit et al., 2007). Though not totally understood, the general mechanism of photocatalysis, including species generation and their potential interactions can be summarized as follows. UV excitation leads to a series of chemical steps that result in the production of reactive oxygen species, including hydroxyl radicals, hydrogen peroxide, and superoxide (Fujishima et al., 2000). In animal cells, the generation of hydroxyl radicals and superoxides are of increased importance and has been linked to causes of cell death following photocatalysis of TiO₂ nanoparticles (Blake et al., 1999). These reactive species are then free to interact with either the

exterior cell membrane, or if UV exposed after uptake, within the cell membrane. This can result in lipid peroxidation and cytotoxicity resulting in cell death.

2.2 In-Vitro Studies Employing TiO₂

Numerous studies have investigated effects of TiO₂ on cancer cell line viability. These studies employ photocatalysis as the method to induce killing, differing mostly in time, duration, and light source power. Depending on the conditions during excitation, ROS such as hydroxyl radicals, superoxides, and hydrogen peroxide can be produced (Fujishima et al. 2000). These highly reactive species frequently attack biological molecules including lipids by abstracting hydrogen, in a process known as lipid peroxidation (Halliwell and Chirico, 1993). In-vitro cell killing via photocatalysis has been demonstrated on HeLa cells (cervical carcinoma) (Cai et al., 1992), T-24 (bladder carcinoma) (Sakai et al., 1995), A-357 (melanoma) (Seo et al., 2007), Ls-174-t (colon carcinoma) (Zhang and Sun, 2004), and U937 (leukemia) (Huang et al., 1997). Most studies investigating the photocatalytic activity of TiO₂ use the P25 phase. This phase has been shown to be the most potent and efficient photocatalyst compared with other structures of TiO₂, though groups have reported favorable results with lab fabricated TiO₂ (Seo et al., 2007).

The assumption common to all studies is that photocatalysis of TiO₂ causes membrane disruption by reactive oxygen species (ROS) leading to cell death. As

previously mentioned, the crystal phase of the TiO₂ particles is related to its ability to photocatalyze. One study using Ls-174-t colon carcinoma (which were shown to be unaffected by TiO₂ exposure in the absence of UV) and a TiO₂ particle concentration of 1mg/mL showed an 80% reduction in cell viability with 30 minutes of UV exposure at 3.7mW/cm² with viability determined by MTT assay. The TiO₂ nanoparticles used in this study were prepared by the researchers and were shown to adhere and accumulate on the cell membrane even in the absence of photoactivation (Zhang and Sun, 2004). A more recent study using melanoma cancer cells was able to show that not only are particles found both on and inside the cell membrane after particle exposure, but also that the cell death due to TiO₂ photocatalysis occurs by both apoptosis and necrosis (Seo et al., 2007).

The other front of *in vitro* TiO₂ research involves examination of toxicity as a result of nano or microparticle exposure without application of photocatalysis. These studies have been focused in the past two decades and deal mostly with phagocytic cell types or other cells with an increased possibility of exposure to small particulates.

2.3 In-Vivo Studies

Only a few studies have attempted to incorporate the benefits of photocatalysis and TiO₂ into a working method for treatment of cancerous masses. After tumor induction, the addition of a TiO₂ nanoparticle solution combined with UV exposure led

to a significant reduction in tumor growth (Kubota et al., 1994; Kubota et al., 1995). Similar effects were seen in an *in vivo* model HeLa cells exposed to TiO₂ and UV (Cai et al., 1992). Though some positive results have been reported, the need for surgical exposure of the tumor combined with UV treatment may restrict this method to superficial tumors and is further limited by the fact that TiO₂ may disseminate throughout the body possibly causing complications during treatment. Also, it appears that photocatalytic treatment with TiO₂ appears to be limited to tumors below a certain size limit (Cai et al., 1992). Recently, researchers have tested on the use of an endoscope, providing increased access to cancer masses, to facilitate the TiO₂ photocatalysis process for *in vivo* cancer treatment (Fujishima et al., 2000).

A rapidly expanding body of work has been devoted to the effects of TiO₂ nanoparticles after exposure via an assortment of routes. Inhalation studies have shown inconsistent results, with results showing particle deposition in the lungs and lymph nodes with the degree of inflammatory response differing among species tested (Bermudez et al., 2002). TiO₂ nanoparticles injected into the peritoneal cavity led to deposition in the peritoneum, liver, lung, and spleen; though deposition did not lead to excessive inflammatory reactions at the sites of deposition (Olmedo et al., 2002). TiO₂ particles disseminated in the lungs have shown the potential to interact with alveolar macrophages causing elevations in ROS (Olmedo et al., 2005). This response in the lungs has been shown to be transient with resolution within a few days after initial exposure (Rehn et al., 2003; Warheit et al., 2006).

Recently these results have come under question, specifically related to the effects of chemical phase composition as well as surface properties of TiO₂. It was recently shown that TiO₂ (P25, 80% anatase 20% rutile) nanoparticles induce lung inflammation, cytotoxicity, and cell proliferation as well as adverse lung tissue effects following intratracheal exposure in comparison with rutile TiO₂ (Warheit et al., 2007). The enhanced ability of the anatase phase of TiO₂ to generate ROS may lead to oxidative damage and undesired cell stimulation, which could lead to powerful inflammatory effects once deposited in tissues (Donaldson et al., 1998).

CHAPTER 3

THE EFFECTS OF SURFACE FUNCTIONALITY ON CELLULAR RESPONSES

Though the influence of surface hydrophobicity on protein adsorption and cellular responses is well documented, recent studies have revealed that surface functional groups also affect protein and cell behavior (Beyer et al., 1997; Scotchford et al., 2002; Tang et al., 1998; Keselowsky et al., 2004). Substantial research efforts have been placed on studying the influence of surface functionality in the cellular response to biomaterials. Surface functional groups can also influence cell growth (Tidwell et al., 1997; Ohya et al., 2004) as well as cellular uptake of nanoparticles (Wilhelm et al., 2003; Shikata et al., 2002), likely due to the fact that surface chemical functionality affects adsorbed protein and subsequent protein:cell interactions. The most common functionalities investigated with relation to biomaterial interactions are the carboxyl (-COOH), hydroxyl (-OH), amino (-NH₂), and methyl (-CH₃) groups. Many recent reports have investigated the effect of these groups on the binding of adhesive proteins and subsequent cellular interactions.

3.1 Hydrophobic and Hydrophilic Surface Functionalities

The wettability that functionality provides to a surface is an important parameter when considering the interactions of unmodified and modified nanoparticles inside the body. The $-CH_3$ group is the major component of commonly used polymeric materials and provides a hydrophobic surface on biomaterials. It is generally accepted that the hydrophobic $-CH_3$ functionality promotes protein adsorption, usually in conformations unfavorable for desired cellular interactions (Keselowsky et al., 2004). As a result, $-CH_3$ functionality has also shown increased fibrinogen binding, platelet accumulation and thus poor blood compatibility (Lestelius et al., 1997). A study measuring the adhesion strength of fibrinogen, albumin, and immunoglobulin G (IgG) showed that all three proteins adhered with the highest strength to $-CH_3$ bearing surfaces (Kidoaki and Matsuda, 1999). Overall, these results suggest that $-CH_3$ surfaces will likely have unfavorable surface reactions with cells due to the magnitude of tightly bound proteins.

In general, hydrophilic functionality provides low interfacial free energy resulting in reduced protein adsorption, cell adhesion, and blood compatibility (Wang et al., 2005; Wang et al., 2004). It is well established that proteins tend to bind to hydrophilic surfaces in a lower amount and less tightly than to hydrophobic surfaces (Higuchi et al., 2002; MacDonald et al., 2002; Hess and Vogel, 2001). Such reduction of protein adsorption affects subsequent cellular responses. In general, micro and nanoparticles

with a hydrophilic shell show decreased protein adsorption and increased circulation time (Redhead et al., 2001).

The hydroxyl group functionality (-OH) represents a neutral, hydrophilic surface. Particles modified with a PEG shell, with a hydrophilic -OH surface functionality, can reduce particle uptake in phagocytes (Choi et al., 2003). The -OH functionality has also been shown to have reduced plasma protein adsorption (Lestilius et al., 1997). It has been suggested that the charge neutrality and hydrophilic nature of the -OH functionality has low protein affinity, and thus protein repelling properties (Lindblad et al., 1997). However, hydrophilic poly(vinyl alcohol) (PVA) surface modification with -OH functionality used in a study comparing uncoated poly(lactic-co-glycolic acid) (PLGA) versus -OH coated PLGA, showed that the addition of the hydrophilic coating led to a 2 fold increase in cellular uptake (Win and Feng, 2005). Fibronectin (FN) adsorbed onto -OH functional groups also show high levels of $\alpha_5\beta_1$ levels leading to increased cell adhesion strength as well as increased levels of structural signaling components related to focal adhesions (Keselowsky et al., 2004). This contradiction highlights the importance of the specific membrane properties of a particular cell type in dictating particle cell interactions.

3.2 Negatively and Positively Charged Surface Functionalities

Equally important to surface wettability, surface charge likely plays an important role in particle:cell interactions, especially given that cell membranes generally have a net negative charge. Therefore the tendencies of negatively and positively charged particle surface coatings may lead to alternative interactions with the cell membrane.

A recent study, examining the effects of altering surface charge on particle uptake, showed that increasing $-NH_2$ group functionality, with a net positive charge, on the particle surface has a correlation to cell uptake (Lorenz et al., 2006). This phenomenon was also shown to be cell dependant, with cancerous cell lines taking up more particles with an increase in $-NH_2$ group density on the surface. Studies using FN show favorable protein conformations after adsorption to the positively charge $-NH_2$ surface (Liu et al., 2005; Keselowsky et al., 2004). Particularly, $-NH_2$ surfaces promote the exposure of high density bound receptors as well as focal adhesion components by adsorbed fibronectin (Keselowsky et al., 2004). These favorable protein adsorption profiles lead to favorable endothelial cell interactions (Liu et al., 2005). It is suggested that the favorable interactions of the cationic $-NH_2$ surface with the cell membrane allow quicker passage into cells.

Studies investigating negatively charged functionalities, such as -COOH , and their effects on protein adsorption have shown that fibronectin and albumin are more easily eluted from surfaces coated with -COOH (Tidwell et al., 1997). This was tied into the fact that this functionality, compared to other common surface functional groups, adsorbed FN and albumin at ratio significantly different to other coatings. In addition, studies investigating the effect of cell adhesion peptides with -COOH functionality showed high levels of two FN domains ($\alpha_5\beta_1$ and $\alpha_v\beta_3$) associated with structural and signaling components related to focal adhesions, similar to the -OH functionality (Keselowsky et al., 2004). Favorable protein expression on the surface of -COOH functionalized nanoparticles may then lead to an increased reactivity with cell membranes. A recent study has shown that -COOH functional surface enhances cell uptake and the amount of nanoparticle uptake could be correlated to the amount of -COOH functionality on the nanoparticle surface. Such interesting phenomenon might be due to favorable interactions of the cell with the negatively charged coating (Holzapfel et al., 2006). However studies have also shown that excessive concentration of -COOH on the surface results in a higher negative charge on the surface, which may cause unfavorable membrane interactions between particles and cell membranes (Ohya et al., 2004).

A recent study has also suggested that surface functionality can also affect both the rate and mechanism of cell uptake. Using dendrimers with terminal groups of -NH_2 , -OH , and PEG, results have shown that -NH_2 surfaces were able to enter cancer cells at

a higher rate than -OH and PEG functionalized surfaces. It is likely that the -OH and PEG functionalities, due to their anionic nature, increase affinity to the cell membrane, and then taken in by an endocytotic route (Kannan et al., 2004). Another study investigating -OH, -NH₂, and -COOH terminal groups on dendrimers showed that -COOH and -OH functional surfaces tend to have increased residence times in vivo, which may be attributed to their ability to resist recognition by the body through protein adsorption, as well as cell uptake properties due to surface functionality (Vandamme and Brodbeck, 2005).

CHAPTER 4

EVALUATION OF CANCER CELL TOXICITY OF UNCOATED AND SURFACE MODIFIED TiO₂ NANOPARTICLES

The goal of this study is to establish the toxicity of TiO₂ to various cancer cells as well as investigate whether changing the surface functionality of TiO₂ has any effect on nanoparticle toxicity to the cancer cell lines. Thus this chapter will be divided into two sections. In the first section (4.1), we will investigate the viability of different cancer cell lines after exposure to different concentrations of unmodified TiO₂. In the second section (4.2), we will establish viability through the use of particles with modified surface chemistry at different concentrations with the cell lines.

4.1 Toxicity of Unmodified TiO₂

After conducting a literature review regarding TiO₂ nanoparticles and their reported interactions, we believe that TiO₂ nanoparticles (especially in the case of particles with anatase structure) may interact with cancer cell membranes, leading to toxicity. These interactions may be enhanced in cancer cells compared to normal cells due to increased permeability of the cancer.

Therefore we aim to establish the relationships between TiO₂ particle concentration and viability for different cancer cell lines.

4.1.1 Introduction

As previously mentioned, the photocatalytic effects of TiO₂ have been thoroughly investigated, with results showing that efficient destruction of different cell types can be achieved, possibly via generation of ROS. The non-photocatalytic effects of TiO₂ on cell viability have been demonstrated, though inconclusively. It is generally accepted that the rutile phase is non-toxic, but the more reactive anatase phase may have some level of toxicity at moderate exposure concentration.

With this in mind, we have chosen to investigate the cancer toxicity of the P25 (80% anatase 20% rutile) formulation of TiO₂. For this study, four cancer cell lines and one fibroblast control were investigated for their toxicity to TiO₂ at a log scale of concentrations for comparison with previously published data.

HYPOTHESIS 1: Exposure of uncoated TiO₂ nanoparticles may lead to cancer cell death.

4.1.2 Materials and Methods

Particle preparation: TiO₂ particles (P25 standard 80% anatase, 20% rutile), acquired from the DeGussa Corporation (Düsseldorf, Germany), were of very uniform size, with an average diameter of 21nm. In order to prevent the involvement of photocatalysis in this experiment, particle solutions were prepared with serum to allow passivation of serum proteins to the particles for 24 hours. This measure has been shown to eliminate/significantly reduce photocatalytic effects of TiO₂ (Xia et al., 2006). Prior to culture, particle solutions were briefly sonicated to resuspend the particles. TiO₂ solutions were then added to cell seeded well plates at desired concentrations.

Cell lines: For viability studies, TiO₂ nanoparticle solutions were prepared and stored overnight in Dulbecco's Modified Essential Media (DMEM) supplemented with 10% fetal calf serum (FCS). Cell lines were purchased from the ATCC and included B16F10 and B16F1 melanoma cell lines, JHU prostate cancer line, LLC lung carcinoma, and 3T3 fibroblasts. B16F10 is mouse skin melanoma commonly used to study metastasis *in vivo*. B16F1 is also a mouse skin melanoma that has tumorigenic properties in mice and is commonly used to induce tumors and study metastasis. JHU is a rat prostate cancer line that is tumorigenic *in vivo* in rats commonly used to study prostate cancer metastasis. LLC is the only semi-adherent cell used in the study. It is a mouse lung carcinoma line that is tumorigenic *in vivo* commonly used to study metastasis as well as the mechanism of cancer therapeutic agents. 3T3 is a mouse

fibroblast line that is commonly used in tissue engineering and other applications as a control cell line. All cultures were recovered from cryopreserved stock and expanded for 3 passages at American Type Cell Culture (ATCC) recommended passage rates prior to well plate seeding. All cells were propagated in DMEM with 10% FCS and 1% antibiotics.

Cell viability study: For all viability studies, cells were grown to confluence in 24 well plates prior to particle exposure. Following exposure to various amounts of particles, cells were allowed to incubate for 24 hours prior to viability staining. The viability of particle-incubated cells was conducted using Viability/Cytotoxicity staining method following a modification of the manufacture's protocol (Molecular Probes, Eugene, OR). The kit allows the simultaneous quantification of live and dead cells based on intracellular esterase activity and plasma membrane integrity, respectively. The kit is commonly used to assess apoptotic cell death as well as cell-mediated toxicity. The kit has two functional components, calcein AM which is enzymatically converted to a fluorescent green upon entry of live cells and Ethidium homodimer-1 (EthD-1) which enters cells with damaged membranes and undergoes a significant red fluorescent enhancement upon binding to nucleic acids.

Briefly, 1 μ M calcein AM and 2 μ M EthD-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 of 1 μ M calcein 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/). Image J was equipped with a cell counter plugin to track both live and dead cells within each image. Cell viability was determined by dividing the live cells by total number of live and dead cells to achieve a percent viability. The viabilities for a given condition were then averaged and analyzed for statistical significance in EXCEL.

Statistical analyses: Statistical comparison was conducted using EXCEL equipped with a data analysis plugin provided in the software. Student t tests were conducted using the data analysis tool to compare data sets for statistical significance. Differences were considered statistically significant when $p < 0.05$.

4.1.3 Results

Influence of particle concentrations on cell survival: 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, B16F1 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-26 were intermediate between that of the B16F1 and LLC cells, being 75% and 50%, respectively (Figure 1).

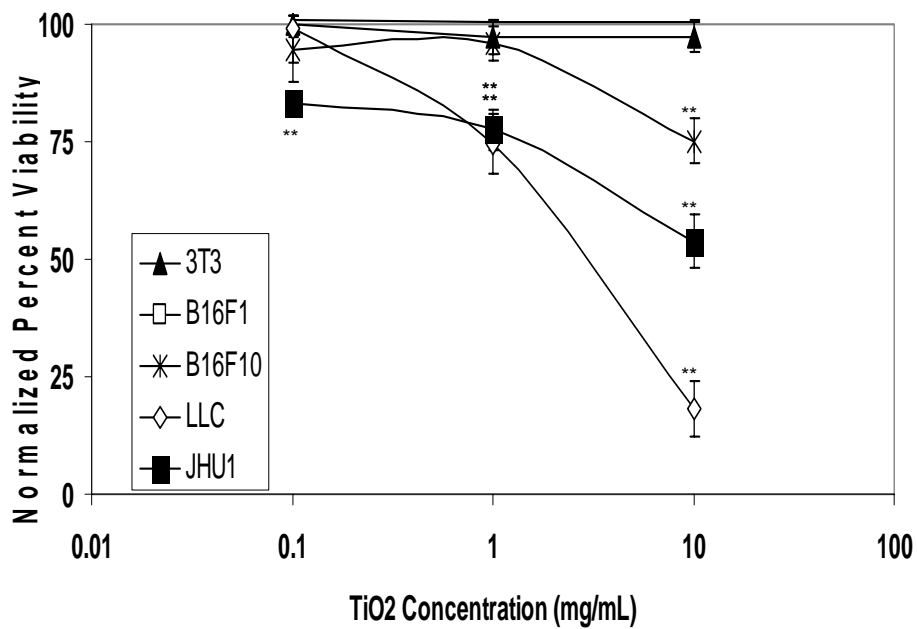


Fig 1 The influence of TiO₂ particle concentration on survival rates of cells. Vertical lines denote \pm 1SD (n=4 for all test samples and cells). Significant of differences between cancer cells vs. 3T3 cells (\blacktriangle): **, P<0.05).

4.2 Toxicity of Surface Modified TiO₂

Based on the viability results unmodified TiO₂ nanoparticles (Figure 1), we see that indeed there are some cell type dependant interactions between TiO₂ nanoparticles and cancer cells. Since these effects are likely due interactions between the reactive TiO₂ surface and the cancer cell membranes, adjusting the surface chemistry may lead to changes in the toxicity of the nanoparticles for each cell type. In this section we will attempt to modify the particle surfaces to investigate whether there are changes in the toxicity of the modified nanoparticles compared with the unmodified TiO₂ nanoparticles.

4.2.1 Introduction

In the first investigation, we were able to determine that TiO₂ nanoparticles indeed possessed some degree of cancer killing property, especially with the JHU and LLC which showed decreased viability at relatively low concentrations. We next sought to determine whether altering the surface functionality of TiO₂ could modulate or reduce cancer cell survival.

It has previously been established that hydrophobic biomaterials evoke undesired inflammatory responses, caused by protein adhesion and denaturation (Hu et al., 2001; Tang and Hu, 2005). Subsequently, it was seen that by altering the

hydrophobic surface of the materials, the compatibility of the materials could be enhanced (Wang et al., 2004; Wang et al., 2005). It has been shown that the addition of different chemical functional groups to the surface led to such benefits as: reduced protein adsorption leading to decreases in inflammatory cell adhesion (Tidwell et al., 1997; Tang et al., 1998), protein adsorption and surface rearrangement in favorable conformations reducing inflammatory cell activation (Keselowsky et al., 2004), adhesion and proliferation of desired cell types (Scotchford et al., 2002), enhanced cell differentiation (Piana et al., 2007), and increased cellular uptake of nanoparticles (Kannan et al., 2004).

The unmodified surface of TiO₂ nanoparticles can be characterized as hydrophilic. However, the processes involving protein adsorption and subsequent cellular interactions can be analyzed more specifically by considering the actual chemical functionality that provides the properties to a surface. We therefore sought to modify the TiO₂ particle surfaces with well defined chemical entities displaying unique chemical functionalities on their surface to determine if the toxic effects of TiO₂ could be modulated or enhanced by surface modification.

Though many surface chemical groups have been investigated, a core group of surface functionalities have been investigated most often, mainly due to the differences in properties of the groups. These core groups are the amine (-NH₂), hydroxyl (-OH),

and carboxyl (-COOH) groups. Group properties at physiological pH are briefly summarized below:

- -NH₂ – Positively charge
- -OH – Hydrophilic, neutral charge
- -COOH – Hydrophilic, negatively charged

Recently, a group was able to show that nanoparticle surfaces, functionalized with -NH₂ and -OH, were taken up in cancer cells by differing mechanisms at different rates (Kannan et al., 2004). Results highlighting the effects of -COOH on cellular uptake have also been reported (Holzapfel et al., 2006). It has been suggested that the surface charge, as well as functionality, lead to different interactions with the cell membrane. The mechanisms underlying these interactions are not completely understood and thus the possible benefits of functional coating in relation to cell targeting and particle:membrane interactions have not been identified. We therefore have attempted to quantify the positive and negative results of surface functionalizing TiO₂ nanoparticles on cancer cell viability by functionalization using radio frequency glow discharge (RFGD) plasma polymerization.

HYPOTHESIS 2: FUNCTIONALIZED TiO₂ NANOPARTICLES HAVE IMPROVED CANCER TOXICITY EFFECT.

4.2.2 Materials and Methods

Particle preparation: The particles used in this study were functionalized and characterized by the lab of Dr. Richard Timmons. TiO₂ particles (P25 standard 80% anatase, 20% rutile), acquired from the DeGussa Corporation, were of very uniform size, with an average diameter of 21nm. 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.

RFGD plasma polymerization 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. 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 thus 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 (Susut and Timmons, 2005), including nanoparticles (Cho et al., 2006). 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. 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)

Cell culture procedures: For this study, we limited the cells lines investigated to 3T3 mouse fibroblasts, B16F1 mouse melanoma, JHU rat prostate cancer, and LLC mouse lung cancer cells. B16F10 mouse melanoma results for surface functional TiO₂ were similar to B16F1, with larger deviations, as seen in the uncoated TiO₂ experiment. Therefore B16F10 was omitted from the study. The cell culture procedures and particle preparation for cell culture were carried out as previously described (Section 4.2).

Cell viability test: For viability analysis: cell culture, particle exposure and Viability/Cytotoxicity staining were carried out identically to the first experiment as described in Section (4.2). Briefly the two functional dye components, calcein AM which is enzymatically converted to a fluorescent green upon entry of live cells and Ethidium homodimer-1 (EthD-1) which enters cells with damaged membranes and undergoes a significant red fluorescent enhancement upon binding to nucleic acids, were used to label live and dead cells.

Analysis was also carried out identical to the first experiment detailed in Section (4.2). Briefly 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/). Image J was equipped with a cell counter plugin to track both live and dead cells within each image. Cell viability was determined by dividing the live cells by total number of live and dead cells to achieve a percent viability. The viabilities for a given condition were then averaged and analyzed for statistical significance in EXCEL as previously documented (Section 4.2).

Cell membrane imaging: To determine the interaction of TiO₂ particles in cell culture, cell membranes were stained with FM1-43 membrane stain (FM1-43 Membrane Stain, Molecular Probes [Invitrogen], Carlsbad, CA). The presence of nanoparticles can be visualized with unique auto-fluorescence only found on –COOH coated particles. 3T3 cells were incubated with –COOH particles for 3 hours. At the end of the studies, the cells were stained with FM1-43 and DAPI then observed using fluorescence microscope.

The culture and staining procedure were as follows. Cells were recovered from cryopreservation (Section 4.2) and cultured on coverlips to confluence. Upon reaching confluence, TiO₂ particle solutions (prepared as described in Section 4.2) were added to the cultures and allowed to incubate for 3 hours. After 3 hour incubation, the media

was removed and the cell layer was gently rinsed in PBS to remove non-adherent particles and cells. The coverslips were then stained with DAPI dissolved in PBS at 300nM. Following nuclear labeling with DAPI, cell membranes were stained with FM1-43. FM1-43 was diluted in HBSS to a concentration of 5 μ g/mL, added to coverslips and put at 4°C for 1 minute. The coverslips were then mounted to slides and immediately imaged with phase contrast and –COOH autofluorescence (FITC green filter), FM1-43 (Texas Red filter), and DAPI (blue DAPI filter).

4.2.3 Results

The dose effects of TiO₂ nanoparticles with various functionalities on cell survival were evaluated using Live/Dead Viability/Cytotoxicity staining (Figure 2). The most pronounced effects of surface functionalized TiO₂ were seen with LLC (Figure 2A) and JHU (Figure 2B). The effect of surface functional groups on cell survival is highlighted at the concentration of 0.1mg/mL for JHU cells (Figure 2B). 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 enhances 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. Similar to responses of JHU cells, the -COOH functional group substantially reduces particle-mediated toxicity to LLC cells (Figure 2A). 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%). Representative viability images of LLC cells can be seen in (Figure 3). 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 2C & 2D).

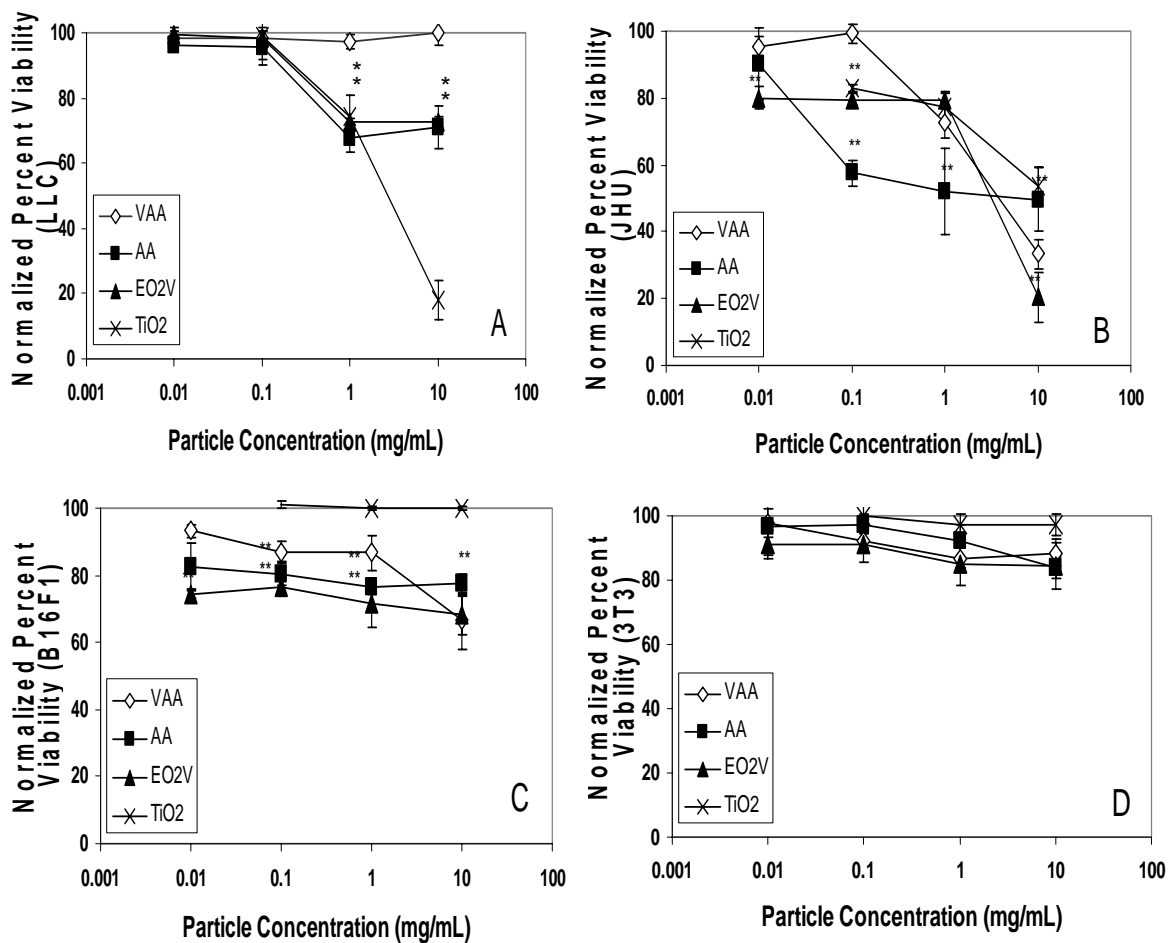


Fig 2 Effect of coated particle concentrations on viabilities of different cell types, (A) LLC (B) JHU-26 (C) B16F1 (D) 3T3. The vertical lines denote \pm 1SD (n=4 for all test samples and cells). Significant of differences between -NH₂ coated and -OH coated particles vs. -COOH coated particles (o): **, P<0.05).

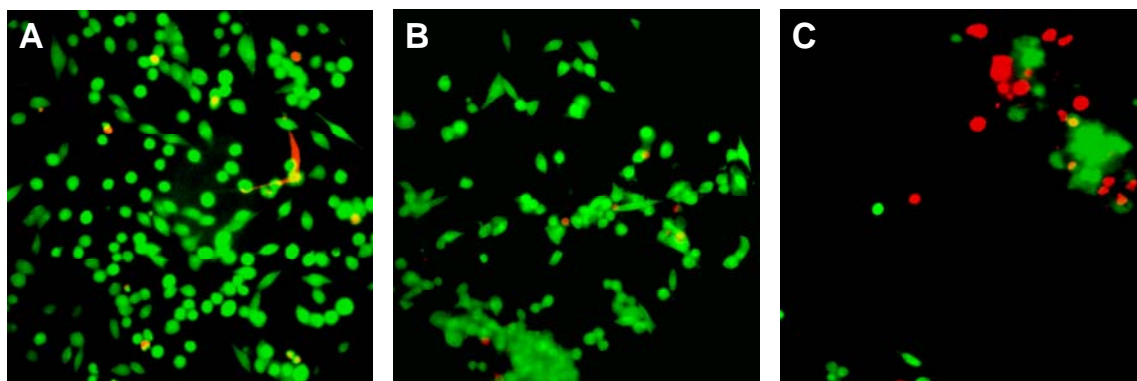


Fig 3 Representative Viability Stains of LLC Cells Exposed to Functionalized TiO₂ Particles. Cultures were incubated overnight. Positive live stains appear green. Positive dead cells appear as a red stained nucleus. (A) LLC control culture cells. (B) Cells cultured with VAA functionalized particles (-COOH functionality). (C) Cells cultured with EO2V functionalized particles (-OH) functionality.

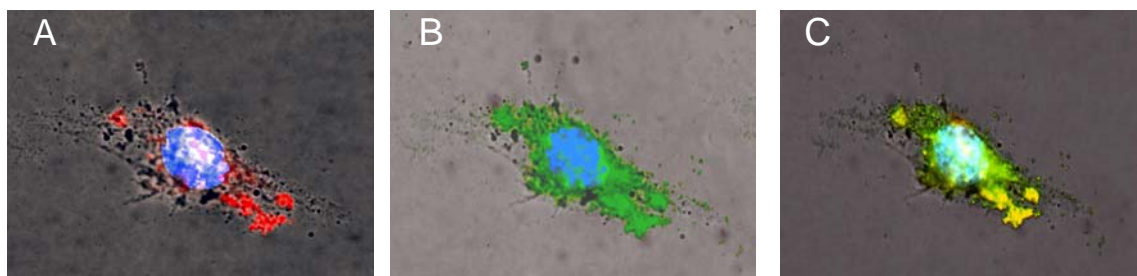


Fig 4 Visualization of the interaction between functionalized (VAA, in 0.01mg/mL) TiO₂ particles and 3T3 cells. Nucleus and membranes was stained with DAPI (blue) and FMI-43 (red), respectively. Particles emitted fluorescence light (green). By overlapping various images, the location and interaction of particles with cells can be clearly identified. (A) Cells with nucleus (blue) encompassed with intact membranes (red). (B) Cells with nucleus (blue) were covered with substantial amount of particles (green). (C) Particles (green) coincided with the cell membranes (red) showing yellow areas of overlap.

4.2.4 Discussion

The first part of this study was designed 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* (Hussain et al., 2005; Okada et al., 2006), *in vitro* inhalation models (Warne et al., 2004), and *in vivo* models (Olmedo et al., 2005; Wang et al., 2006). 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 LLC 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 (Warheit et al., 2007). 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 (Bermudez et al., 2002; Urban et al., 2000; Olemdo et al., 2002). The specific interactions between TiO₂ particles and

proteins are not totally understood. Studies have revealed that TiO₂ particles have a net negative charge (at pH = 7) (Topoglidis et al., 2003) and also bind preferentially to amino acids containing -OH, -NH, and -NH₂ in their side chains (Tran et al., 2006). Many studies have observed TiO₂ adherence to cell membranes as well as uptake into cells. Therefore, changes in cell viability when comparing different particle culture conditions can be attributed to differential interactions between the surface and the individual cell types. These observations indicate that surface properties of TiO₂ particle may affect cell:particle interaction.

Surface functionality has been shown to affect cell:particle interactions (Barbosa et al., 2006). Although it has been suggested that surface functionality should be the determining factor concerning cell uptake and subsequent activity inside the cell (Wilhelm et al., 2003), 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 TiO₂ particles can be altered. Three functional groups with various surface charges (-OH, -NH₂, 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 at 1mg/mL. These findings are in agreement with recent findings that TiO₂ nanoparticle surface functionality (hydrophilic verses hydrophobic) had insignificant effects on cell toxicity in an intratracheal rat model (Warheit et al., 2006; Rehn et al., 2003). These

differences may be due to protein composition of the cell membrane and how these proteins interact with the TiO₂ 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. In contrast, surface functionality exerts medium influence on Lewis lung carcinoma toxicity, possibly due to increased interaction between the TiO₂ 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. The JHU prostate tumor cell line showed a significant susceptibility to the -NH₂ group. Perhaps, the positive charged amine group prompts a destructive impact on the negatively charged membrane of the cells. While positive charged -NH₂ coating shows a significantly low viability for JHU cells, negative charged -COOH coated particles substantially increase JHU cell viability, a phenomenon also seen in the case of LLC cells. It appears that positive surface charge may enhance particle accumulation on cell membrane and subsequently particle uptake by cells (Lorenz et al., 2006; Holzapfel et al., 2006).

To further investigate the interactions of -COOH functionalized particles, 3T3 cells were cultured on coverslips and exposed to -COOH particles. Upon merging the stain images of 3T3 cells exposed to -COOH functionalized particles, it was seen that the particles appear to aggregate at the cell membrane with a slight presence around the

periphery of the nucleus, possibly suggesting particle uptake inside the cell membrane. Results have shown that –COOH functionality can be correlated with increased cell uptake. The uptake of –COOH particles may be facilitated differently by cells, as suggested by uptake studies investigating particles with different functionalities (Kannan et al., 2004). This uptake mechanism may be the difference for the observed preservation of cell viability after exposure.

CHAPTER 5

DISRUPTION OF CELL MEMBRANE FLUIDITY INDUCES CANCER CELL DEATH

5.1 Introduction

Previous results suggest that TiO₂ particles either attach to the cell membrane or enter the cell. We hypothesize that TiO₂ is disrupting the fluidity of the cell membrane, leading to the generation of openings in the cell membrane. Ideally, the particles could be labeled with a fluorescent dye to track the movement of the particles after addition to the media. However, the chemical addition of fluorescent compounds may alter the surface functionality and obscure their cellular interactions while also possibly causing toxicity.

As described in previous chapter, we have determined that the –COOH functionalized particles omit auto-fluorescence, allowing us to visualize and image the particles. However, the other coatings do have this property. Therefore, in order to preserve the surface functionality of the particles, we sought to label the cell membrane, hoping to visualize any disruption after particle exposure. In order to visual the cell membrane after particle exposure, FM1-43 lipophilic dye can be used to monitor the cell membrane. FM1-43 has been used to track endocytosis and recycling of cell

membranes after uptake.

Openings in the membrane may lead to release of cytoplasmic contents into the surrounding media. We sought to test this hypothesis by conducting both a lactate dehydrogenase (LDH) and [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) cell proliferation assay. LDH is a cytoplasm enzyme and LDH reduction is often associated with cell membrane damage and cell death (Lobner, 2000). Therefore a correlation between viability, membrane fluidity, cell proliferative ability, and LDH results may hint at the mechanism causing cell death.

HYPOTHESIS 3: TiO₂ PARTICLES INTERRUPT CELL MEMBRANE FLUIDITY WHICH LEADS TO CANCER CELL DEATH.

5.2 Materials and Methods

Particle preparation: All TiO₂ nanoparticle solutions, uncoated and surface functionalized, were prepared as previously explained in Sections (4.2 and 4.6). Exception was made in the case of culture to be used for LDH activity studies. In order to achieve accurate enzyme results, particles solutions were prepared in Hank's Balanced Salt Solution (HBSS) with 1% FCS to reduce the possible effects of serum background during LDH assay. HBSS with 1% FCS particle solutions were prepared

and stored overnight to passivate the particles with serum proteins to limit photocatalytic effects. TiO₂ particle solutions were briefly sonicated prior to cell culture.

Cell culture: The LLC cell line was used exclusively in both the LDH and cell proliferation test (MTS assay) experiments due to the fact that LLC cells had the most distinguishable and consistent results when exposed to uncoated and surface functionalized TiO₂. Expansion and pre-experimental treatments were identical to those methods described in Sections (4.2 and 4.6).

Cell membrane integrity: LLC cancer cell survival was quantified with two methods, Lactic dehydrogenase assay and cell proliferation test (MTS assay). For LDH studies, following sonication, the cell culture media was removed from the well plates, and particle solutions in HBSS at desired concentrations were added to cell seeded well plates. For enzyme assay experiments cell were grown to confluence prior to particle exposure in 24 well plates. Particle exposed cells were allowed to incubate for 3 hours before enzyme assay (the maximum time period before cell death can be observed due to serum deprivation).

At the conclusion of the experimental exposure period, 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. Untreated samples exposed to the

same culture conditions without the addition of particle solutions were used as controls. Samples of both lysate and supernatant for each particle type were then analyzed by spectroscopy. The activity of LDH was measured spectrophotometrically by assaying reduced nicotinamide adenine dinucleotide oxidation @340 nm during LDH-catalyzed reduction of pyruvate to lactate (Tang et al., 1993).

LDH enzyme assay conditions were conducted as follows. After preparation of vials containing lysate and supernatant samples collected from LLC cells exposed to different concentrations of uncoated TiO₂ (.01mg/mL to 10mg/mL) and surface functionalized TiO₂ (all samples at 1mg/mL), test solutions at approximately 1mL were prepared. Test solutions consisted of 100µL of sample, 725µL of phosphate buffer, 15 µL of NADH, and 50µL of sodium pyruvate. After the addition of sodium pyruvate (which begins the reaction), the samples were immediately read at @340 nm. A calibration curve was then used to determine mU of LDH activity from recorded absorbance readings.

Cell proliferation testing: For verification of Live/Dead viability results for VAA coated TiO₂, a cell proliferation test (MTS assay) was conducted on LLC cells grown to confluence in 96 well plates (Promega, Madison, Wisconsin). Upon reaching confluence, media containing 1mg/mL TiO₂, functionalized or uncoated, was added to cultures and allowed to incubate for 3 hours at 37° C and 5% CO₂. After 3 hours, 20µL of Aqueous One Solution was added to begin the assay. Manufactures suggested

protocol was followed concerning solution incubation time (4 hours) and microplate settings. Samples were compared to control, untreated sample optical densities (OD). Sample optical densities were read at 490nm with background at 630nm. Background readings were taken of culture media with 20 μ L of Aqueous One Solution and subtracted from sample readings.

Cell membrane staining: Following LDH and cell proliferation test (MTS assay) experiments, cells were stained 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.

Statistical analyses: Statistical comparison (achieved on the basis of variable cell number) was carried out according to Student t- test. Differences were considered statistically significant when $p < 0.05$.

5.3 Results

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. The LLC cell line

was chosen for these assays as it had the most distinct response to both uncoated and surface functionalized TiO₂. 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 5). Upon assaying uncoated and surface functionalized TiO₂ at a concentration of 1mg/mL, the same general trend as seen in viability staining was observed. Supernatant levels of LDH activity were lowest compared to all other particle types, though lysate levels were slightly lower than expected, falling below control and uncoated TiO₂ levels (Figure 6).

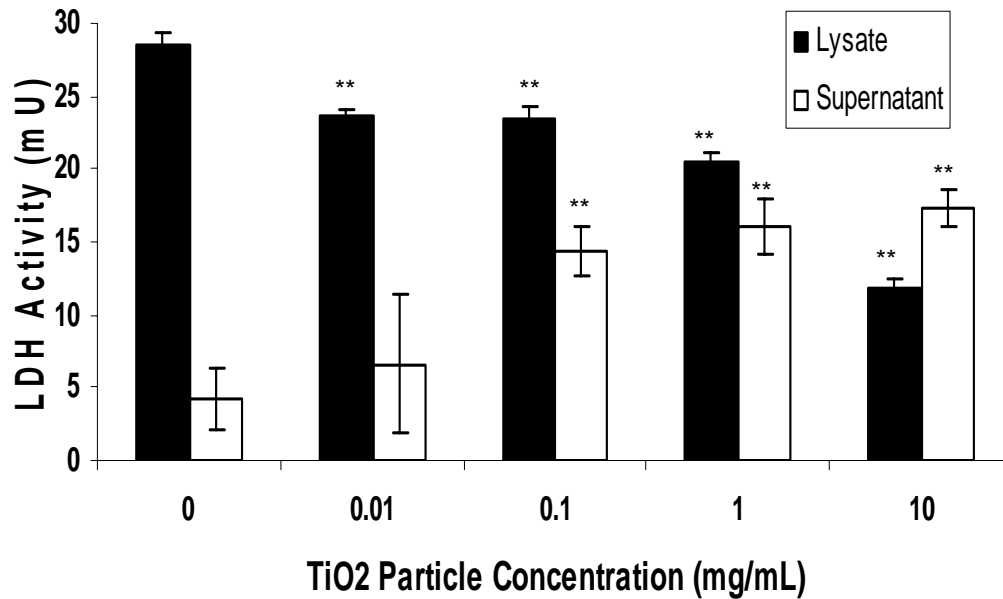


Fig 5 LDH release from LLC cells following unmodified TiO₂ exposure. Cells with 70-90% confluence were exposed TiO₂ particles in media consisting of HBSS with 1% FCS. After incubation for 3 hours, the supernatants were collected and the adherent cells were then lysed with 0.5% Triton X-100. The LDH activities of supernatants and adherent cell lysates were then determined spectrophotometrically. The vertical lines denote \pm 1SD (n=3 for all test samples and cells). Significant of differences between particle incubated cells vs. untreated cells: **, P<0.01).

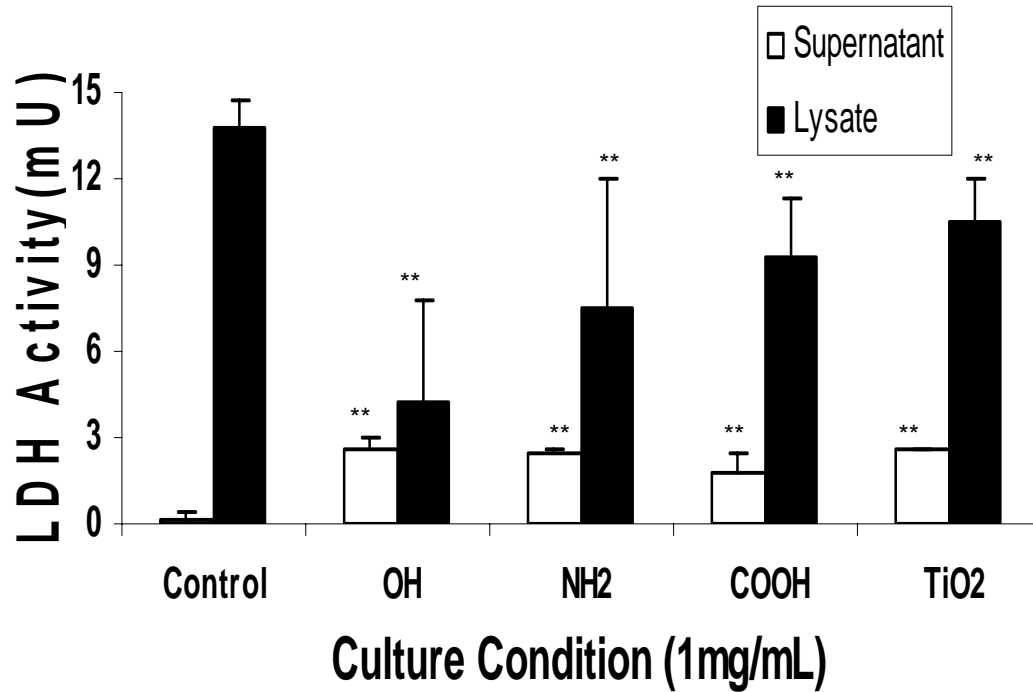


Fig 6 LDH release from LLC cells following exposure to functionalized TiO₂ nanoparticles at 1mg/mL. Cells with 70-90% confluence were exposed TiO₂ nanoparticles in media consisting of HBSS with 1% FCS. After incubation for 3 hours, the supernatants were collected and the adherent cells were then lysed with 0.5% Triton X-100. The LDH activities of supernatants and adherent cell lysates were then determined spectrophotometrically. The vertical lines denote \pm 1SD (n=3 for all test samples and cells). Significant of differences between particle incubated cells vs. untreated cells: **, P<0.05)

For further verification, similar studies were carried out using a cell proliferation test (MTS 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 of P25 TiO₂ toxicity and that –COOH functionality reduced toxicity of TiO₂ to LLC cells (Figure 7 and Figure 8). 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%).

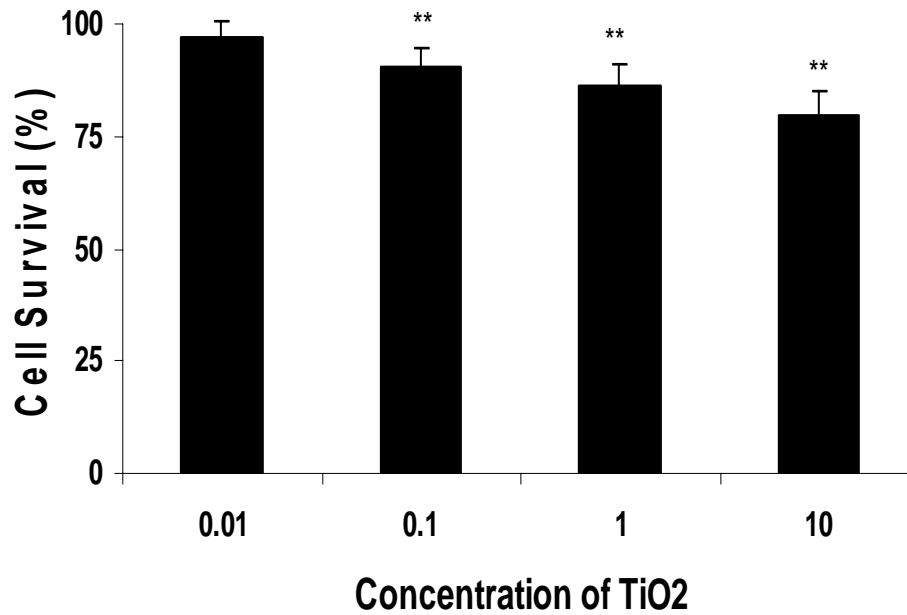


Fig 7 Cell proliferation test (MTS assay) on particle-exposed LLC cells was carried out to compare with cell viability and LDH results. LLC cells were incubated with various concentrations of unmodified TiO₂ particles. After incubation for 3 hours, the activity of adherent cells was then quantified with MTS assay kit (Aqueous One cell solution, Promega). The viable cells were then determined spectrophotometrically. The vertical lines denote \pm 1SD (n=5 for all test samples). Significant of differences between particle incubated cells vs. untreated cells: **, P<0.05).

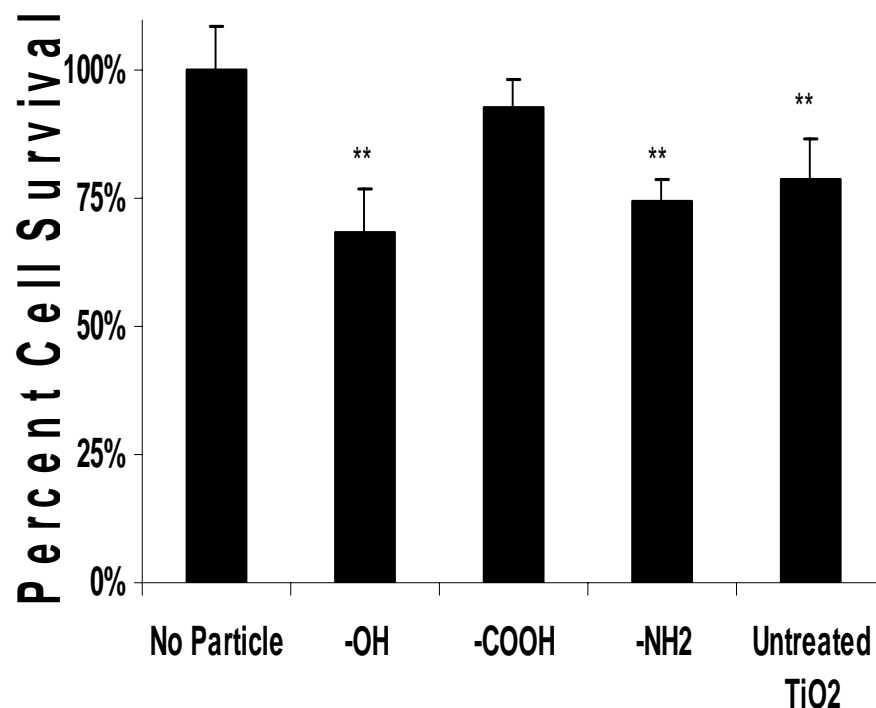


Fig 8 Cell proliferation test (MTS assay) on particle-exposed LLC cells was carried out to compare with cell viability and LDH release results. LLC cells were incubated with variously functionalized TiO₂ particles in media consisting of HBSS with 1% FCS. After incubation for 3 hours, the activity of adherent cells was then quantified with MTS assay kit (Aqueous One cell solution, Promega). The viable cells were then determined spectrophotometrically. The vertical lines denote \pm 1SD (n=5 for all test samples). Significant of differences between particle incubated cells vs. untreated cells: **, P<0.01).

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 9A). On the other hand, the spotty, rough cell membranes were found in cultures with uncoated TiO₂ particles (Figure 9B). 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 9C & 9D). This suggests that cell:particle interactions, other than particle deposition on cell membranes, are responsible to cell death. Observation of the cells under higher magnification (X400), confirmed that LLC cells served a focal point for large numbers of TiO₂ particles (Figure 10). 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.

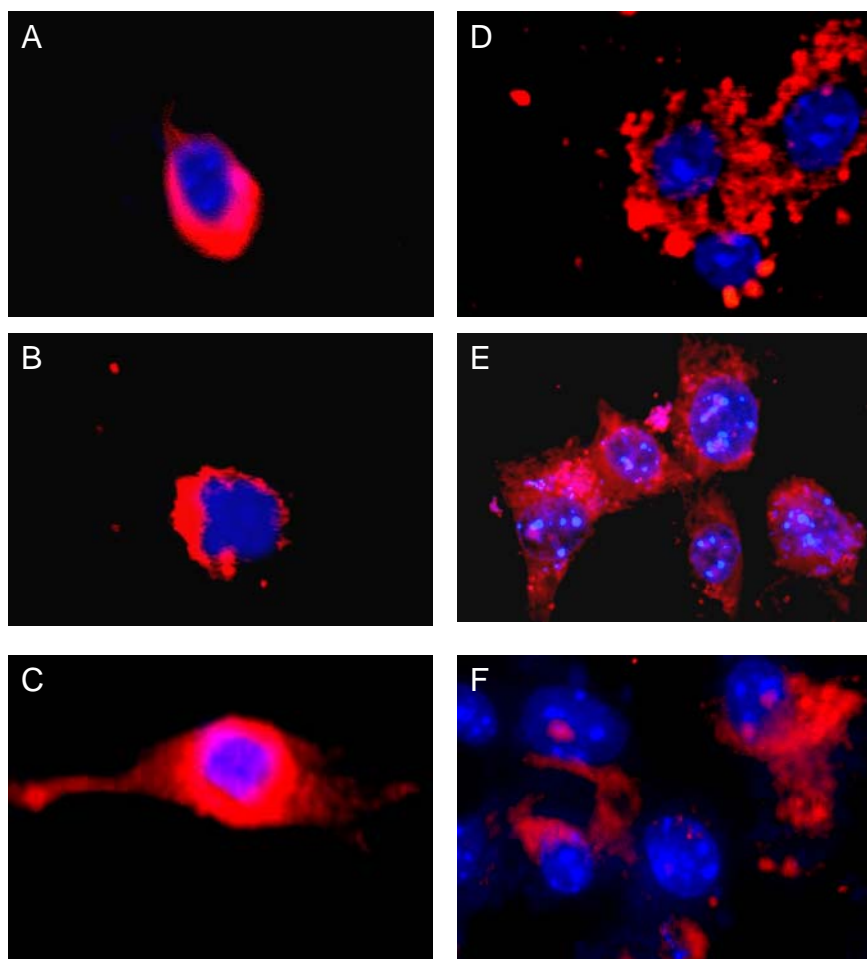


Fig 9 Effect of particle:cell interactions on cell membrane integrity. Various cancer cells were cultured with TiO_2 particles (1mg/ml) for three hours. The cell membranes were then stained with FM1-43 membrane stain (red) as well as nuclear DAPI staining (blue). (A) Untreated LLC cell showed continuous 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 fragmented membranes.

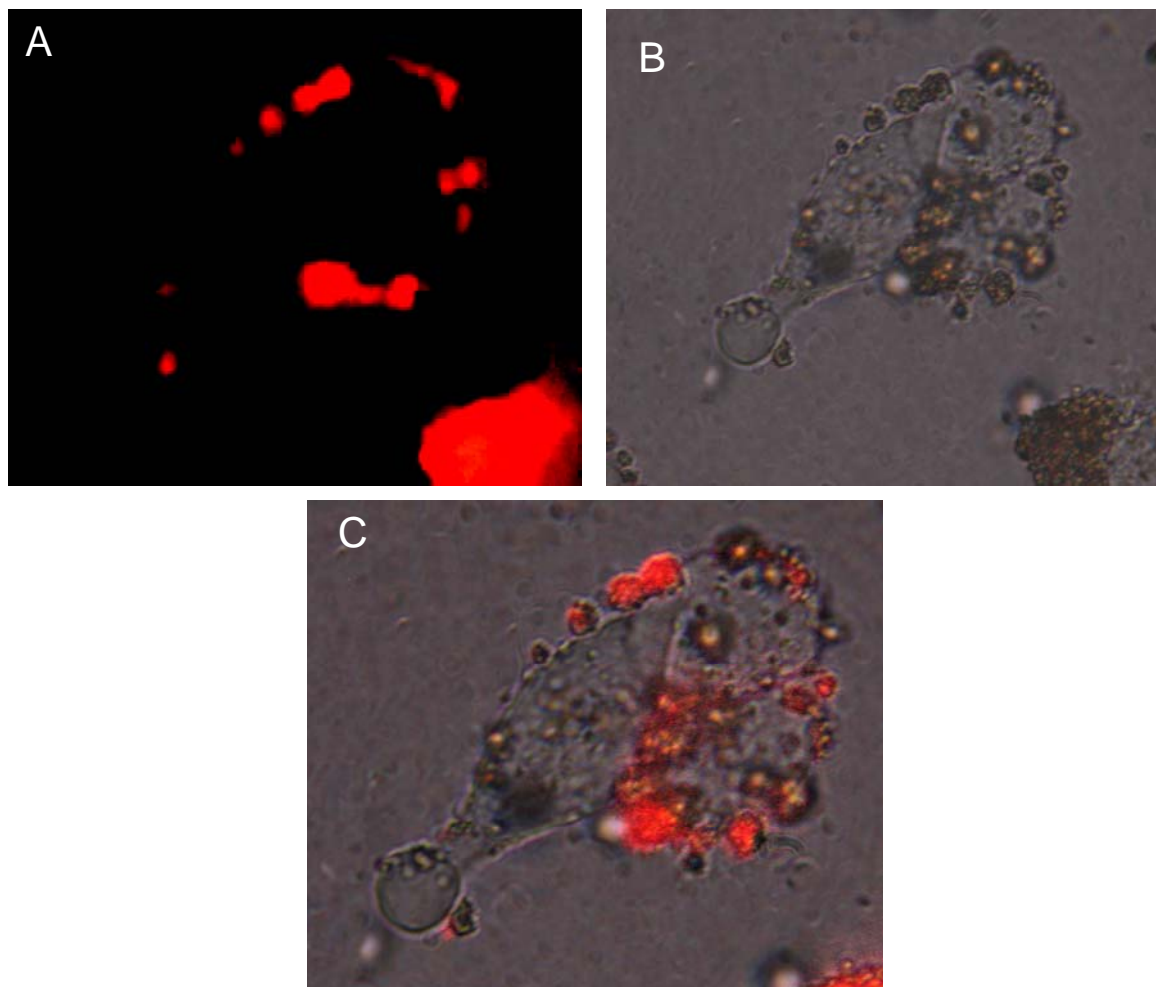


Fig 10 Particles influence on cell membrane integrity. (A) The disrupted cell membrane of TiO_2 exposed LLC cells were stained with FM1-43 membrane stain (red). (B) TiO_2 particles can be visualized surrounding cells under phase contrast optical microscope. (C) By overlapping membrane stain images and optical image, membranes can be seen to coincide with the particles indicating potential involvement of particles on cell membrane disintegration.

5.4 Discussion

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 (Avivi-Green et al., 2002). 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 (Wamer et al., 1997; Blake et al., 1999). Second, TiO₂ uptake was decreased in alveolar macrophages in the absence of calcium, preventing influx of calcium into damaged cell membranes (Blake et al., 1999; Stringer et al., 1995). Third, results have suggested that particle-mediated production of hydroxyl radical is responsible for the interruption of pulmonary cell and cancer cell membrane (Donaldson et al., 1998; Blake et al., 1999).

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 has suggested that the disruption of cell membranes often lead to cell death

(Pelicano et al., 2004; Blake et al., 1999). 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 (Hussain et al., 2005). Indeed, there is an excellent relationship between LDH release and cell death (Live/Dead stain), further 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 (Bousiff et al., 1995). 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 lead 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 (Bousiff et al., 1995).

CHAPTER 6

CONCLUSIONS

- TiO₂ nanoparticles trigger cancer cell death that is cell type dependent among the cell lines tested in this work, including B16F10, LLC, 3T3, and JHU.
- TiO₂ nanoparticles-induced cancer cell death is concentration dependent. List the specific concentration for every cell lines in referencing to 3T3 cells.
- This toxicity can be altered by the addition of a specific chemically functionalized surface. Depending on the membrane properties of the specific cell type, these functionalities can increase or decrease TiO₂ nanoparticle toxicity *in vitro*. The –COOH functionalized particles were shown to maintain viability near control levels for all cells tested. In the case of JHU cells, the NH₂ coating at low to moderate particle concentrations caused a drastic reduction in JHU cell viability. With 3T3 and B16F1 cells, which were for the most part unaffected by TiO₂ exposure, -OH surface coated surfaces had the highest decrease in viability for both cell types.

- The basis of both unmodified and surface functionalized TiO₂ nanoparticles appears to be linked to their interactions with the cell membrane. Upon binding to and/or uptake through the cell membrane, the particles cause a disruption in membrane fluidity, leading to a permanent disruption of the cell membrane, inhibiting proliferation and subsequently leading to cell death.

CHAPTER 7

FUTURE RECOMMENDATIONS

- The *in vivo* effects of these particles would likely provide interesting and relevant results. Induction of cancerous masses, followed by injection of functionalized TiO₂ in conjunction with histological analysis could provide interesting information on tumor growth suppression and retention of the particles in the cancerous mass
- The photocatalytic abilities of surface functionalized TiO₂ have not been investigated. It would be interesting to determine if surface functionality has an effect on the photocatalytic abilities of TiO₂.
- It may be possible that the effects seen in this investigation, in conjunction with photocatalysis, may provide an effect superior to TiO₂ and photocatalysis alone.
- Addition work could be done to more precisely determine the mechanism of cell destruction and the possible interactions and uptake mechanisms of each coated particle type.
- We could also investigate drug release from other surface functionalized nanoparticles.

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BIOGRAPHICAL INFORMATION

Paul Todd Thevenot received a Bachelor of Science in Biological Engineering from the Louisiana State University Baton Rouge, Louisiana in May 2005. Paul was born in Lake Charles, LA on November 2, 1981. Following fulfillment of degree requirements, Paul passed the Fundamentals of Engineering Exam and was certified as an Engineering Intern in the State of Louisiana. Paul was accepted in to the Biomedical Engineering program at the University of Texas at Arlington in the Fall of 2005.