Particle Characterization & Centrifugation
Nanotoxicity Application Note

Beckman Coulter tools enable quantification of cellular toxicity caused by nanoparticles; reducing time to remove aggregated particles and quantifying cell toxicity in the presence of single-walled carbon nanotubes. Improve nanotechnology workflow and accuracy.

Abstract

Nanomedicine is a field common to materials scientists, chemists, biomedical engineers, biologists and medical scientists. The following application note will help researchers in this field gain insight into the inherent shortcomings that are present in traditional cellular toxicity assays, and how these shortcomings are magnified when the toxicity assay involves nanomaterial. Researchers will also benefit from understanding how Beckman Coulter centrifugation and particle characterization instruments can significantly improve the accuracy and workflow in both preparing nanoparticles and assessing the nanoparticles *in vitro* toxicity.

Introduction

Nanotechnology biomedicine is an emerging field still in its nascent phase. Nanoparticles, including quantum dots (QDs), carbon nanotubes (CNTs), and graphene, have many unique spectral features that have specific application for *in vivo* imaging and drug delivery[1]. QDs,[2,3] CNTs,[1] and graphene[1] all fluoresce in the advantageous biological window allowing for deeper imaging with better sensitivity[4,5]; the strongly red-shifted Raman signal of CNTs has also been exploited for *in vivo* imaging[6]. High surface area and strong light absorption of CNTs and graphene also make them excellent drug delivery and photothermal therapy agents[1]. However, toxicity characterization of biologically focused nanomaterial poses a challenge to researchers because of these unique properties. The intrinsic fluorescence and absorption of each of these nanomaterials overlap with the absorption and fluorescence region used typically *in vitro* cell toxicity assays, leading to inaccurate and misleading results[7-9]. Even more confounding is the fact that absorption, fluorescence, and toxicity[10,11] properties of these materials are greatly altered when they are aggregated together compared with individually solubilized; thus, toxicity studies comparing aggregated vs. non-aggregated have systemic bias. Aggregated nanoparticles are typically removed by long centrifugation processes, i.e., CNTs typically undergo six-hour centrifugations at 22,000 $x\ g$ to pellet aggregates[12-16]. This application note highlights how the Beckman Coulter Optima MAX-XP ultracentrifuge was used to effectively reduce the time to remove the aggregated nanotubes, while the Vi-CELL XR from Beckman Coulter quantified cell toxicity in the presence of single-walled carbon nanotubes (SWCNT).

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Carbon Nanotube Preparation

Single-walled carbon nanotubes (Sigma-Aldrich) were mixed with 0.2% 1, 2-Distearoyl-phosphatidylethanolamine-methyl-polyethyleneglycol (DSPE-mPEG, 5 kDa molecular weight, Laysan Bio) in 10 mL of water. The solution was bath-sonicated for 30 minutes to create well-dispersed carbon nanotubes following previously established procedures. Using a TLA-120.2 rotor in an Optima MAX-XP Ultracentrifuge, 5 mL of SWCNT solution was centrifuged in open-top polycarbonate centrifuge tubes (Beckman Coulter P/N 343778) at 22°C, 55,000 RPM ($\sim$131,000 x g) for two minutes. The top 650 μL of supernatant was collected with care to avoid disturbing the pelleted aggregates. The ultracentrifuged SWCNT (referred to as UCF'd SWCNT) and the remaining, uncentrifuged SWCNT (referred to as As-Made SWCNT) were concentrated using 10 kDa, Amicon Ultra 0.5 mL Centrifugal Filters (Millipore) with a Beckman Coulter Microfuge 20 microcentrifuge. The concentration was quantified using a UV-Vis-NIR spectrophotometer (Paradigm, Molecular Devices) and the established mass extinction coefficient of SWCNTs at 808 nm of 46.5 L/g*cm.[2] After concentration, UCF'd SWCNTs and As-Made SWCNTs were diluted using deionized water to concentrations of 0.6 mg/mL, 0.3 mg/mL, and 0.06 mg/mL.
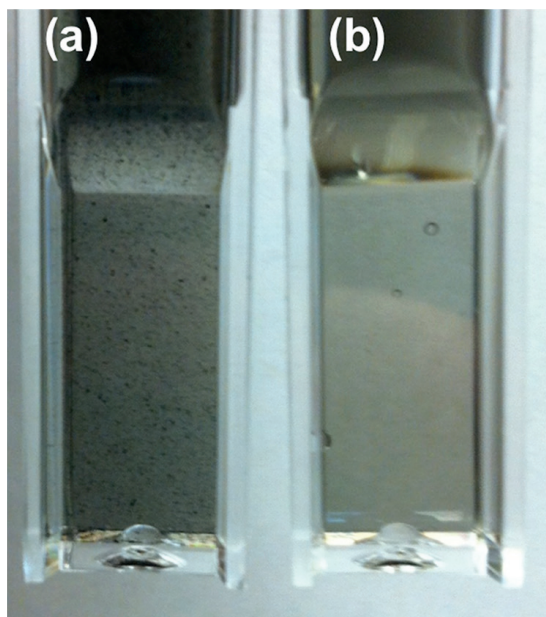
Toxicity Assay

MCF-7 breast cancer cells were plated at a density of 0.08×10^6 per well in a 24-well plate with 900 μL of RPMI/10% FBS (Invitrogen) 24 hours before the addition of nanotubes. Cell viability and growth were confirmed using one of the wells before the addition of nanotubes. 100 μL of SWCNT samples were added to wells on the second day. There were six SWCNT groups (n=2/group) in total: 0.06 mg/mL UCF'd SWCNTs; 0.06 mg/mL As-Made SWCNTs; 0.03 mg/mL UCF'd SWCNTs; 0.03 mg/mL As-Made SWCNTs; 0.006 mg/mL UCF'd SWCNTs; and 0.006 mg/mL As-Made SWCNTs. 100 μL of DSPE-mPEG only sample was added to wells serving as a control. There were three surfactant buffer control groups (n=2/group) in total: 0.2 mg/mL DSPE-mPEG; 0.02 mg/mL DSPE-mPEG; and 0.002 mg/mL DSPE-mPEG. Finally, control 1 (n=1) was a complete control, with cells left untouched, and control 2 (n=1) had 100 μL of sterilized water added. After 24 hours, all the wells were washed with PBS, trypsinized and mixed in 1 mL of PBS for counting in the Vi-CELL XR. A new cell type was created in the Vi-CELL XR software to minimize the counting of aggregated nanotubes as cells. Percentages of viable cells were used to compare cell viability and difference in the two solutions.
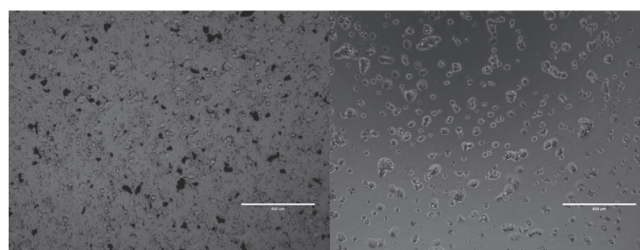
MCF-7 cells after 24 hours of addition of As-Made SWNT   MCF-7 cells after 24 hours of addition of UCF'd SWNT

Figure 2. Cell Imaging. MCF-7 cells were imaged under an optical microscope after 24 hours of incubation with SWCNT. The cells, incubated with either 0.06 mg/mL As-Made SWNT (left image) or 0.06 mg/mL ultracentrifuged SWNT (right image), have not yet reached confluence. Black aggregates of SWNT can be seen in the image on the left; these aggregates are difficult to wash away without washing away the cells as well. The aggregates have absorption and fluorescence properties that will skew traditional toxicity assays.

Figure 1. Images of SWCNT. Optical images of single-walled carbon nanotube (a) without centrifugation and (b) with ultracentrifugation for two minutes at 55,000 RPM (-131,000 x g). Note the presence of black, aggregated SWCNT in the sample that was not ultracentrifuged.
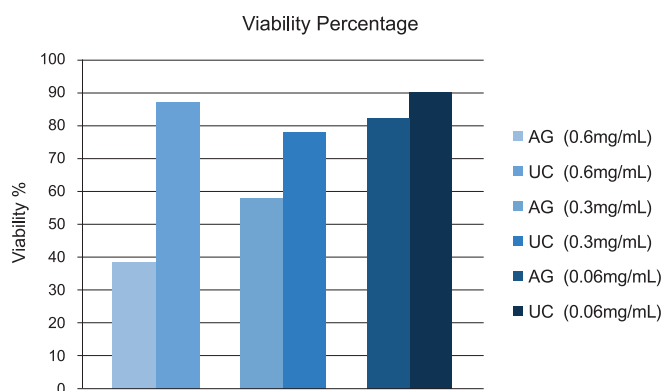
Viability Percentage

Figure 3. Viability Results. At all concentrations, ultracentrifuged SWCNT (designated by UC) had minimal toxicity; 75% or more of the MCF-7 cells remained viable 24 hours after incubation. Contrastingly, SWCNT that were not centrifuged and contained aggregated species (designated by AG) had increasing toxicity toward MCF-7 cells that scaled with increasing concentration. At a stock concentration of 0.6 mg/mL, corresponding to a concentration in solution with cells at 0.06 mg/mL, the aggregated SWCNTs had greater than 50% cell death.

Conclusion/Discussion

Aggregated nanoparticles pose a difficult problem in nano-biomedicine.[10,11] In this study, the toxicity of As-Made SWCNTs (which contained visible aggregates) was examined; however, this data is representative of most nanoparticles. The SWCNTs were separated into two groups—one group was As-Made, without any purification step to remove aggregates, while the second group underwent ultracentrifugation in the Beckman Coulter Optima MAX-XP ultracentrifuge. While centrifugation procedures have been shown to effectively remove aggregated nanoparticles and are a standard for purification of SWCNTs, the long centrifugation times (six hours or more) at low speeds (5,000 × g to 22,000 × g)[12-16] are a hindrance to research workflow. Our new ultracentrifugation method demonstrates that a two-minute, high-speed ultracentrifugation achieves the same biocompatibility and individual solubilized SWCNTs[15] as the longer centrifugation time—a 180-fold time savings to researchers.

Optical images and dynamic light scattering data taken using the DelsaMax PRO are evidence that all aggregated SWCNT have been removed by the rapid ultracentrifugation. The toxicity data gathered in this study was possible due to the use of Vi-CELL XR; the strong absorption of the aggregates would confound typical MTT and MMP toxicity assays. The Vi-CELL XR was programmed to specifically look for spherical cells with defined outlines in a sharply delineated size range, ensuring that counting

of carbon nanotube aggregates as either viable or dead cells was minimized. The Vi-CELL XR optimization is important because of the large number of aggregates present even after cell washing.

As a control, ultracentrifuged nanotubes were run without cells; in this trial, the Vi-CELL XR did not count a single live or dead cell. Aggregates show increased toxicity over ultracentrifuged SWCNT, which can be attributed to poor surfactant coverage and larger size of aggregated SWCNT. Aggregated SWCNT have more exposed surfactant-free surface; this increased surface availability of SWCNT directly contributes to an increase in reactive oxygen species (ROS).[7] Furthermore, aggregated or As-Made SWCNTs are much larger on a whole, as demonstrated by the dynamic light scattering data; the increased SWCNT size can block cell-signaling pathways or disrupt cellular action, inhibiting cell growth.[7] Thus, it is important that aggregated nanoparticles are removed before being used *in vitro* or *in vivo*.[10]

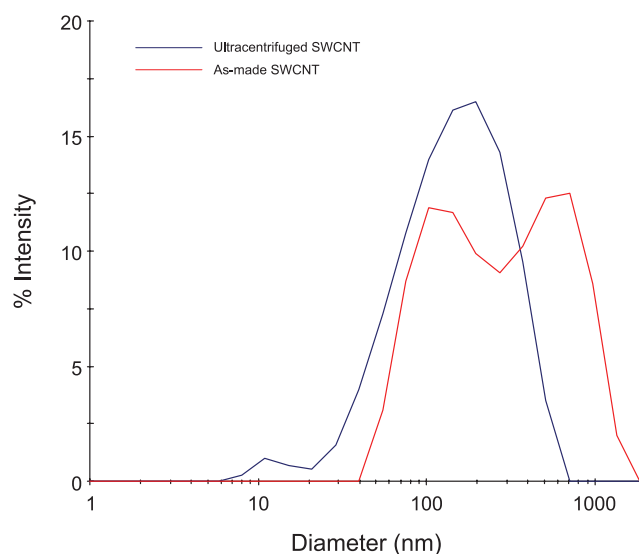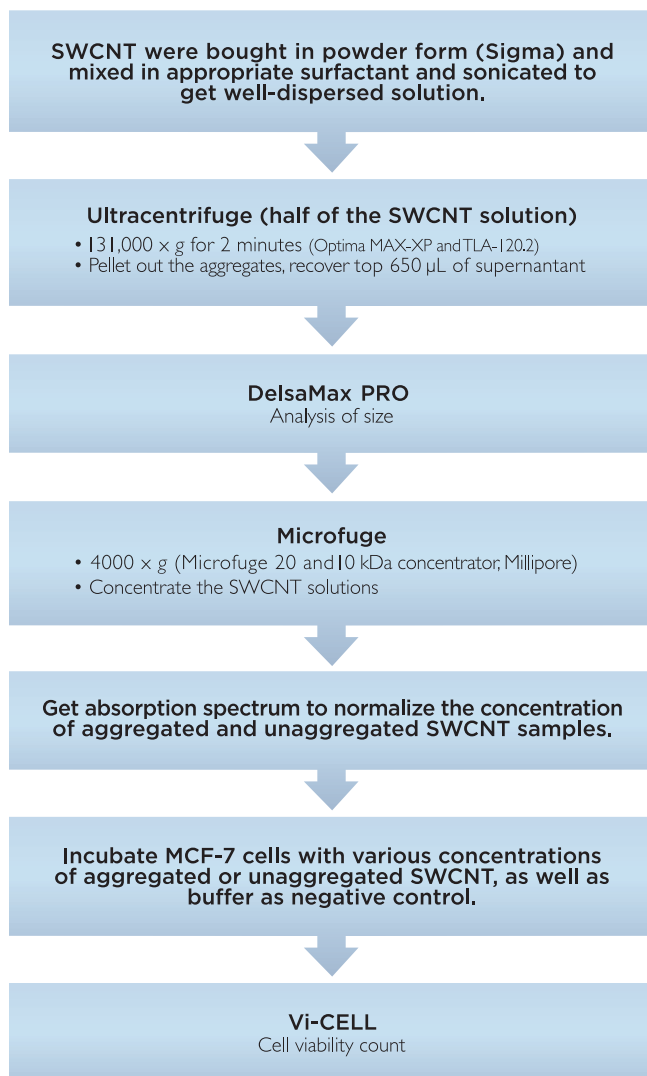| Item	Diameter (nm)	Polydispersity (nm)	Zeta Potential (mV)	PALS Cell Temp (°C)
Ultracentrifuged SWCNT	100.0	28.5	-24.18	25.0
As-made SWCNT	239.7	68.4	-23.54	25.0

Figure 4. Size Distribution Data. Single-walled carbon nanotubes, after sonication in surfactant, still have a number of aggregated species. Size distribution, determined by Dynamic Light Scattering on the DelsaMax PRO, showed two broad species (red line). The first size range, roughly 100 nm in diameter, represents individually solubilized carbon nanotubes. The second species, containing mostly aggregated carbon nanotubes, has a diameter peak closer to one micron in size. After a two-minute ultracentrifugation, the SWNT demonstrate only a single broad species at 100 nm, indicating that virtually all aggregates have been removed. This is further indicated by a 59% decrease in polydispersity. Interestingly, the zeta potential remains unchanged between aggregated and centrifuged carbon nanotubes. This is most likely due to the fact that steric repulsion, from the Poly(ethylene glycol) surfactant, provides most of the stability to the carbon nanotubes, while electrostatic repulsion does not play a major role.

Flowchart

SWCNT were bought in powder form (Sigma) and mixed in appropriate surfactant and sonicated to get well-dispersed solution.

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Ultracentrifuge (half of the SWCNT solution)
- 131,000 x g for 2 minutes (Optima MAX-XP and TLA-120.2)
- Pellet out the aggregates, recover top 650 μL of supernantant

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DelsaMax PRO
Analysis of size

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Microfuge
- 4000 x g (Microfuge 20 and 10 kDa concentrator, Millipore)
- Concentrate the SWCNT solutions

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Get absorption spectrum to normalize the concentration of aggregated and unaggregated SWCNT samples.

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Incubate MCF-7 cells with various concentrations of aggregated or unaggregated SWCNT, as well as buffer as negative control.

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Vi-CELL
Cell viability count

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