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In vitro interactions between splenocytes and dansylamide dye-embedded nanoparticles detected by flow cytometry

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Abstract

Engineered nanoparticles (NPs) possess a range of biological activity. *In vitro* methods for assessing toxicity and efficacy would be enhanced by simultaneous quantitative information on the behavior of NPs in culture systems and signals of cell response. We have developed a method for visualizing NPs within cells using standard flow cytometric techniques and uniquely designed spherical siloxane NPs with an embedded (covalently bound) dansylamide dye. This method allowed NP visualization without obscuring detection of relevant biomarkers of cell subtype, activation state, and other events relevant to assessing bioactivity. We determined that NPs penetrated cells and induced a range of biological signals consistent with activation and costimulation. These results indicate that NPs may affect cell function at concentrations below those inducing cytotoxicity or apoptosis and demonstrate a novel method to image both localization of NPs and cell-level effects.

Keywords

nanotoxicology; in vitro; flow cytometry; cellular function

INTRODUCTION

The pace of research and development in nanotechnology has generated a large number of unique materials with potential application in many fields, including medicine. Engineered nanoparticles (NPs) are already in use for *in vivo* and *in vitro* imaging, and considerable research is currently directed towards the development of highly specific delivery systems and other functions with biomedical applications. Our current research efforts are focused in this latter area, in the fabrication of NPs that can be safely and effectively utilized to deliver materials –including dyes, drugs, genes, and other materials – to specific cellular and intracellular sites.

For these experiments, we utilized NPs currently under development for application in biomedicine specifically as imaging and diagnostic agents that combine biocompatibility and

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stability. Present alternatives include quantum dots containing toxic cadmium^{1–6} and organic dyes with limited oxidative stability or those that leach from NPs once they are introduced into an aqueous or biological environment. Silica-based NPs offer a more biocompatible matrix upon which to build imaging/diagnostic agents^{7–11} and also avoid the concerns of NPs containing metals such as cadmium. However, little is known about the immunotoxic properties of silica-based NP, which is of potential concern given the literature on silica and autoimmune disease (reviewed by¹²) as well as silicone and immunopathology.¹³ To date, no immunotoxic testing of silicate/silicone-based NPs has been undertaken.

Like other laboratories, we approach the assessment of potential unwanted bioactivity of NPs through the use of *in vitro* systems.¹⁴ This approach can assist in defining conditions of exposure as well as relevant signals of cellular response. There is a considerable literature on *in vitro* methods for nanotoxicology and a range of events have been associated with *in vitro* exposure of different cell systems to several types of NPs, including generation of oxidative stress,^{15, 16} alterations in cell signaling pathways (detected by microarray),¹⁷ and changes in protein synthesis.^{18, 19} However, NPs may behave anomalously within *in vitro* systems by aggregating or clumping.²⁰ In some cases bioactivity has been detected in the presence of substantial cell loss through cytotoxicity. Since these are conditions that would not be tolerable in biomedical applications, we have devised methods that permit the simultaneous observation of NP behavior, cellular integrity, and biological responses.

The purpose of this study was to investigate the applicability of fluorescence activated cell sorting (FACS) methods to visualize NPs and simultaneously determine biological effects of NPs under realistic conditions of exposure. Currently, most investigators are utilizing microscopy to visualize NPs in cells.^{21, 22} This method allows for a static determination of localization of NPs on fixed (dead) cells with little room for concurrent determination of effects on cellular proteins. That is, it cannot provide information on the dynamic affects of NPs exposure on living cells. Some recent studies have used flow cytometry for visualization purposes,^{23, 24} but not for simultaneous detection of the biological state of exposed cells. We chose to utilize flow cytometric methods which are well described for the ability to visualize individual cells; but we have used the additional capacity of FACS to collect information on biological responses of cells exposed to NPs. FACS is limited only by the number of fluorophores (emission spectra) that the particular instrument is able to distinguish (based upon the lasers available for excitation and filters available for detection) and the availability of antibodies to the particular cellular protein of interest with their associated fluorophores.

For this initial study, we utilized a uniquely designed silicate-silicone (siloxane), spherical NP (approximately 200nm diameter) with an embedded dansylamide dye and exposed murine splenic lymphocytes *in vitro*. The dye is incorporated into the NPs during their construction and is covalently bound within the matrix of the material. We specifically did not encapsulate the dye within the core of the NP in order to avoid potential leaching through the NP shell. Rather, we created a matrix of siloxane in which the Si molecule of the dansylamide dye is allowed to covalently bind during the formulation process. Extensive washing of the NP following generation removes excess unbound dye.

Exposure was defined in terms of number of NPs/volume (i.e. concentration). We examined the uptake of NPs by three subsets of immune cells, macrophages, T and B cells, and we collected data on cellular markers of costimulation, activation, and regulation. We also used these methods to determine changes in cell numbers and induction of apoptosis (programmed cell death) associated with NP treatment.

METHODS

Creation of nanoparticles

The NPs utilized in this study were formed in an emulsion where 0.5g of the hydrophobic phase, PDMS [high molecular weight poly(dimethylsiloxane)]²⁵ was dissolved in 2ml of the sol-gel precursor TEOS (tetraethyl orthosilicate) and 70ml of ethanol. Then, 0.05g of Dansyl-aminopropyltriethoxysilane (dansylamide dye, structure shown in Figure 1A, obtained from Gelest) was present in the solution, and ultimately became covalently bonded to the crosslinked silica network. Control experiments established that this networking was essential for retention of the dye inside the particles (data not shown). However, not all the added dye was required to become covalently linked within the particles (and we might not expect it all to become incorporated); we only needed sufficient amount of dye within the NPs in order to visualize their fluorescence on the FACS. After adding 10ml of NH₄OH to initiate the polymerization process, 0.4g of surfactant Dow 190 (silicone ethylene oxide/propylene oxide copolymer) was added. The reaction was allowed to continue for 12 hours at room temperature. After the reaction was completed, NPs were washed with ethanol and deionized water to remove surfactant and centrifuged 5 times. The final particle concentration was 1.25×10^{11} particles per 3.5mg/ml as determined by averaging the number of counts from the field of view in 20 SEM images of the particles when 3.5mg/ml NPs was created in a monolayer for imaging.

The size and shape of the NPs were examined under scanning electron microscopy and Edax x-ray micro analysis system (SEM JEOL 6700 F). The NPs created in this study were found to be spherical, on the order of 200nm in diameter, dispersed, and uniform in size and shape (Figure 1B). Fluorescence and UV-vis reflectance spectra of dye doped NP were measured with a Hitachi F-4500 spectrophotometer and Varian Cary 50 Bio UV-Vis spectrophotometer, respectively. As shown in Figure 2, the fluorescence emission of the dansylamide-doped NPs was at 460–480 nm, blue-shifted by 20–40 nm relative to solutions of the unpolymerized dansyl dye. The signal is within a wavelength that permitted us to also simultaneously detect the fluorophores used to tag the cellular functional signals described below. Edax results showed that there was no contamination as only the expected elements (Si, C, O, etc) were found (Figure 3).

In vitro cell culture

For these studies we utilized splenic lymphocytes isolated from BALB/c female mice (8–10 weeks old) purchased from The Jackson Laboratories (Bar Harbor, ME) since these cells are frequently used in immunology and immunotoxicology following a Johns Hopkins Bloomberg School of Public Health Institutional Animal Care and Use Committee approved protocol. Splenocytes were isolated utilizing standard procedures.²⁶ Briefly, mice were euthanized with avertin anesthesia (intraperitoneal dose, 40ug/ml per mouse) followed by cervical dislocation and rapid removal of spleens under sterile conditions. Spleens were manually homogenized between sterile glass slides in 2ml of media (RPMI). Red blood cells were lysed with cell lysis buffer (BD Biosciences) on ice and lymphocytes washed with phosphate buffered saline (PBS) and collected by centrifugation. Individual cell suspensions containing mixed splenic lymphocytes (including T cells, B cells, and macrophages) were aliquoted into 24 well culture plates at a final concentration of 1×10^6 cells/ml. In order to examine potential modulatory effects of NPs, we examined responses to NPs (in comparison to controls) in the presence and absence of generic stimulation by 50nM lipopolysaccharide (LPS), a bacterial cell wall component. Cultures were treated with 6.25×10^7 particles based on the particle concentration of 1.25×10^{11} per 3.5mg/ml described above. In vitro exposures were continued for 48 hours at 37°C and 5% CO₂ and then cells were harvested by gentle pipetting, washed, and stained with monoclonal antibodies for FACS analysis. We conducted preliminary experiments to determine a concentration of NP in culture that did not induce overt cell death within 48 hours

of culture, using a range of concentrations from 6.25×10^5 up to 6.25×10^9 particles per 1ml culture (data not shown). We chose to utilize a concentration that did not induce massive cell death in order to observe the more subtle effects of NP in culture on live cells. Each individual culture condition was created in triplicate and the entire experiment repeated twice on separate days. Data are shown from one independent experiment.

FACS staining and analysis

Cells were stained with the following monoclonal antibodies (BD Pharmingen or eBioscience) diluted in 1% fetal bovine serum (FBS) in PBS: CD3 (clone 17A2), CD4 (clone RM4-5), CD8s (Ly-2, clone 53-6.7), CD11b (clone M1/70), CD19 (clone 1D3), CD80 (B7-1, clone 16-10A1), CD86 (B7-2, clone GL1), CTLA-4 (CD152, clone UC10-4B9), and toll-like receptor (TLR) 4 (clone MTS5510). For intracellular staining, cells were fixed and permeabilized using BD Cytotfix/perm (BD Pharmingen). For analysis of apoptosis, cells were stained with Annexin-V and 7-AAD (vital dye) according to manufacturer's protocols (BD Pharmingen) in conjunction with monoclonal antibodies for identifying cell subsets (such as CD19 for B cells). Cell fluorescence was measured using a LSR II flow cytometer equipped with four lasers (488, 633, violet, UV) and data analyzed using FlowJo software (Tree Star, Inc.). The BD LSR II instrument is capable of visualizing 12 separate fluorophores on a single cell, which makes it optimal for detecting multiple signals, including the NP as well as cell subtypes and biomarkers of cell response. Results are presented as the average for three experimental replicates from one experiment day $\pm$ standard error. Statistical differences between treatment groups (cultures treated with NP or not) or between cell populations containing NP or not were determined by Student's t-test.

RESULTS

The NPs were well behaved in biological media as well as water; under the conditions of these experiments, there was no clumping or aggregation observed. The FACS analysis clearly demonstrated that the NPs were able to penetrate all three cell types (B cells defined as CD19⁺, T cells defined as CD3⁺CD4⁺, and macrophages defined as CD11b⁺), as shown in Figure 4 (dansylamide NP fluoresce in the indo-violet range). Not all cells had NPs, which could have been a result of the limited time frame of the experiments, although we believe this to be unlikely. However, differences in the percent of cells positive for NP within each NP treated culture did not change significantly from 24 to 48 hours of incubation (data not shown).

The FACS analysis also demonstrated that these *in vitro* conditions did not cause loss of cells (in terms of cell number) or increases in apoptosis (as measured by Annexin-V). Figure 5A shows a representative plot of the data from one treatment well with media alone and from one treatment well with NP. We also examined changes in cell number and apoptosis in three cell types—while there was an increase in cell loss and apoptosis over increasing NP concentration (data not shown), these events were not different in cells treated with NPs as shown in Figure 5B. Thus we confirmed that any detectable signals of alterations in cell status would be independent of these nonspecific events, and also potentially more relevant to the expected conditions of application, in which cell loss would not be acceptable.

We also identified several changes in cells exposed to NPs, as compared to controls in terms of markers of activation/costimulation. Representative FACS dot plots for B cells (CD19⁺) co-staining for CD86 (top 2 panels, Figure 6A) or TLR4 (bottom 2 panels, Figure 6B) are shown and demonstrate that with the addition of NPs to the cell cultures, the expression level of these cellular biomarkers changed. Expression level of markers of activation and costimulation varied by cell type, as shown in Figure 6B, since T cells, B cells, and macrophages all had different expression patterns for the biomarkers investigated. In T cells, NP treatment increased the expression of CTLA-4, an important marker of regulatory cells. In B cells, costimulatory

molecule CD86 and activation marker TLR4 were both significantly increased in cells containing NP. In macrophages, the costimulatory molecule CD86 was also increased.

DISCUSSION

This paper contains two new findings; it represents the first application of FACS methodology not solely for imaging or localizing NPs, but also for detecting signals related to immunoactive properties of NPs and it presents important findings on the biological activity of NPs. These results confirm the potential applicability of this technique to research on the intended and unwanted biological properties of NPs. We demonstrated that NPs can enter cells under conditions in which NPs do not clump or aggregate and that the concentration of NPs used in the *in vitro* culture system did not induce cell death. These findings are similar to those published by Fuller *et al.*,²³ using quantum dots, but dissimilar from those of Wörle-Knirsch²⁰ whose *in vitro* exposures resulted in altered behavior of the nanowires. The conditions of our experiments permit us to define effects at the cell phenotype level and to distinguish changes in activation state from nonspecific responses to cell loss or overt cell death. Importantly, we were able to generate consistent data over multiple *in vitro* treatments because we based our experimental conditions on the actual number of particles added per unit volume (not simply a dilution of stock, as many other investigators report their data).

In this preliminary study we also found that those cells containing NPs expressed upregulated biomarkers for cellular activation. In particular, NP exposure upregulated expression of the costimulatory molecule CD86 on both B cells and macrophages and TLR4 on B cells. The increase of the regulatory marker CTLA4 on T cells is also an indication of a general activating effect of NP treatment. The findings increase our knowledge of the bioactivity of NPs, by providing information on markers of activation and regulation in the absence of cytotoxicity or apoptosis. Interpretation of these observed changes in biomarkers will depend upon further analysis of these systems, including microarray analysis of the cells and supernatants (released proteins) from these *in vitro* experiments.

Overall, these results suggest that FACS can significantly increase the information that can be obtained from *in vitro* studies of NPs in cell systems. Close collaboration among investigators in nanotechnology and immunotoxicology facilitates the development of such materials. Working together, we were able to design and utilize dye-embedded NPs in these experiments. The dansylamide dye was selected to facilitate co-detection by FACS of other signals (biomarkers) indicative of cell phenotype, cell survival, and cell response. With this strategy we were able to utilize FACS for simultaneous observation of all these events, including localization of NPs in cells by cell type, biomarkers of cytotoxicity and apoptosis, as well as a complex set of biomarkers of cellular response.

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A.

B.

Figure 1. Structure of dansylamide and SEM image of silica/PDMS dansylamide-doped nanoparticles

(A) chemical structure of Dansyl-aminopropyltriethoxysilane and (B) Scanning electron micrograph of silica/PDMS dansylamide-doped NP.

Figure 2. Normalized fluorescence emission spectra of Dansyl dye and Dansyl dye-doped NP
Fluorescence emissions of dansylamide dye alone and Dansylamide dye-doped NP (both 50/50 water/ethanol) were measured using Hitachi F-4500 spectrophotometer. Intensity (height of peak) of the emission has been normalized to correct for the different sample concentrations.

Figure 3. Engineered siloxane NPs with embedded dye are uncontaminated

The Energy Dispersive X-ray Spectroscopy (EDX) surface analysis of platinum sputtered NPs (platinum is not part of the NPs) shows the chemical composition of the sample.

Figure 4. Presence of dansylamide-doped NPs in macrophages, T and B cells

A representative dot plot of cells cultured with NP and the resulting dansylamide emission (in the violet range) is shown for each of the three cell types examined. Each dot on the dot plot represents a single cell with a specific emission frequency in the combination of fluorophore measured on the X and Y axes. For these plots, the Y-axis reports increased fluorescence in the wavelength emitted by the NPs. Therefore, positivity along the Y-axis was used to determine presence of NP within cells after culture for 48 hours with NP and LPS. That is, the farther up the Y-axis a dot is plotted, the more highly the cell is fluorescing in that particular wavelength and thus the more NPs inside the cell. Positivity along the X-axis indicates here whether a cell has the particular cellular subset marker to which the monoclonal antibody (with its associated fluorophore) is bound. Thus, a cell that is in the upper right corner of the dot plot would be considered positive for both the NP and the biomarker of cell subset. The two rectangle-shaped gates (one on the top half of each plot and one on the bottom half) show the cells that are gated to be NP⁺ and those that are gated to be NP^{negative} and the percentage of gated cells (of total lymphocyte gate) are shown in each gate.

Figure 5. The viability and survival of lymphocytes was not altered with NP treatment
 (A) 7-AAD (vital dye) and Annexin-V staining was used to analyze the viability of cells after culture with LPS (50nM) $\pm$ NP. The percentages of stained and unstained cells are shown in each quadrant. One replicate of B cell-gated (positive for CD19) cells is shown for each culture condition (with or without NP). (B) The average percentage of cells staining positive for Annexin-V and negative for 7-AAD (thus considered to be undergoing apoptosis) $\pm$ standard error (for three replicate cultures) is shown for each cell type analyzed (B cells- CD19⁺; T cells-CD3⁺CD4⁺; and macrophages- CD11b⁺).

Figure 6. NP treatment changes cell markers of activation and stimulation

(A) Representative dot plots of B cells ($CD19^+$ along the X-axis) co-staining for the costimulatory molecule CD86 or the regulatory molecule TLR4. The panels on the left are cells only from the NP^{negative} gate (described above Figure 3A, lower rectangle gate) and the panels on right are cells only from the NP^+ gate (described above Figure 3A, upper rectangle gate). Fewer dots (cells) are shown in the NP^+ panels because fewer cells contained NP than did not. (B) Graphically, the average percentage of T cells (defined as $CD3^+CD4^+$), B cells (defined as $CD19^+$), and macrophages (defined as $CD11b^+$) co-staining for the listed costimulatory, activation, and regulatory molecules with or without NP are shown $\pm$ standard error. Statistically significant differences between populations ($\pm NP$) were determined by Student's t-test: * $p < 0.05$ ** $p < 0.01$.