Carbon Nanotubes as Molecular Nanocarriers for Antibody Delivery and Photothermal Ablation of Breast Cancer Cells

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ABSTRACT

In this paper we demonstrate that single wall nanotubes functionalized with Her2 and IGF1R specific antibodies showed selective attachment to breast cancer cells compared to nanotubes functionalized with non-specific antibodies. Confocal microscopy revealed that the nanotubes treated with specific antibodies were well attached on top of the cell membrane. After the complexes were attached to specific cancer cells, nanotubes were excited by ~808 nm infrared photons at ~800 mW/cm^2 for 3 minutes. Viability after photo thermal therapy was determined by Trypan blue exclusion. Cells incubated with nanotube/non-specific antibody hybrids were still alive after photothermal treatment due to the impermeability of the cell membrane. Quantitative analysis demonstrated that all the cells treated with SWCNT/IGF1R and Her2 specific antibody complex were completely destroyed, while that of SWCNT/non-specific antibody hybrids remained alive. The energy estimated to kill cancer cell was estimated to be ~200 nW, too small to kill neighboring normal cells. Past techniques have shown the transport of DNA using nanotubes and NIR agents to kill cells. This study demonstrates that nanotubes could be used as nanocarriers for antibodies that are specific to group of breast cancer cells and as well as energy transducers for thermal ablation of breast cancer cells.

Key Words: Photothermal ablation, Carbon nanotubes, antibody, Molecular targeting, Nanocarrier, Cancer

1. INTRODUCTION

Despite outstanding progress in the area of cancer biology, significant challenges remain in administering highly selective, targeted anti-cancer therapy. It is striking to note that only 1 to 10 parts per 100,000 intravenously administered monoclonal antibodies reach their parenchymal targets *in vivo* [1]. Nanoparticles [2-3], nanoshells [4-5] and nanotubes [6-7] have been shown in the past to be quite applicable for cancer imaging and therapy. Subcellular nanostructures exhibiting advanced physical properties for biological applications can have an important impact on the introduction and delivery of DNA, proteins, and drug molecules into the living cells [7]. In addition, the unique thermal properties [8], and the subcellular size of nanostructures makes them very attractive for multi-component targeting of surface receptors and photo-thermal killing of cells. Circulating cancer cells often express characteristic cell surface markers. Human MCF7 ER+ breast cancer cells exhibit high expression of the insulin-like growth factor 1 receptor (IGF1R), while BT474 ER- cancer cells have lower expression of IGF1R but high expression of human endothelial receptor 2 (Her2) [9]. In this paper we demonstrate molecular targeting combined with photo-thermal cell killing of breast cancer cells. It was found that co-localizing the nanotubes to the surface of the cell membrane was just enough to cause cell death during photo-thermal therapy.

2. EXPERIMENTAL

1 mg/mL of SWCNT (Nano-Lab, Lot number FH-P 071706) in phosphate buffered saline solution (PBS) was agitated for one hour under room temperature in ultrasonicator (Fisher Scientific FS60H) to break the SWCNT bundles into individual nanotubes. The solution was then microcentrifuged at 10,000×g for 15 minutes. Sediments containing mostly large conjugations of SWCNT were discarded and supernatants were collected. To closely examine the dispersion of SWCNT, samples were prepared for transmission electron microscope by dropping 1 μL of such solution on top of a TEM grid. TEM images of the SWCNT were taken from different parts of the solution to ensure that SWCNT were uniformly dispersed within the solution.

5 mg 1-pyrenebutanoyl succinimide was dissolved in 50 mL of methanol with gentle agitation under room temperature. 1 mL solution was mixed with 1 mL SWCNT solution in PBS and kept at 20ºC for 30 minutes to allow the reaction between the reactants. 5 mg PEG (Acros Organic, M.W. 8000) was diluted in the solution to form a self assembly monolayer (SAM) on unoccupied sites so that the SWCNT were insulated from outside liquid to prevent undesirable binding with other bio-molecules. The already functionalized SWCNT were then filtered, collected, triple rinsed, re-suspended in PBS and kept at 4ºC for storage. Such a protocol ensures adhesion of SWCNT and 1-pyrenebutanoyl succinimide, the removal of excess reagents, and allows the complex to be stored for long periods. Non-

specific mouse anti human myeloma IgG, anti-IGF1R mouse monoclonal antibody and anti-Her2 (Merck Bioscience, Calbiochem Inc.) were prepared by diluting 1 mg/mL monoclonal antibody solution with PBS. Following this, 1 mL of SWCNT solution was mixed with 1 mL of monoclonal antibody solution (IGF1R or Her2) and the solution was allowed to incubate for one hour at room temperature and microcentrifuged. The sample was triple rinsed to remove excess antibodies in the incubation liquid. These procedures yield two different solutions containing SWCNT-anti-IGF1R antibodies and SWCNT-anti-Her2 antibodies conjugates. Samples for microscopy were then prepared by dropping a small droplet on top of the TEM grids and several samples were investigated for antibody functionalization using TEM to ensure the repeatability of the antibody adsorbing effect.

300U Alexa Fluor 488 phalloidin and 555 (Invitrogen) were dissolved by 1.5 mL methanol and kept at -20ºC for storage. 10 mL SWCNT in PBS at 0.1 mg/mL was mixed with 10 µL Alexa Fluor 488 to deliver green color under confocal laser excitation. Similarly, 40 µL pure antibody solutions at 1 mg/mL were dyed red with 10 µL Alexa Fluor 555. The two mixtures were kept at room temperature for 30 minutes to allow the optimum reaction. The two solutions were mixed following previously mentioned protocol to form Fluor 488-SWCNT-antibody-Fluor 555 complexes for further cell endocytosis.

In order to remove the excessive fluorescent dyes the solution was microcentrifuged at 10,000×g for 5 minutes. Bottom solution containing mostly the heavy Fluor 488-SWCNT-antibody-Fluor 555 conjugates was collected and re-suspended in PBS.

Human BT474 and MCF7 breast cancer cells (ATCC, Germantown MD) were incubated in DMEM supplemented with 2 mM glutamine, 5000 U/mL penicillin, 50 µg/mL streptomycin and 10% fetal bovine serum at 37ºC under 5% CO2 for 48 hours before experiments. MCF7 cells were further supplemented with 7.5 nM 17-β-estradiol (Sigma). Cells released by EDTA went on microcentrifuge at 100×g for 5 minutes. Precipitates containing the cells were collected and re-suspended in PBS. 4 µL of SWCNT-anti-IGF1R antibody solution was mixed with 4 µL of cells, incubated for one hour. Following this, 4 µL of this solution was then mixed with the same amount of SWCNT-anti-Her2 antibody solution, incubated for an hour and then micro-centrifuged.

Following incubation of SWCNT-antibody complex with the cancer cells, laser light of 808 nm at 800 mW/cm^2 was dosed for 3 minutes to all the samples. After laser excitation, membrane permeability was investigated by adding 0.4% Trypan blue in PBS. Optical microscopy was used to record the images of cell viability, membrane permeability and nanotube binding. The lasers used were tunable from 50 mW/cm^2 to 1 W/cm^2 and intensities were calibrated by power meters.

3. RESULTS AND DISCUSSION

Transmission electron microscopy (TEM) of the antibody functionalized SWCNT gives us direct confirmation that the antibodies were attached to the SWCNT on many different sites as imaged in Figure 1. This shows that each atom on the surface of the nanotube is potential site and act as nano-carriers for drug delivery. One of the intriguing observations that were made was the attachment of nanotubes atop the cell membrane using confocal microscopy. Cancer cells incubated with fluorescent specific-antibody-SWCNT complex were imaged by confocal microscopy in Figure 2.

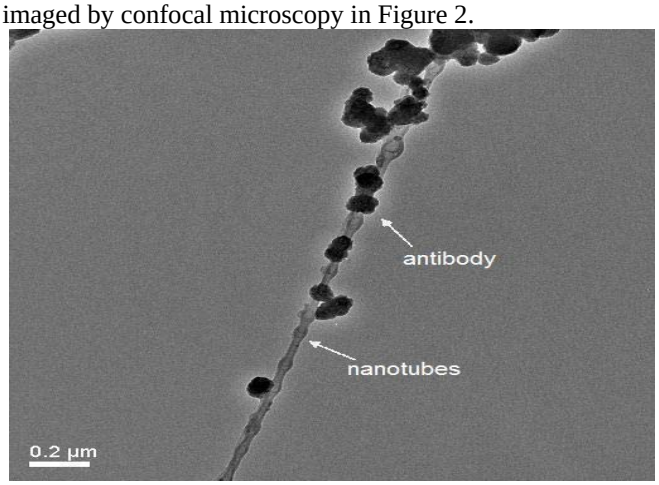

Figure 1: TEM image of nanotube nano-carrier of antibodies

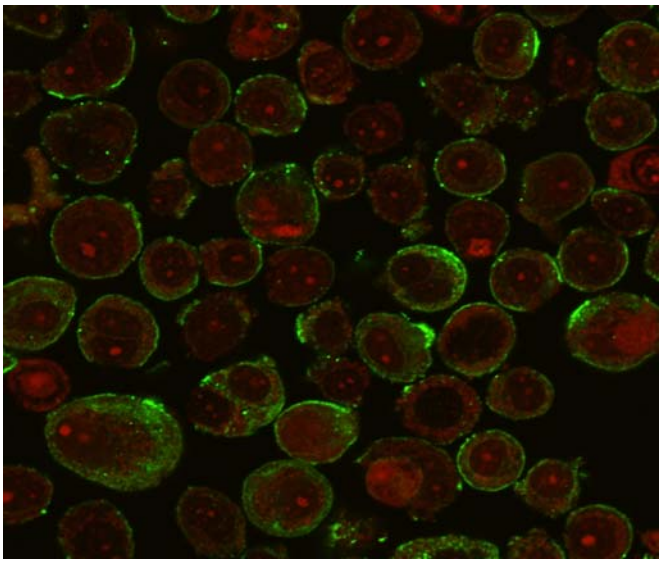

Figure 2: Confocal microscopy of nanotube-antibody hybrids on top of the cell membrane

Images were obtained at the same spot but using different filters. SWCNT functionalized with Alexa Fluor 488 appeared green on top of the cell and on the edge of the cell membrane while Alexa Fluor 555 delivered red color to the antibodies and observed mostly inside the cells. Control studies using confocal microscopy indicated that when nanotubes that were terminated with PEG molecules to prevent binding with biomolecules and treated with IGF1R specific antibodies that were tagged with Alexa Fluor 555, and incubated with MCF7 cells, showed no green fluorescence on the cells illustrating no binding of SWNT on the membrane and no internalization [10]. In the second

control experiment the cells were only incubated with SWCNT tagged with Alexa Fluor 488 and then micro-centrifuged to remove excess dye, triple rinsed and imaged using confocal microscopy. There was no fluorescence in these confocal experiments. The absence of green fluorescence demonstrated that nanotube by themselves do not get internalized within this period of time. Finally, SWNT tagged with Alexa 488 and IGF1R specific antibody tagged with Alexa 555 dye were mixed and kept at room temperature for 30 minutes. The suspension was then incubated with MCF7 cancer cells for 30 minutes, micro-centrifuged, rinsed and imaged using confocal microscopy. All these images showed the green fluorescence of nanotube both inside and on top of the cells as in Figure 2. Figure 3 (a) shows the cells that were only treated by NIR dosing for 3 minutes and Trypan blue for investigating membrane permeability and cell death. As observed, Trypan blue did not penetrate into the cell membrane and only can be seen on the background indicating no damage to the cell membrane.

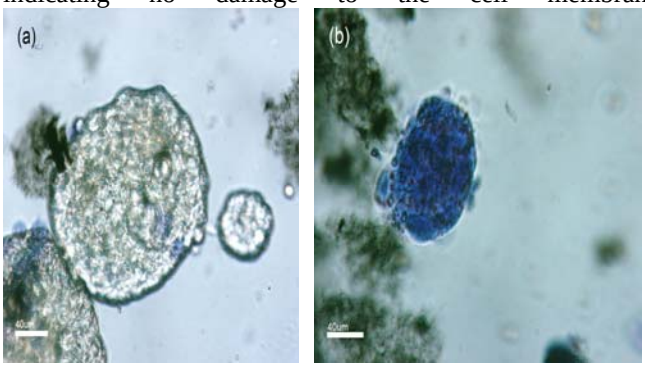

Figure 3: (a) MCF7 cancer cells treated without nanotube-nonspecific antibody complexes survived the NIR dosing at 800 mW/cm^2 for 3 minutes, (b) MCF7 cancer cells treated with nanotube-anti-IGF1-Her2 antibody complex showing that all the cells died after NIR dosing at 800 mW/cm^2 for 3 minutes. The cells appear blue in color indicating cell death.

The cells that only received NIR light without internalized SWCNT survived after photon dosing showing high degree of transparency of biological systems to NIR light. In sharp contrast, in Figure 3 (b), the cells incubated with SWCNT-anti-Her-anti-IGF1 hybrids, were all bright blue, indicating membrane damage and cell death. We repeated these experiments several times and obtained similar results. When antibodies attach to their corresponding receptors in cancer cells, stresses are generated due to the release in free energy. Specific antibody-antigen binding creates much higher change in free energy compared to non-specific-antibody antigen binding [11]. This higher change in free energy can create large compressive stresses (large enough to bend a micro-cantilever [11]) on the surface of the receptor which in turn can intimately contact the nanotube to the cell membrane. Thermal excitement of nanotubes through photons can then kill the cell as the membrane is compromised. This study just goes to show that adhesion of nanotube to the cell membrane is just enough to cause cell death through photothermal therapy. Carbon nanotubes are also sticky systems and tagging antibodies to the surface ensures their targeting to the surface receptors. Antibodies incubated with the cells acted as biological transport carriers to realize the attachment of SWCNT to the cell membrane. Shining NIR laser light heated the nanotubes inside the cells. The localized photothermal effect produced heat to destroy the cells completely. NIR was also used as it has been shown that cells are transparent to 700-1100 nm wavelength of light. Temperature measurements of the samples with SWCNT dispersed in solution did not show more than 30°C increases for light intensities that are used here bringing the actual temperature of the sample to 55°C. This was confirmed by both thermocouples in solution as well as using infrared thermometers. The energy used in the destruction of cancer cells was estimated to be ~200 nW per cell, too low to create any damage for the normal cells.

4. CONCLUSIONS

In this paper, we have demonstrated multi-component molecular targeting of surface receptors (IGF1R and Her2) and NIR dosing of cancer cells using SWCNT. While previous studies have shown the transport of DNA into cells using nanotubes, in this study we show multi-component molecular targeting of both IGF1R and Her2 surface markers in cancer cells using single wall carbon nanotubes. IGF1 and Her2 specific antibodies conjugated to the SWCNT were used to target their corresponding receptors in cells and internalize the SWCNT. The cells were then dosed with NIR 808 nm photons at ~800 mW/cm^2 for 3 minutes. Cells that were treated with non-specific antibody-SWCNT hybrids survived the NIR dosing compared to the dead cells with anti-IGF1-anti-Her2-SWCNT hybrids. The amount of energy consumed for cell killing was estimated to be as small as ~200 nW per cell, which is an order of magnitude smaller than competing techniques. Quantitative estimates showed that cells incubated with SWCNT-anti-Her-anti-IGF1R antibodies were completely destroyed while 80% of the non-specific-antibody-SWCNT hybrids treated cells were still alive. These results indicate that SWCNT could be used as biological transport agents and the high optical absorbance in the NIR are capable of killing cancer cells with minimum collateral damage.

5. ACKNOWLEDGEMENTS

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