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RNA Polymer Translocation with Single-Walled Carbon Nanotubes

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ABSTRACT

The translocation of single-walled carbon nanotubes (SWNTs) across MCF7 breast cancer cells was demonstrated with radioisotope labeling. Hybrids of SWNT−RNA polymer poly(rU) were formed through a nonspecific binding mechanism which could allow for the dissociation of the poly(rU) from the SWNTs upon delivery. The cellular uptake of the hybrids was examined by confocal fluorescence microscopy. Through cell growth and MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assays, we showed the negligible cytotoxicity of SWNTs (up to 0.5 mg/mL) to MCF7 cells.

Gene delivery and transfection, the introduction and expression of foreign DNA,[1] continue to draw strong interest because of their potential for gene therapy and disease prevention.[2] One of the greatest challenges for gene delivery is the physicochemical properties of DNA such as its negative charge and hydrodynamic volume.[2] Transporters for DNA need to be developed for gene delivery and gene transfection.[3] Solutions to this problem have included viral (retroviral, lentiviral, and adenoviral) vectors and nonviral transfection vectors[2] including cationic lipids,[4] polyethylenimine (PEI), and other cationic polymers.[2,5] To date the viral transfection vectors are the most effective due to their natural ability to introduce foreign genetic information into cells.[2] However, these viral vectors often provoke immune responses from cells which prevent successful gene delivery.[6] Nonviral transfection vectors can avoid this problem but are often hindered by low efficiency rates for nuclear membrane penetration and gene expression.[6] Even though a few of the cationic polymers such as PEI boast relatively high efficiency rates for nonviral vectors, they exhibit cytotoxicity.[6]

Since none of the current transfection vectors are ideal, alternative vectors must be sought through the exploration of new materials and methods.[6] One possible transfection vector is single-walled carbon nanotubes (SWNTs), which possess versatile electronic and mechanical properties and have found numerous applications in nanotechnology and material science.[7] This is due to their large surface area, stability, flexibility, and biocompatibility,[8−12] which make SWNTs prospects for effective drug and gene delivery and therapies.[13,14] Recently, P. Pantarotto et al. showed that fluorescently labeled SWNTs covalently bound with bioactive peptides can penetrate cellular and nuclear membranes.[15] The mechanism for the translocation of SWNTs has yet to be determined and therefore can only be speculated upon. Kam et al. reported that SWNT−streptavidin conjugates appeared within promyelocytic leukemia and T cells via the endocytosis pathway.[3] Regarding the cytotoxicity of SWNTs, it was attributed to the transition metal catalysts for SWNT synthesis, iron and nickel, which at an SWNT concentration of 0.06 μg/μL or above caused the death of human epidermal keratinocytes (HaCaT).[16] Pantarotto et al. reported that 90% of the fibroblast cells remained viable when incubated with FITC-labeled SWNTs of 5 μM.[15] Kam et al. suggested that cytotoxicity for human promyelocytic leukemia cells is negligible when the concentration of SWNTs is below 1.25 μM.[3]

To explore the possibilities of SWNTs as transporters for gene delivery and transfection, we examined for the first time the translocation of nucleic acid, RNA polymer poly(rU), into breast cancer cells (MCF7). The nonspecific binding mechanism for SWNTs and poly(rU) in our study, as opposed to the covalent binding scheme by Pantarotto et al. and Kam et al.[3,15] in their respective peptide and protein delivery studies, may offer more options and flexibility for the release of the load carried by SWNTs upon delivery. The sectioning property of confocal fluorescence microscopy in our study allows for axial discrimination of fluorescently labeled SWNT−poly(rU) hybrids on cell membrane, within cytoplasm or within the nucleus. In addition to confocal fluorescence imaging of SWNT−poly(rU) incubated with MCF7 breast cancer cells, we performed radioisotope label-

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ing, cell enumeration, and MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay, which measures cellular metabolic activity through absorption. These additional studies quantitatively evaluate the direct cellular uptake and the cytotoxicity of SWNTs.

In our studies, SWNT bundles were synthesized using the arc-deposition method with the dominant SWNT diameter of ~1.4 nm.[17] For environmental protection the cytotoxicity of SWNTs was evaluated by cell growth and MTS assays for MCF7 cells. The SWNT bundles were probe-sonicated (VC 130 PB, Sonics & Materials) at 8 W for 90 min on ice in 10% fetal bovine serum (FBS)/phosphate buffer saline (PBS) buffer. The SWNTs at a concentration of 0.4 mg/mL were filtered through a 0.45 μm filter before incubating with MCF7. Both control cells and cells treated with SWNTs were enumerated at 24, 48, and 72 h. The cell growth medium was RPMI supplemented with 10% FBS and 1% penicillin streptomycin. After the designated time of incubation, cells were released by trypsin−EDTA treatment (5 min) and counted with hemocytometry. Compared with the raw SWNTs used in the study by Shvedova et al.,[16] the concentration of SWNTs was kept lower than 0.4 mg/mL in our studies. Also, precautions were taken to prevent contamination in the course of the preparation of SWNTs, including filtration to remove the bacteria and sterilization of the instruments in contact with the sample. The MTS assay was preformed after treating MCF7 cells with various concentrations of SWNTs (0, 0.0125, 0.025, 0.05, 0.1, 0.125, 0.25, 0.5, and 1 mg/mL). After 24 h of incubation, the growth medium was removed from the 96-well plate and 200 μL PBS was used to wash the cells twice. Approximately 200 μL RPMI-1640 medium without phenol red and 25 μL of MTS were added to the each cell well, followed by incubation at 37 °C for 3 h. The absorbance in each well was measured using a spectrophotometric plate reader (Benchmark Microplate Reader, Bio-Rad) at a wavelength of 490 nm.

The translocation of SWNTs into cells was confirmed by radioisotope labeling assay. SWNTs were probe-sonicated in RPMI-1640 growth medium and incubated with radioactive thymidine [methyl-3H] overnight. The radioactively labeled SWNTs were collected by centrifugation and resuspended in PBS before incubating with MCF7 cells. After the incubation, the cells were released by trypsin−EDTA and recollected as pellet by centrifugation. The pellet of MCF7 cells was washed thoroughly with PBS buffer twice to remove excess SWNTs and thymidine bound on the cell surface. The scintillation counts (Beckman Coulter, LS6500) read from the MCF7 cells, as opposed to those from the supernatants and the PBS washing buffer, yielded the translocation efficiencies of the radioactively labeled SWNTs at 1.3%, 6.3%, 10.7%, and 15.4% for incubation times of 0.5, 1, 2, and 4 h, respectively. This result confirms that SWNTs alone can penetrate through cellular membranes with an increased efficiency over time. This agrees with the general understanding that cell membrane intakes small hydrophobic particles.

For the imaging experiment, the SWNT−poly(rU) hybrids were prepared as follows. A mixture of 0.5 mg/mL poly(rU) and 0.125 mg/mL SWNTs in TE (10 mM Tris-HCl, 1 mM EDTA) buffer was probe-sonicated. As with the binding of SWNTs and DNA,[18] the hydrophobic nitrogenous bases of poly(rU) were expected to bind to the hydrophobic surface of SWNTs. The bases of poly(rU) could also form π-stacking with the carbon rings on the SWNT surface, and the π-stacking was found dominating the hydrophobic interactions in fluorescence microscopy.[19] The binding affinity between SWNTs and poly(rU) hybrids was evident when, upon binding, the Raman *G* band was broadened and the radial breathing mode was suppressed for an isolated SWNT.[19] This nonspecific binding scheme results in the exposure of the charged phosphate backbone of poly(rU), which aids in the solubilization of SWNTs as diffusing hybrids observed using EPI fluorescence microscopy.[20] From scanning electron microscopy and single-molecule diffusion studies, the prepared SWNT−poly(rU) hybrids were characterized to be in small bundles or isolated form with an average length of 400 nm.[20]

To visualize the SWNT−poly(rU) hybrids, poly(rU) was fluorescently labeled with propidium iodide (PI, Sigma), which has an excitation peak at 535 nm and an emission peak at 617 nm. PI is known to intercalate between the base pairs of nucleic acids. PI is also membrane impermeable and therefore is generally excluded by viable cells. To achieve fluorescent labeling, PI at a concentration of 0.05 mL/mL in TE buffer was incubated with SWNT−poly(rU) hybrids at a volume ratio of 1:20 for at least 15 min. The excess PI and unbound poly(rU) were removed by cassette dialysis (Slide-A-Lyzer, 10 000 MWCO, Pierce) for a total of 36 h with three changes of buffer solution.

Approximately 10 000 MCF7 cells were deposited in each well of an eight-chambered slide to form a sparsely distributed layer of cells. This ensures good exposure of cell membranes to SWNT−poly(rU) hybrids. Cells were cultured directly on the chamber glass slide using RPMI-1640 growth medium at 37 °C under 5% CO_2. All the cells were incubated for at least 24 h until approximately 60% confluence was achieved. Then 20 μL of PI, PI+poly(rU), or PI-labeled SWNT−poly(rU) hybrids (0.05 mL/mL) were added to the chamber slide. The cells were incubated for 2 h, 3 h, or 4 h. After incubation, the cells were washed twice with 200 μL growth medium and kept in 400 μL PBS before imaging with the confocal fluorescence microscope (LSM510, Zeiss, objective NA = 1.3, oil). The fluorescence from PI was collected in the TRITC channel, and the background was recorded from the bright-field channel of the microscope.

The confocal fluorescence images of the two controls, PI and PI+poly(rU), showed little to no fluorescence after incubation. The MCF7 cells appeared elongated and healthy (Figure 1). By contrast the PI control (Figure 1a) showed one instance of intense fluorescence from a PI stained circular unhealthy cell. The minimal fluorescence intensity surrounding the cellular membrane for the PI+poly(rU) (Figure 1b) was probably due to electrostatic interactions between the charged poly(rU) and cellular membrane.

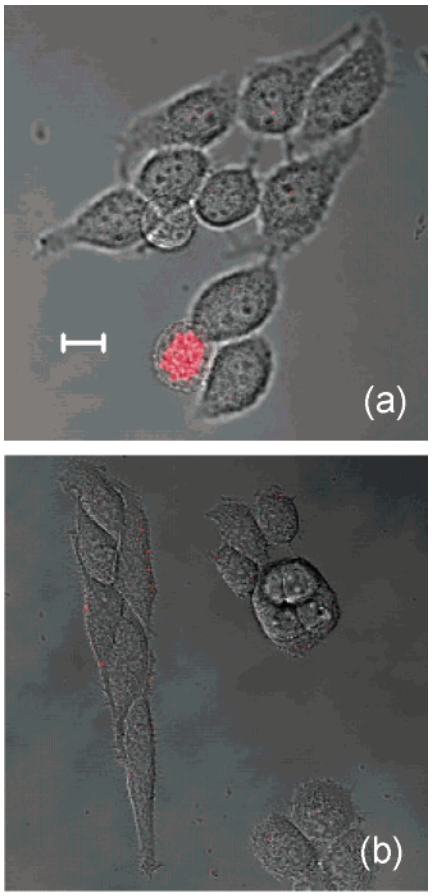

Figure 1. Control experiments showing little to no uptake of (a) PI except in the dying circular cell and (b) PI+poly(rU). The scale bar is 10 μm.

The confocal fluorescence images of the MCF7 cells that were incubated with the SWNT−poly(rU) hybrids for 2 h showed translocation into the cytoplasm without accumulation of SWNT−poly(rU) hybrids on the outside of the cellular membrane. As shown in Figure 2, MCF7 cells appear to retain the SWNT−poly(rU) hybrids after an incubation of 3 h. All the images were taken from a stack of slices scanned through the cells with a full scanning depth of approximately 10 μm and a scanning step of 1.01 μm. Smaller red spots with low intensities as noticed in Figure 2a−e are speculated to be small soluble SWNT−poly(rU) hybrids and/or PI-labeled poly(rU) dissociated from SWNTs after translocation. They gradually disappear in Figure 2f−h, which implies the uneven distribution of SWNT−poly(rU) hybrids in cytoplasm and/or possibly the defocusing of the hybrids. In Figure 2b, a more prominent spot, as indicated by the arrow, shows the uptake of a SWNT−poly(rU) hybrid in the cytoplasm. While Figure 2d shows an instance of a SWNT−poly(rU) hybrid appearing in the vicinity of cell membrane, it is unclear, however, whether the exact location of the hybrid is within or on the cell membrane. In Figure 2f,g, the fluorescent spot was observed to co-localize with the nucleus, which supports the possibility that the SWNT−poly(rU) hybrid could have penetrated the nuclear membrane. The observed fluorescence in Figure 2f,g could also be from PI-labeled poly(rU) released from SWNTs due to dissociation kinetics and the change of pH between cytosol and nucleus. The released nucleic acids may be used as templates for transfection at a later stage. It should be pointed out that, from the energetic viewpoint, the fluorescence in the nucleus is unlikely to be from free PI or PI dissociated from poly(rU) and then re-intercalated with host DNA or RNA. The binding dynamics of SWNTs and nucleic acids need further investigation but are beyond the scope of this letter. The strong fluorescence in Figure 2f,g fades gradually in Figure 2e,h, which further indicates that the SWNT−poly(rU) hybrid was localized within the cell. From the depth information, we estimate the length of this large SWNT−poly(rU) hybrid to be less than 2 μm. This is consistent with the length distribution of SWNTs obtained with probe sonication confirming that the fluorescence observed was from the SWNT−poly(rU) hybrid. After 4 h of incubation, the SWNTs were still present within the MCF7 cytoplasm and nucleus.

The uptake of the SWNT−poly(rU) is hypothesized to be a result of amphipathic properties of both the cellular membrane and the SWNT−poly(rU) hybrids. Due to thermal agitation, lateral diffusion of the phospholipids within the biomembrane may contribute to the translocation of SWNT−poly(rU) hybrids by allowing hydrophobic interactions between SWNTs[16,21,22] and the hydrocarbon chains within the bilayer. The uptake of the SWNTs by the nuclear membrane is attributed to passive ratchet diffusion.[23] Cell mitosis might also play a significant role in the internalization of the SWNT−poly(rU) hybrids. During cell division, the nuclear envelope breaks down into multiple small vesicles early in mitosis, which may allow the translocation of SWNT−poly(rU) hybrids. In telophase, the last mitotic stage, the nuclear envelope reforms, possibly incorporating the SWNT−poly(rU) hybrids. Once the SWNT−poly(rU) hybrids are within the MCF7 cells, it is possible that endosomes in the cytoplasm store SWNTs after poly(rU) translocation.

Our cell growth studies and MTS assay on MCF7 cells suggest SWNTs do not cause significant cytotoxicity. Figure 3 shows cell growth for MCF7 cells with and without SWNTs of concentration 0.4 mg/mL. A similar result was observed for d2C keratinocyte cells (data not shown). In both cases, no significant growth effect was observed for SWNT-treated MCF7 or d2C cells over a 3 day period. The cell count for the MCF7 cells with SWNTs showed only minimal decrease in the number of cells for day 2 and day 3; however, the overall trend mirrored the cell count for the control MCF7 cells. MTS assay for cellular metabolism (Figure 4) on the absorbance of MCF7 cells was performed on cultures without SWNTs (control) and with SWNTs of various concentrations. The absorbance remained similar for the control cells (0.631) and those of various concentrations of SWNTs (0.607 to 0.631). The highest concentrations of SWNTs at 1 and 0.5 mg/mL have the largest drop in absorbance of 0.024 and 0.021, respectively. At concentrations lower than 0.5 mg/mL, the absorbance varies by less than 0.01. Our results suggest that SWNTs of a concentration as high as 1 mg/mL have no effect on metabolism and therefore cell viability.

In summary, we have demonstrated the delivery of RNA polymer poly(rU) using SWNTs as transporters. The trans-

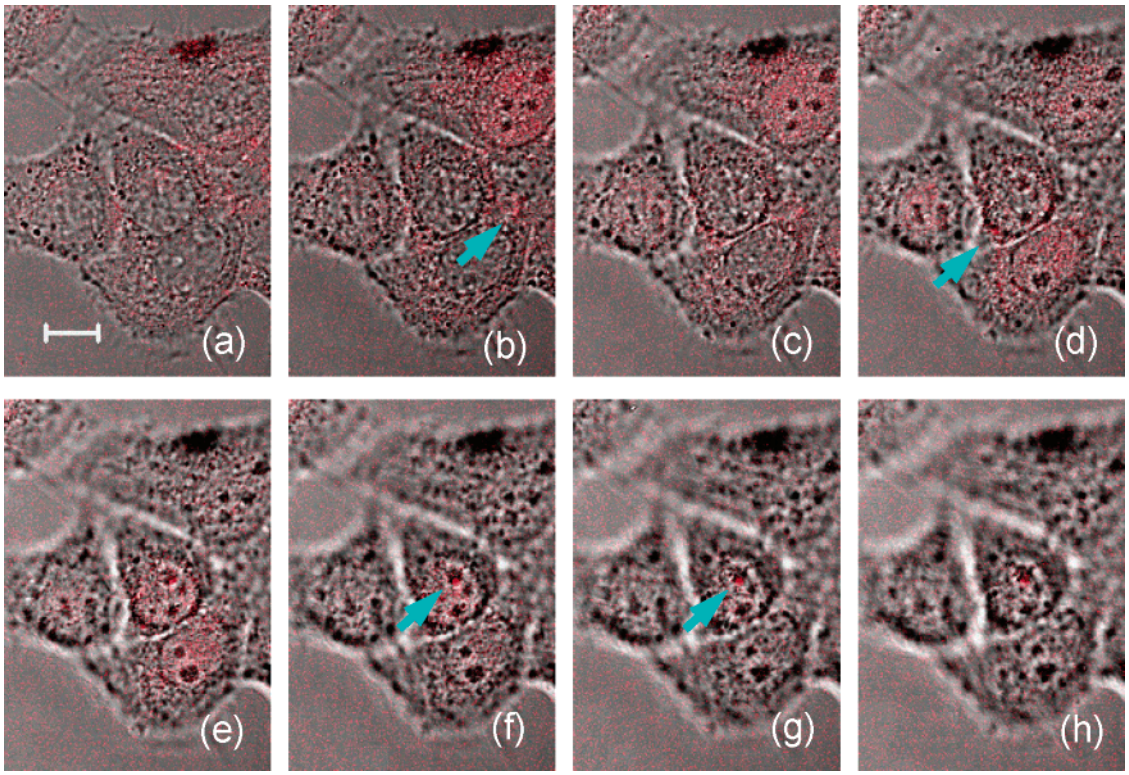

Figure 2. Confocal fluorescence images of MCF7 cells incubated with 0.05 mL/mL of PI labeled SWNT−poly(rU) for 3 h. Images a−h were acquired at different depths (z = 0.5, 1.51, 2.52, 3.53, 4.54, 5.55, 6.56, 7.57 μm), across the z axis normal to the chamber slide surface. The arrows point to the fluorescent spots where large SWNT−poly(rU) hybrids are localized. The scale bar is 10 μm.

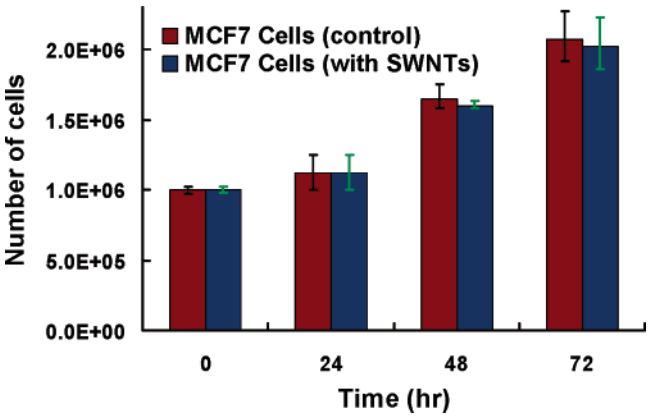

Figure 3. Cell growth curves for MCF7 breast cancer cells without (control, red) SWNTs and (blue) with SWNTs of 0.04 mg/mL. Total incubation time was 72 h with observations every 24 h.

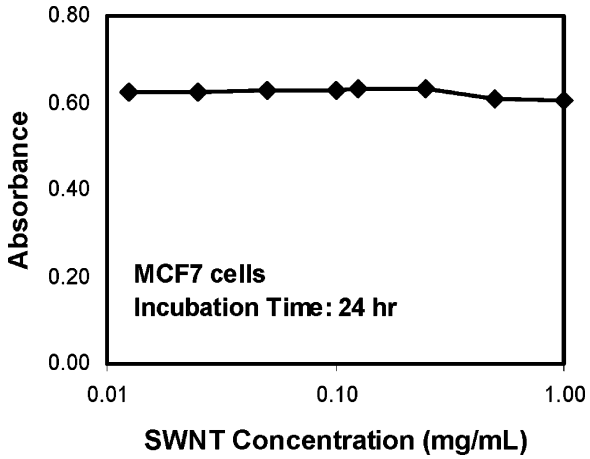

Figure 4. MTS assay on MCF7 cell absorbance vs SWNT concentration. The control cell absorbance was measured to be 0.630. The cell absorbance ranges from 0.607 to 0.631 for SWNT concentrations of 0.0125 mg/mL to 1 mg/mL.

location of SWNTs into MCF7 cells was confirmed by radioisotope labeling assay. Based on the sectioning property of confocal microscopy, fluorescently labeled SWNT−poly(rU) hybrids were found across the cellular and the nuclear membranes of MCF7 cells while the controls were excluded. Both cell growth and MTS studies have shown no cytotoxicity in either MCF7 breast cancer cells or d2C keratinocytes for concentrations up to 1 mg/mL. These studies show the potential of using SWNTs as transporters for gene delivery. Further biophysical and biochemical studies need to be conducted in order to better decipher the mechanisms of SWNT translocation across cellular and nuclear membranes. Toward the final goal of gene therapy and disease prevention, assays on gene transfection using SWNT transporters need to be performed. These research activities will promote the integration of SWNTs with biomedicine.

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