Published in final edited form as:

J Appl Toxicol. 2016 April ; 36(4): 579–585. doi:10.1002/jat.3203.

Developing *Xenopus* Embryos Recover by Compacting and Expelling Single-Wall Carbon Nanotubes

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Abstract

Single-wall carbon nanotubes are high aspect ratio nanomaterials that are being developed for use in materials, technological and biological applications due to their high mechanical stiffness, optical properties, and chemical inertness. Because of their prevalence, it is inevitable that biological systems will be exposed to nanotubes, yet studies of the effects of nanotubes on developing embryos have been inconclusive and are lacking for single-wall carbon nanotubes exposed to the widely studied model organism *Xenopus laevis* (African clawed frog).

Microinjection of experimental substances into the *Xenopus* embryo is a standard technique for toxicology studies and cellular lineage tracing. Here we report the surprising finding that superficial ($12.5 \pm 7.5 \mu\text{m}$ below the membrane) microinjection of nanotubes dispersed with Pluronic F127 into one-to-two cell *Xenopus* embryos resulted in the formation and expulsion of compacted, nanotube-filled, punctate masses, at the blastula to mid-gastrula developmental stages, which we call “boluses”. Such expulsion of microinjected materials by *Xenopus* embryos has not been reported before and is dramatically different from the typical distribution of the materials throughout the progeny of the microinjected cells. Previous studies of microinjections of nanomaterials such as nanodiamonds, quantum dots or spherical nanoparticles report that nanomaterials often induce toxicity and remain localized within the embryos. In contrast, our results demonstrate an active recovery pathway for embryos after exposure to Pluronic F127-

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site: Holt_SI.pdf: Additional Raman nanotube heat maps and nanotube Raman spectra analysis and confocal Z-scan of nanotube G-band intensity within a bolus. Holt_Movie_M1.avi: A time-lapse movie of nanotube expulsion collected using a stereomicroscope.

coated nanotubes, which we speculate is due to a combined effect of the membrane activity of the dispersing agent, Pluronic F127, and the large aspect ratio of nanotubes.

Keywords

Xenopus laevis; carbon nanotube; Raman spectroscopy; nanoparticle toxicity; development

Introduction

Single-wall carbon nanotubes are a high aspect ratio nanomaterial whose molecular structure leads to desirable mechanical, chemical, thermal and optical properties which uniquely qualify nanotubes for technological, materials and/or biological applications (Heister et al. 2013; Jariwala et al. 2013). As the use of nanotubes increases, they have the potential to interact with biological molecules, cells and systems, and they are likely to possess unique interfaces and interactions with biological systems which may lead to cytotoxicity, particularly in developing organisms (Dobrovolskaia et al. 2009; Fadeel 2012; Heister et al. 2013).

The embryo of the African clawed frog, *Xenopus laevis* (hereafter referred to as *Xenopus*), is an established model system for investigations of how materials interact with living biological tissues. One such *Xenopus*-based assay developed by the U.S. Environmental Protection Agency is FETAX: Frog Embryo Teratogenesis Assay – *Xenopus* (Bacchetta et al. 2012; Delgado et al. 2013; Dumont et al. 1983; Hutchison et al. 2014). Some advantages of the *Xenopus* embryo are that fertilization occurs externally, large numbers of embryos can be readily attained and the single cell fertilized ovum is extremely large (1 mm) and can be manipulated by microinjections. The development process occurs quickly at room temperature with gastrulation beginning nine hours post fertilization and can be visualized by light microscopy in non-sterile conditions. In addition, the system allows for examination of downstream impacts on organism development.

Microinjection is a well-established method to directly expose known amounts of substances into the developing *Xenopus* embryo, having direct implications for developmental toxicity assessment (Gimlich et al. 1985; Lacal et al. 1999; Sive et al. 2010; Weisblat et al. 1978), and several investigations have utilized microinjection to determine the effects of engineered nanomaterials on *Xenopus* development. These studies reported that the toxicity of nanomaterials depended on their size, shape and interfacial properties (Giannaccini et al. ; Jackson et al. 2013). For example, nearly spherical carboxylated nanodiamonds have been found to cause embryo toxicity and teratogenicity while hydroxyl-functionalized nanodiamonds were not toxic to embryos (Marcon et al. 2010). Spherical quantum dots encapsulated in phospholipid micelles were found to be non-toxic when injected into one-cell *Xenopus* embryos up to a cellular uptake of 5×10^9 nanocrystals/cell and remained diffusely localized throughout the cells up until the mid-blastula transition when the quantum dots concentrated in the cell nuclei (Dubertret et al. 2002).

Studies of embryos exposed to carbon nanotubes in their growth media have shown that developing larvae stage embryos suffer from nanotube dose-dependent toxic effects

(Mouchet et al. 2011, 2010, 2008; Saria et al. 2014). For example, when *Xenopus* larvae were maintained in a suspension containing high concentrations of aggregated multi-wall carbon nanotubes, the aggregates led to oxidative stress and accumulated in the gills and digestive tract, leading to dose-dependent toxicity that typically became significant at an exposure concentration of $\sim 10 \mu\text{g/mL}$ (Bourdiol et al. 2013; Mouchet et al. 2008; Saria et al. 2014). Short multi-wall carbon nanotubes were found to result in severe toxicity when microinjected into zebrafish embryos, but full-length multi-wall carbon nanotubes were not toxic (Cheng et al. 2012). The same group also demonstrated that microinjections of purified nanotubes into zebrafish embryos were minimally toxic (Cheng et al. 2009, 2011). It has also been reported that microinjections of dispersed multi-wall carbon nanotubes did not alter *Drosophila* development (Liu et al. 2014). Unfortunately, differences in animal models and nanotube administration routes, exposure doses, purities, dispersion states and interfacial properties have made understanding how nanotubes affect developing embryos inconclusive. Furthermore, microinjection of single-wall carbon nanotubes into early *Xenopus* embryos has not been explored.

In this work, we determined the effects of single-wall carbon nanotubes on developing *Xenopus* embryos by superficially microinjecting nanotube dispersions into the cytoplasm at the one or two cell stage and collecting time-lapse stereoscopic images immediately after injection. The time-lapse sequences revealed that small ($\sim 50 \mu\text{m}$) optically dark masses were being expelled through the embryo's superficial epithelium. Using optical stereo-microscopy we measured the localization and distribution of the dark masses, hereafter referred to as boluses, in the embryo, and using phase contrast and confocal Raman microscopies we characterized the concentration and dispersion state of nanotubes in the boluses. Developing embryos concentrated the injected nanotubes before finally expelling them to just under the vitelline or outer "fertilization membrane". This differential processing and expulsion of nanotubes by embryos has never been observed before with other nanomaterials including spherical nanoparticles, quantum dots and nanodiamonds, and reveals a robust recovery pathway for embryos after exposure to high aspect ratio nanotubes stabilized with a membrane active dispersing agent.

Materials and Methods

Nanotube Dispersions

Single-wall carbon nanotubes that were synthesized by high pressure carbon monoxide conversion synthesis (HiPCO) were purchased from Unidym. The raw HiPCO material contained $< 35 \text{ wt.}\%$ residual Fe and trace Co catalyst and the rest was carbon that included nanotubes and carbonaceous impurities such as amorphous carbon and fullerenes. The raw HiPCO material was purified according to previously published methods that involved heating the raw material in air in the presence of H_2O_2 for 3–6 h to remove carbonaceous impurities followed by acid refluxing with 2–3M HNO_3 for 20 min to remove metal impurities (Islam et al. 2005; Johnston et al. 2005). The acid in the nanotube materials was then neutralized with NaOH and further washed with deionized water. We repeated acid refluxing and neutralization 3 times. The HiPCO material was then annealed by heating in vacuum to $1,150^\circ\text{C}$ for 3 h to remove any defects on the nanotubes that were created during

the acid refluxing step. The metal impurities were further removed through magnetic fractionation which involved dispersing the nanotubes with sodium dodecylbenzene sulfonate and flowed over a magnetic field gradient. Purification did not alter the nanotubes' properties or structures, and the final purified sample contained <5 wt.% carbonaceous impurities, ~0.3% metallic impurities and the rest SWCNTs as determined by thermogravimetric analysis and wide-angle X-ray scattering (Islam et al. 2005; Johnston et al. 2005).

The purified nanotubes were sorted by length according to previously reported methods. First, the nanotubes were dispersed in water using sodium deoxycholate (DOC) (Islam et al. 2003). The dispersion of DOC-stabilized nanotubes contained a mixture of individual and bundles of nanotubes. To separate individually dispersed nanotubes from bundles, the dispersion was centrifuged at 21,000×g for 2 h, and the supernatant was collected (Arnold et al. 2006). The supernatant was subjected to density gradient ultracentrifugation to separate nanotubes by length, and the 10th–12th fractions were collected. These fractions contained nanotubes of lengths of 145 ± 17 nm (Fagan et al. 2008). These nanotubes were pelleted using ultracentrifugation, and the sodium deoxycholate was burned off by heating to 300 °C in wet air in the presence of H₂O₂, followed by multiple wash steps (Holt et al. 2010).

These highly purified, length fractionated nanotubes were then dispersed in water using Pluronic F127 (BASF) as previously reported (Holt et al. 2010). Dispersions were prepared at 1 mg/mL nanotubes and 10 mg/mL Pluronic F127 by 2 h of probe tip sonication (Fisher Scientific, Sonic Dismembrator Model 100, 3 mm probe tip) at 6 W followed by centrifugation at 21,000×g for 30 min (Holt et al. 2010). Supernatant concentration was determined via a known absorbance coefficient of 2.6 (absorbance · mL) / (mg · mm) at 930 nm (Fagan et al. 2008; Holt et al. 2010). Pluronic F127-dispersed nanotubes were sterilized in sealed glass containers by direct exposure to ultraviolet radiation (UVP Black-Ray, 100 W long wave UV lamp, Model B 100 AP/R) for 1 h prior to microinjections.

Embryos, Nanotube Microinjection and Imaging

Xenopus embryos were obtained by standard methods (Kay 1991), fertilized *in vitro*, dejellied in 2 wt.% cysteine and cultured in (1/3)× Modified Barth's Saline (MBS; 29 mM NaCl, 0.27 mM MgSO₄, 0.8 mM NaHCO₃, 0.33 mM KCl, 0.11 mM Ca(NO₃)₂, 0.14 mM CaCl₂ and 3.3 mM HEPES) (Sive et al. 2000) at 22 °C. Staging of embryos was performed according to the table of Nieuwkoop and Faber (Laboratorium 1967). Microinjection is a well-established, common technique to directly introduce small amounts of material into the *Xenopus* embryo (Gimlich et al. 1985; Lacal et al. 1999; Sive et al. 2010; Weisblat et al. 1978). Embryos were superficially injected in the animal region at the one or two cell stage with 2, 4, 8 and 16 nL of nanotubes at concentrations ranging from 600 – 850 µg/mL. For toxicity studies, embryos were cultured to tailbud (stage 27) or until death. For development studies, embryos were cultured to late gastrula (stage 12).

To perform a superficial microinjection, the microneedle was pushed against the embryo, indenting the embryo until penetration was achieved. The penetration length was determined by stereoscopic imaging of the needle during penetration into the embryo. Lengths were calibrated using a micrometer. After penetration, the needle was 125 ± 25 µm deep from the

membrane. The needle was then retracted until the tip was $12.5 \pm 7.5 \mu\text{m}$ below the membrane at which point nanotube dispersion was delivered. Each embryo was injected at 4 different locations in the animal region (i.e., for an 8 nL injection, 2 nL were injected in 4 locations) to promote better distribution of the nanotubes throughout the cellular progeny, which may not happen if the entire volume of nanotube dispersions were injected through a single location.

Embryos were kept in 3% Ficoll solution made in $(1/3) \times \text{MBS}$ during microinjection then transferred back to regular $(1/3) \times \text{MBS}$ after healing (30 min). Embryos were transferred to mesh-bottom dishes after microinjection to stabilize them for time-lapse stereomicroscopy. Vitelline membranes of embryos were removed after imaging with forceps (Fine Science Tools Inc., Foster City CA) to isolate nanotube boluses. Boluses were stored in phosphate buffered saline until imaging.

Microinjection Testing

To determine appropriate volumes and masses for injection into embryos, we used nanotubes dispersed with Pluronic F127 at concentrations ranging from 600 – 850 $\mu\text{g/mL}$ and superficially injected different volumes spanning 2 – 16 nL into ~20 embryos of the same clutch, that is eggs from the same mother. These nanotube dispersion dosages corresponded to $\sim 5 \times 10^9$ to $\sim 40 \times 10^9$ number of nanotubes and 10 mg/mL of Pluronic F127 per embryo. The slightly different nanotube concentrations arose from different dispersion batches. Another set of embryos from the same clutch was also injected with 2 – 16 nL of 10 mg/mL Pluronic F127 solution as control ($n = 25$). Cleavage defects, for example failure of embryos to undergo appropriate cellular divisions, or gastrulation defects, such as malformation of the three germ layers which are endoderm, mesoderm and ectoderm, were identified according to established definitions (Sive et al. 2000). It is known that Pluronic F127 is membrane active (Dumortier et al. 2006; Wu et al. 2009; Yaron et al. 2011), and the observed developmental defects for microinjections volumes $> 4 \text{ nL}$ were likely a result of Pluronic F127 destabilizing embryonic membranes. All subsequent experiments reported here involve a microinjection volume of 4 nL of nanotube dispersion.

Raman Spectroscopy and Imaging

We isolated the expelled boluses and imaged them with phase contrast microscopy to determine their basic physical features. We also analyzed the boluses with Raman spectroscopy and imaged them with confocal Raman microscopy to determine spatially-resolved nanotube localization, concentration and dispersion state (Boyer et al. 2013; Holt et al. 2011, 2012a, 2012b). Specifically, 20 μL of boluses maintained in phosphate buffered saline were pipetted onto a glass microscope slide (Fisher Scientific), covered with a #1.5 cover glass (Fisher Scientific) and sealed with epoxy. Expelled boluses were imaged on an inVia confocal Raman microscope (Renishaw) with a 100 $\times$, 1.4 numerical aperture oil-immersion objective via phase-contrast and Raman spectroscopy. The 785 nm (1.58 eV) laser power density was kept less than 90 kW/cm^2 to prevent sample degradation. Large (hundreds of μm) fields of view were generated via concatenations of individual fields of view in the Raman spectrometer software, WiRE 3.4 (Renishaw).

Nanotubes possess an intense Raman peak at $\sim 1591\text{ cm}^{-1}$ which is known as the G-band (Dresselhaus et al. 2005). We have previously shown that nanotube concentration can be determined from the G-band intensity using a calibration curve (Holt et al. 2011, 2012a). We used this method to determine the local concentration of nanotubes within the boluses by acquiring point-by-point Raman spectra, scanning over the entire field of view. Heat maps of G-band intensity were generated using in-house MATLAB® (The MathWorks, Inc.) code and represent G-band counts above baseline, thresholded to include only points with > 5 signal-to-noise ratio and converted to concentration using the previously reported calibration curve (Holt et al. 2011, 2012a).

Radial breathing modes (RBMs) correspond to coordinated nanotube vibrations in the radial direction and typically occur between $100 - 300\text{ cm}^{-1}$ (Dresselhaus et al. 2005). The locations and intensities of the Raman RBMs of nanotubes have been used to effectively obtain the degree of bundling of nanotubes (Dresselhaus et al. 2005; Heller et al. 2004; Holt et al. 2012a). For our experimental system of nanotubes with diameters of $\sim 1\text{ nm}$ and a Raman laser line of 785 nm (1.58 eV), nanotube Raman RBM intensity below 250 cm^{-1} arises from individualized nanotubes whereas RBM intensity above 250 cm^{-1} arises from nanotube bundles (Dresselhaus et al. 2005; Heller et al. 2004). In this study, we defined “bundle fraction” (BF) as the ratio of maximum bundle RBM intensity ($I_{\text{max, RBM} > 250\text{ cm}^{-1}}$) to the sum of $I_{\text{max, RBM} > 250\text{ cm}^{-1}}$ and maximum individual RBM intensity ($I_{\text{max, RBM} < 250\text{ cm}^{-1}}$):

$$BF \equiv \frac{I_{\text{max, RBM} > 250\text{ cm}^{-1}}}{I_{\text{max, RBM} > 250\text{ cm}^{-1}} + I_{\text{max, RBM} < 250\text{ cm}^{-1}}}. \quad (1)$$

Heat maps of bundle fraction, the ratio of individual RBM to G-band, and the ratio of bundle RBM to G-band were generated in MATLAB®. Data points were only included if the signal-to-noise ratio of the G-band was > 5 and the signal-to-noise ratio of both $I_{\text{max, RBM} > 250\text{ cm}^{-1}}$ and $I_{\text{max, RBM} < 250\text{ cm}^{-1}}$ were > 3 (Boyer et al. 2013; Holt et al. 2011, 2012a, 2012b).

Results

Real-time observation of the effects of nanotubes on embryo development and nanotube expulsion

We began our investigation of how microinjections of nanotubes affect developing *Xenopus* embryos by first identifying a nanotube concentration range that did not induce any negative responses by the embryos. Microinjection of Pluronic F127 alone or nanotubes dispersed with Pluronic F127 at volumes of 8 nL or greater resulted in early cleavage defects or gastrulation defects. Microinjection of 4 nL or less of Pluronic F127 only and nanotube dispersions with Pluronic F127 resulted in normal development to early tailbud (stage 27).

We next examined embryo development post nanotube microinjection using stereomicroscopy. One or two cell embryos were injected with 4 nL of nanotube dispersion (4 microinjection sites, 1 nL each), and then embryos were mounted and imaged over time

(Fig. 1 and Supporting Information, Movie M1). The nanotube distribution within the embryos immediately following microinjection could not be determined with stereomicroscopy due to the opacity of the embryos. Rounds of blastomere cleavage in the early embryo proceeded normally, every 30 min. Surprisingly, after approximately 6 rounds of division (stage 7), dark black punctate regions began to emerge, which we refer to as boluses began to appear at the surface of the animal epithelium and remained trapped between the embryo and its vitelline membrane or outer “fertilization membrane” (Fig. 1 and Supplemental Information, Movie M1). These boluses became more pronounced as embryo development progressed as can be seen in the images of embryos at late blastula (stage 8) and mid gastrula (stage 11) (Fig. 1 and Supplemental Information, Movie M1). Embryos were cultured to gastrula stage (stage 11 – 12) at which time the vitelline membranes of the embryos were microsurgically removed. Upon removal of the membrane, the boluses were separated from the embryo and collected for analysis. The number of boluses for each embryo was the same as the number of microinjection sites plus or minus one (4 ± 1). Microinjection of embryos with nanotubes at 700 $\mu\text{g/mL}$ dispersed with bovine serum albumin did not result in the formation of boluses.

Nanotube compaction and localization in the boluses

Phase contrast imaging demonstrated that the expelled boluses were typically a few hundreds of μm in diameter, possessed highly phase-dense regions and were self-contained (Fig. 2(A) and Supplemental Information, Fig. S1(A, F)). Raman microscopy revealed that the boluses were filled with nanotubes. Although the injected nanotube dispersion concentrations ranged from 600 – 850 $\mu\text{g/mL}$, the expelled boluses possessed substantially higher local nanotube concentrations, with many locations having concentrations $>3,000$ $\mu\text{g/mL}$ while some approached 30 mg/mL . The local concentration and spatial distribution of nanotubes within a confocal slice of a representative bolus are shown as a heat map based on nanotube G-band intensity in Fig. 2(B) and Supplemental Information Fig. S1(B, E). The nanotube concentrations were heterogeneous throughout the boluses, with local concentrations ranging from ~ 0.005 – ~ 30 mg/mL , having an average of 2.8 ± 1.5 mg/mL . Despite a high concentration gradient between the expelled boluses (~ 3 mg/mL) and the surrounding phosphate buffered saline solution without a detectable concentration of nanotubes (< 0.001 mg/mL), we did not observe any nanotubes escaping from the expelled boluses to the surroundings, which suggested that the boluses had robust structural integrity.

Nanotube dispersion state in the boluses

Since nanotubes were concentrated and expelled as boluses, we wanted to determine if the originally well dispersed nanotubes had become bundled together within the boluses. Therefore, we examined if the embryos changed the dispersion state of the nanotubes in the boluses from the dispersion state of the injected nanotube solution by comparing nanotube bundle fraction in the boluses to that of the injected nanotube dispersion using Raman spectroscopy.

We first examined a powder of the purified and length-selected nanotubes that were ultimately dispersed with Pluronic F127 and injected into the embryos. In this powder form (labelled undispersed in Fig. 3(A)), the nanotubes are expected to be in a highly bundled

state due to strong inter-nanotube van der Waals interactions between bare nanotubes. Indeed, the powder possessed a bundle fraction of 0.72 (Fig. 3(B)). In contrast, nanotubes dispersed with Pluronic F127 in ultrapure water (labelled dispersed in Fig. 3(A)), which were expected to contain well dispersed nanotubes and was later injected into the embryos, had a bundle fraction of only 0.19. Interestingly, nanotubes contained within the boluses (labelled expelled in Fig. 3(A)) were not substantially more bundled as the average bundle fraction was only 0.22, even though nanotubes were significantly more concentrated compared to the injected nanotube dispersion concentrations. Furthermore, the other nanotube Raman peaks for the boluses were similar to the initial dispersion (Fig. 3(A)).

Finally, we also investigated the local bundle fraction using confocal Raman imaging microscopy. Fig. 3(C) shows a heat map of bundle fraction for the same bolus shown in Fig. 2. Visually, bundle fraction (Fig. 3(C)) did not correlate with nanotube concentration (Fig. 2(B, C)), and the correlation coefficient was -0.07 . To show more boluses, similar heat maps are shown in Supplemental Information, Fig. S1. Overall, although nanotube concentrations within the boluses ranged from $\sim 0.005 - \sim 30$ mg/mL spatially with some regions having $50\times$ higher nanotube concentration than the injected nanotube dispersion, the bundle fraction of nanotubes within the boluses was 0.22 ± 0.04 and ranged from $0.08 - 0.58$. Since the boluses were not more bundled compared to the microinjected nanotubes, the boluses did not consist of nanotubes that had bundled together.

Implications of the high nanotube concentration in the boluses

To determine if the embryos expelled all of the injected nanotubes, we estimated what nanotube mass would be present in the boluses assuming no loss of injected nanotube mass. We estimated the volume of the nanotube regions within the boluses by using the nanotube Raman signal of the imaged X–Y planes and determined thickness from confocal Z-stacks of nanotube Raman signal. For depth slices acquired near the center of the boluses, the nanotube G-band intensity *versus* Z-position peak width at half maximum was 3.5 ± 0.5 μm (Supplemental Information, Fig. S2). Knowing the initial microinjection volume and nanotube concentration along with the estimated bolus volume, we calculated a nanotube concentration of $3,000 \pm 1,600$ $\mu\text{g/mL}$ within the boluses, assuming no loss of nanotubes. Although our Raman-determined local concentrations were calculated from confocally acquired spectra and do not necessarily reflect the concentration of the entire bolus, the calculated nanotube concentration within boluses is $2,800 \pm 1,500$ $\mu\text{g/mL}$.

Discussion

Nanotubes dispersed with Pluronic F127 have been previously shown to associate with cellular membranes (Yaron et al. 2011). Since Pluronic F127 is membrane active, being able to insert into membranes and alter their fluidity and defects (Dumortier et al. 2006; Wu et al. 2009), it could help fluidize membranes that then envelop the microinjection dose, allowing for bolus formation around the injected nanotube suspension. Unfortunately, obtaining Raman spectra of Pluronic F127 from within the embryo was not feasible and prohibited our imaging of its localization within the boluses. Since our preliminary experiments of microinjections of nanotubes dispersed with bovine serum albumin did not lead to bolus

formation and other studies with varying nanostructured materials, including carbon-based materials, have not reported bolus formation, we speculate that the membrane activity of Pluronic F127 contributed to nanotube expulsion by *Xenopus* embryos.

Bundling of nanotubes was possible because the Pluronic F127 dispersing agent was physically adsorbed to the nanotubes, and it could have dissociated from the nanotubes or exchanged with other molecules within the embryo (Cherukuri et al. 2006; Frise et al. 2012), allowing for bundling of nanotubes via van der Waals interactions that have been estimated to have a strength of $40 k_B T/\text{nm}$ (Girifalco et al. 2000). We note that uncoated nanotubes are highly hydrophobic and readily bundle in aqueous conditions. However, since the nanotube bundle fraction did not change, we contend that the nanotubes remained well dispersed. Note, though, that bundle fraction is not capable of determining if Pluronic F127 were displaced from the nanotube.

The estimated average nanotube concentrations within the boluses, assuming that all nanotubes in the microinjected volume were compacted within the boluses, were similar to the experimentally determined average concentration of nanotubes within the bolus based on Raman confocal slices, which suggests that the embryos expelled all the nanotube mass via boluses. We acknowledge that our assumption that the average nanotube concentration of a confocal slice reflected the average nanotube concentration of the entire bolus may not be realistic since we cannot exclude the possibility that some nanotubes were incorporated into the embryo. However, since all analyzed boluses possessed similarly high nanotube concentrations, we suggest that nanotubes were preferentially expelled from the embryos. Any remaining nanotube mass within the embryos would be below our detection limit of 0.001 mg/mL and would be substantially less than the mass expelled through boluses.

As nanotubes are being developed for numerous applications, understanding the implications of embryonic exposure to nanotubes is critical for developing safe nanotechnologies and toxicity analysis (Campagnolo et al. 2012; Giannaccini et al. 2014). Here, we observed a recovery pathway for developing *Xenopus* embryos to expel high aspect ratio nanotubes dispersed with membrane active Pluronic F127 that were injected $12.5 \pm 7.5 \mu\text{m}$ below the embryo membrane at the one-to-two cell stage. The discovery of the ability of *Xenopus* embryos to expel injected nanotubes through boluses represents a previously unknown pathway for developing embryos to discard foreign nanomaterials, as microinjections of other macromolecules and nanomaterials such as nanodiamonds and quantum dots become diffusely localized throughout the developing organisms. Therefore, we suggest that developing embryos may be more tolerant of intracellular exposure to certain types of nanomaterials than previously thought, further supporting the perspective that development is relatively stable (Hamdoun et al. 2007), and nanomaterial/embryo interactions are likely dictated by a combination of shape, membrane activity and interfacial properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Science Foundation (CMMI-1335417 (M.F.I.); CAREER IOS-0845775 and CMMI-1100515 (L.A.D.); and CBET-0954421 and CMMI-1300476 (K.N.D.)), the National Institutes of Health (R01 HD044750 and R21 ES019259 (L.A.D.)).

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Figure 1.

Real-time observation of nanotube expulsion. Still images from a time-lapse stereomicroscopy movie (Supplemental Information, Movie M1) of *Xenopus* embryo development after microinjection of nanotubes at the one or two cell stage. As the embryos developed through different stages, nanotubes were concentrated into small regions (as highlighted by arrows) and ultimately expelled as nanotube-filled boluses. The stage names correspond to stages 4, 7, 9 and 11, which correspond to 2.25, 4.00, 7.00 and 11.75 h post fertilization, respectively. The scale bar is 200 μm .

Figure 2.

Phase contrast and Raman imaging of expelled boluses from *Xenopus* embryos after nanotube microinjection. **(A)** A phase contrast image of a representative bolus indicated a highly phase-dense structure. **(B)** Raman imaging using the intensity of the Raman active G-band of nanotubes demonstrated that the boluses had heterogeneous nanotube concentrations (heat map indicates local nanotube concentration), with many regions being more concentrated than the microinjected dose (600 μg/mL), reaching almost 30 mg/mL. **(C)** The overlay of the nanotube concentration heat map with the phase contrast image revealed that the highest nanotube concentrations were located at highly phase-dense regions.

Figure 3.

Embryos did not alter nanotube dispersion state. **(A)** Comparison of Raman spectra from nanotube powder (undispersed), nanotubes dispersed with Pluronic F127 in ultrapure water (dispersed) and nanotubes in the expelled boluses (expelled) provided a quantitative determination of nanotube dispersion state after being injected into the embryos. Each spectrum was normalized to its Raman G-band peak intensity at $\sim 1591\text{ cm}^{-1}$ and was offset for display clarity. **(B)** A zoomed-in view of the RBMs (gray, dashed box of (A)) and quantification of bundle fraction demonstrated that undispersed nanotubes had a high bundle RBM peak relative to the individual RBMs (and G-band). Dispersed and expelled nanotubes had similar RBMs and bundle fractions. Note that “BF” means bundle fraction, “Indiv.” means individual RBMs (*i.e.*, $I_{\text{max, RBM}} < 250\text{ cm}^{-1}$) and “Bundle” means RBM intensity from bundled nanotubes (*i.e.*, $I_{\text{max, RBM}} > 250\text{ cm}^{-1}$). **(C)** A heat map of the local nanotube bundle fraction of the bolus shown in Fig. 2.