articles

molecular pharmaceutics

Rapid Photothermal Intracellular Drug Delivery Using Multiwalled Carbon Nanotubes

Nicole H. Levi-Polyachenko,*,† Eric J. Merkel,‡ Bradley T. Jones,‡ David L. Carroll,§ and John H. Stewart IV||

Department of Plastic and Reconstructive Surgery, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157, Department of Chemistry, Wake Forest University, Winston-Salem, North Carolina 27109, Center for Nanotechnology and Molecular Materials, Department of Physics, Wake Forest University, Winston-Salem, North Carolina 27109, and Department of General Surgery, Section of Surgical Oncology, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157

Received December 2, 2008; Revised Manuscript Received May 22, 2009; Accepted June 22, 2009

Abstract: Carbon nanotubes are unique materials that absorb infrared (IR) radiation, especially between 700 and 1100 nm, where body tissues are most transparent. Absorbed IR promotes molecular oscillation leading to efficient heating of the surrounding environment. A method to enhance drug localization for peritoneal malignancies is perfusion of warm (40−42 °C) chemotherapeutic agents in the abdomen. However, all tissues in the peritoneal cavity are subjected to enhanced drug delivery due to increased cell membrane permeability at hyperthermic temperatures. Here we show that rapid heating (within ten seconds) of colorectal cancer cells to 42 °C, using infrared stimulation of nanotubes as a heat source, in the presence of the drugs oxaliplatin or mitomycin C, is as effective as two hours of radiative heating at 42 °C for the treatment of peritoneal dissemination of colorectal cancer. We demonstrate increased cell membrane permeability due to hyperthermia from multiwalled carbon nanotubes in close proximity to cell membranes and that the amount of drug internalized by colorectal cancer cells heated quickly using carbon nanotubes equals levels achieved during routine application of hyperthermia at 42 °C. This approach has the potential to be used as a rapid bench to bedside clinical therapeutic agent with significant impact for localizing chemotherapy agents during the surgical management of peritoneal dissemination of colorectal cancer.

Keywords: Hyperthermia; multiwalled carbon nanotubes; colorectal cancer

Introduction

According to the American Cancer Society, 153,000 cases of colorectal cancer (CRC) were diagnosed in 2006, leading to 65,000 deaths from metastatic disease of the peritoneum and liver.[1] A treatment that overcomes ineffective drug localization of systemic chemotherapy is intraperitoneal hyperthermic chemotherapy (IPHC).[1−3] IPHC involves surgical removal of accessible tumor followed by a two hour

* Corresponding author. Department of Plastic and Reconstructive Surgery, Wake Forest University School of Medicine, Winston-Salem, NC 27157. Phone: (336) 713-8052. Fax: (336) 713-6524. E-mail: nlevi@wfubmc.edu.

† Department of Plastic and Reconstructive Surgery, Wake Forest University School of Medicine.

‡ Department of Chemistry, Wake Forest University.

§ Center for Nanotechnology and Molecular Materials, Department of Physics, Wake Forest University.

|| Department of General Surgery, Section of Surgical Oncology, Wake Forest University School of Medicine.

(1) Levine, E. A.; Stewart, J. H.; Russell, G. B.; Geisinger, K. R.; Loggie, B. L.; Shen, P. Cytoreductive Surgery and Intraperitoneal Hyperthermic Chemotherapy for Peritoneal Surface Malignancy: Experience With 501 Procedures. *J. Am. Coll. Surgeons* **2007**, *204*, 943–953.

(2) Stewart, J. H.; Shen, P.; Russell, G. B.; Bradley, R. F.; Hundley, J. C.; Loggie, B. L.; Geisinger, K. R.; Levine, E. A. Appendiceal Neoplasms With Peritoneal Dissemination: Outcomes After Cytoreductive Surgery and Intraperitoneal Hyperthermic Chemotherapy. *Ann. Surg. Oncol.* **2006**, *13*, 624–634.

10.1021/mp800250e CCC: $40.75
Published on Web 06/22/2009

perfusion of the peritoneum with chemotherapeutic agent warmed to 40−42 °C.[2−5] Delivery of drugs with hyperthermia increases cellular metabolism and membrane permeability for enhanced drug uptake by cells.[5] Although IPHC has significantly improved patient outcomes,[1,3] the procedure has significant limitations, including lengthening the time that a patient must be anesthetized and requiring liters of drug perfusate.[1,3] We therefore propose to use the novel paradigm of treating cancer with hyperthermic chemotherapy using localized carbon nanotubes stimulated with infrared light.

Carbon nanotubes have special electrical, optical, and thermal characteristics due to the arrangement of the carbon atoms confined in nanometer sized volumes.[6−9] Due to their shape (high aspect ratio since the tube diameter is much smaller than tube length), they can influence the electric field in their localized area, which enhances absorption of electromagnetic energy and generates rapid heating of the tube.[10−13] Single-walled nanotubes with short (∼150 nm) lengths have been used in ablation studies; however, nanotubes behave as dipole antennas so the most efficient radiation coupling should occur for tubes longer than half the wavelength of the incident light. Multiwalled nanotubes (MWNT) have also been used for thermal ablation.[14,15] Based on previous studies and antenna theory, MWNT may be more effective thermal generators, and we have experimentally determined that this is the case.[15,16] The infrared source used in this work has a wavelength of 1064 nm; therefore, we grew MWNT with lengths longer than 532 nm. Iron is a common catalyst for growth of MWNT, and intratubular iron has the potential for MRI imaging so the location of nanotubes *in vivo* can be determined.[17−19] MWNT grown with variable amounts of iron were examined to determine the effect that catalysts have on thermodynamic potential. Infrared absorption is dependent upon nanoparticle concentration so the quantity of MWNT to achieve maximum temperature was evaluated for different MWNT.

Hyperthermia, defined as temperatures above 37 °C, is used clinically to treat a variety of malignancies.[20−24] Irreversible cell damage is incurred for temperatures above 45 °C; therefore, clinical IPHC procedures use mild hyperthermia between 40 and 42 °C for peritoneal perfusion.

(3) Stewart, J. H.; Shen, P.; Levine, E. A. Intraperitoneal Hyperthermic Chemotherapy for Peritoneal Surface Malignancy: Current Status and Future Directions. *Ann. Surg. Oncol.* **2005**, *12*, 765–777.

(4) Loggie, B. W.; Fleming, R. A.; Geisinger, K. R. Cytologic Assessment Before and After Intraperitoneal Hyperthermic Chemotherapy for Peritoneal Carcinomatosis. *Acta Cytol.* **1996**, *40*, 1154–1158.

(5) Shen, P.; Hawksworth, J.; Lovato, J.; Loggie, B. W.; Geisinger, K. R.; Fleming, R. A.; Levine, E. A. Cytoreductive Surgery and Intraperitoneal Hyperthermic Chemotherapy With Mitomycin C for Peritoneal Carcinomatosis From Nonappendiceal Colorectal Carcinoma. *Ann. Surg. Oncol.* **2004**, *11*, 178–186.

(6) Kroto, H. W.; Heath, J. R.; Obrien, S. C.; Curl, R. F.; Smalley, R. E. C-60 - Buckminsterfullerene. *Nature* **1985**, *318*, 162–163.

(7) Hepplestone, S. P.; Srivastava, G. P. The Intrinsic Lifetime of Low-Frequency Zone-Centre Phonon Modes in Silicon Nanowires and Carbon Nanotubes. *Appl. Surf. Sci.* **2006**, *252*, 7726–7729.

(8) Dresselhaus, M. S.; Dresselhaus, G.; Eklund, P. C. Science of Fullerenes and Carbon Nanotubes; Academic Press Inc.: San Diego, CA, 1996.

(9) Mittal, J. P. Excited-States and Electron-Transfer Reactions of Fullerenes. *Pure Appl. Chem.* **1995**, *67*, 103–110.

(10) Burke, P. J.; Li, S. D.; Yu, Z. Quantitative Theory of Nanowire and Nanotube Antenna Performance. *IEEE Trans. Nanotechnol.* **2006**, *5*, 314–334.

(11) Hanson, G. W. Fundamental Transmitting Properties of Carbon Nanotube Antennas. *IEEE Trans. Antennas Propag.* **2005**, *53*, 3426–3435.

(12) Kempa, K.; Rybczynski, J.; Huang, Z. P.; Gregorczyk, K.; Vidan, A.; Kimball, B.; Carlson, J.; Benham, G.; Wang, Y.; Herczynski, A.; Ren, Z. F. Carbon Nanotubes As Optical Antennae. *Adv. Mater.* **2007**, *19*, 421+.

(13) Wang, Y.; Kempa, K.; Kimball, B.; Carlson, J. B.; Benham, G.; Li, W. Z.; Kempa, T.; Rybczynski, J.; Herczynski, A.; Ren, Z. F. Receiving and Transmitting Light-Like Radio Waves: Antenna Effect in Arrays of Aligned Carbon Nanotubes. *Appl. Phys. Lett.* **2004**, *85*, 2607–2609.

(14) Biris, A. S.; Bolder, D.; Palmer, J.; Monroe, W. T.; Mahmood, M.; Dervishi, E.; Xu, Y.; Li, Z.; Galanzha, E.; Zharov, V. Nanophotothermolysis of Multiple Scattered Cancer Cells With Carbon Nanotubes Guided by Time-Resolved Infrared Thermal Imaging. *J. Biomed. Optics* **2009**, *14*, 021007.

(15) Torti, S.; Byrne, F.; Whelan, O.; Levi, N.; Ucer, B.; Schmid, M.; Torti, F.; Akman, S.; Liu, J.; Ajayan, P.; Nalamasu, O.; Carroll, D. Thermal Ablation Therapeutics Based on CNx Multi-Walled Nanotubes. *Int. J. Nanomed.* **2007**, *2*, 707–714.

(16) Berger, C.; Poncharal, P.; Yi, Y.; de Heer, W. Ballistic Conduction in Multiwalled Carbon Nanotubes. *J. Nanosci. Nanotechnol.* **2003**, *3*, 171–177.

(17) Choi, J. H.; Nguyen, F. T.; Barone, P. W.; Heller, D. A.; Moll, A. E.; Patel, D.; Boppart, S. A.; Strano, M. S. Multimodal Biomedical Imaging With Asymmetric Single-Walled Carbon Nanotube/Iron Oxide Nanoparticle Complexes. *Nano Lett.* **2007**, *7*, 861–867.

(18) Caruthers, S. D.; Wickline, S. A.; Lanza, G. M. Nanotechnological Applications in Medicine. *Curr. Opin. Biotechnol.* **2007**, *18*, 26–30.

(19) Monteiro-Riviere, N. A.; Inman, A. O. Challenges for Assessing Carbon Nanomaterial Toxicity to the Skin. *Carbon* **2006**, *44*, 1070–1078.

(20) Hanajiri, K.; Maruyama, T.; Kaneko, Y.; Mitsui, H.; Watanabe, S.; Sata, M.; Nagai, R.; Kashima, T.; Shibahara, J.; Omata, M.; Matsumoto, Y. Microbubble-Induced Increase in Ablation of Liver Tumors by High-Intensity Focused Ultrasound. *Hepatol. Res.* **2006**, *36*, 308–314.

(21) Rouviere, O.; Mege-Lechevallier, F.; Chapelon, J. Y.; Gelet, A.; Bouvier, R.; Boutitie, F.; Lyonnet, D. Evaluation of Color Doppler in Guiding Prostate Biopsy After HIFU Ablation. *Eur. Urol.* **2006**, *50*, 490–497.

(22) Wu, F.; Wang, Z. B.; Cao, Y. D.; Xu, Z. L.; Zhou, Q.; Zhu, H.; Chen, W. Z. Heat Fixation of Cancer Cells Ablated With High-Intensity-Focused Ultrasound in Patients With Breast Cancer. *Am. J. Surg.* **2006**, *192*, 179–184.

(23) Bastide, C.; Garcia, S.; Anfossi, E.; Ragni, E.; Rossi, D. Histologic Evaluation of Radiofrequency Ablation in Renal Cancer. *Eur. J. Surg. Oncol.* **2006**, *32*, 980–983.

(24) Parekh, D. J.; Chiang, L. W.; Herrell, S. D. In Vivo Assessment of Radio Frequency Induced Thermal Damage of Kidney Using Optical Spectroscopy. *J. Urol.* **2006**, *176*, 1626–1630.

Hyperthermic chemotherapy techniques are most effective clinically when the area of interest is warmed for a few hours.[2,4,5] In IPHC procedures, nodules are removed and the peritoneum is prewarmed and then perfused with heated chemotherapeutic agent. Hyperthermia increases cellular metabolism and membrane permeability for enhanced drug uptake by cells.[5] Our *in vitro* hyperthermia procedures were designed to reach the target temperature of 42 °C as rapidly as possible using the most efficient nanotubes. Oxaliplatin and mitomycin C (MMC) are two chemotherapeutic agents used in IPHC treatment of peritoneal dissemination of colorectal cancer for which clinical dosing parameters are well established. Therefore, we examined the potential of carbon nanotubes, stimulated by infrared light, to increase the efficiency of delivery of these agents to CRC cells.

Current methods being developed for using carbon nanotubes to eliminate tumors include thermal ablation therapy, where the nanotube is excited by infrared radiation,[25−27] and intracellular drug transport.[26,28] Carbon nanotubes have an absorption peak in the infrared spectrum between 700 and 1100 nm, which is known to be the region of the electromagnetic spectrum where tissue is most transparent.[29] Due to their small size, nanotubes can heat localized areas and have been shown able to be excreted from the body naturally, making them an acceptable choice for treatment of cancer.[30] We use hyperthermia below the ablation threshold (less than 45 °C) to raise the local temperature and enhance drug uptake by malignant cells, spare surrounding tissues excessive thermal impact and avoid increased drug uptake in sensitive tissues. Current IPHC technique involves an open or closed abdominal hyperthermic chemoperfusion. During the initial surgical stage, tumor nodules are removed (tumor debulking or cytoreduction). However, metastatic peritoneal cancer often involves a great many small nodules that cannot be removed surgically, but will be eliminated by hyperthermic penetration of a chemotherapeutic agent. Patients who undergo incomplete debulking have poor outcomes than for complete debulking, resulting in poor penetration of the drug, subsequent tumor growth and residual tumor masses. MWNT near tumor nodules, such as in the peritoneum, can generate hyperthermia directly adjacent to tumors to enhance chemotherapeutic uptake by malignant cells. MWNT could be localized to tumor nodules by labeling of the nanotubes with moieties for attachment of nanotubes to tumor surfaces. Rapid hyperthermic chemotherapy can be delivered simply by proximity of nanotubes and chemotherapeutic agent near cell surfaces when an infrared light illuminates the area. Hyperthermia can be localized to tumor nodules using nanotubes since the surgeon can see or feel the nodules, and during the open abdomen debulking procedure can apply infrared light to tumor nodules that are surrounded by a solution of chemotherapeutic agent and nanotubes. The adult human abdomen does not hold liters of fluid, but liters of drug are needed for use with current intraperitoneal perfusion apparatus. We expect that about 0.5 L of chemotherapeutic agent would be needed using nanotube induced hyperthermia since perfusion circuitry would be eliminated. The peritoneal cavity would just need to be filled once with a drug/nanotube solution, and then infrared light applied to tumor nodules bathed in the solution. The time for tumor debulking can be as long as ten hours because of the need to remove many nodules that are too large for hyperthermic chemoperfusion to be effective. IPHC using nanotubes could aid in drug penetration so that more nodules would respond to the delivered drug and fewer nodules would need to be removed, thus significantly reducing the time for tumor debulking and time that the patient would be anesthetized. Improvement in drug uptake may result in an enhanced IPHC technique using carbon nanotubes with reduced treatment times, and localization of the chemotherapeutic agent.

Materials and Methods

Cells and Reagents. RKO and HCT 116 colorectal cancer cell lines were purchased from American Type Culture Collection (ATCC) and cultured in McCoy's medium, supplemented with 2.5 μg/mL amphotericin, 1% L-glutamine, 1% penicillin/streptomycin and 10% fetal bovine serum. Cells were plated into 48-well tissue culture dishes at a seeding density of 10,000−20,000 cells per well. Oxaliplatin and mitomycin c were purchased from Sigma-Aldrich.

Nanotube Preparation. Multiwalled nanotubes were grown by chemical vapor deposition (CVD) methods according to Czerw et al.[31] Catalyst amounts were varied by addition of ferrocene at the beginning of growth. Fifty milligrams of as-grown carbon nanotubes were shortened and cleaned by sonication in 90 mL of sulfuric acid and 30 mL of nitric concentrated acids for twenty hours. Length of these nanotubes was approximately 2 μm as determined by transmission electron microscopy. Nanotube (NT) suspensions were prepared by adding 1 mg of MWNT per 1 mL of

(25) Monch, I.; Meye, A.; Leonhardt, A.; Kramer, K.; Kozhuharova, R.; Gemming, T.; Wirth, M. P.; Buchner, B. Ferromagnetic Filled Carbon Nanotubes and Nanoparticles: Synthesis and Lipid-Mediated Delivery into Human Tumor Cells. *J. Magn. Magn. Mater.* **2005**, *290*, 276–278.

(26) Kam, N. W. S.; O'Connell, M.; Wisdom, J. A.; Dai, H. J. Carbon Nanotubes As Multifunctional Biological Transporters and Near-Infrared Agents for Selective Cancer Cell Destruction. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 11600–11605.

(27) O'Neal, D. P.; Hirsch, L. R.; Halas, N. J.; Payne, J. D.; West, J. L. Photo-Thermal Tumor Ablation in Mice Using Near Infrared-Absorbing Nanoparticles. *Cancer Lett.* **2004**, *209*, 171–176.

(28) Kam, N. W. S.; Dai, H. J. Single Walled Carbon Nanotubes for Transport and Delivery of Biological Cargos. *Phys. Status Solidi B* **2006**, *243*, 3561–3566.

(29) Weissleder, R. *Nat. Biotechnol.* **2001**, *19*, 319.

(30) Singh, R.; Pantarotto, D.; Lacerda, L.; Pastorin, G.; Klumpp, C.; Prato, M.; Bianco, A.; Kostarelos, K. Tissue Biodistribution and Blood Clearance Rates of Intravenously Administered Carbon Nanotube Radiotracers. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 3357–3362.

(31) Czerw, R.; Liu, J. W.; Carroll, D. L. Electronic Effects in Scanning Tunnelling Microscopy of Metal-Filled Multiwalled Carbon Nanotubes. *New J. Phys.* **2004**, *6*, http://www.njp.org/. DOI: 10.1088/1367-2630/6/1/031.

water with 0.1% biocompatible Pluronic surfactant, F-127; these suspensions were briefly sonicated to aid in dispersion, and the solution pH balanced prior to tissue culture use. The nanotube suspension was added to medium at a concentration of 100 μL of NT stock per 1 mL of medium, for a final concentration of 100 μg per mL of media. Although 2 μm long MWNT is a nanotube length longer than needed as predicted by antenna theory, we have found that longer nanotubes generate more heat with the same amount of laser stimulation than do shorter nanotubes.[15] Nanotube length was chosen based on a convenient time for acid-treating and hence shortening of the nanotubes. So long as the nanotubes were longer than 532 nm, based on antenna theory, they would be acceptable for use. The laser protocols for *in vitro* cell culture were developed experimentally by preparing concentration dilutions of MWNT with surfactant coating in water.

Heating Methods. Adherent cells were used to eliminate any cell settling or turbidity that may impact infrared absorption. Three hundred microliters of nanotube/media solution was added for temperature testing and heating of the cells. Cells were placed on a hot water bottle to maintain a temperature of 37 °C during laser application. A Nd:YAG laser (1064 nm) operating at 3 W of power was used to apply infrared stimulation to the nanotubes, with a beam diameter of 1 cm. A thermocouple measured the temperature of nanotube/medium solutions immediately after laser application. The time for laser exposure to raise the temperature of the wells to 42 °C was found to vary between 8 and 11 s per well, depending on ambient room temperature.

Drug Treatments. Five treatment variables included a control of medium, a water dilution (230 μL/1 mL) of the medium with 0.1% F-127, 300 μM oxaliplatin, 100 μg/1 mL of medium nanotubes, and 300 μM oxaliplatin plus 100 μg of nanotubes per 1 mL of medium. Medium with treatments was added immediately prior to laser exposure. RKO colorectal cancer cells were treated with 0.1% F-127, 0.1% F-127 plus 40 μM MMC, 100 μg of nanotubes, 100 μg of nanotubes plus 40 μM MMC, or a control with no treatment.

Cell Viability Assays. One 48-well plate (with the treatment options) was held at 37 °C, and another 48-well plate was held at 42 °C. A third plate was held at 37 °C until laser application. Infrared treatment was applied three times, for 8−11 s per application, over a time period of two hours. Following incubation, treatment medium was replaced with fresh medium and the three plates were incubated at 37 °C for forty-eight hours. Following incubation, cell viability was quantified over a three hour period using Promega's CellTiter 96 AQ_{ueous} assay kit.

As an additional viability assay, RKO cells underwent calcein/ethidium staining forty-eight hours post-treatment. Cells were washed with cold PBS to remove adherent nanotubes and serum from the medium. Solutions of calcein (2 μM) and ethidium homodimer (4 μM) in PBS were added to the cells. The plates were evaluated on an inverted Olympus fluorescent microscope in the fluorescein and rhodamine channels.

Elemental Analysis for Oxaliplatin. Because oxaliplatin contains a platinum metal atom at the core of the molecule, drug uptake by colorectal cancer cells can be quantified by elemental analysis for platinum. The platinum concentration of treated cells was determined by inductively coupled plasma (ICP) atomic emission spectrometry, using a Prodigy High Dispersion ICP instrument (Teledyne Leeman Laboratories, Hudson, NH). The ICP system employed a concentric nebulizer, a solution flow rate of 1.4 mL/min, and an RF power of 1.2 kW. A calibration curve was generated using 5 standards, 0 ppb, 300 ppb, 700 ppb, 1000 ppb, and 2000 ppb, prepared by serial dilutions of a 1000 ppm $PtCl_2 \cdot 6H_2O$ (platinum(II) chloride hexahydrate salt dissolved in deionized water) stock. A 1000 ppb spike of gold was added to all standards and samples to be used as an internal standard. The gold emission line at 242.795 nm was monitored along with the platinum emission line at 214.423 nm. Using this method, reference solutions of known Pt concentration were analyzed with better than 99% accuracy. Ten million RKO and HCT116 colorectal cancer cells were prepared for ICP elemental analysis (EA) by treating with 300 μM oxaliplatin and 100 μg of nanotubes per mL of medium and warming to 37 and 42 °C for two hours, or 42 °C rapidly using infrared absorption by the nanotubes. Following treatments, cells were washed with ice cold PBS and shaken on an orbital shaker in a cold room for twenty minutes to remove nanotubes. Cells were trypsinized, counted, then digested in 500 μL of concentrated nitric acid. Following overnight digestion at room temperature, 500 μL of ammonium hydroxide was then added to neutralize, and water was added to bring the total sample volume to 4 mL. The amount of platinum was determined for each sample and the results normalized against the number of cells in each sample.

Results

MWNT Concentration. Cleaning and shortening of MWNT in acids releases iron that is distributed throughout the length of the nanotube during growth. Elemental analysis confirms the differences between nanotubes grown with variable amounts of iron catalyst before and after acid treatments (Figure 1, Table 1). Electron microscopy observation of nanotubes wrapped in surfactant following laser application reveals that nanotubes generate enough heat at their surface to ablate the surfactant (Figure 1a, b). This result corroborates findings by other authors[26] and leads to moderate nanotube aggregation following laser application. MWNT reach a saturation limit at 0.1 mg/mL, above which there is no significant increase in temperature, regardless of the amount of catalyst (Figure 1c). Based on this result, we concluded that multiwalled nanotubes with minimal catalyst should be used for subsequent hyperthermia procedures and that the maximal amounts of nanotubes should be 100 μg of nanotubes per milliliter of treatment medium.

Viability of Colorectal Cancer Cell Populations. Cell viability is analogous for nanotube induced rapid hyperthermia and current clinical protocols. Cell viability assays

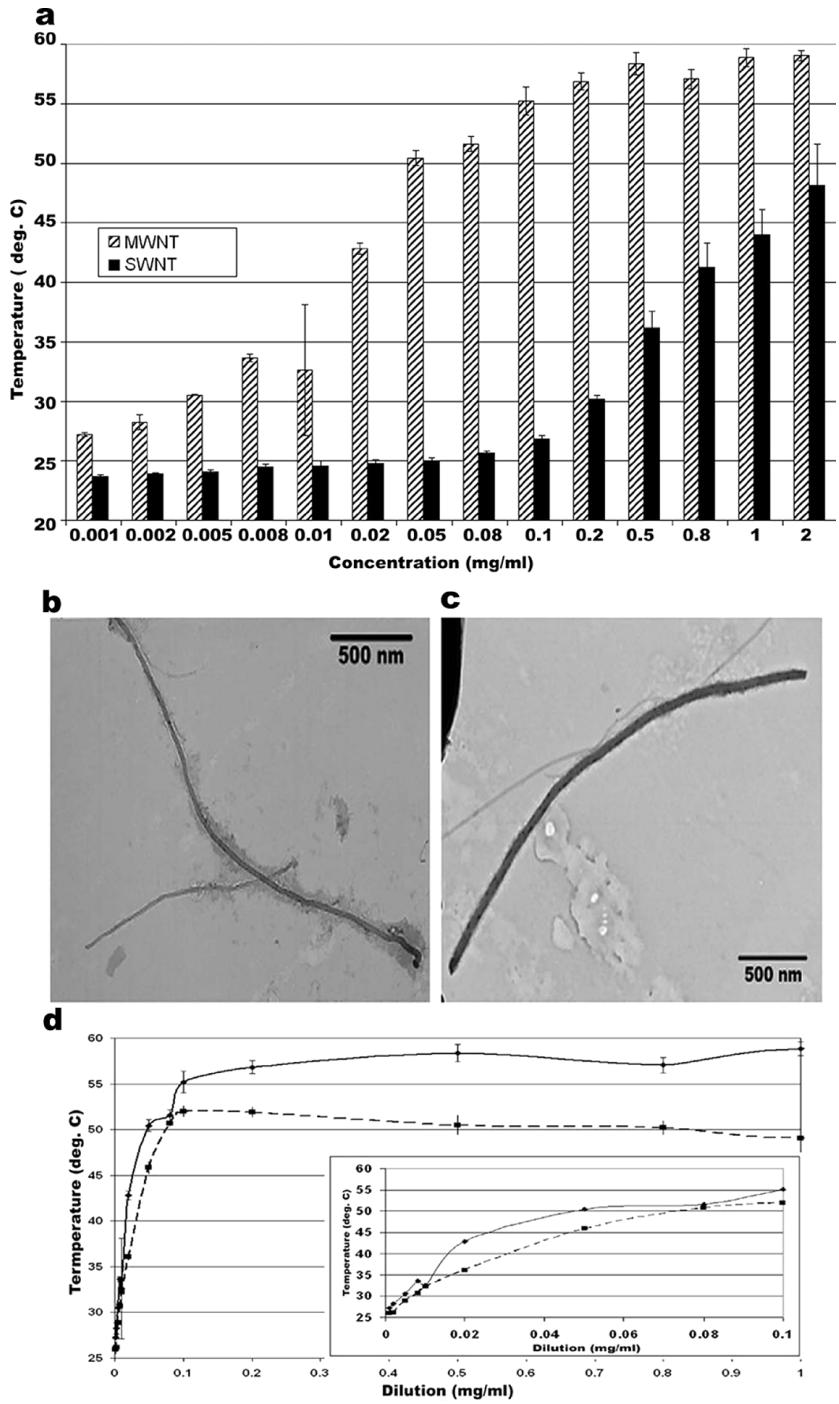

Figure 1. (a) Comparison of temperature increases of single-wall nanotubes compared to multiwall nanotubes, at concentration dilutions in water with Pluronic surfactant. (b) CVD-MWNT wrapped with Pluronic F-127 before laser application. (c) After 30 s of laser application, the surfactant was removed from the outside of the nanotubes. (d) At all dilutions of nanotubes, MWNT grown with 60 mg (—◆—) iron catalyst have higher temperatures than MWNT grown with 400 mg (- - -■- - -) of iron catalyst. The inset in panel d is an enlarged view of the graph between 0 and 0.1 mg/mL. Panel d also shows that the absorption cutoff beyond which there is no further increase in temperature is 0.1 mg/mL of MWNT in solution.

provide an analysis of cellular response to rapid temperature increases with or without chemotherapeutic agents. Cell viability after laser treatment without nanotubes of CRC cells in the presence or absence of oxaliplatin corresponds most strongly with that seen after treatment at 37 °C. However, once nanotubes are employed to induce hyperthermia to 42 °C via infrared absorption, cell viability most closely matches the number of cells treated at 42 °C for two hours (Figure 2a, b). A significant reduction in cell viability was seen in RKO cancer cells treated with nanotubes, MMC, and laser

Table 1. Amount of Iron Catalyst (%w/w) for CVD-MWNT Grown with 60 mg or 400 mg of Iron before and after Cleaning and Cutting with Acid Treatment

	pre-acids	post-acids
60 mg	4.65	1.26
400 mg	7.31	2.05

relative to treatment with these agents alone (Figure 2c). Our data also confirms previous reports that oxaliplatin is a more effective chemotherapeutic agent against colorectal carcinoma *in vitro* than MMC.[32]

Calcein/ethidium cell staining corroborates the trend observed in the MTS assay (Figure 3). RKO cells treated with oxaliplatin and no nanotubes but with laser application have cell viability similar to those treated with oxaliplatin at 37 °C. When cells are treated with oxaliplatin, nanotubes, and laser, the cell population is significantly reduced relative to cells heated at 37 °C, and comparable to cells treated at 42 °C.

Quantification of Intracellular Drug Delivery. Elemental analysis of platinum within CRC cells confirms that equivalent amounts of oxaliplatin are retained in the cells during rapid hyperthermia via nanotubes compared to two hours of hyperthermia at 42 °C. Intracellular platinum content serves as a proxy for oxaliplatin uptake. In the RKO and HCT 116 colorectal cell lines, infrared stimulation of nanotubes results in rapid heating of the medium and increased intracellular platinum concentrations that are comparable to those found in cells treated with oxaliplatin for two hours at 42 °C (Figure 4a, b). There was a higher concentration of platinum observed in HCT 116 cells because the cells are larger and can hold more oxaliplatin.

Discussion

Cell viability assays and elemental analysis for intracellular platinum concentration corroborate that hyperthermia can be induced within seconds using carbon nanotubes and that the described methods produce results statistically equivalent to currently used IPHC protocols. The present work suggests that nanotubes are useful for hyperthermic chemotherapy delivery. Rapid heating of the bulk nanotube solution containing oxaliplatin or mitomycin C aids in cellular uptake of the drugs. Our methods are shown to be effective in two different colorectal cancer cell lines. Cell viability assays demonstrate that when cells are treated with infrared light alone, or oxaliplatin or MMC alone, the results are comparable to cell incubation at 37 °C. When nanotubes are added and infrared stimulation is applied for approximately ten seconds, cell survival is reduced to a level comparable to cells incubated at 42 °C for two hours. Oxaliplatin uptake is

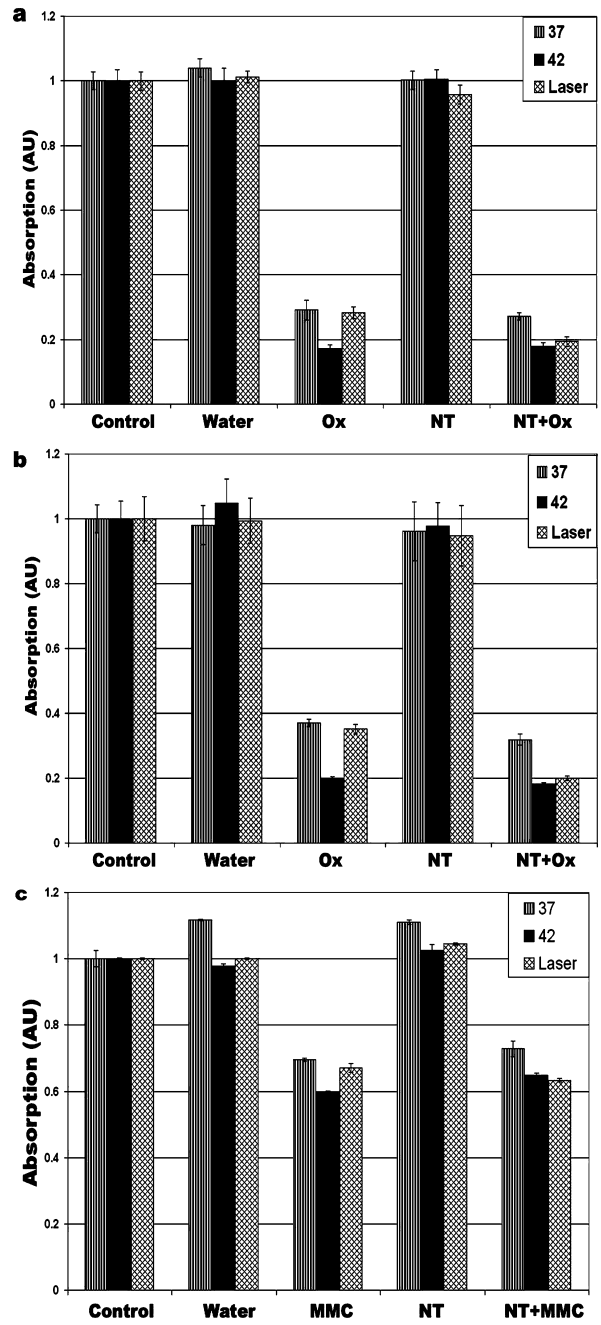

Figure 2. HCT 116 (a) and RKO (b) colorectal cancer cell lines' response to nanotubes, laser application and oxaliplatin. Ox: oxaliplatin. NT: nanotubes. When nanotubes are added to the system, with oxaliplatin, and laser stimulation is applied, the cell population statistics fall most closely in line with cells treated at 42 °C for two hours. Without nanotubes, but with IR stimulation, cell population statistics most closely align with cells treated at 37 °C. This result indicates that nanotubes are necessary for hyperthermia induced chemotherapy. (c) Cell viability of RKO cells treated with 40 μM MMC at 37 °C, 42 or 42 °C rapidly via carbon nanotube heating. MMC: mitomycin. This chart shows that hyperthermic chemotherapy using nanotubes is beneficial for use with MMC.

increased after achieving rapid hyperthermia with nanotubes. Most importantly, the time for increased drug uptake and

(32) Elias, D.; Benizri, E.; DiPietrantonio, D.; Menegon, P.; Malka, D.; Raynard, B. Comparison of Two Kinds of Intraperitoneal Chemotherapy Following Complete Cytoreductive Surgery of Colorectal Peritoneal Carcinomatosis. *Ann. Surg. Oncol.* **2007**, *14*, 509–514.

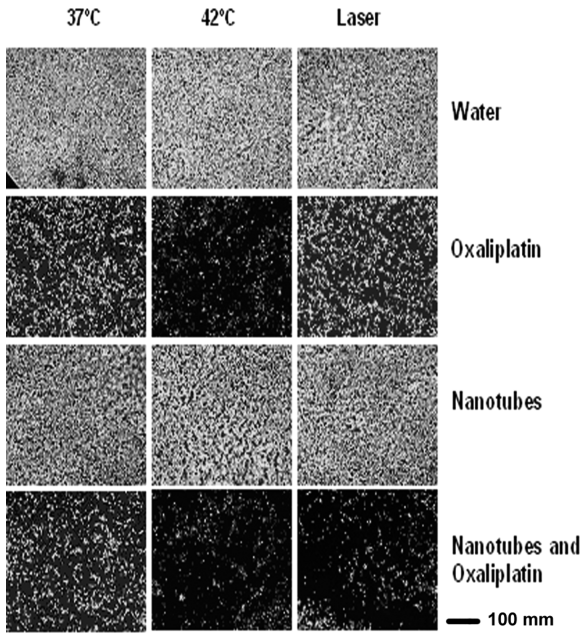

Figure 3. Live RKO cell populations for treatments with oxaliplatin at 37 and 42 °C compared to rapid induction of hyperthermia via infrared laser applied to carbon nanotubes.

decreased cell viability is reduced to seconds using nanotubes compared to two hours of hyperthermia at 42 °C.

Since only low hyperthermic temperatures (42 °C) are employed using near-infrared stimulation of the nanotubes, the chances for thermal damage or increased heat shock protein expression (which can lead to more aggressive recurrence) is reduced. Furthermore, the energy fluences, dependent on the power of the laser and the time of application, used here are much lower than those used by other authors for ablation studies. For example, Kam et al. used 168 J/cm^2 with single-wall carbon nanotubes,[26] O'Neal et al. used 720 J/cm^2 with gold nanoshells,[27] and the fluence we have used with multiwall carbon nanotubes is only 38.4 J/cm^2. Although the rapid hyperthermic chemotherapy method outlined here employs multiwall carbon nanotubes as the absorbing source, other nanoparticles including single-wall carbon nanotubes, nanohorns or metallic nanoshells may be used as the heat generating particle.

The method we have developed demonstrates the efficacy of nanotubes stimulated by infrared radiation in the presence of a chemotherapeutic agent. The method is quite simple since no attachment of the nanoparticle to the drug is required. Yet, the effect is significant increase in the amount of agent that is retained in the cells. The evaluations of nanotubes and other nanoparticles reported to date have focused on therapeutic delivery or thermal ablation. However, most cancer treatments involve a combination of surgery, chemotherapy, and radiation. Intraperitoneal hyperthermic chemoperfusion using carbon nanotubes may be utilized as a rapid bench-to-bedside technique since it can be applied during surgical procedures, all the nanomaterial can be removed from the body following delivery, and hyperthermia can be localized.

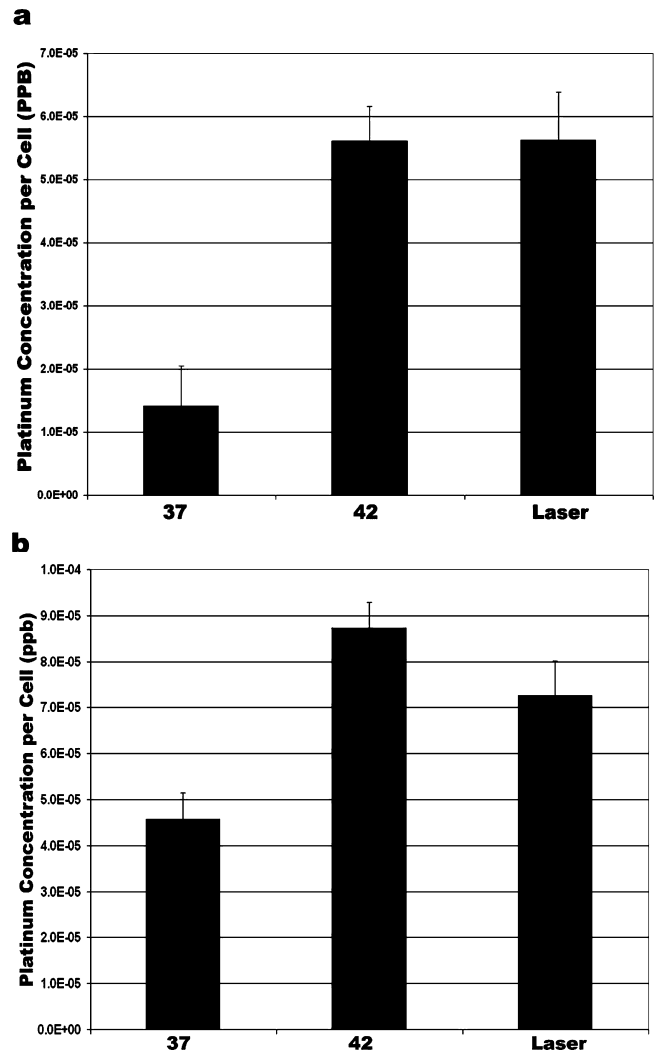

Figure 4. The amount of platinum per RKO (a) or HCT 116 (b) colorectal cancer cell treated at 37, 42, or 42 °C rapidly by infrared laser stimulation of nanotubes. Platinum amounts are increased if cells are raised to 42 °C quickly using ten second infrared stimulation of nanotubes or if incubated at 42 °C for two hours. In either case, more platinum gets into these cells than cells treated at 37 °C.

MWNT induced chemotherapy for metastatic peritoneal cancer can be clinically applied during open abdominal procedures by filling the abdomen with a solution of nanotubes and chemotherapeutic agent and applying infrared light only to the tumor nodules. MWNT would be directly introduced into the treatment region and not intravenously introduced due to the lack of systemic delivery of therapeutic agents from the bloodstream to the peritoneum. It is beneficial to minimize the surgical time to reduce the time that a patient is anesthetized, to maximize recovery and minimize complications due to prolonged anesthesia. For example, to surgically remove a tumor nodule may take five minutes but laser application would take only a few seconds. This would significantly reduce the overall time of the surgery. Furthermore, the nanotubes could be easily removed from the abdomen following hyperthermic chemotherapy

delivery by flushing the abdomen with saline after the procedure. Future evolutions for nanotube usage for dissemination of metastatic peritoneal cancers include (1) targeting nanotubes to specific tumor types using antibodies, bacteriophages, or other moieties such as folic acid; or (2) using nanotubes to induce hyperthermic chemotherapy in a closed abdominal procedure using laproscopic techniques and a fiber optic infrared source. IPHC using carbon nanotubes has the clinical potential to reduce treatment times for hyperthermic chemotherapy by localizing heat, thus aiding in the penetration of chemotherapeutics into malignant tumor cells, and hence reducing the overall treatment time and increasing the effectiveness of treatment and patient survival.

Acknowledgment. B.T.J. and E.J.M. gratefully acknowledge financial support from Teledyne Leeman Laboratories. J.H.S. and N.H.L.-P. gratefully acknowledge funding from the Wake Forest University School of Medicine—Department of General Surgery Research Fund.

MP800250E