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Photoluminescent carbon nanotubes interrogate the permeability of multicellular tumor spheroids

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Abstract

Nanomaterials have been extensively investigated for cancer drug delivery and imaging applications. Nanoparticles that show promise in two-dimensional cell culture systems often fail in more complex environments, possibly due to the lack of penetration in dense, three-dimensional structures. Multicellular tumor spheroids are an emerging model system to investigate interactions of nanoparticles with 3D *in vitro* cell culture environments. Using the intrinsic near-infrared emission of semiconducting carbon nanotubes to optically reconstruct their localization within a three-dimensional volume, we resolved the relative permeability of two different multicellular tumor spheroids. Nanotube photoluminescence revealed that nanotubes rapidly internalized into MCF-7 breast cancer cell-derived spheroids, whereas they exhibited little penetration into spheroids derived from SK-136, a cell line that we developed from murine liver cancer. Characterization of the spheroids by electron microscopy and immunohistochemistry revealed large differences in the extracellular matrix and interstitial spacing, which correlated directly with nanotube penetration. This platform portends a new approach to characterize the permeability of living multicellular environments.

1. Introduction

The use of nanomaterials for cancer drug delivery aims to increase tumor drug concentration while avoiding healthy tissues in order to improve anti-tumor efficacy and reduce dose-limiting toxicities [1,2]. Although nanomedicines remain promising, with rapid advancement on multiple fronts including nanoparticle formulation, targeting specificity, and multifunctional abilities, their clinical potential has not yet been fulfilled [3]. Although drug carrier nanoparticles in clinical use do reduce off-target drug toxicity, this has not translated to improved therapeutic efficacy in patients [4].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.carbon.2015.08.024>.

A major reason for the limited success of drug carrier nanoparticles is poor penetration into tumor tissues caused by dense extracellular matrix (ECM) components and closely-packed cells which hinder diffusion of nanoparticles [5]. High affinities of targeted nanoparticles to cancer cells or the ECM may further reduce transport into the tumor interstitium [6].

Two-dimensional monolayers cannot model the extracellular environment or cellular tight junctions present in tumor tissue, leading to technologies which may not function as predicted from *in vitro* assays [7,8]. Additionally, two-dimensional cell culture lacks other aspects encountered *in vivo*, such as a hypoxic tumor microenvironment and a three-dimensional nutritional gradient [9,10].

Spheroid monocultures have emerged as a test platform to bridge the gap between 2D cell cultures and *in vivo* studies. Useful for studying particle penetration in tumor tissue mimics, spheroids as small as 150 μm across can exhibit physiological cell-to-cell and cell-to-matrix interactions [11]. The arrangement of a population of cells in a three-dimensional conformation, embedded within multiple ECM components, introduces a spatial heterogeneity in the microenvironment of each cell [12]. In particular, cell adhesion and mechanical forces acting on each cell can significantly differ in 2D and 3D. One of the four main types of spherical tumor models used is the multicellular tumor spheroid (MCTS). The MCTSs are generated by culturing cancer cells in fetal bovine serum (FBS)-supplemented media in an environment that promotes cell-to-cell interactions over cell-to-substrate interactions [13]. A standard approach involves culturing cells in an ultra-low attachment flask, where the adhesive forces between cells are stronger than the interaction with the flask surface.

A one-dimensional, nanoscale form of carbon [14], single-walled carbon nanotubes (SWCNT) provide a unique structural topology for probing the accessibility of a complex object – with a diameter of approximately 1 nm and lengths ranging from 50 to 1000 nm, SWCNT have a highly unusual aspect ratio when compared to standard fluorescent beads and nanoparticles [15]. This small size in one dimension allows nanotubes to interact with a cell membrane tip first, as a nano-needle [16,17]. A recent study demonstrated that SWCNT diffusion coefficients inside a hepato-cellular carcinoma tumor spheroid was comparable to similarly charged molecules with 10,000 times lower molecular weights [18].

Semiconducting carbon nanotubes exhibit intrinsic [19], and uniquely photostable [20], near infrared photoluminescence (PL) [21]. Upon excitation with visible light (400–800 nm), nanotubes emit fluorescence in the near infrared range (900–1700 nm). Living tissues exhibit attenuated autofluorescence, scattering, and absorption in this range [15]. These properties have been exploited to achieve single-nanotube detection *in vivo* [22], measurements through whole animals [23], and non-invasive imaging through a mouse skull [24].

In this work, we employed photoluminescent carbon nanotubes to interrogate the permeability of two different types of tumor spheroids. We developed tumor spheroids from a new murine cell line, SK-136, derived from an orthotopic model of myc-driven liver cancer [25]. We then used the near-infrared emission of semiconducting carbon nanotubes to

measure the relative permeability of SK-136 spheroids, as well as MCF-7 spheroids, derived from a breast cancer cell line. Photoluminescence microscopy revealed that the nanotubes rapidly internalized into MCF-7 spheroids, whereas they exhibited little penetration into SK-136 spheroids. Characterization of the spheroids by electron microscopy and immunohistochemistry revealed large differences in the extracellular matrix and interstitial spacing, which corresponded with nanotube penetration.

2. Experimental

2.1. Preparation of functionalized nanotubes

Single walled carbon nanotubes prepared by the high-pressure carbon monoxide (HiPco) process were purchased from Unidym (Sunnyvale, CA). The nanotubes arrived in a wet “mud” form consisting of both SWCNTs and carbonaceous impurities. Nanotubes were dispersed by sonicating 1 mg of SWCNT, weighed using an ultramicrobalance (Mettler Toledo) with 1 mL of a 1% solution of the anionic surfactant sodium deoxycholate (SDC, Sigma Aldrich, MO) in phosphate-buffered saline (PBS, Life Technologies). The nanotube-SDC mixture was sonicated for 30 min with a probe tip ultrasonicator (Sonics & Materials, Sonics Vibracell) using a 1/8" Tapered Microtip (Sonic & Materials) for 30 min at 40% of maximum amplitude. During sonication, nanotubes were cooled to 4 °C with a CoolRack (Biocision). Dispersed nanotubes were separated from the non-dispersed fraction by ultracentrifugation at 280,000g for 30 min (Sorvall Discovery 90SE). The supernatant was stored at 4 °C as the SDC-SWCNT stock solution.

2.2. Characterization of nanotube physical and chemical properties

The concentration of the SDC-SWCNT stock solution was determined using the Beer–Lambert Law. Absorbance was measured with a UV–Vis–nIR spectrophotometer (Jasco V-670). The absorption spectrum of the nanotube solution was acquired after 50× dilution in PBS. The concentration was calculated using the absorbance value at 907 nm and the corresponding extinction coefficient of $0.02554 \text{ L mg}^{-1} \text{ cm}^{-1}$ [26]. The length distribution of the SDC-SWCNT sample was measured using atomic force microscopy (AFM). A stock solution of SDC-CNTs at 50 mg/L in 100 mM NaCl was diluted 20× in DI H₂O. Free SDC was removed using a 100 kDa Amicon centrifuge filter (Millipore). The filtered SDC-SWCNT sample was deposited on AP-mica for 4 min before rinsing with 10 mL of DI H₂O and blowing dry with argon gas. AP-mica was produced by treating freshly-cleaved mica with aminopropyltriethoxysilane (AP-mica) via the vapor deposition method [27]. An Asylum-MFP-3D-BIO AFM with Olympus AC240TS probe was used to image in AC mode. The data was captured with 2.93 nm/pixel *xy* resolution and 15.63 pm *z* resolution. Nanotube lengths were acquired from over 900 nanotube images. The images were processed using ImageJ software [28].

2.3. Characterization of nanotube photoluminescence

Fluorescence excitation/emission spectra of SDC-SWCNTs were acquired using an apparatus consisting of a tunable white light laser source, inverted microscope, and InGaAs nIR detector. A SuperK EXTREME supercontinuum white light laser source (NKT Photonics) was used with a Varia variable bandpass filter accessory capable of tuning the

output from 500 to 825 nm with a bandwidth of 20 nm. A longpass dichroic mirror (900 nm) was used to filter the excitation beam. The light path was shaped and fed into the back of an inverted IX-71 microscope (Olympus) to pass through a 20× nIR objective (Olympus LCPlan N 0.45 IR) and illuminate a 100 µL aqueous sample of nanotubes at a concentration of 0.2 mg/L in a 96-well plate (Greiner). Emission from the nanotube sample was collected through the 20× objective and passed through a dichroic mirror (875 nm, Semrock), and f/# matched to an Isoplan SCT-320 spectrograph (f/4.6, Princeton Instruments) with a slit width of 410 µm. The light was dispersed using a 86 g/mm grating with 950 nm blaze wavelength. The light was collected by a NIRvana 640 × 512 pixel InGaAs array (Princeton Instruments). Following acquisition, the data was processed to apply spectral corrections for wavelength-dependent excitation power, non-linearity in the InGaAs detector response, and background subtraction.

2.4. Imaging of carbon nanotube photoluminescence within multicellular tumor spheroids (MCTSs)

To image tumor spheres with nanotubes, MCTSs were transferred to a standard tissue-culture treated 24-well plate. This surface was not ultra-low attachment and induced the MCTS to bind in approximately 30 min. The immobilized MCTSs remained stationary during image acquisition to facilitate image deconvolution. Near-infrared fluorescence microscopy was performed using an apparatus consisting of an inverted Olympus IX-71 microscope with 20× nIR objective (Olympus LCPlan N 0.45 IR), beam-shaping optics and 256 × 320 pixel InGaAs array (Photon Etc.). The sample was excited using a continuous wave 730 nm diode laser with an output measuring 780 mW at the sample (Frankfurt Laser, Germany). The excitation beam was shaped to ensure a homogeneous intensity profile, and speckle from the laser was removed with a custom fiber shaker (Photon Etc.). The sample emission was separated from the excitation beam with a 880 nm long-pass dichroic mirror (Semrock). z-Stacks of images were obtained using a piezo-controlled stage (Applied Scientific Instrumentation). To collect emission from SDC-SWCNTs in multicellular tumor spheroids (MCTSs), z-stacks were acquired by collecting images using 500 nm z-axis steps with 0.5 s exposure time. All nIR fluorescence imaging experiments in this work were performed on live MCTSs.

2.5. Image deconvolution and analysis

Near-infrared images of MCTSs were collected with a z-plane spacing of 500 nm, which satisfies the Nyquist criterion for 20× imaging of a sample in water. The z-stacks were corrected for non-uniform excitation across the image field. Voxel parameters were set using Bitplane Imaris 8.0.2 software (1 pixel ~ 1.33 µm), and 3D deconvolution was performed with a blind PSF algorithm with AutoQuant X3 Image Deconvolution Software. The deconvolved image stacks were analyzed using the FIJI program.

Analysis for nanotube fluorescence intensity was performed using a radial intensity profiler FIJI (Fiji Is Just ImageJ) plugin. Other analysis including maximum-intensity-projection, hyperstack-color coding to z-axis distance, and white space detection were performed using FIJI plugins.

2.6. Cell line generation and reagents

All reagents were purchased from Sigma–Aldrich unless stated otherwise. All cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% deactivated FBS, penicillin, streptomycin and 2 mM L-glutamine. To generate high-grade tumor spheroids, we developed a murine cell line, SK-136, derived from an orthotopic model of liver cancer. The cells were generated and harvested from c-MYC/ β -catenin amplified hepatoblastoma cells harvested from FVB mice using a method described previously [29]. The cells were plated on ultra-low attachment 96-well plates (Corning) and incubated for 3 days. The wells were examined with an inverted light microscope to identify the formation of multicellular tumor spheroids. The tumor spheres were centrifuged, trypsinized, and seeded in 75 cm² tissue culture-treated flasks with DMEM. This process was repeated 3 times to generate a sub-clone of spheroid-forming cells.

2.7. Formation of multicellular tumor spheroids (MCTSS)

Both MCF-7 and SK-136 cell lines were expanded on regular tissue culture treated T75 flasks with DMEM growth medium supplemented with 10% deactivated FBS, penicillin, streptomycin and 2 mM L-glutamine. When cells reached 80% confluence, they were harvested with trypsin–EDTA solution (0.25%) and centrifuged for 5 min at 1200 RCF. The cells were suspended in 1 mL of growth medium. Aliquots of 0.1 mL of the cell suspensions were introduced to ultra-low attachment cell culture flasks (Corning®) at a concentration of 50,000 cells in 10 mL of complete DMEM. The formation of nearly geometrically perfect spheroids could be observed as fast as 24 h. After 3 days, the contents of the flask were divided into three. The cell culture media was adjusted to 10 mL and kept for 6 days before further experiments.

2.8. Characterization of multicellular tumor spheroids

The spheroids were placed into 1 mL tubes. The spheres were allowed to settle down by gravity for 2 min and the media was replaced with the fresh media. The spheroids were placed on poly-L-lysine-coated plastic coverslips (Thermonex). The spheroids were then fixed in 2.5% paraformaldehyde (PFA) in 0.075 M cacodylate buffer for 1 h, rinsed in cacodylate buffer, and dehydrated in a graded series of alcohols: 50%, 75%, 95% through absolute alcohol. The samples were then critical-point dried in a Denton Critical Point Dryer JCP-1. The coverslips were attached to SEM stubs and sputter coated with gold/palladium in a Denton Vacuum Desk 1V. Images were obtained with a Zeiss Scanning Field Emission Supra 25 Scanning Electron Microscope. To characterize the internal structure of the spheres, 9 day-old tumor spheroids were fixed in 2% PFA and embedded in paraffin. 10 μ m slices were stained with anti-mouse Ki67 antibody (R&D) or hematoxylin and eosin stain (H&E). H&E images were quantified in FIJI by first applying a color deconvolution to separate the images into three channels. The dominant channel was converted to grayscale, and standard thresholding analysis was used by applying the same threshold cutoff value for all the images. The amount of interstitial space per image was defined as the ratio of white space within an ROI over the total area of each ROI.

3. Results and discussion

3.1. Preparation and characterization of surfactant-solubilized carbon nanotubes

Single-walled carbon nanotubes produced via the high-pressure carbon monoxide process (HiPco) are formed as strongly aggregated carbon nanotube bundles mixed with carbonaceous impurities and metallic catalyst particles [30]. Sonication of the raw carbon nanotube material with an aqueous surfactant, followed by centrifugation, is an efficient method to individually suspend single walled carbon nanotubes and subsequently remove nanotube bundles and impurities [21]. We used sodium deoxycholate (SDC), an anionic biosurfactant that noncovalently coats the nanotube surface to suspend single-walled carbon nanotube complexes [31] (referred to as SDC-SWCNT in this work). SDC-coated nanotubes exhibit intense photoluminescence and are stable for at least 6 months in aqueous environments [31,32].

The absorption spectrum of SDC-SWCNT in PBS (Fig. 1A) exhibited the sharp electronic absorption peaks associated with well-dispersed samples of individually-suspended carbon nanotubes [33]. The stock concentration of SDC-SWCNT was calculated to be 45.5 mg/L and was stored at 4 °C in PBS.

We generated a photoluminescence excitation/emission plot of the SDC-SWCNT sample by acquiring the emission from the bulk solution as a function of the excitation wavelength. As seen in Fig. 1B, each chirality appears as a distinct local intensity maximum. A HiPco sample is composed of over 30 nanotube species, with each species represented by a unique (n,m) chiral index [34]. Using reference values obtained via spectrofluorimetry of surfactant-suspended HiPco nanotubes [35], we identified 17 of the emissive chiralities observable in water. We did not detect spectral changes associated with sonication-induced damage [36].

The subsequent imaging experiments were conducted with a 730 nm laser for optical excitation of the SDC-SWCNT sample. A subset of the nanotube chiralities were found to emit strongly at this excitation wavelength (Fig. 1C). The bulk of the emission resulted from the (10,2), (9,4), (7,6), (8,6) and the (8,7) nanotube species. The diameters of these 5 nanotube chiralities range from 0.88 to 1.03 nm [35].

We characterized the length of the SDC-SWCNT sample via atomic force microscopy (AFM). The SDC-SWCNT sample was plated at an optimum density to allow easy discrimination of single nanotubes. The end-to-end distances of over 900 individual nanotubes were measured (Fig. 2A). A histogram of the lengths of SDC-SWCNT (Fig. 2B) indicates a relatively short nanotube population, with a median length of 232 nm. This length is comparable to that previously identified as optimum for cellular uptake of SWCNTs – approximately 320 nm [38]. The 232 nm mean length of our SDC-SWCNT sample is longer than the experimentally-determined exciton–diffusion length, and can be imaged with single-nanotube sensitivity [39].

3.2. Generation of multicellular tumor spheroid (MCTS) models

Multicellular tumor spheroids grown from SK-136 cells and MCF-7 cells were incubated in an ultra-low attachment flask for 7–10 days. Transmitted light images of the spheroids

showed that they were comparable in size, confirmed by SEM imaging (Fig. 3). By controlling the initial cell-seeding density and the number of days in culture (details in Section 2.7), we could effectively control the size of the spheroids formed. For the nanotube penetration assays, we imaged SK-136 and MCF-7-derived spheroids that were similar in overall size, approximately $\sim 100\ \mu\text{m}$ in diameter. Spheroids of this size were obtained by seeding 50,000 cells in 10 mL of complete media, and allowing the spheres to form for 6 days. This size range was used to balance the realistic physiological properties that result as a function of MCTS size, such as physiological cell-to-cell and cell-to-matrix interactions [11], with the attenuation of near-infrared fluorescence through thick tissues [40].

3.3. Imaging of SWCNT fluorescence through live MCTS

To confirm the ability to detect SDC-SWCNTs embedded through the volume of a tumor spheroid, we incubated 0.25 mg/L of the SDC-SWCNT complexes with SK-136 cells in an ultra-low attachment flask (Fig. 4A). After 3 days, we observed well-formed spheroids, suggesting that extended incubation durations of SK-136 MCTS with SDC-SWCNTs did not hamper the ability of these cells to self-assemble into spherical assemblies. Interestingly, we saw no free nanotubes in solution, indicating that the entire nanotube population in the flask had associated with the SK-136 cells.

We conducted widefield fluorescence imaging with deconvolution to spatially localize SDC-SWCNT emission within the MCTS volume. Deconvolved widefield fluorescence imaging has several advantages over confocal imaging, including faster acquisition time and greater dynamic range [41]. We achieved high signal-to-noise images with 0.5 s exposure time. Each z -stack of SDC-SWCNT embedded within an MCTS was sampled at $0.5\ \mu\text{m}$ intervals and ranged from ~ 30 to $60\ \mu\text{m}$ in total z -axis distance. At 0.5 s exposure, the total time required to image each MCTS was less than 1 min. The short imaging time minimized artifacts caused by the movement of SDC-SWCNTs within the MCTSs and slight movement due to the spheroids themselves.

For the 3D imaging of the SK-136 MCTS, we acquired 140 images at $0.5\ \mu\text{m}$ spacing for a total z -axis distance of $70\ \mu\text{m}$ (Fig. 4B). We color-coded each frame by its distance from the center of the spheroid in the z -dimension, i.e. frames close to the center of the spheroid were colored magenta while frames further away above or below were colored cyan. The resulting image shows magenta-colored emission from the spatial (xy) center of the image, indicating that SDC-SWCNTs were present in the center of the sphere.

3.4. Spatial distribution of SWCNTs within live MCTS models

We examined the initial interactions between SDC-SWCNT complexes and MCTSs by incubating fully-formed tumor spheroids with 0.25 mg/L of the nanotubes in a 24-well plate. After 30 min of incubation at $37\ ^\circ\text{C}$, we removed free nanotubes from solution by repeated washing. The media containing nanotubes was replaced with fresh media. We confirmed, via a Hoechst penetration assay, that 0.005% SDC did not affect the integrity of the cell membrane (Fig. S1). This is the concentration of free SDC in the working dilution of SDC-SWCNTs. After two washes, no free SDC-SWCNTs were observed in solution. At this point, we performed near-infrared fluorescence microscopy. As the nanotubes themselves

are fluorescent and label-free, the detection of nIR fluorescence is unambiguously associated with the presence of nanotubes. No nIR background fluorescence was detected from MCTS in the absence of SDC-SWCNTs (Fig. S2).

We found strong nanotube emission on the perimeter of the SK-136-derived spheroids (Fig. 5A). The outer surface of the SK-136 MCTSs appeared to interact strongly with SDC-SWCNTs, and little penetration was observed within the MCTS, as evident from the lack of magenta-colored fluorescence in the color-coded images. In contrast, SDC-SWCNTs in contact with MCF-7 spheroids appeared as punctate spots, with no particular preference for the perimeter of the spheroid (Fig. 5B). Additionally, the corresponding color-coded image suggests a higher degree of penetration by the SDC-SWCNTs into the spheroid volume, even at this early time point.

We performed a second set of experiments to probe the permeability of the spheroids, where SDC-SWCNTs were incubated with fully-formed tumor spheroids for 4 h at 37 °C. Free nanotubes were removed by washing away the media and replacing with fresh media. We calculated the radial intensity profile for each spheroid (Fig. S3A). Essentially, the radial intensity profile value at a distance x is the intensity within a circle of radius x , divided by the area. As x increases from zero to the full radius of the spheroid, the radial intensity profile maps the fluorescence intensity as a function of distance from the center.

In the three SK-136 spheroids imaged (Fig. 6A), the nanotubes clearly localized on the outer surfaces. The central image through the sphere clearly showed few nanotubes in the center compared to the periphery. The radial density profiles quantified the same observation. This result is consistent with conclusion that the SDC-SWCNTs predominantly localized on the perimeter of SK-136 MCTS with only slight penetration into the interior of the spheroid.

In striking contrast to the SK-136 spheroids, nanotubes exhibited significant penetration into the interior of the MCF-7 MCTSs (Fig. 6B). Accordingly, the radial intensity profiles show nanotube emission to be distributed throughout the spheroid at essentially all distances from the center of the spheroid. We quantified the distribution by calculating the intensity associated with a circle of half the radius of each spheroid i.e. how much nanotube fluorescence is localized within the inner half of each MCTS (Fig. S3B). In SK-136 MCTSs, approximately $30 \pm 5\%$ of the total fluorescence localized within the inner half of the spheroid. In comparison, $46 \pm 8\%$ of the total fluorescence appeared in the inner half of the MCF-7 spheroids. This difference was validated with a two-sample t -test showing a p -value < 0.01 .

We also observed nanotube localization as a function of z -axis position. For a large SK-136 spheroid (Fig. 7A), we color-coded each frame by its z -position. We observed no magenta color within the interior of the spheroid, denoting little signal emanating from the center of the spheroid. A montage of frames separated by 5 μm in z -axis distance confirmed that nanotube emission was limited to the outer surface of this spheroid (Fig. 7B). This result is distinct from MCF-7 spheroids, where magenta-coded emission is clearly visible (Fig. 7C). The corresponding image (Fig. 7D) confirms nanotube emission from within the interior of

the spheroid. Lastly, we quantified the diameters of the SK-136 and MCF-7 spheroids, confirming that they were comparable in size (Fig. 7E).

The nanotube penetration data suggests several conclusions regarding the permeability of the two tumor spheroids. The accumulation on the outer surface of SK-136 MCTSs suggests a relatively high affinity for the nanotubes. Nanotubes appeared to bind to the surface and remain localized there, even after 4 h. In contrast, little binding occurred on the surfaces of MCF-7 MCTSs at both short and long incubation times. Furthermore, the cores of the MCF-7 MCTSs were significantly more accessible, with nanotubes localizing even in the center of the spheroids. In a 2D assay where SDC-SWCNTs were incubated with monolayers of SK-136 and MCF-7 cells, no difference was observed in the relative affinity of SDC-SWCNT for the two cell types (Fig. S4).

3.5. Characterization of the surface and interior of MCTS models

To better understand the differential penetration of nanotubes in these two spheroid models, we employed electron microscopy to analyze the surface of the spheroids (Fig. 8). We observed a dense fibrous matrix on the surface of SK-136s, while MCF-7 spheres exhibited short protrusions. In addition, the SK-136 cells appeared to be tightly fused to one another, while MCF-7 cells appeared distinct. The morphological differences corroborate the relative impermeability of the SK-136 spheroids to the nanotubes. In contrast, the MCF-7 spheroid surface showed no such matrix and was highly permeable to the nanotubes.

In addition to the surface, we characterized the interior structure of the tumor spheroids by histology. H&E staining of 10 μm sections of the spheres showed that SK-136 tissue is denser and has no observable interstitial space (Fig. 9A). The images show that the SK-136 cells are tightly packed together, while MCF-7 spheroids exhibited lower intercellular density and 10-fold more interstitial space as calculated by image analysis of spheroid slices ($11.3 \pm 2.6\%$ for MCF7 and $0.5 \pm 0.15\%$ for SK-136, Fig. 9B). The images of MCF-7 spheroids show interstitial channels several microns long, which is orders of magnitude larger than the radius of gyration for SWCNT with the length distribution used in this work [37].

The spheroid tissues were stained to measure proliferation using the Ki67 antibody. Images showed active proliferation of MCF-7 MCTSs throughout the spheroids, but SK-136 spheroids exhibited greater proliferation at their edges. This result suggests that the abundant interstitial space in the MCF-7 spheroids promotes cell proliferation, likely due to increased distribution of nutrients and oxygen. In contrast, the reduced proliferation in the center of the SK-136 spheroids possibly stems from the lack of interstitial space, leading to insufficient transport of nutrients and oxygen.

4. Conclusions

In this work, the intrinsic near-infrared fluorescence of single walled carbon nanotubes was exploited to interrogate the permeability of multicellular tumor spheroids (MCTS). We developed a tumor spheroid model of liver cancer cells, composed of a sub-clone of cells selected specifically for their ability to form spheroids (SK-136). These tumor spheroids

were compared with MCF-7, a breast-cancer cell line which forms spheroids under low adhesion conditions. Widefield near-infrared fluorescence microscopy in live cells spatially resolved the locations of nanotubes associated with MCTSs. We found that the nanotubes exhibited little penetration into the SK-136 spheroids while remaining on the spheroid surface. In contrast, nanotubes penetrated to the center of MCF-7 spheroids likely due to the large degree of interstitial space measured using conventional histological techniques. We present the use of near-infrared fluorescent carbon nanotubes as a validated and qualitative method to interrogate the permeability of live tumor spheroids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Optical characterization of the SDC-SWCNT sample. (A) Absorption spectrum of SDC-SWCNTs in PBS. (B) Photoluminescence excitation/emission plot with each nanotube chirality labeled. (C) Emission from the sample illuminated with 730 nm excitation.

Fig. 2. Atomic force microscopy characterization of the SDC-SWCNT sample. (A) Individual nanotubes adsorbed on a mica surface. (B) Length distribution measured from approximately 900 nanotubes.

Fig. 3. Transmitted light and SEM images of representative SK-136 and MCF-7 multicellular tumor spheroids. Scale bar is 50 μm.

Fig. 4.

Imaging of carbon nanotube photoluminescence through live MCTSs. (A) Experimental schematic: growth of SK-136 cells in the presence of SDC-SWCNT to form MCTSs with SDC-SWCNTs embedded throughout the volume of the spheroids. (B) Transmitted light image and a color-coded z -axis projection of an SK-136 MCTS incubated with SDC-SWCNTs for 3 days. Scale bar is 50 μm . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Near-infrared fluorescence imaging of SDC-SWCNTs in tumor spheroids after 30 min of incubation. Transmitted light images, maximum intensity projection of SWCNT fluorescence from a 60 μm z-stack, and a color-coded image of a z-stack of (A) SK-136 MCTS and a (B) MCF-7 MCTS. Scale bar is 50 μm . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6.

Near-infrared fluorescence imaging of carbon nanotubes in tumor spheroids. Broadband fluorescence images through the central slices (midpoint of the MCTS in terms of z -axis distance) of (A) SK-136 spheroids and (B) MCF-7 spheroids. Below each image is the radial intensity profile for the corresponding spheroid, plotting the total intensity as a function of the radial distance from the center. Scale bar is 50 μm . (C) Relative contribution of near-infrared emission from the center of the spheroid. p -Value calculated by a two-sample t -test, ** indicates $p < 0.01$, $n = 5$ for each cell type.

Fig. 7.

3D imaging and spatial distribution of SDC-SWCNTs in live tumor spheroids. (A) The transmitted light image of an SK-136 MCTS next to z-axis coded image. Scale bar is 50 μm . (B) Images acquired through the z-axis of the spheroid acquired in 5 μm steps. (C) The transmitted light and z-axis coded images of a representative MCF-7-derived spheroid. Scale bar is 50 μm . (D) Images acquired through the z-axis of the MCF-7 spheroid. (E) The difference in the mean areas of the two tumor spheroid types was not statistically significant. *p*-Value was calculated using a two-sample *t*-test, $n = 5$ for each cell type.

Fig. 8.

SEM images of 9 day-old SK-136 and MCF-7 spheroids. (A) Low magnification images highlight the difference in surface morphology, as individual cells were not distinguishable in SK-136 MCTTs. In contrast, single cells were discernible in MCF-7 spheroids. (B) In high magnification SEM, SK-136 spheroids presented an apparently dense extracellular matrix on their surface. Scale bar is 4 μ m.

Fig. 9.

(A) Top: H&E staining of SK-136 and MCF-7 MCTS cross sections, showing the greater interstitial spacing between cells in MCF-7 spheroids (white regions between purple cells, indicated with arrows). Bottom: IHC staining for proliferation using Ki67 antibody. (B) Quantification of interstitial space percentage for MCF-7 and SK-136 MCTS. Error bars denote standard deviation, $n = 6$. p -Value was calculated by a two-sample t -test, and ** signifies $p < 0.01$. Scale bar is 10 μm . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)