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PROBING INTERACTIONS OF SINGLE-WALLED CARBON NANOTUBES AT THE NANO-BIO INTERFACE

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PROBING INTERACTIONS OF SINGLE-WALLED
CARBON NANOTUBES AT THE NANO-BIO INTERFACE

BY
MITCHELL GRAVELEY

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
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ABSTRACT

Non-covalent hybrids of single-stranded DNA and single-walled carbon nanotubes (DNA-SWCNTs) have demonstrated applications in biomedical imaging and sensing due to their enhanced biocompatibility and photostable, environmentally-responsive near-infrared (NIR) fluorescence. Significant progress has been made in developing robust biological probes based on the sensitive optical properties of SWCNTs, however biological environments introduce complex and dynamic conditions that interact with, and ultimately modulate, their intrinsic properties. These fundamental interactions within biological settings can determine whether a nanomaterial is biocompatible and robust, or cytotoxic and disruptive to biological processes. Thus, a mechanistic understanding of such interactions and their effect on cellular function is crucial to the design of nanoscale technology for biomedical purposes.

In this dissertation, several approaches were applied to study the fundamental interactions which occur at the interface between nanomaterials and biological systems (i.e., at the nano-bio interface). We exploited the optical capabilities of DNA-SWCNTs using spectroscopic and microscopy techniques, most notably via near-infrared fluorescence spectroscopy, hyperspectral fluorescence microscopy, and confocal Raman microscopy. Additionally, we developed an advanced hyperspectral immunofluorescence assay to acquire spectral data directly from fluorescently labeled organelles.

The physical and optical stability of DNA-SWCNTs within a biological environment was first assessed as a function of DNA sequence. Short DNA functionalized SWCNTs exhibited reduced physical and optical stability despite higher uptake and exocytosis rates compared to long DNA sequences in a mammalian cell line. Further
analysis in primary human cells revealed irreversible aggregation of DNA-SWCNTs occurred during intracellular processing upon localization to lysosomes, however the DNA sequence was not a factor in these processes. Additionally, a machine learning model was trained to predict subcellular localization using the Raman spectrum of internalized DNA-SWCNTs, enabling endosomal mapping using a single marker. Next, a hyperspectral counting method was developed and applied to accurately quantify endosomal loading, revealing both inter and intra-cellular heterogeneity in SWCNT uptake. Moreover, initially-aggregated DNA-SWCNT dispersions were found to inhibit intracellular accumulation in three distinct cell lines despite equal uptake compared to a singly-disperse sample, further highlighting the complex nature of these interactions. Finally, we established and optimized the first generation of wearable textile biosensors for continuous, wireless monitoring of oxidative stress in wound healing applications.
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Chapter 1

1.1. Introduction

The rise of nanotechnology in the 21st century has impacted virtually all areas of science and engineering, scaling from medicine to electronics to environmental applications. At the nanometer scale, physical and chemical properties of materials can be vastly different from their bulk form\(^1\) and, in certain cases, offer significant advantages in comparison. In the case of single-walled carbon nanotubes (SWCNTs), the nanoscale structure imparts a catalogue of unique optical, chemical, and mechanical properties,\(^2\) attracting substantial attention among scientific communities across a diverse range of disciplines. In biomedical applications, SWCNTs wrapped with single-stranded DNA provide a biocompatible platform for deep tissue imaging\(^2\,^{3}\) and biosensing applications.\(^4\,^{5}\) The principles which guide such applications, in addition to the research presented in this dissertation, are outlined in the following sections.

1.2. Physical and Electrical Properties of Single-Walled Carbon Nanotubes

Single-walled carbon nanotubes (SWCNTs) are one among many different allotropes of carbon, however their unique shape and chemical structure generates a highly desirable and diverse set of characteristics. The most significant factor in determining the electronic structure is the angle at which the graphitic lattice is joined to form the cylindrical structure.\(^6\) The exact nature of this is defined by two scalar components, \(n\) and \(m\), which are geometrically derived from the chiral vector that joins ends of graphitic layer (Fig. 1.1a). Together, the indices \((n,m)\) are used to define the individual chiral species which are formed during SWCNT synthesis.\(^6\)
Figure 1.1. Physical and electronic structures of SWCNTs. (a) Structure of a graphene lattice showing possible roll-up vectors (red arrow) and the corresponding \((n,m)\) chiral indices.\(^2\) (b) Band diagram of a metallic and (c) semiconducting SWCNT. \(^2\)

The electronic structure of SWCNTs can be generalized by their semiconducting or metallic character, however optical transition energies between valence and conduction bands is unique to each chirality.\(^6\) The low dimensionality and high electron density of a single SWCNT promotes quantum confinement of excitons along the radial direction of a tube.\(^7\) The electronic density of states diagram is thus characterized by sharp maxima known as Van Hove singularities in both valence and conduction bands (Fig. 1.1b-c). The energy differential between the singularities, known as the electronic transition energy \(E_{ii}\), where \(i = 1, 2, etc.\), establishes the optical features of each SWCNT chirality.\(^8\)
In the case of semiconducting chiralities, an energy gap between valence and conduction bands enables band-gap photoluminescence (fluorescence) upon photon absorption if the photon energy matches the $E_{ii}$ of the SWCNT. As a result, variations in $E_{ii}$ across each chirality produce many different excitation/emission profiles (Fig. 1.2a). Moreover, as all carbon atoms reside on the surface, SWCNT fluorescence is sensitive to the local environment. An additional result of their electronic structure is that SWCNT Raman scattering is subject to a resonance condition which, if satisfied, produces resonance Raman scattering (RRS) and increases signal intensity by a factor of $\sim 10^3$ compared to standard Raman scattering. For any SWCNT, if the incident laser energy ($E_L$) matches the energy separation between two electronic states ($\Delta E$), resonance enhancement occurs.

The SWCNT Raman spectrum contains many features which can inform different physical and electronic characteristics, most notably the radial breathing mode (RBM) and G-band. The RBM arises from carbon atom vibrations in the radial direction of the tube and typically occurs between 120 and 350 cm$^{-1}$ (Fig. 1.2b). The RBM frequency ($\omega_{\text{RBM}}$) is inversely proportional to the tube diameter, which itself is proportional to the mass of atoms along the tube circumference. Therefore, it is possible to determine the ($n,m$) assignment of a single SWCNT based on its $\omega_{\text{RBM}}$ and the $E_L$ which maximizes the RBM peak intensity. The G-band is a Raman signature of $C-C$ bond stretching in $sp^2$ carbon materials. It is a combination of two features in SWCNTs, a $G^+$ and $G^-$ component, which can be associated with vibrations along the SWCNT axis and vibration in the circumferential direction, respectively. The total G-band peak intensity is directly related to the mass or concentration of SWCNTs, and thus can be used for various quantitative applications.
1.3. Surface Functionalization, Biocompatibility, and the Potential for Cytotoxicity

In their raw as-produced form, SWCNTs are highly hydrophobic and form bundles with nearby nanotubes, rendering them ineffective for biological applications and in certain cases mildly toxic.\textsuperscript{17-19} Several approaches have enabled SWCNTs to be suspended in an aqueous solution, most often by introducing hydrophilic components \textit{via} surface functionalization. Covalent surface functionalization can significantly enhance water solubility by introducing heteroatoms (N and O) or functional groups (carboxylation) to the pristine \textit{sp}^2 structure.\textsuperscript{2, 20} However, disruption of the \textit{\pi} network directly impacts the electronic structure and can significantly reduce SWCNT fluorescence.\textsuperscript{2}

In contrast, non-covalent functionalization retains the unique optical properties of SWCNTs that are necessary in their biological applications.\textsuperscript{21} Various types of amphiphilic polymers and surfactant molecules can disperse SWCNTs into an aqueous solution while interacting with the SWCNT sidewall by some combination of \textit{\pi} – \textit{\pi} stacking and
hydrophobic interactions. The resultant SWCNT is thus individually dispersed without the significant structural damage which can impact the intrinsic optical properties. Biocompatibility is also dependent on the non-covalent functionalization. Surfactants have been used to stabilize SWCNTs for biological applications, however studies have shown their removal from the SWCNT by proteins after internalization in mammalian cells. Moreover, potential toxicity due to excess surfactant and biological instability have led to the development of more biocompatible approaches.

Single-stranded DNA can disperse single SWCNTs into a stable suspension to create a DNA-SWCNT hybrid with enhanced biocompatibility. π – π stacking of the DNA bases onto the SWCNT sidewall stabilizes the hybrid, while the hydrophilic phosphate backbone allows for aqueous solubility. However, the supramolecular structure which forms is dependent on both the SWCNT chirality and DNA base pair. As a result, certain types of DNA-SWCNTs have been identified to detect specific analytes both in live cells and in vivo. Moreover, the DNA has been shown to enhance the biocompatibility of SWCNTs. Heller et. al. first showed that DNA-SWCNTs could be internalized by murine myoblast cells, where they would remain for weeks without any indication of cellular toxicity. A follow up study in vivo assessed the short- and long-term biodistribution and biocompatibility of DNA-SWCNTs, concluding no risk of acute or long-term health effects due to DNA-SWCNT exposure.

1.4. Experimental Approaches for Bio-Nano Investigations

SWCNTs can be characterized using several spectroscopic methods due to their optical properties. Techniques such as absorbance, fluorescence, and Raman spectroscopies are suitable for solutions containing SWCNTs and provide relevant
information which could characterize various physical and environmental properties.\textsuperscript{6} However, biological studies typically occur within more complex environments, such as cells and tissues, and thus standard spectroscopic methods are unsuitable. Visualization of SWCNTs can be achieved within these systems using near-infrared (NIR) fluorescence microscopy. While useful for SWCNT localization, fluorescence microscopy alone fails to utilize the key optical properties previously described. Hyperspectral microscopy, \textit{i.e.}, spectrally resolved microscopy, is thus advantageous for thorough investigation of SWCNTs.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.3.png}
\caption{Raman intensity maps of a single RAW 264.7 macrophage 32.4 min after introducing nanotubes. Each map represents intensities from a single Raman mode.\textsuperscript{35}}
\end{figure}
NIR hyperspectral fluorescence microscopy of SWCNTs continuously collects the emission over a range of wavelengths, allowing both the intensity and peak position of each chirality to be resolved simultaneously.\textsuperscript{36} This is ideal both for investigating changes to the SWCNT’s local environment within biological systems\textsuperscript{37} and developing/characterizing biosensors for specific processes.\textsuperscript{4, 5, 30, 31} Confocal Raman microscopy is another method which can spatially resolve the many SWCNT Raman features by producing hyperspectral Raman images. SWCNTs have been imaged \textit{in vitro} using confocal Raman microscopy to determine their concentration,\textsuperscript{38} length,\textsuperscript{39} surface functionalization,\textsuperscript{40} and cell type\textsuperscript{41} dependences on cellular uptake, while spectral signatures from multiple nanotube species have also been observed in live cells.\textsuperscript{35} Together, these powerful hyperspectral techniques have the capabilities to probe various complex problems within biological systems.
1.5. References


Chapter 2

Biomolecular Functionalization of a Nanomaterial to Control Stability and Retention within Live Cells

by

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2.1. Abstract

Non-covalent hybrids of single-stranded DNA and single-walled carbon nanotubes (SWCNTs) have demonstrated applications in biomedical imaging and sensing due to their enhanced biocompatibility and photostable, environmentally-responsive near-infrared (NIR) fluorescence. The fundamental properties of such DNA-SWCNTs have been studied to determine the correlative relationships between oligonucleotide sequence and length, SWCNT species, and the physical attributes of the resultant hybrids. However, intracellular environments introduce harsh conditions that can change the physical identities of the hybrid nanomaterials, thus altering their intrinsic optical properties. Here, through visible and NIR fluorescence imaging in addition to confocal Raman microscopy, we show that the oligonucleotide length controls the relative uptake, intracellular optical stability, and retention of DNA-SWCNTs in mammalian cells. While the absolute NIR fluorescence intensity of DNA-SWCNTs in murine macrophages increases with increasing oligonucleotide length (from 12 to 60 nucleotides), we found that shorter oligonucleotide DNA-SWCNTs undergo a greater magnitude of spectral shift and are more rapidly internalized and expelled from the cell after 24 hours. Furthermore, by labeling the DNA with a fluorophore that dequenches upon removal from the SWCNT surface, we found that shorter oligonucleotide strands are displaced from the SWCNT within the cell, altering the physical identity and changing the fate of the internalized nanomaterial. Finally, through a pharmacological inhibition study, we identified the mechanism of SWCNT expulsion from the cells as lysosomal exocytosis. These findings provide a fundamental understanding of the interactions between SWCNTs and live cells as well as evidence suggesting the ability to control the biological fate of the nanomaterials merely by varying the type of DNA wrapping.
2.2. Introduction

Single-walled carbon nanotubes (SWCNTs) have attracted substantial attention in the nanotechnology field due to their unique set of electrical,\(^1\) physical,\(^2\) and optical properties.\(^3\) Their electronic band gap energies are dependent on their chiral identity, denoted by integers \((n,m)\), and vary based on diameter and rollup angle,\(^4\) resulting in semiconducting species which exhibit band gap photoluminescence.\(^3\) Although highly hydrophobic in their raw as-produced form, non-covalent functionalization of SWCNTs using surfactants\(^5\textsuperscript{-6}\) or amphiphilic biomolecules\(^7\textsuperscript{-9}\) has been shown to effectively disperse SWCNTs into aqueous solutions while preserving their intrinsic optical properties. Single-stranded DNA can non-covalently functionalize SWCNTs via \(\pi\)-stacking of hydrophobic bases onto the SWCNT sidewall, while the hydrophilic phosphate backbone allows for significantly enhanced aqueous solubility.\(^10\) These DNA-SWCNT hybrids have shown promise as biological imaging\(^11\) and sensing probes\(^12\) due to their near-infrared (NIR) photoluminescence which is tunable, photostable, and sensitive to their local environment.\(^13\textsuperscript{-16}\)

Hybrids of DNA and SWCNTs are preferred over other non-covalent approaches due to their enhanced biocompatibility,\(^17\) ability to sort single \((n,m)\)-chiralities from parent mixtures,\(^18\textsuperscript{-19}\) and the potential for sensing imparted by the inherent diversity of oligonucleotide sequence.\(^20\) Specific sequence formulations of DNA-SWCNTs have been recently used to detect miRNA \textit{in vivo}\(^21\) in addition to reporting lipid concentrations in live cells\(^22\) and animals,\(^23\) while other approaches have used similar oligonucleotide surface modifications for DNA or siRNA delivery both \textit{in vivo}\(^24\textsuperscript{-25}\) and in plants for controllable gene regulation\(^26\textsuperscript{-27}\). Although these advances are promising displays of the utility of
nanoscale technology, fundamental questions relating the identity of these sensors after prolonged exposure within the biological environment remain largely unexplored. The potential instability of such DNA-SWCNT sensors has direct implications on their ability to perform a designated task, yet the indirect consequence is a nanomaterial with altered properties from its original state. The changed identity of such nanomaterials can cause concerns about toxicity and the unknown effects imparted on the immediate biological environment. While many types of DNA-SWCNTs have been studied extensively in situ both computationally and experimentally, their direct translation to more complex biological systems cannot be assumed.

Nanomaterials can be designed to enter the body via ingestion, injection, inhalation etc., yet macrophages are typically the first cells to detect and internalize foreign molecules regardless of entry method. Macrophages are the immune system’s first line of defense, whether as a primary response to a wound or to engulf foreign substances such as nanoparticles that enter the bloodstream. Various studies have shown that macrophages internalize DNA-SWCNTs via endocytosis and phagocytosis through the endolysosomal pathway, eventually leading to localization within the lysosomes and accumulation in the liver macrophages of mice in vivo. Once entrapped within the lysosomes, SWCNTs can remain for days where they experience biologically low pH and exposure to more than 60 hydrolases meant for catabolic degradation. In these conditions, surface modifications can play a large role on a nanoparticle’s ultimate fate, whether degradation, exocytosis, or lysosomal escape. Given their extremely high surface area to volume ratio, small changes in surface functionalization of SWCNTs can make a major impact on their functionality and stability in such environments.
While oligonucleotide length determines the intrinsic stability of the resultant hybrid with a SWCNT in water, little is known how this length of DNA can affect the stability of SWCNTs in complex intracellular environments. Herein, we present an investigation of the physical and optical stabilities of (GT)$_n$-SWCNTs, where $n$ is the number of sequence repeats, upon internalization into murine macrophages. Near-infrared hyperspectral microscopy in live cells revealed strong correlations between oligonucleotide length, NIR fluorescence intensity, and spectral stability of the examined SWCNTs. All DNA-SWCNT combinations displayed emission shifts to lower energies (i.e. red-shifts) upon interacting with the cells, however several chiralities of (GT)$_6$-SWCNTs exhibited significant blue-shifts over the course of 24 hours, indicating molecular adsorption and/or DNA displacement. We quantified SWCNT concentrations in cells using confocal Raman microscopy, which can detect all SWCNTs including non-fluorescent species, and revealed significant differences in both internalization and lysosome-mediated expulsion of (GT)$_6$- and (GT)$_{30}$-SWCNTs over 24 hours. Finally, we used fluorophore labeled DNA to probe the condition of the SWCNT hybrids as they were processed through the endolysosomal pathway.

2.3. Results and Discussion

To study the effects of single-stranded DNA length on the intracellular optical properties of DNA-SWCNTs, we first non-covalently functionalized HiPco SWCNTs with one of five different (GT)$_n$ oligonucleotides, where $n = 6, 9, 12, 15,$ or $30$ repeats (Fig. S2.1, Table S1). Murine macrophages (RAW 264.7 cell line) were incubated with 1 mg/L of each (GT)$_n$-SWCNT sample for 30 minutes under standard cell culture conditions and replenished with fresh media (hereby referred to as a “pulse” of DNA-SWCNTs). The
majority of cells exhibited substantial NIR broadband fluorescence (ca. 900-1600 nm) when excited by a 730 nm laser (Fig. 2.1a). In agreement with previous studies,15,22,43 NIR fluorescence movies confirmed the internalization of the SWCNTs into endosomal vesicles, which were actively translocated around the cell and could be easily distinguished from background cellular autofluorescence (Movies S1, S2). The NIR fluorescence images were acquired 0, 6, or 24 hours after an initial pulse to assess the DNA length and temporal dependencies on intracellular fluorescence intensity (Fig. 2.1b). In general, the observed NIR fluorescence intensities visibly increased with increasing oligonucleotide length and decreased in time after initial loading into the cells. Histograms constructed from pixel intensity values of the 0- and 24-hour images confirmed that the temporal decreases in intensities were similar amongst all sequences (Fig. 2.1c). Interestingly, the initial intensity distributions were much broader in longer oligonucleotide sequences, suggesting more heterogeneity in the optical response to internalization of these SWCNTs. To quantify the images, the average fluorescence intensities were extracted using a global thresholding analysis to examine the NIR fluorescence from only SWCNTs contained within the cells. We observed significant increases in NIR fluorescence intensities as a function of DNA length (Fig. 2.1d) at each time point. Pearson correlation coefficients ($r_p$) were determined to be 0.846, 0.885, and 0.850 at 0, 6, and 24 hours respectively, confirming that the correlation was linear and statistically significant ($p < 0.001$ for all) between sequence length and fluorescence intensity at any given time point. To mitigate variations in fluorescence quantum yield (Fig. S2.2), the images from Fig. 2.1b were normalized to each (GT)$_n$-SWCNT’s average 0 hour intensity (Fig. S2.3a) and the percent change in initial intensity was quantified (Fig. S2.3b). While most DNA-SWCNTs demonstrated
significant fluorescence quenching in time, (GT)$_6$-SWCNTs first increased nearly 25% at 6 hours before decreasing to more than 25% below the initial intensity after 24 hours. Among other factors, it is known that the fluorescence intensity of a SWCNT is inversely correlated to the density of water in the immediate vicinity. Thus, a removal of water from the surface of (GT)$_6$-SWCNTs through the adsorption of other amphiphilic molecules can explain the increase in intensity observed at 6 hours. Altogether, we propose that these discrepancies in intracellular fluorescence are affected by (1) variations in DNA-SWCNT interaction with and internalization into cells as a function of DNA length, (2) variations in the optical stability of the (GT)$_n$-SWCNT hybrids after interacting with and/or internalizing into the cells, or (3) variable rates of cellular expulsion. Throughout the letter, we will carefully examine these hypotheses.
Figure 2.1. Length-dependent intracellular fluorescence of DNA-SWCNTs. (a) NIR fluorescence image of live macrophages pulsed with (GT)$_{15}$-SWCNTs, along with respective transmitted light image and merged NIR/transmitted light image. Scale bar = 40µm. (b) NIR fluorescence images of macrophages after 30-minute pulse of (GT)$_n$-SWCNTs, imaged over the course of 24 hours. Scale bar = 20µm. (c) Histograms corresponding to the 0- and 24-hour (GT)$_n$-SWCNT images in (b), and (d) average intracellular fluorescence intensities for all examined DNA sequences 0-, 6-, or 24-hours after (GT)$_n$-SWCNT pulse. Experiments were performed in triplicate and are represented as mean ± s.d. (* p < 0.05, ** p < 0.01, according to two-tailed two-sample t-test).

The relationship between stability and fluorescence of DNA-SWCNTs is highly dependent on SWCNT chirality as well as oligonucleotide length. Therefore, we employed NIR hyperspectral fluorescence microscopy to assess the chirality-resolved intracellular stability of the (GT)$_n$-SWCNTs. Using a 730 nm excitation laser, we were able to resolve four distinct bands in the NIR region corresponding to the emission spectra...
of the four brightest SWCNT chiralities, (10,2), (9,4), (8,6), and (8,7) (Fig. S2.4a). Hyperspectral images were acquired immediately following a 30-minute pulse of each (GT)$_n$-SWCNT and after an additional 24 hours of incubation in SWCNT-free cell media (Fig. S2.4b-f). Upon internalization, we observed two common characteristics of all fluorescence spectra: (1) an initial red-shift (i.e. increase in wavelength) of every chirality compared to the spectra acquired in cell culture media and (2) increased intensities of longer wavelength chiralities relative to shorter. To explain the first finding, a red-shift in SWCNT emission spectra can be caused by charged species that interact with the phosphate backbone of DNA and induce a conformational change, ultimately modulating the dielectric environment of the SWCNT and thus shifting SWCNT emission to longer wavelengths. Surface proteins present on cell membranes with high charge densities have been shown to promote this red-shift upon first contact with DNA-SWCNTs before endocytosis. Additionally, the exposure of DNA-SWCNTs to serum-containing cell culture media can produce aggregation that causes spectral modulation by protein-DNA electrostatic interactions. We attribute the initial red-shift observed to a combination of these factors directly following a pulse of (GT)$_n$-SWCNTs, in which the macrophages contained both membrane-bound particles that had not yet been internalized as well as newly-formed endosomal vesicles that essentially forced DNA-SWCNTs to form small aggregate complexes with other phagocytosed proteins and cargo. Regarding the second finding, changes in the ratiometric intensities between shorter and longer emission wavelength SWCNTs have been described by inter-nanotube exciton energy transfer (INEET), a phenomenon that behaves similarly to Förster resonance energy transfer and could be the result of closely packed DNA-SWCNTs contained within lysosomes.
To further compare the NIR fluorescence stabilities across SWCNT chiralities, we converted the emission center wavelengths to energies (meV) and computed the change in emission energy relative to solution controls acquired in cell culture media. Of all the examined oligonucleotide lengths, only the (GT)$_6$-SWCNTs exhibited significant increases in emission energies in multiple chiralities over 24 hours of intracellular processing (Fig. 2.2a, Table S2), while the other DNA-SWCNTs commonly displayed a moderate loss of energy over the same period of time despite presumably identical intracellular conditions. Previous studies have demonstrated that this increase in emission energy could arise from endosomal lipids binding to the exposed SWCNT surface,\textsuperscript{22,23} however the maximum shift in emission energy was not observed at the same time for each chirality, indicating the shifts could be a convolution of multiple physical mechanisms. The influence of a lowered pH to mimic the lysosomal environment was also considered as a potential modulator of SWCNT emission. However, most (GT)$_n$-SWCNTs exposed to cell media with pH of 4.5 experienced random, small (< 2meV) shifts compared to physiological pH (Fig. S2.5). We believe these results indicate that longer oligonucleotides protect the SWCNT surface from competitive molecular adsorption.

To assess spectral shifts in SWCNT emission at the single-cell level, we created hyperspectral maps of the shortest and longest oligonucleotide DNA-SWCNTs (\textit{i.e.} (GT)$_6$ and (GT)$_{30}$, respectively). By fitting each SWCNT-containing pixel of a hyperspectral image to a Lorentzian curve,\textsuperscript{15,22} we were able to overlay transmitted light images with center emission energy maps for the (9,4)-SWCNT, \textit{i.e.} the most abundant and brightest SWCNT under 730 nm excitation in HiPco, and construct histograms for each image to depict the intracellular change in SWCNT emission energy change through time (Fig.
Immediately following a 30-minute pulse (“0 hours”), the average emission energies of (GT)$_6$-SWCNTs and (GT)$_{30}$-SWCNTs were statistically identical. Additionally, by fitting the pixel histograms to a Gaussian distribution, the heterogeneity in the populations could be assessed by examining the full width at half maximum (FWHM). In doing so, we uncovered that the FWHM of (GT)$_6$-SWCNTs immediately after internalization was more than double that of (GT)$_{30}$-SWCNTs. While the emission energy of (GT)$_{30}$-SWCNTs showed little change in time, (GT)$_6$-SWCNTs displayed an 8 meV increase in emission energy and ~50% decrease in FWHM after 6 hours. We believe these DNA-length dependent NIR fluorescence modulations are the result of variations in the relative abundance of oligonucleotide strand ends surrounding each SWCNT. For a given weight of DNA in a DNA-SWCNT hybrid, (GT)$_6$-SWCNTs have 5 times the number of oligonucleotide strand ends than (GT)$_{30}$-SWCNTs assuming a similar degree of surface coverage. We propose that these strand ends can act as initiation sites for amphiphilic biomolecules to interact with and adsorb onto the exposed nanotube surfaces, leading to higher overall surface coverages (reduced water densities) and thus greater emission energies.$^{22,44,48}$ Consequently, the wide FWHM initially displayed by (GT)$_6$-SWCNTs was likely the result of individual SWCNTs responding to the varying local environments through progressing stages of the endosomal pathway, while the reduced FWHM and blue-shift after 6 hours can be attributed to molecular adsorption by lysosomal molecules and rearrangement or displacement of the oligonucleotide wrapping on the majority of SWCNTs. Interestingly, after 24 hours the emission energy slightly decreased closer to its initial value while the FWHM increased towards its initial value, revealing that the hybridized (GT)$_6$-SWCNTs observed at 6 hours were ultimately unstable.
Figure 2.2. DNA length- and SWCNT chirality-dependent intracellular NIR fluorescence stabilities. (a) Heat maps representing average intracellular change in SWCNT emission energy compared to controls in cell culture media, delineated by chirality, as a function of DNA-sequence and time. All experiments were performed in triplicate. Overlay of transmitted light and hyperspectral images of RAW 264.7 pulsed for 30-minutes with (b) (GT)$_6$- or (c) (GT)$_{30}$-SWCNTs. Color scale maps to the fitted center emission energy of (9,4)-SWCNTs and histograms represent center emission energies of all SWCNT-containing pixels in each respective image. Bin size = 4 meV. Gaussian functions were fitted to binned data and overlaid with respective $R^2$, FWHM, and center emission energies ($E_c$). Scale bar = 20 µm.

Due to the large variations in spectral stability of the internalized DNA-SWNCTs, we devised an assay to probe their integrity of the hybrids based on the ability of SWCNTs to quench conventional organic fluorophores. We first constructed (GT)$_6$- or (GT)$_{30}$-SWCNTs with a Cy3 dye attached to the 5’ end of the DNA strand. The initially quenched fluorophore could be restored to a brightly fluorescent state via displacement from the SWCNT surface by a competing molecule (Fig. 2.3a). Note, even partial displacement of the DNA strand can accomplish this process, thus Cy3 dequenching kinetics of the two prepared hybrids are similar despite unequal displacement kinetics by sodium deoxycholate (SDC) determined from NIR fluorescence (Fig. 2.3b). When introduced to
macrophage cells in the same 30-minute pulse method, we observed substantially different dequenching behavior between the two sequences (Fig. 2.3c). The Cy3-(GT)₆-SWCNTs significantly dequenched inside of the cells, reaching a maximum intensity 4 hours after the pulse (Fig. 2.3d) and decreased to its initial intensity after 24 hours. In contrast, dequenching was not observed in the Cy3-(GT)₃₀-SWCNTs at any point, resulting in statistically significant differences in the dequenching behavior of the DNA-SWCNTs, i.e. either partial or full displacement of the DNA from the SWCNTs, within the first six hours.

**Figure 2.3.** Intracellular stability of DNA-SWCNT hybrids. (a) Schematic of experimental design. Cy3-DNA is quenched when wrapping is intact on SWCNTs, but highly fluorescent once displaced. (b) Normalized intensity increase as a function of time after Cy3-DNA is displaced with SDC. (c) Overlaid Cy3-DNA and white light images of RAW 264.7 pulsed with Cy3-(GT)₆ or Cy3-(GT)₃₀-SWCNTs for 30 minutes. Scale bar = 10 µm. (d) Average fluorescence intensities (n ≥ 14 cells) normalized to 0-hour intensity. Error bars represent mean ± s.d. Five-pointed stars represent significance between Cy3-(GT)₆ and Cy3-(GT)₃₀, and six pointed stars represent significance versus initial intensities.
< 0.05, **p < 0.01, ***p < 0.001 according to two-tailed two-sample t-test).

While the observed NIR fluorescence of SWCNTs can be modulated by both concentration and local-environment,\textsuperscript{16,48,50} certain Raman signatures of pristine SWCNTs depend only on concentration,\textsuperscript{51-54} allowing for all SWCNT chiralities in a sample to be represented regardless of fluorescence ability. Therefore, we assessed the localized intracellular concentrations of SWCNTs using confocal Raman microscopy. Small regions were scanned in 0.5 µm intervals to obtain Raman maps of macrophages pulsed with 1 mg/L (GT)\textit{6-} or (GT)\textit{30-}SWCNTs for 30 minutes (Fig. 2.4a). The intensity of the G-band spectral feature, indicative of \textit{sp}\textsuperscript{2} carbon,\textsuperscript{52-53} was correlated to known SWCNT concentrations in the construction of a calibration curve in order to obtain a mass of SWCNTs per analyzed cell (Fig. S2.6). Although the local concentrations varied greatly within a single cell, on average the cells pulsed with (GT)\textit{6-}SWCNTs had more than twice the initial intracellular SWCNT weight than those incubated with (GT)\textit{30-}SWCNTs (Fig. 2.4b). After 24 hours of additional incubation in SWCNT-free cell media, the internal SWCNT concentration of cells dosed with (GT)\textit{6-}SWCNTs decreased by more than 75%, while those dosed with (GT)\textit{30-}SWCNTs displayed statistically similar initial and final concentrations. While cellular uptake of nanoparticles can be influenced by a multitude of factors, we attribute the higher uptake of (GT)\textit{6-}SWCNTs to their higher overall density of DNA per SWCNT as compared to (GT)\textit{30-}SWCNTs,\textsuperscript{31} increasing the probability of interactions between DNA and cellular membrane proteins and thus leading to more nanotubes per engulfing phagosome. Conversely, we surmise that changes in the physical identity of internalized (GT)\textit{6-}SWCNTs are inducing the macrophages to exocytose this sample more rapidly than the stable (GT)\textit{30-}SWCNTs.
To corroborate the unexpected results from confocal Raman microscopy, we performed solution-based Raman spectroscopy to determine the SWCNT concentrations in the supernatants at time 0 and 24 hours, representing the delivered dose and the amount of exocytosed SWCNTs, respectively. The relative delivered dose, calculated as the percent decrease in supernatant G-band intensity after a 30-minute pulse incubation with the cells, confirmed that cells internalized a significantly higher amount of \((\text{GT})_6\)-SWCNTs than \((\text{GT})_{30}\)-SWCNTs (Fig. 2.4c). Furthermore, additional agreement with Raman microscopy data was observed at 24 hours when significantly more \((\text{GT})_6\)-SWCNTs were found in the supernatant as compared to \((\text{GT})_{30}\)-SWCNTs (Fig. 2.4d). We believe these results verified that the decreased intracellular concentration of \((\text{GT})_6\)-SWCNTs after 24 hours was the result of exocytosis and not cell-mediated degradation of the SWCNT material.

Finally, we sought to better understand the mechanisms dictating retention versus exocytosis by identifying the main pathway in which \((\text{GT})_6\)-SWCNTs were being expelled from the cells. Typically, the fate of phagocytosed nanomaterials contained within lysosomes is either regulated secretion, in which the contents are further processed and excreted from the Golgi apparatus, or lysosomal exocytosis via direct fusion with the cell membrane.\(^{55-57}\) Therefore, we devised an assay to compare the intracellular DNA-SWCNT concentration of macrophages after treatment with specific pathway inhibiting compounds. Bafilomycin A1 and Nocodazole, both of which inhibit lysosomal exocytosis,\(^{58-59}\) induced retention of \((\text{GT})_6\)-SWCNTs within cells at significantly higher average concentrations than the control after 24 hours (Fig. 2.4e,f), while little effect was seen on the average concentrations of \((\text{GT})_{30}\)-SWCNTs. Conversely, Brefeldin A and Exo1, inhibitors of
Golgi-mediated exocytosis,\textsuperscript{60-61} did not cause a significant increase in average concentration for either DNA-SWCNT hybrid, suggesting that the main clearance mechanism for DNA-SWCNTs is through lysosomal exocytosis.

One of the main functions of lysosomal exocytosis is to secrete various biomolecules such as proteins, enzymes, or antigens for intercellular communication and illicit an immune response from nearby cells if necessary.\textsuperscript{42, 62} Studies have shown that while SWCNTs with various types of surface functionalization can reduce or prevent cytotoxicity,\textsuperscript{63} pristine SWCNTs are recognized as pathogenic substances upon interaction with Toll-like receptors present on the cell membrane of macrophages,\textsuperscript{64} leading to the secretion of inflammatory cytokines as a mechanism of defense.\textsuperscript{65} Therefore, we believe that the ability of the lysosomal environment to remove the DNA from (GT)\textsubscript{6}-SWCNTs causes the cell to identify the altered nanomaterial as it would a non-functionalized SWCNT (Fig. 2.5a). Once the cell has recognized this material as a foreign body, excretion from the cell via lysosomal exocytosis is initiated in order to illicit an immune response from nearby cells, resulting in a diminished intracellular SWCNT concentration. Conversely, the increased stability provided by a longer DNA wrapping prevents major alterations from occurring in the lysosomal environment and avoids triggering exocytosis, resulting in a high degree of cellular retention (Fig. 2.5b).
Figure 2.4. SWCNT concentration maps determined by confocal Raman microscopy. (a) Representative confocal Raman microscopy images showing G-band intensity and white light images of RAW 264.7 cells pulsed with (GT)$_6$- or (GT)$_{30}$-SWCNTs for 30 minutes. Color map represents local SWCNT pixel concentration derived from G-band intensity calibration (60x objective). Scale bar = 10 µm. (b) Average SWCNT concentration ($n \geq 4$ cells) calculated from total pixel concentration within cellular ROIs. (c) Percent of SWCNT dose internalized compared to the initial incubated concentration, determined from cell media supernatant concentration after a 30-minute DNA-SWCNT pulse ($n = 10$). (d) Concentration of exocytosed SWCNTs in cell supernatant 24 hours after pulse ($n = 4$). (e) Representative confocal Raman G-band intensity maps overlaid on white light images of macrophages 24 hours after a 30-minute pulse of (GT)$_6$- or (GT)$_{30}$-SWCNTs. Indicated cells were treated with exocytosis inhibitors Bafilomycin A1 (200 nM), Nocodazole (2 µM), Brefeldin A (500 nM), or Exo1 (50 µM) 2 hours following DNA-SWCNT pulse. Color map represents local SWCNT pixel concentration derived from G-band intensity calibration (10x objective). Scale bar = 75 µm. (f) Average concentration of SWCNT-containing pixels from all confocal Raman area scans described in (e), ($n \geq 3$ area scans). Error bars are represented as mean ± s.d. for all. (*p < 0.05, ** p < 0.01, ***p < 0.001, according to two-tailed two-sample t-test).
Figure 2.5. Schematic depicting the DNA length-dependent intracellular processing of DNA-SWCNTs. (a) (GT)_6-SWCNTs are internalized into macrophages in large amounts and localize to the lysosomes. There, biomolecules displace DNA from the SWCNT surface and induce lysosomal exocytosis from the cells. (b) (GT)_30-SWCNTs are also located to the lysosomes after internalization into the cells but do not experience DNA displacement. These DNA-SWCNTs with enhanced integrity are retained within the cells.
2.4. Conclusions

We propose that the intracellular processing and ultimate fate of (GT)$_n$-SWCNTs are controlled by the differential stabilities of the hybrid nanomaterials in the lysosomal environment, which correlate strongly to the length of a given DNA strand. The observed intracellular fluorescence intensities were shown to increase with increasing oligonucleotide length, while only the shortest DNA-SWCNTs (i.e. (GT)$_6$) displayed instabilities in NIR fluorescence spectra in time. We have shown that (GT)$_{30}$-SWCNTs are mostly retained within the cells over 24 hours with minimal exocytosis, while (GT)$_6$-SWCNTs expelled more than 75% of the internalized cargo over the same time period despite nearly a two-fold higher amount of initial uptake. The correlation between an increase in emission energy and the dequenching of Cy3-(GT)$_6$ strongly suggests that competitive molecular adsorption to the SWCNT sidewall results in a destabilized structure within the lysosome, increasing the probability of complete DNA displacement or degradation from the SWCNT. Without the biocompatibility afforded by the DNA wrapping, the cell is able to recognize a SWCNT as a foreign pathogenic substance and subsequently secrete its lysosomal contents. These findings accentuate the necessity of biocompatible stability when designing any carbon nanotube-based biosensors while highlighting their sensitivity to small changes in surface chemistry.

2.5. Materials and Methods

**DNA-SWCNT Sample Preparation:** Raw single-walled carbon nanotubes produced by the HiPco process (Nanointegris) were used throughout this study. For each dispersion, 1 mg of raw nanotubes was added to 2 mg of (GT)$_n$ (where $n = 6, 9, 12, 15,$ or $30$) oligonucleotide (Integrated DNA Technologies), suspended in 1 mL of 0.1M NaCl.
(Sigma-Aldrich), and ultrasonicated using a 1/8” tapered microtip for 30 min at 40% amplitude (Sonics Vibrad cell VCX-130; Sonics and Materials). The resultant suspensions were ultra-centrifuged (Sorvall Discovery M120 SE) for 30 min at 250,000 xg and the supernatant was collected. Concentrations were determined using a UV/vis/NIR spectrophotometer (Jasco, Tokyo, Japan) and the extinction coefficient of $A_{910} = 0.02554 \text{L mg}^{-1} \text{cm}^{-1}$.15

**Cell Culture:** RAW 264.7 TIB-71 cells (ATCC, Manassas, VA, USA) were cultured under standard incubation conditions at 37 °C and 5% CO$_2$ in cell culture medium containing sterile filtered high-glucose DMEM with 10% heat-inactivated FBS, 2.5% HEPES, 1% L-glutamine, 1% penicillin/ streptomycin, and 0.2% amphotericin B (all acquired from Gibco). For all cell-related studies, cells were allowed to grow until 90% confluency and used up to the 20$^{th}$ passage.

**Near-Infrared Fluorescence Microscopy of Live Cells:** A near-infrared hyperspectral fluorescence microscope, similar to a previously described system,15 was used to obtain fluorescence images and hyperspectral data within live cells. In short, a continuous 730 nm diode laser with 1.5 W output power was injected into a multimode fiber to produce an excitation source, which was reflected on the sample stage of an Olympus IX-73 inverted microscope equipped with a 20X LCPlan N, 20x/0.45 IR objective (Olympus, USA) and a stage incubator (Okolab) to maintain 37 °C and 5% CO$_2$ during imaging. Emission was passed through a volume Bragg Grating and collected with a 2D InGaAs array detector (Photon Etc.) to generate spectral image stacks. For live cell experiments, cells were seeded into tissue culture treated 96-well plates (Fisher Scientific) at a final concentration of 50,000 cells/ well and allowed to culture overnight in an
incubator. The media was removed from each well, replaced with 1 mg/L each (GT)$_n$-SWCNT diluted in media, and incubated for 30 minutes (pulsed) to allow for internalization into the cells. After this pulse, the SWCNT-containing media was removed, the cells were rinsed 3X with sterile PBS (Gibco) and fresh media was replenished. Well plates were mounted on the hyperspectral microscope to obtain broadband images, transmitted light images, and fluorescence hyperspectral images at each given time point. Hyperspectral data were processed and extracted using custom codes written with Matlab software. All Gaussian curve fits were generated using OriginPro 2018.

**Solution-Based Fluorescence Dequenching Assay:** Cy3-(GT)$_n$- or Cy3-(GT)$_{30}$ oligonucleotides were purchased from Integrated DNA Technologies and used in the creation of DNA-SWCNTs (see above). After ultrasonication and ultracentrifugation, the Cy3-DNA-SWCNTs were filtered 3 times using 100 kDa Amicon centrifuge filters (Millipore) to remove free Cy3-DNA from solution, diluted to 2.5 mg/L, and 1 mL was placed in a plastic cuvette under magnetic stirring. The fluorescence intensity of each sample was obtained in 1-second intervals for 3 minutes using a Perkin Elmer LS 55 fluorescence spectrometer set to 532 nm excitation and 569 nm emission with 3 nm bandwidth. A 10 µL aliquot of a 10% sodium deoxycholate solution (Sigma-Aldrich) was spiked into the Cy3-DNA-SWCNTs after a baseline intensity was established for a final concentration of 0.1% SDC in order to temporally displace the Cy3-DNA from the SWCNTs as previously described.33

**Visible Fluorescence Microscopy in Live Cells:** Cy3-(GT)$_n$-SWCNTs and Cy3-(GT)$_{30}$-SWCNTs were first filtered 3 times using 100kDa Amicon centrifuge filters (Millipore) to remove free Cy3-DNA from solution. The cells were seeded onto 35 mm
glass-bottom petri dishes (MatTek) to a final concentration of 500,000 cells/dish and allowed to culture overnight in an incubator. The media was removed from each well, replaced with 1 mg/L of filtered Cy3-(GT)$_6$-SWCNTs or Cy3-(GT)$_{30}$-SWCNTs diluted in media, and incubated for 30 minutes to allow internalization into the cells. The SWCNT-containing media was removed, the cells were rinsed 3X with sterile PBS (Gibco), and fresh media was replenished for each sample. The petri dishes were mounted in a stage incubator (Okolab) on an Olympus IX-73 inverted microscope with a UApO N 100x/1.49 oil immersion objective for epifluorescence imaging with a U-HGLGPS excitation source (Olympus) filtered through a Cy3 filter cube. The fluorescence images were analyzed by extracting average fluorescence intensity values of individual cell ROIs using ImageJ.

**Confocal Raman Microscopy:** Cells were seeded into 35mm glass bottom microwell dishes (MatTek) to a final concentration of 500,000 cells/dish and allowed to culture overnight in incubator. The media was removed from each well, replaced with 1 mg/L (GT)$_6$-SWCNT or (GT)$_{30}$-SWCNT diluted in media, and pulsed for 30 minutes to allow internalization into the cells. The SWCNT-containing media was removed, the cells were rinsed 3X with sterile PBS (Gibco), and fresh media was replenished. The 0-hour samples were immediately fixed using 4% paraformaldehyde in PBS for 10 minutes, rinsed 3X with PBS, and covered with PBS to retain an aqueous environment during imaging. The 24-hour samples were later fixed using the same procedure. The cells were imaged using an inverted WiTec Alpha300 R confocal Raman microscope (WiTec, Germany) equipped with a Nikon CFI-Achro 60x/0.8 air objective, a 785nm laser source set to 35mW sample power, and collected with a CCD detector through a 600 lines/mm grating. The Raman spectra were obtained in 0.5x0.5 µm intervals with 1s integration time to construct
hyperspectral Raman area scans of cellular regions. A calibration curve was obtained by
recording spectra of known SWCNT concentrations serially diluted in a single pixel
volume with identical acquisition settings. Each spectrum was averaged over 20 scans. A
global background subtraction and cosmic-ray removal was performed using Witec
Control 5.0 software on all acquired confocal Raman data and G-band maximum intensities
were extracted and correlated with known concentrations to produce a linear curve fit using
OriginPro 2018 analysis software. The cellular SWCNT concentration data were produced
by relating the G-band linear equations to each SWCNT-containing pixel and intracellular
concentrations were obtained in individual cell ROIs with the correlation
\[
\frac{SWCNT \ weight}{cell} = total \ ROI \ SWCNT \ concentration \ast total \ SWCNT \ pixel \ volume.
\]

**Solution-based Raman Spectroscopy:** RAW 264.7 cells were seeded into tissue
culture treated 96-well plates (Fisher Scientific) at a final concentration of 50,000 cells/well
and allowed to culture overnight in an incubator. The media was removed from each
well, replaced with 200 µL of 1 mg/L each (GT)\textsubscript{n}-SWCNT diluted in media, and incubated
for 30 minutes (pulsed) to allow for internalization into the cells. After this pulse, the
SWCNT-containing media was collected, the cells were rinsed 3X with sterile PBS (Gibco)
and 200µL of fresh media was replenished. 24-hours later, the supernatant was again
collected from the cells. All supernatant was placed into new 96-well plates and Raman
spectra were obtained using a WiTec Alpha300 R confocal Raman microscope (WiTec,
Germany) equipped with a Zeiss Epiplan-Neofluar 10x/0.25 objective, a 785nm laser
source set to 35mW sample power, and collected with a CCD detector through a 600
lines/mm grating. A calibration curve was obtained by recording spectra of known SWCNT
concentrations serially diluted in a single pixel volume with identical acquisition settings.
A global background subtraction and cosmic-ray removal was performed using Witec Control 5.0 software on all acquired confocal Raman data and G-band maximum intensities were extracted and correlated with known concentrations to produce a linear curve fit using OriginPro 2018 analysis software. The intensity of the G-band was extracted from each spectrum and related to G-band linear fit equations to determine average supernatant SWCNT concentrations.

**Pharmacological Inhibition of Exocytosis Pathways:** RAW 264.7 cells were cultured and dosed with (GT)$_{6}$- or (GT)$_{30}$-SWCNTs following the same procedure previously described, however 2 hours after SWCNT removal cells were spiked with either 200 nM Bafilomycin A1, 58 2 µM Nocodazole, 59 500 nM Brefeldin A, 61 50 µM Exo1, 60 or an equal volume of media. At 24 hours after the initial SWCNT dose (22 hours post inhibitor treatment), cells were fixed using 4% paraformaldehyde in PBS for 10 minutes, rinsed 3X with PBS, and covered with PBS to retain an aqueous environment during imaging. Large cellular regions were scanned in 10 µm intervals using a WiTec Alpha300 R confocal Raman microscope (WiTec, Germany) equipped with a Zeiss Epiplan-Neofluar 10x/0.25 objective, a 785nm laser source set to 35mW sample power, and collected with a CCD detector through a 600 lines/mm grating with a 0.5s integration time. A calibration curve was obtained by recording spectra of known SWCNT concentrations serially diluted in a single pixel volume with identical acquisition settings. A global background subtraction and cosmic-ray removal was performed using Witec Control 5.0 software on all acquired confocal Raman data and G-band maximum intensities were extracted and correlated with known concentrations to produce a linear curve fit using OriginPro 2018 analysis software. The cellular SWCNT concentration data were produced by relating the
G-band linear equations to each SWCNT-containing pixel and average cellular SWCNT concentrations were extracted from each area scan using a custom Matlab script.

**Statistical Analysis:** All statistical measures for hypothesis testing were carried out using two-sample two-tailed unequal variance t-tests in Microsoft Office Excel 2016. All curve fitting and related statistics were performed in OriginPro 2018.
2.6. Acknowledgements

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2.7. References


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Chapter 3

Multispectral Fingerprinting Resolves Dynamics of Nanomaterial Trafficking in Primary Endothelial Cells

by

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3.1. Abstract

Intracellular vesicle trafficking involves a complex series of biological pathways used to sort, recycle, and degrade extracellular components, including engineered nanomaterials (ENMs) which gain cellular entry via active endocytic processes. A recent emphasis on routes of ENM uptake has established key physicochemical properties which direct certain mechanisms, yet relatively few studies have identified their effect on intracellular trafficking processes past entry and initial subcellular localization. Here, we developed and applied an approach where single-walled carbon nanotubes (SWCNTs) play a dual role - that of an ENM undergoing intracellular processing, in addition to functioning as the signal transduction element reporting these events in individual cells with single organelle resolution. We used the unique optical properties exhibited by non-covalent hybrids of single-stranded DNA and SWCNTs (DNA-SWCNTs) to report the progression of intracellular processing events via two orthogonal hyperspectral imaging approaches of near-infrared (NIR) fluorescence and resonance Raman scattering. A positive correlation between fluorescence and G-band intensities was uncovered within single cells, while exciton energy transfer and eventual aggregation of DNA-SWCNTs were observed to scale with increasing time after internalization. An analysis pipeline was developed to colocalize and deconvolute the fluorescence and Raman spectra of subcellular regions of interest (ROIs), allowing for single-chirality component spectra to be obtained with sub-micron spatial resolution. This approach uncovered correlations between DNA-SWCNT concentration, dielectric modulation, and irreversible aggregation within single intracellular vesicles. An immunofluorescence assay was designed to directly observe the DNA-SWCNTs in labeled endosomal vesicles, uncovering a distinct relationship between the physical state of organelle-bound DNA-SWCNTs and the dynamic luminal conditions.
during endosomal maturation processes. Finally, we trained a machine learning algorithm to predict endosome type using the Raman spectra of the vesicle-bound DNA-SWCNTs, enabling major components in the endocytic pathway to be simultaneously visualized using a single intracellular reporter.

3.2. Introduction

Intracellular trafficking is a highly regulated yet diverse system of pathways involving the entry, translocation, and localization of cargo internalized by endocytic cells.\textsuperscript{1-3} The endosomal maturation process, which initializes the main cellular degradation pathway, entails a dramatic series of physicochemical changes; a drop in luminal pH, influx of lysosomal enzymes, and change of ionic environment all promote digestion of vesicle contents.\textsuperscript{3} Most types of engineered nanomaterials (ENMs) gain cellular entry through active endocytic processes,\textsuperscript{4} where they are trafficked through these endosomal pathways before accumulating in lysosomal vesicles.\textsuperscript{5, 6} The key mechanisms of entry and localization of ENMs in biological systems have been extensively studied.\textsuperscript{7} The differential uptake of ENMs based on their size,\textsuperscript{8} shape,\textsuperscript{9} and surface chemistry\textsuperscript{10} have provided insight on targeting, while the formation of a protein corona on the ENM surface is a dynamic process that can further direct biological interactions.\textsuperscript{11, 12} In contrast, the pathways in which these ENMs are subject to after internalization, as well as the effects they might have on these pathways, lack the same depth of clarity despite the importance of these native processes. Proper lysosomal function and trafficking are essential in multiple metabolic pathways which regulate basic cellular functions including autophagy, nutrient degradation, and catabolite export.\textsuperscript{6} In addition, dysfunction of the endosomal-lysosomal pathways has been implicated in Alzheimer’s disease,\textsuperscript{13} lysosomal storage disorders,\textsuperscript{14, 15}
and infectious diseases.\textsuperscript{16} As EMNs are developed for biomedical applications, it is crucial to understand their environmental interactions in complex biological systems at the single-organelle level in order to properly assess their impact on key cellular functions. Intracellular trafficking is accurately described by a range of characteristics across a population of vesicles due to the asynchronous nature of the endocytic pathway,\textsuperscript{3} and thus cell-averaged analysis leads to systematic and compound errors. By tracking the fate of individual endosomal pathway reporters, a more accurate representation of the distribution of processes can be obtained to provide a deeper understanding of the dynamic ENM trafficking system.

The continuous evolution of endocytic vesicles presents a challenging system to investigate since experimental strategies are often limited by the optical capabilities of a given ENM. Fluorescence microscopy and organelle colocalization have routinely been used, providing valuable insight on the spatial and temporal localization of internalized ENMs,\textsuperscript{17, 18} however traditional fluorophores lack environmental responsivity. Hyperspectral microscopy and confocal Raman imaging, which can both resolve spectral data with spatial resolution, are two approaches that can provide information about physical and chemical components within a system. Near-infrared (NIR) hyperspectral fluorescence imaging has enabled environmental sensing within endosomal vesicles of live cells,\textsuperscript{5, 19, 20} while Raman probes have been designed to report intracellular aggregation,\textsuperscript{21} pH,\textsuperscript{22} as well as the molecular composition within various endosomal vesicles.\textsuperscript{23} These approaches provide robust intracellular data which can characterize the complex ENM interactions in biological settings, however more suitable methods for processing and interpreting these
highly dimensional datasets must be developed to enable widespread adoption of these techniques.\textsuperscript{24} 

Single-walled carbon nanotubes (SWCNTs) are among the distinctive materials which exhibit both NIR fluorescence and resonance Raman scattering as intrinsic optical properties,\textsuperscript{25} making them exceptional reporters of physical and environmental changes. The electronic structure of a given SWCNT, including its metallic or semiconducting character,\textsuperscript{26} is dependent on its chiral identity (denoted by the integers \((n,m)\)), which varies by diameter and roll-up angle. Each chirality possesses unique optical transition energies between valence and conduction bands \((E_{ii}, \text{where } i = 1, 2, \text{etc.})\),\textsuperscript{27} and as a result, the intensity of the Raman spectrum from SWCNTs with \(E_{ii}\) resonant with the laser excitation is significantly enhanced by resonance Raman scattering.\textsuperscript{28} At the same time, semiconducting SWCNTs exhibit band gap fluorescence when excited at their \(E_{22}\) resonances (500-900 nm),\textsuperscript{29} however the two observed spectra provide unique information which can detail their physical state and local environment. The Raman spectrum contains multiple features, most notably the radial breathing mode (RBM, 150 – 350 cm\(^{-1}\)) and G-band (\(~1589\text{ cm}^{-1}\)), which can be used to characterize the chiral composition,\textsuperscript{27} concentration,\textsuperscript{30} aggregation state,\textsuperscript{31} and surface chemistry\textsuperscript{32} of a SWCNT mixture. SWCNTs emit fluorescence in the NIR range (\(~900 – 1400\text{ nm}\)), where absorbance and scattering effects from biological samples are at a minimum,\textsuperscript{33} to produce a multi-peak spectrum of all excitable chiralities at a given excitation wavelength. Because SWCNTs exhibit solvatochromism,\textsuperscript{34} the emission from each chirality is subject to position and intensity modulation in response to environmental changes, including analyte binding.\textsuperscript{35}
changes in charge density, aggregation, pH, ionic environment, and reactive oxygen species.

Single-stranded DNA, which can disperse single SWCNTs into a stable aqueous suspension, provides a biocompatible surface functionalization while preserving their advantageous optical properties. DNA-SWCNTs are internalized by cells via energy dependent endocytosis and are reported to localize to intracellular vesicles in the endolysosomal pathway, making them exceptional candidates to nonspecifically target these trafficking processes. Therefore, we propose that simultaneous characterization of (1) the intracellular environmental conditions and (2) the ENMs physical condition can be achieved using DNA-SWCNTs, allowing for a multispectral characterization of the intracellular trafficking processes. Here, we report the internalization and intracellular processing of DNA-SWCNTs within individual cells using a dual-hyperspectral colocalization technique, which correlated intracellular fluorescence and Raman spectra. In tandem, the responsive NIR fluorescence and multi-featured Raman scattering from DNA-SWCNTs detail the changing intracellular environment and the resultant condition of the SWCNT hybrids in primary endothelial cells. We observed a temporal increase in local concentration of DNA-SWCNTs, inducing exciton energy transfer (EET) and aggregation at two distinct rates within concentrated regions due to vesicle coalescence during intracellular processing. Pharmacological inhibitors of endosomal maturation effectively eliminated these events, confirming these processes were responsible, while a DNA sequence dependence was generally not observed. Common regions of interests (ROIs) were identified within individual cells to colocalize the subcellular regions and spectral deconvolution was performed to obtain single-chirality component spectra. A
relationship between concentration, aggregation, and NIR fluorescence modulation was identified within nanoscale regions, exhibiting heterogeneity which varied in time. These dynamic parameters were related to endosomal trafficking using an immunofluorescence assay to colocalize DNA-SWCNT Raman spectra with labeled endosomal organelles, delineating the temporal accumulation and aggregation within lysosomal vesicles. Finally, a machine learning algorithm was applied to identify endosomes and lysosomes based on the Raman spectrum of encapsulated DNA-SWCNTs, enabling the major components of the endocytic pathway to be simultaneously resolved within single cells.

3.3. Results and Discussion

3.3.1. Co-dependence of Fluorescence and G-band Intensities

To develop a spectral model of nanomaterial trafficking, we first identified two formulations of DNA-SWCNTs which were previously shown to induce differential cellular responses when internalized by macrophages as potential intracellular reporters.\textsuperscript{20} HiPco SWCNTs were aqueously dispersed with (GT)$_6$ or (GT)$_{30}$ oligonucleotides via probe-tip sonication and high-speed ultracentrifugation, resulting in highly purified, monodisperse DNA-SWCNT suspensions.\textsuperscript{44} The presence of multiple peaks in both the visible and NIR range of their absorbance spectrum (Fig. S3.1a) confirmed that both ssDNA sequences had suspended a multi-chiral mixture with strong optical absorbance. Excitation with a 730 nm laser produced bright fluorescence in the NIR range from multiple chiralities (Fig. S3.1b), while the apparent differences in peak emission wavelengths were explained by the DNA sequence and nanotube chirality dependence on the hybrid structures.\textsuperscript{45} A 1.58 eV (785 nm) laser source was used to acquire the Raman spectrum of both DNA-SWCNTs (Fig. S3.1c,d), producing sharp peaks in both the radial
breathing mode range (RBM, 150 – 350 cm\(^{-1}\)) and the G-band (~1589 cm\(^{-1}\)). Furthermore, the low intensity of the D-band (~1350 cm\(^{-1}\)) from both samples confirmed the removal of catalyst impurities and amorphous carbon from the raw HiPco materials.\(^{46}\)

Human umbilical vein endothelial cells (HUVEC primary cell line), a common \textit{in vitro} model used to study neovascularization,\(^{47}\) were chosen to represent the endothelium, which would contact any ENMs delivered through intravenous injection. First, HUVEC cultured in grid-labeled glass bottom petri dishes were incubated with 1 mg-L\(^{-1}\) of either (GT)\(_6\)- or (GT)\(_{30}\)-SWCNTs for 1 hour under standard cell culture conditions, after which the SWCNT-containing media was removed and the cells were rinsed with phosphate buffered saline (PBS). Next, the cells were either fixed with paraformaldehyde (considered the 0 hour (h) time point) or replenished with fresh media and allowed to incubate for additional time before fixation. Multiple cells from each condition, identifiable by their location within the grid-labeled culture area, were then imaged at 100\(\times\) magnification using both NIR hyperspectral fluorescence and confocal Raman microscopes. Near identical images of the internalized DNA-SWCNTs were constructed from the broadband NIR fluorescence and G-band spectral regions (Fig. 3.1a-c), each of which depicted distinct subcellular regions containing the (GT)\(_{30}\)-SWCNTs. Histograms of the NIR fluorescence (Fig. 3.1d) and G-band intensities (Fig. 3.1e) were constructed using pixel values from the entire dataset, revealing common temporal changes in the intensity distributions. To quantify these trends, the average intensity fold changes with respect to 0h averages were computed (Fig. 3.1f), showing nearly identical increases of fluorescence and G-band intensities at 6h followed by differential reductions from 6-24h. The G-band intensity, which is linearly dependent on SWCNT concentration,\(^{30}\) could only increase over 6h due
to localized concentration increases in the cell, which we hypothesized could be due to fusion of intracellular vesicles over time. Moreover, we suspect the reduced fluorescence at 24h could indicate an intracellular quenching mechanism such as DNA-SWCNT aggregation. Similar results were obtained from cells incubated with (GT)$_6$-SWCNTs throughout this study, indicating no clear dependence on DNA sequence. These results can be found in supporting information.

**Figure 3.1.** Fluorescence intensity and local concentration of DNA-SWCNTs are co-dependent within single cells. (a) Transmitted light, (b) broadband NIR fluorescence (950-1350 nm), and (c) G-band Raman intensity micrographs of individual cells dosed with 1 mg-L$^{-1}$ (GT)$_{30}$-SWCNTs for 1h and incubated in fresh media for indicated times. (d) Fluorescence intensity and (e) G-band intensity histograms of SWCNT-containing pixels from all examined cells at each time point. The distributions are fitted to log-normal curves and the widths are estimated by the log standard deviation parameter ($\sigma$). (f) Fold change of average fluorescence and G-band intensities with respect to 0h averages. Error bars represent mean ± s.d. with $n \geq 4$ cells per condition. Five pointed stars between columns represent significance between fluorescence and G-band intensities and six pointed stars above columns represent significance versus 0h values. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ according to two-tailed two-sample t-test).
3.3.2. Spectral Features Identify Intracellular Aggregation of DNA-SWCNTs

While the broadband fluorescence intensity from DNA-SWCNTs is the integrated sum of all excited chiralities, the intensity from each individual chirality can be differentially affected.\(^45\) By splitting the fluorescence spectrum from internalized DNA-SWCNTs into emission wavelength bands (Fig. 3.2a), the integrated intensities corresponding to chiralities emitting over various wavelength windows could be quantified independently (Table S1).\(^36\) The integrated intensity from each band was normalized by the total intensity from each cell and the average normalized intensities were computed (Fig. 3.2b), illustrating the relative intensity change of each band over time. We found that bands with lower emission energies (higher wavelength) generally increased over time, while the intensities of the two highest energy bands either decreased or remained constant, revealing certain chirality dependences. The ratiometric intensity of band 4 divided by band 1 (Fig. 3.2c) provided a metric of this trend, which could be fitted to an exponential curve with respect to time. The decreasing and increasing of high and low energy emission intensities, respectively, are characteristics observed from exciton energy transfer (EET) between individual SWCNT chiralities in close proximity,\(^43, 48\) indicating a progressive degree of DNA-SWCNT flocculation occurring in time due to intracellular processing.

The same band deconvolution process was applied to the two dominant regions of the RBM spectrum (Fig. 3.2d), and the average normalized intensity from cells at each time point were determined (Fig. 3.2e), revealing a monotonic increase of band 2 with increasing incubation time. The ratiometric intensity of band 2 divided by band 1 (Fig. 3.2f) quantified the intensity changes, displaying a linear increase in time. To explain these findings, we acquired Raman spectra of DNA-SWCNTs both in solution and aggregated out of solution (Fig. S3.3) and identified the contributing chiralities present in each band
along with their $E_{22}$ transition energies when dispersed in a solution (Table S2). In general, chiralities in band 1 were within the excitation resonance range of the 1.58 eV laser, resulting in higher intensity RBM features compared to band 2 when in solution. However, the dramatic increase of band 2 intensities upon aggregation is explained by a decrease in $E_{22}$ transition energies, bringing these chiralities into resonance with the laser while simultaneously shifting band 1 chiralities out of resonance. Therefore, we attribute the increase of band 2 intensity over time to intracellular aggregation of DNA-SWCNTs, in agreement with previous findings, and propose the ratiometric intensity of band 2 divided by band 1 could be used to quantify the degree of aggregation. The fluorescence and RBM band ratios established could potentially discern between tightly compacted and irreversibly aggregated DNA-SWCNTs, respectively, due to their differing responses to complete SWCNT bundling. The fluorescence intensity of DNA-SWCNTs rapidly decreases upon formation of hard aggregates (i.e., direct contact between exposed SWCNT surfaces), eventually causing EET to reach a maximum level before becoming undetectable due to fluorescence quenching. In contrast, the RBM remains optically active regardless of dispersion quality, and thus a transition from closely packed DNA-SWCNTs to directly aggregated SWCNT bundles could be identified as the point where fluorescence band 4/1 plateaus and RBM band 2/1 continues to increase.
Figure 3.2. Temporal resolution of DNA-SWCNT spectral features indicates aggregation within subcellular regions. (a) Average fluorescence spectrum of (GT)$_{30}$-SWCNTs in single cells after variable lengths of intracellular processing, normalized to the total integrated intensity of each spectrum. Fluorescence bands are indicated by shaded regions. (b) Average normalized fluorescence band intensities from (GT)$_{30}$-SWCNTs in single cells after variable lengths of intracellular processing. Each spectrum was normalized by the total cell intensity, and average normalized band intensities are reported. (c) Ratiometric intensity of fluorescence band 4 divided by band 1, with exponential fit, as a function of time. (d) RBM region of the average Raman spectrum of (GT)$_{30}$-SWCNTs in single cells after variable lengths of intracellular processing, normalized to the total integrated intensity of each spectrum. RBM bands are indicated by shaded regions. (e) Average normalized RBM band intensities from (GT)$_{30}$-SWCNTs in single cells after variable lengths of intracellular processing. Each spectrum was normalized by the total cell RBM intensity, and average normalized band intensities are reported. (f) Ratiometric intensity of RBM band 2 divided by band 1, with linear fit, as a function of time. Error bars represent mean ± s.d. for all, with $n \geq 4$ cells per condition. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ according to two-tailed two-sample t-test).

3.3.3. Inhibition of Endosomal Maturation Reduces Spectral Changes

To confirm the observed spectral changes were induced via vesicle trafficking and endosomal maturation, we investigated the effect of inhibiting these native processes using two mechanistically different pharmacological inhibitors. HUVEC cells were incubated
with DNA-SWCNTs following the same procedure previously described, however the cells were treated with 10 µg-mL\(^{-1}\) Nocodazole (NOC), which polymerizes microtubules and inhibits vesicle motility,\(^{52}\) or 100 µM Chloroquine (CQ), which elevates the luminal pH of endosomal vesicles,\(^{53}\) for 6h following DNA-SWCNT removal. The fold change of G-band and fluorescence intensities with respect to 0h averages were computed for cells treated with both compounds (Fig. 3.3a,b), revealing inhibited increases of G-band intensity from both treatments and a reduction of fluorescence from CQ when compared to the 6h control condition. We extended this analysis to examine the effect of pharmacological inhibition on intracellular EET and aggregation by calculating the fluorescence band 4 divided by band 1 intensity ratio (Fig. 3.3c) and RBM band 2 divided by band 1 intensity ratio (Fig. 3.3d). Significant inhibition of EET occurred from both treatments, while a high degree of variability in aggregation from individual cells was observed. Notably, the two treatments differentially affected the processes of intracellular trafficking and endosomal maturation, resulting in spectral similarities between 0h or 3h untreated cells and 6h CQ or NOC treated cells, respectively (Fig. 3.3e,f). This could be explained by their differing mechanisms of action; CQ prevents endosomal maturation and vesicle fusion by directly inhibiting endosomal acidification,\(^{54}\) while NOC does not inhibit the initial acidification of endosomes,\(^{55}\) but rather prevents cargo from reaching and fusing with more acidic organelles.\(^{52}\) This could allow the initial steps of vesicle maturation to occur during treatment with NOC, while initiation of these processes was immediately inhibited following treatment with CQ.
Figure 3.3. Spectral response to inhibition of endosomal progression. (a) Fold change of G-band and (b) fluorescence intensities, with respect to 0h controls, from intracellular (GT)$_{30}$-SWCNTs after 6h of incubation with Nocodazole (NOC, 10 μg·mL$^{-1}$) or Chloroquine (CQ, 100 μM). Averages from untreated cells at 0h or 6h are shown as blue or red lines, respectively. (c) Ratiometric intensity of fluorescence band 4 divided by band 1 and (d) RBM band 2 divided by band 1 from inhibitor-treated cells after 6h. Error bars represent mean ± s.d. for all, with $n \geq 4$ cells per condition. Stars above error bars represent significance versus 6h untreated cells. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ according to two-tailed two-sample t-test). (e) Average intracellular fluorescence and (f) RBM spectra from inhibitor-treated cells after 6h compared with spectra from untreated cells at indicated times. Each spectrum was normalized to the total integrated intensity.
3.3.4. Segmentation and Colocalization of Intracellular ROIs

Spectra acquired within whole cells can provide ensemble measurements of internalized DNA-SWCNTs, yet the endocytic system is heterogeneous by nature due to a lack of synchrony between processes occurring simultaneously,\(^3\) potentially resulting in measurement bias towards more abundant processes while eliminating observation of rare occurrences within single vesicles.\(^{17}\) To overcome this issue, we developed a method which could segment a single cell into multiple regions of interest (ROIs) while colocalizing the signals from fluorescence and Raman spectra. First, NIR fluorescence and G-band intensity images (Fig. 3.4a) were constructed, roughly colocalized, and binarized to create two equally sized template images. The template images were then segmented into separate matching ROIs (Fig. 3.4b), which were individually confirmed and adjusted manually to account for processing errors and minor discrepancies in ROI locations. The pixels within each ROI were then averaged to create a single fluorescence and Raman spectrum belonging to each subcellular region (Fig. 3.4c). Finally, the full fluorescence spectrum and the RBM of the Raman spectrum were deconvoluted to their chirality components using simultaneous multi-peak fitting algorithms modeled by Voigt\(^56\) and Lorentz\(^57\) line shapes, respectively, while the G-band was fit independently to a single Lorentz curve. The fits were restricted by known peak characteristics from literature,\(^{49, 57}\) and the whole process was carefully monitored to avoid erroneous and over-fitting of spectra.
Figure 3.4. Colocalization of single-cell NIR fluorescence and Raman signals. (a) Transmitted light and brightfield images merged with broadband fluorescence and G-band intensity images, respectively, of a single cell incubated with DNA-SWCNTs. (b) Segmented ROI masks determined from the fluorescence and G-band intensity images in (a). Inset shows magnified fluorescence and G-band intensity pixels corresponding to the indicated region in the masked image. (c) Deconvoluted fluorescence spectrum and RBM range of the Raman spectrum from the outlined cellular ROI in (b). Peaks from the fluorescence and RBM spectrum were fit to Voigt or Lorentz line shapes, respectively.
3.3.5. Fluorescence Modulation of Concentration Subcellular Regions

With the highly improved spatial resolution, we first revisited the correlation between fluorescence intensity and G-band intensity using the colocalized subcellular ROIs. Figure 3.5a shows fluorescence intensity as a function of G-band intensity of all intracellular ROIs containing (GT)$_{30}$-SWCNTs at various time points. A linear regression was performed, and the Pearson coefficient ($r_p$) was calculated for each dataset, revealing a statistically significant positive correlation ($r_p > 0.4, p < 1e-4$) at each time point. The sustained positive relationship between fluorescence intensity and DNA-SWCNT concentration confirmed a common mechanism could explain their temporal fluctuations, including the simultaneous increases observed from 0h to 6h. Since it is not possible for more DNA-SWCNTs to enter the cells after their initial dosing, we conclude that this is a signal of coalescence between vesicles of the endolysosomal pathway.

We next investigated the relationship between sub-cellular DNA-SWCNT concentration and fluorescence emission modulation. Hyperspectral maps of (9,4)-SWCNT emission wavelength and G-band intensity were constructed from the ROIs of cells imaged at 0h or 6h (Fig. 3.5b,c), revealing that red shifted regions were generally correlated to more concentrated areas regardless of incubation time. To quantify this trend, scatter plots were created to compare these two measurements from all ROIs at 0h or 6h time points (Fig. 3.5d,e). Average values from 0h data were used to split the ROI population into four quadrants, thus providing a quantitative measure of their change due to intracellular processing events. The majority of DNA-SWCNT-containing ROIs had simultaneously red shifted and increased in concentration after 6h, however these shifts were completely prevented in cells treated with NOC or CQ (Fig. S3.7), confirming the mechanism related to vesicle trafficking processes. The same trend was observed
comparing the G-band intensity to (8,6)-SWCNT emission wavelength (Fig. S3.9), revealing no apparent dependencies on SWCNT chirality. We partially attribute this correlation to the formation of DNA-SWCNT-protein aggregates once the nanotube concentration reaches a certain threshold, in which densely packed proteins can increasingly perturb the DNA wrapping to increase accessibility of the nanotube surface. This ultimately modulates the local dielectric environment, thus red shifting the SWCNT emission. At the same time, mature late endosomes and endolysosomes undergo a series of changes in their luminal environments, including a fluctuation of ion concentrations and an increase in negative surface charges, which could further decrease the fluorescence emission energy. We surmise that a combination of these factors could be contributing to the observed ROI characteristics.

Figure 3.5. Fluorescence modulation from DNA-SWCNTs within concentrated subcellular regions. (a) (GT)$_{30}$-SWCNT fluorescence intensity as a function of G-band intensity from all intracellular ROIs, with linear fits, at indicated time points. Pearson correlation coefficients, displayed in parentheses, were calculated from scatter data at each time point. Transmitted light images, (9,4)-SWCNT emission maps, and G-band intensity maps of individual cells at (b) 0h or (c) 6h time points. Color scale range encompasses 20
– 80% of values from each ROI map. (d) G-band intensity as a function of (9,4)-SWCNT emission wavelength from all 0h or (e) 6h intracellular ROIs. Average values from 0h data, represented as dashed lines, were used to compute the percent of ROIs in each quadrant.

3.3.6. Intracellular Aggregate Formation is Time Dependent

The RBM of a DNA-SWCNT Raman spectrum is directly related to the resonance of a chirality’s transition energy with the excitation laser source. In the case of SWCNT aggregation, a global decrease of transition energies causes distinctive changes to components of the RBM spectrum (Fig. S3.11). More specifically, we observed that chiralities with \( E_{22} < E_{laser} \) \( (E_{22} > E_{laser}) \) displayed a substantial intensity decrease (increase) upon aggregation in control experiments (Fig. S3.12a), providing a viable basis to probe the dynamics of intracellular aggregation using fitted RBM data (Fig. 3.6a). To visualize the temporal progression of chirality components in all ROI spectra, we constructed a heat map illustrating the relative intracellular intensity change of each chirality with respect to solution intensities (Fig. 3.6b) and included the aggregated controls as a reference. Each chirality was grouped based on its solution \( E_{22} \) value, revealing a clear trend as almost every chirality experienced an intensity change that suggested some amount of intracellular aggregation.

Next, we devised an intracellular aggregation measurement based on the RBM intensity changes observed upon SWCNT aggregation. We identified the ratiometric RBM intensity of \((10,2)/(10,5)\) as a suitable metric for a number of reasons: (1) distinguishable RBM peaks from both chiralities are present in aggregated and solution controls, (2) the \( E_{22} \) of \((10,5)\)-SWCNTs in solution \((\sim 1.58 \text{ eV})\) is directly in resonance with the laser and can only decrease with aggregate formation, (3) the \( E_{22} \) of \((10,2)\)-SWCNTs in solution \((\sim 1.69 \text{ eV})\) is greater than the laser energy and would move into resonance upon aggregate formation, however the expected shift \((\sim 70 \text{ meV})\) due to complete bundling could not
decrease the transition below the laser energy. Therefore, the RBM (10,2)/(10,5) intensity ratio (hereby referred to as the “RBM aggregate ratio”) could directly relate chirality intensities to their transition energies to provide a quantitative measure of aggregation. Significantly different values of the RBM aggregate ratio were calculated from DNA-SWCNT controls of solution and aggregated spectra (Fig. S3.12b), providing reference points to compare against the cellular data. The RMB aggregate ratio was then calculated for every intracellular ROI containing (GT)$_{30}$-SWCNTs and box plots were constructed for the full dataset (Fig. 3.6c), revealing significant differences between the distributions which increased and broadened in time. We then investigated whether a relationship could be identified between the degree of aggregation, environmental conditions within ROIs, and time of DNA-SWCNT processing within the cells. Scatter plots of the RBM aggregate ratio as a function of (9,4)-SWCNT emission wavelength were constructed from ROIs after 0h, 6h, or 24h of incubation with internalized (GT)$_{30}$-SWCNTs (Fig. 3.6d-f). Again, each set of ROIs were split into four populations based on median values from 0h data. The percentage of ROIs with red shifted and increased RBM ratios effectively doubled from 0h to 6h, yet this number plateaued with additional incubation time. At the same time, an increasing number of ROIs became quenched over time (Fig. S3.13). The majority of quenched ROIs, however, exhibited substantial aggregation, as shown in the right-hand column scatter plots of each time point. These spectral characteristics could be indicative of increasingly harsh environmental conditions which the DNA-SWCNTs were subjected to during later stages in the processing pathway, as evidenced by sequential red shifting, aggregate formation, and fluorescence quenching due to excessive aggregation.  $^{50,51}$
Figure 3.6. Intracellular aggregate formation is time dependent. (a) The RBM peak intensity of a single SWCNT depends on its transition energy ($E_{22}$) and the excitation energy ($E_{\text{laser}}$). Aggregation shifts the optical transition to lower energies ($\Delta E_{22}$), resulting in selective intensity enhancement for chiralities brought into resonance ($E_{22}^{\text{soln}} \geq E_{\text{laser}}$) and intensity reduction for chiralities brought out of resonance ($E_{22}^{\text{soln}} \geq E_{\text{laser}}$) with the excitation. (b) Heat map representing the change of intracellular (GT)$_{30}$-SWCNT RBM intensities from solution as a function of chirality and time. Control intensities of intentionally aggregated (GT)$_{30}$-SWCNTs are displayed as a reference. The chirality intensities from each ROI or control replicate were normalized by the total RBM intensity and average values are reported. (c) The ratio of RBM (10,2)/(10,5) intensities of all intracellular ROIs as a function of time. Boxes represent 25-75% of the data, small white squares represent the mean, trend lines connect medians, and dashed lines indicate values from aggregated or solution controls. One-way ANOVA with Tukey post hoc analysis was performed (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 1e^{-4}$). The ratio of RBM (10,2)/(10,5) intensities as a function of (9,4)-SWCNT emission wavelength of all (d) 0h, (e) 6h, or (f) 24h ROIs. Boxed column scatter plots on the right-hand side depict RBM ratio values from ROIs with poorly fitting or quenched fluorescence. Median values from 0h data, represented as dashed lines, were used to compute the percent of ROIs in each quadrant. Shaded regions indicate the RBM (10,2)/(10,5) intensity threshold identified from aggregated controls.

3.3.7. Immunofluorescence Colocalization Identifies Dynamics of Intracellular Trafficking

To corroborate the observed spectral changes with specific organelles from the endosomal pathway, we devised an immunofluorescence assay to colocalize fluorescent antibody markers with Raman spectra from intracellular DNA-SWCNTs. We identified
four specific protein markers to distinguish key intracellular compartments using fluorescent antibody labels. These protein markers included early endosome antigen 1 (EEA1), Ras-related proteins 7 and 11a (RAB7, RAB11a), and lysosomal-associated membrane protein 1 (LAMP1), which corresponded to early endosomes, late endosomes, recycling endosomes, and lysosomes, respectively. HUVEC cells which had been incubated with (GT)$_{30}$-SWCNTs in the same method previously described were fixed, labeled with one of four organelle markers, stained with a fluorophore-conjugated secondary antibody, and imaged with a confocal Raman microscope. Antibody fluorescence was collected by scanning single-cell areas using a low power (10 mW) 532 nm laser excitation source. Although DNA-SWCNTs produce distinct Raman spectra using a 10 mW 532 nm excitation source, control experiments confirmed that the reduced laser power used to acquire fluorescence data could not produce detectable Raman scattering (Fig. S3.15). Next, the sample area was rescanned using a 785 nm laser excitation source to collect hyperspectral Raman maps from the intracellular DNA-SWCNTs within the same area. The immunofluorescence data, generated as a 3D hyperspectral cube, was converted into a confocal fluorescence image by integrating along the spectral dimension, while the corresponding Raman intensity image was produced by integrating the RBM range of the spectral dimension (Fig. S3.16).

Figure 3.7a-d shows representative images of antibody fluorescence intensity, DNA-SWCNT Raman intensity, and merged two-channel images for each investigated condition. Early endosomes and lysosomes appeared to colocalize with the DNA-SWCNTs the most at initial and later time points, respectively, while little colocalization was observed with recycling and late endosomes. To quantify the degree of colocalization
between images, we applied a global series of image processing techniques (see methods for details) to each set of antibody fluorescence or Raman images to create binary representations of the intracellular vesicles and internalized DNA-SWCNTs (Fig. S3.17). The binary images of DNA-SWCNTs were then split into individual ROIs, and the fraction of ROIs which colocalized with the immunofluorescence labels was determined for each cell after various incubation times (Fig. 3.7e). We note that the total fraction of colocalized ROIs exceeded 1 by varying amounts at each time point, however this could be interpreted as partial overlap between the different endosomal markers.\textsuperscript{18,64} Trends were most apparent among early endosomes and lysosomes, in which the fraction of colocalized ROIs monotonically decreased or increased with incubation time, respectively. Small amounts of DNA-SWCNTs colocalized with late endosomes throughout all time points, while recycling endosomes did not appear to play a major role in these processes at any time point. ROI colocalization with lysosomes significantly increased over a 24h incubation time, after which ~80% of all nanotube ROIs were contained within lysosomal organelles. These findings corroborate with previous reports\textsuperscript{5,19} to confirm that DNA-SWCNTs progress through the endosomal pathway before accumulating within lysosomal vesicles. Additionally, we have previously shown that a fraction of DNA-SWCNTs can be released by a cell to the surrounding media through lysosome-mediated exocytosis,\textsuperscript{20} a process in which a lysosome fuses with the plasma membrane to release its contents. The presence of DNA-SWCNTs in early endosomes after 24 hours could be an indication that small amounts of DNA-SWCNTs were released from a cell and subsequently re-endocytosed.
Figure 3.7. Immunofluorescence identifies dynamics of endosomal trafficking. Fluorescence images of cells labeled with (a) EEA1 (early endosome), (b) RAB11a (recycling endosome), (c) RAB7 (late endosome), or (d) LAMP1 (lysosome) antibody markers. Left panels (green) show immunofluorescence markers, middle panels (red) show Raman maps of (GT)$_{30}$-SWCNTs, and right panels show the overlay. (e) Average fraction of SWCNT ROIs colocalized with each vesicle marker with respect to incubation time. Error bars represent mean ± s.d. for all, with $n = 7$ cells per condition. One-way ANOVA with Tukey post hoc analysis was performed ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).
3.3.8. The Lysosomal Environment Induces DNA-SWCNT Aggregation

Next, we extended the colocalization analysis to directly observe the full Raman spectrum from DNA-SWCNTs contained in labeled organelles. For each endosomal marker, we pooled the Raman spectrum from every immunofluorescence-labeled ROI to compare the average and population characteristics. The average Raman spectrum from each set of colocalized ROIs is shown in Figure 3.8a. Differences between spectra were most apparent in the RBM region (Fig. 3.8b), which exhibited sequentially increasing high wavenumber peak intensities as the endocytic vesicle type progressed from early/recycling endosomes to late endosomes and lysosomes. To quantify these changes in the entire ROI population, we calculated the RBM aggregate ratio of each ROI and constructed a box plot to show aggregation as a function of vesicle type (Fig. 3.8c). The RBM aggregate ratio was relatively constant between the early/ recycling/ late endosomes, however a clear and statistically significant increase in aggregation occurred once the DNA-SWCNTs progressed to the lysosomes. Notably, the G-band intensity was mostly unaffected by vesicle type, increasing instead with the overall incubation time (Fig. S3.18). These findings delineate lysosomes as the final intracellular destination for DNA-SWCNTs, where the catabolic environmental conditions promote irreversible bundling between nanotube surfaces. Moreover, the concentration of DNA-SWCNTs within the lysosomes does not appear to contribute to this type of direct-contact aggregation.
Figure 3.8. DNA-SWCNTs aggregate within lysosomes. (a) Average Raman spectrum and (b) RBM spectrum of (GT)\textsubscript{30}-SWCNTs colocalized with endosomal markers. Each spectrum was normalized by the total integrated intensity. (c) The ratio of RBM (10,2)/(10,5) intensities of all ROIs colocalized with endosomal markers. Boxes represent 25-75% of the data, small white squares represent the mean, black line represents the median, and whiskers represent mean ± s.d. One-way ANOVA with Tukey post hoc analysis was performed (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 1e-4).

3.3.9. Endosomal Mapping via DNA-SWCNT Intracellular Reporters

In addition to the RBM changes displayed in Figure 3.8b, a number of minor but clear differences could be identified between various Raman features of immunofluorescence-labeled DNA-SWCNTs (Fig. S3.19). We speculated that the unique Raman profiles obtained from each type of organelle could act as their spectral marker, enabling classes of endosomal vesicle to be identified by the encapsulated DNA-SWCNTs. To investigate this concept, we implemented an artificial neural network (ANN), a machine learning algorithm with exceptional performance in data classification and pattern recognition applications.\textsuperscript{65} Using the Raman spectra of immunofluorescence-labeled ROIs as the training dataset (see methods for details), the model was built to recognize early endosomes, late endosomes, and lysosomes based on the DNA-SWCNT Raman spectrum of an input ROI (Figure 3.9a). The model was validated with a 10-fold cross-validation and
performance was assessed from the resulting confusion matrix and receiver operating characteristic (ROC) curves (Figure S3.20, Table S3), suggesting the ability to identify vesicle localization with an accuracy of over 84%. The model was then applied to classify the intracellular localization of the non-labeled dataset of (GT)\textsubscript{30}-SWCNT ROIs using the Raman spectrum as the input variable. Endosomal maps were constructed by coloring each ROI by vesicle classification (Fig. 3.9b), enabling simultaneous resolution of DNA-SWCNT localization through key stages of the endosomal trafficking pathway. The percent of ROIs classified to each vesicle type was calculated for ROI populations with respect to time (Fig. 3.9c), showing similar trends to those observed in figure 3.7e. Additionally, a bar chart was constructed to summarize ROI classification for all conditions examined (Fig. 3.9d). Notably, the classification of ROIs from cells dosed with CQ and NOC most closely resembled 0h and 3h untreated populations, respectively, corroborating with the observations from Figure 3.3e-f. To confirm that characteristics from the model-classified ROIs matched the original immunofluorescence-labeled data, the average RBM ratio was compared between each type of vesicle (Figure 3.9e). Similar values were obtained for each endosomal marker, suggesting the model had predicted the same characteristics identified from the immunofluorescence-labeled data.

Next, the SWCNT fluorescence characteristics of model-classified ROIs were examined. The average NIR fluorescence spectrum from ROIs assigned to each endosomal marker exhibited unique features (Fig. 3.9f), suggesting the DNA-SWCNTs were responsive to the dynamic environmental conditions. Several fluorescence characteristics were identified and compared to elucidate the environmental differences between endosomal vesicles. The integrated fluorescence intensity was observed to remain constant.
between early and late endosomes before significantly increasing within the lysosomes (Fig. 3.9g). Chirality-dependent intensity changes, specifically the intensity decrease (increase) of low (high) wavelength peaks, were quantified using the ratio of fluorescence (8,6)/(6,5) fitted peak intensities (Fig. 3.9h). The ratiometric intensity progressively increased in each sequential endosomal vesicle, with mean values from early endosomes being significantly lower than late endosomes and lysosomes. Histograms of the (9,4)-SWCNT emission wavelength were fitted to Gaussian distributions to assess the differences in dielectric environment (Fig. 3.9i). Relative to the emission wavelength of early endosomes, a blue-shift and a red-shift was identified from late endosomes and lysosomes, respectively.

Although the trend between vesicles varied considerably across the examined fluorescence characteristics, these observations could be explained by a combination of physical and environmental factors. To provide additional context, the same fluorescence properties were calculated from control fluorescence spectra of (GT)₃₀-SWCNTs acquired in a series of biologically relevant conditions, namely varying pH, salt concentration, and protein corona (Fig. S3.21-22). A heat map was constructed to summarize these effects with respect to stock DNA-SWCNT conditions (Fig. S3.23). Lysosome-classified ROIs exhibited the highest fluorescence intensities and fluorescence (8,6)/(6,5) intensities. Compared to early and late endosomes, the lysosomal lumen possesses high concentrations of Ca²⁺ along with an abundance of enzymes, other proteins, and amino acid catabolites, all of which could promote these intensity changes. In addition, the ratiometric (8,6)/(6,5) intensity is responsive to exciton energy transfer (EET) between adjacent DNA-SWCNTs, hence lysosomal aggregation can contribute to the high ratiometric intensities. On average,
the emission wavelengths from (9,4)-SWCNTs were also the longest (red-shifted compared to early endosomes) in lysosome-classified ROIs. While the luminal pH (~4.5) caused a blue-shift of (9,4)-SWCNT emission wavelength in control experiments, we hypothesize that other lysosomal components preferentially interact with the DNA wrapping, thus controlling the local dielectric and red-shifting the fluorescence emission. For example, the presence of divalent cations, amphiphilic proteins, lipids, and charged residues could strongly interact with DNA-SWCNTs and prevent the effects of lowered pH. Late endosome-classified ROIs also exhibited significantly higher fluorescence (8,6)/(6,5) intensity ratios than early endosomes, however the average emission wavelength from (9,4)-SWCNTs was blue-shifted by ~1.4 nm. Since late endosomes are derived from the vacuolar domains of early endosomes, concentrated levels of endocytosed proteins are selectively retained from the tubular endosome structure, thus DNA-SWCNT-protein interactions could increase the fluorescence (8,6)/(6,5) intensity. At the same time, the rapid drop in pH upon late endosome formation in the absence of interactive lysosomal components could explain the blue-shift observed from late endosome-classified DNA-SWCNTs.
Figure 3.9. Machine learning facilitates endosomal mapping via DNA-SWCNT Raman spectra. (a) Schematic depicting the general process of building and applying an artificial neural network. Full Raman spectra from ROIs colocalized with endosomal markers were pre-processed by principal component analysis (PCA) and input to the untrained neural network (left) to establish and connect weighted variables to differentiate input classes. Next, the unknown dataset was pre-processed, input to the trained neural network (right), and categorized to the vesicle type with the highest classification probability. (b) Endosomal maps overlayed on transmitted light images of single cells at various time points, depicting the predicted ROI class output from the convolutional neural network. (c) The percent of ROIs categorized as early endosomes (EE), late endosomes (LE), or lysosomes (LY) as a function of time. (d) Stacked bar graph showing the percent of ROIs categorized as each vesicle type. (e) The average RBM (10,2)/(10,5) intensity of endosomal vesicles from immunofluorescence-labeled spectra and model-classified spectra. A two-tailed two-sample t-test was performed between groups to determine significance ($p > 0.05$ for all). (f) The average fluorescence spectrum of each predicted vesicle type, normalized by total intensity. Box plots depicting (g) integrated fluorescence intensity and (h) fluorescence (8,6)/(6,5) intensity from model-classified ROIs. Boxes represent 25 – 75% of the data, small white squares represent means, black lines represent medians, and whiskers represent mean ± s.d. One-way ANOVA with Tukey post hoc analysis was performed ($*p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 1e-4$). (i) Histograms show the (9,4)-SWCNT emission wavelength from all categorized ROIs. Bin size = 2 nm. Gaussian functions were fitted to binned data and overlayed with the fitted center wavelength indicated.
To illustrate these findings in terms of sequential trafficking events, we propose a schematic (Fig. 3.10) to describe the intracellular processes which ultimately control the fate of internalized nanomaterials. An emphasis is placed on the endosomal maturation process, in which a highly coordinated series of events dramatically transform the endosomal vesicles, thus altering their physicochemical properties and controlling the luminal environment. The DNA-SWCNTs first enter the cell through active endocytic processes (i), where they are transported into early endosomes along with a cohort of endocytic cargo. Continuous recycling of transport vesicles sends the majority of internalized cargo back to the plasma membrane, however DNA-SWCNTs are retained by the early endosome to be included in the degradation pathway. Conversion into a late endosome is followed by a rapid decrease in luminal pH (ii), thus initiating the endosomal maturation process. Next, the encapsulated DNA-SWCNTs experience a coordinated series of physicochemical transformations (iii), including luminal acidification, ion flux across the endosomal membrane, and changes in size and morphology. DNA-SWCNTs identify late endosome containment with blue-shifted fluorescence emission (a) and chirality-dependent intensity modulation (b), while temporal fusion of DNA-SWCNT-containing vesicles drives an increase in luminal nanotube concentrations (e). Acidification continues until lysosomal proteins, hydrolases, and other components are activated, eventually transforming the late endosome into a lysosome (iv). Here, the components of lysosomal environment bind to and interact with the DNA wrapping, promoting increasingly complex interactions between the DNA-SWCNTs and luminal biomolecules to ultimately modulate the dielectric environment and induce a red shift of DNA-SWCNT fluorescence emission (c). After a period of time in the lysosomes (v), internalized DNA-
SWCNTs aggregate (d) and NIR fluorescence emission is quenched, marking the end of the trafficking pathway. In contrast, pharmacological inhibition of endosome maturation effectively suppresses these key spectral changes by preventing endosome acidification (CQ) or physically obstructing progression (NOC) by reducing endosome motility.

**Figure 3.10.** Schematic summarizing DNA-SWCNT trafficking through the endosomal pathway. (i-v) Depiction of sequential steps of DNA-SWCNT intracellular processing and (a-e) the identifiable spectral changes resulting from these processes.

3.4. Conclusions

Herein, we developed a correlative approach to simultaneously study the intracellular fate of internalized nanomaterials and dynamics of the endosomal maturation processes, enabling a comprehensive analysis of DNA-SWCNT trafficking in the context of the endosomal pathway. The fluorescence and Raman spectra from whole cells were first examined, revealing an increase of fluorescence and G-band intensities from 0h to 6h of equal magnitude. At the same time, intensity changes from fluorescence emission bands suggested the occurrence of EET between closely packed DNA-SWCNT chiralities. Although the extent of EET plateaued at 6h, direct aggregation of internalized DNA-SWCNTs was indicated by changes in RBM band intensities, which monotonically scaled with incubation time. To confirm these events were induced by progression of the
intracellular trafficking pathway, cells were treated with two pharmacological inhibitors of vesicle maturation, both of which suppressed the identified spectral changes over 6h via distinct mechanisms of action.

We developed a segmentation process which could colocalize the Raman and fluorescence spectrum of internalized DNA-SWCNTs within nanoscale regions. A simultaneous multi-peak fitting algorithm, which provided single-chirality resolution of multicomponent spectra, was used to quantify the relevant spectral features and characterize the conditions of each cellular ROI. This approach determined correlations between fluorescence intensity, DNA-SWCNT concentration, fluorescence emission wavelength, and aggregate formation within cellular ROIs for the first time, illustrating the effect of changing intracellular conditions on the internalized DNA-SWCNTs. Immunofluorescence markers for specific endosomal vesicles were applied and colocalized with DNA-SWCNT Raman spectra, enabling spectral signatures to be directly observed from nanotubes encapsulated by specific organelles. Significant colocalization with lysosomal markers confirmed temporal accumulation of DNA-SWCNTs, while colocalized spectral data implicated lysosomes in the irreversible aggregation observed between bare nanotube surfaces, presumably an effect of the catabolic environmental conditions.

Finally, we trained a machine learning algorithm to predict endocytic vesicle type using the Raman spectrum of DNA-SWCNTs contained within. ROIs from non-labeled hyperspectral datasets were classified to one of three endocytic organelles and endosomal maps were constructed, enabling major components in the endocytic pathway to be simultaneously visualized in whole cells. Additionally, SWCNT fluorescence properties
were examined across the model-classified endosomal vesicles and interpreted with respect to known luminal conditions, thereby relating the endosomal maturation process with the observed spectral dynamics. The approaches detailed in this study could be extrapolated to investigate multiple aspects of ENM-cell interactions. We envision that the spectral immunofluorescence colocalization assay, for example, could be adapted to study intracellular dynamics of other types of ENMs, including the multitude of surface enhanced Raman scattering (SERS) reporters designed for advanced biological applications. Furthermore, this work demonstrates the potential of machine learning techniques for data classification at the single-organelle level, providing a versatile framework to connect multivariate data from complex biological systems.

3.5. Materials and Methods

**DNA-SWCNT Sample Preparation:** Raw single-walled carbon nanotubes produced by the HiPco process (Nanointegris) were used throughout this study. For each dispersion, 2 mg of (GT)$_6$ or (GT)$_{30}$ oligonucleotide (Integrated DNA Technologies) was added to 1 mg of raw nanotubes, suspended in 1 mL of 0.1M NaCl (Sigma-Aldrich), and ultrasonicated using a 1/8” tapered microtip for 30 min at 40% amplitude (Sonics Vibracell VCX-130; Sonics and Materials). The resultant suspensions were ultra-centrifuged (Sorvall Discovery M120 SE) for 30 min at 250,000 × g and the top ~80% of the supernatant was collected. Concentrations were determined using a UV/vis/NIR spectrophotometer (Jasco, Tokyo, Japan) and the extinction coefficient of $A_{910} = 0.02554$ L mg$^{-1}$ cm$^{-1}$.$^{56}$

**Cell Culture:** HUVEC cells (ATCC, Manassas, VA, USA) were cultured under standard incubation conditions at 37 °C and 5% CO$_2$ in endothelial growth media (EGM
BulletKit CC-3124, Lonza). For all imaging experiments, cells were seeded into grid labeled collagen-coated 35mm glass bottom microwell dishes (MatTek) to a final concentration of 5,000 cells/cm² and allowed to culture for at least 48 hours, with regular media replacement every 24 hours. To dose the cells, the media was removed from each culture dish, replaced with 1 mg·L⁻¹ (GT)₆-SWCNT or (GT)₃₀-SWCNT diluted in media, and incubated for 1 hour to allow internalization into the cells. The SWCNT-containing media was removed, the cells were rinsed 3X with sterile phosphate buffered saline (PBS, Gibco), and fresh media was replenished. The 0h samples were immediately fixed using 4% paraformaldehyde in PBS for 10 minutes, rinsed 3X with PBS, and covered with PBS to retain an aqueous environment during imaging. The 3h, 6h, and 24h samples were later fixed using the same procedure.

**Near-Infrared Fluorescence Microscopy:** A near-infrared hyperspectral fluorescence microscope, similar to a previously described system, was used to obtain the hyperspectral fluorescence images from fixed cell samples. Briefly, a continuous 730 nm diode laser with 1.5 W output power was injected into a multimode fiber to produce an excitation source, which was reflected on the sample stage of an Olympus IX-73 inverted microscope equipped with a UApo N 100×/1.49 oil immersion IR objective (Olympus, USA). Emission was passed through a volume Bragg Grating and collected with a 2D InGaAs array detector (Photon Etc.) to generate spectral image stacks. Fixed cell samples were mounted on the hyperspectral microscope to obtain transmitted light images and hyperspectral images from internalized DNA-SWCNTs in individual cells at each time point. Hyperspectral data were processed and extracted using custom codes written with Matlab software.
**Confocal Raman Microscopy:** Each cell sample was imaged with an inverted WiTec Alpha300 R confocal-Raman microscope (WiTec, Germany) equipped with a Zeiss Epiplan-Neofluar Pol Oil 100×/1.3 objective, a 785 nm laser source set to 35 mW sample power, and collected with a UHTS 300 spectrograph (600 lines/mm grating) coupled with an Andor DR32400 CCD detector (−61 °C, 1650 x 200 pixels). Small cellular areas were scanned, and spectra were obtained in 0.29 × 0.29 μm intervals using 0.2 s integration time per spectrum to construct hyperspectral images of individual cells. Global background subtraction and cosmic-ray removal were performed on each scan using Witec Project 5.2 software. Hyperspectral data was extracted and processed using custom codes written with Matlab software.

**Pharmacological Inhibition of Endosomal Maturation:** HUVEC cells were cultured and dosed with (GT)_6-SWCNTs or (GT)_30-SWCNTs following the same procedure previously described, however the media used to replenish the cells after DNA-SWCNT removal and PBS rinsing was spiked with 10 µg-mL⁻¹ Nocodazole (NOC) or 100 µM Chloroquine (CQ). The cells were incubated for 6 hours following the addition of inhibitors before fixation in 4% paraformaldehyde in PBS for 10 minutes. The cells were then imaged following the same procedure used for the untreated cells.

**ROI Colocalization:** ROI colocalization was carried out on all hyperspectral ‘cubes’ (i.e., three-dimensional datasets in which x and y dimensions are spatial coordinates, the z dimension is the spectral coordinate, and the pixel value corresponds to the spectral intensity) following initial background subtraction and cosmic-ray removal steps. Using custom Matlab codes, composite fluorescence images were created by integrating the entire spectral dimension and composite Raman images were created by
integrating the G-band or RBM regions of the spectrum. The fluorescence and Raman images were first roughly colocalized by applying an intensity threshold to each image, binarizing and segmenting each image individually, determining the intensity-weighted centroid of each segmented ROI, and iteratively overlaying the images to find the coordinates which minimize the root-mean-square deviation (RMSD) of similar ROIs. Next, the composite images and cubes were cropped and imported to the open-source image processing software FIJI. The G-band composite images were segmented into ROIs, which were manually adjusted to ensure consistency in the segmentation process, and compared with the RBM region of the Raman cube. The ROIs determined from the Raman data were then transferred to the fluorescence images, where each ROI was manually adjusted to account for minor discrepancies in their location and shape. Once all ROIs were determined for a cell, their locations were imported to Matlab for further analysis. Of note, the resolution of the confocal Raman area scans was experimentally optimized prior to data acquisition to match the pixel size of the hyperspectral fluorescence microscope, and thus the spatial resolution of the two cubes was essentially the same. ROI location adjustments mainly accounted for minor rotations of the imaging field as the result of mounting on two separate instruments, and ROIs which could not be clearly identified as the same were disregarded. In certain cases, ROIs which exhibited strong Raman intensities in both the G-band and RBM regions displayed little to no fluorescence intensity from the same spatial location. These ROIs were considered to be colocalized and accurate only if their G-band and RBM integrated intensities were comparable with other ROIs in the same image and other nearby ROIs which exhibited fluorescence were colocalized with Raman signal. The appearance of visible, dark spots within these ROIs in transmitted light images obtained
with the hyperspectral fluorescence microscope were also used to verify the presence of DNA-SWCNTs which were quenched. An example of quenched fluorescence from cellular ROIs is provided in the supporting information. Any and all ROIs which could not be definitively colocalized were disregarded from further data analysis.

**Multi-Peak Fitting of the Fluorescence and RBM Spectra:** The colocalized ROI data for the fluorescence and Raman cubes were used to obtain average spectra from each ROI, which was processed with a custom Matlab pipeline. First, the average fluorescence and Raman spectrum from each ROI were calculated by averaging pixel intensity values in their x-y direction and extracting the spectral z dimension from each cube. The fluorescence spectrum from each ROI was fitted to an additive combination of Voigt line shapes corresponding to the single chirality component spectra, and only chiralities which were identified to significantly contribute to the fluorescence spectrum were included in the fitting process. The peak center wavelength and width parameters of each chirality were allowed to vary independently, but each parameter was limited within the same set of constraints. The area under the curve and global offset were restricted to non-negative values. The radial breathing mode of the Raman spectrum in each ROI was fit to an additive combination of Lorentz line shapes corresponding to the single chirality component spectra. The chiralities which were included in the fits were chosen based on (1) their resonance with the excitation laser, determined by empirical Kataura plots found in the literature,49 (2) their presence when spectra were obtained from solution controls, and (3) their presence when spectra were obtained from aggregated samples. Peak centers were initially specified and allowed to shift within a very small window, however each spectrum was restricted to a single full width and half maximum for all peaks.57 The area under the
curve and global offset were restricted to non-negative values. $r^2 > 0.95$ was used as a cutoff to remove poor-fitting ROI data from further analyses.

**Primary and Secondary Antibodies:** Rabbit anti-EEA1 (# MA5-14794), rabbit anti-Rab7 (# PA5-52369), and rabbit anti-Rab11a (# 71-5300) primary antibodies and goat anti-rabbit IgG Alexa Fluor 532 (# A-11009) secondary antibodies were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Rabbit anti-LAMP1 (# ab24170) was purchased from Abcam (Cambridge, Ma, USA).

**Immunofluorescence Staining Procedures:** HUVEC cells were cultured and dosed with $(GT)_{30}$-SWCNTs using the same procedure previously described. Two separate protocols were used for immunofluorescence staining procedures. (1) Samples stained against EEA1, RAB7, and RAB11a were fixed with 4% PFA for 15 minutes and rinsed 3 times with PBS. Cells were permeabilized and blocked with saturation solution (PBS; 10% goat serum (Gibco); 0.05% saponin) for 45 minutes. Primary antibodies, diluted in saturation solution (1:200), were allowed to incubate overnight while kept in a humid container at 4°C, after which samples were thoroughly washed in PBS and allowed to sit for 5 minutes. Cells were subsequently incubated with secondary antibodies, diluted in PBS (1:1000), for 30 minutes at room temperature, washed 3x with PBS, and covered with a layer of PBS throughout imaging. (2) Samples stained against LAMP1 were fixed with -20°C methanol for 5 minutes and washed multiple times in PBS. The first rinse was added before methanol removal to prevent rapid dehydration, and the final rinse was allowed to sit for 5 minutes. Samples were incubated in blocking buffer (PBS; 10% goat serum) for 45 minutes. Primary antibodies, diluted in blocking buffer (1:200), were allowed to incubate overnight while kept in a humid container at 4°C, after which samples were
thoroughly washed in PBS and allowed to sit for 5 minutes. Cells were subsequently incubated with secondary antibodies, diluted in PBS (1:1000), for 30 minutes at room temperature, washed 3x with PBS, and covered with a layer of PBS throughout imaging.

**Immunofluorescence Data Acquisition:** Samples were imaged with an inverted WiTec Alpha300 R confocal-Raman microscope (WiTec, Germany) equipped with a Zeiss Epiplan-Neofluar Pol Oil 100×/1.3 objective. Single cells were scanned in 0.25×0.25 μm intervals using a 532 nm laser source set to 10 μW sample power to collect confocal fluorescence data. The same region was immediately rescanned using a 785 nm laser source set to 35 mW sample power to collect confocal Raman data. We accounted for chromatic aberration by applying a pre-determined offset in the z-direction between scans. The offset was acquired from a series of depth scans on silicon substrates, in which the intensity profile of a reference peak was compared and matched between 532 nm and 785 nm spectra in the z-direction. Global background subtraction and cosmic-ray removal were performed on each scan using Witec Project 5.2 software. Hyperspectral data was extracted and processed using custom codes written with Matlab software.

**Immunofluorescence Image Processing:** Raw fluorescence images were constructed from immunofluorescence hyperspectral datasets by calculating the total spectral intensity from all datapoints between 300 cm⁻¹ (~540 nm) and 1350 cm⁻¹ (~570 nm). The following processes were then applied to create binary fluorescence images. Note that specific intensity values, morphological operation values, etc., were globally applied to images from the same immunofluorescence marker, however each stain was optimized independently. A global intensity value was subtracted to remove background signal and a top hat filter was applied to remove objects much larger than the organelles from the
images. Next, a global threshold was applied to create binary fluorescence images. A watershed transform was performed to divide individual organelle structures, and finally binary image opening was applied to create binary fluorescence representations of each organelle label. DNA-SWCNT Raman images were constructed from confocal Raman hyperspectral datasets by calculating the total spectral intensity from all datapoints between 200 cm\(^{-1}\) and 300 cm\(^{-1}\). The following processes were then applied to create binary DNA-SWCNT images, and the same global settings were used regardless of the corresponding immunofluorescence marker. A global threshold was applied to create binary images and remove background signal. A watershed transform was initially performed to divide large DNA-SWCNT-containing regions. Binary erosion was used to shrink connections between regions of separate ROIs in close proximity, and a second watershed transform was applied to completely separate close ROIs which could not be initially distinguished. Finally, each DNA-SWCNT ROI was given a label before proceeding to the colocalization analysis. All images were constructed using custom Matlab codes and binary image operations were performed with FIJI.

**Quantitative Colocalization Analysis:** Colocalization analysis was performed by assessing each DNA-SWCNT ROI independently with respect to the corresponding binary fluorescence image. The intensity-weighted centroid position was calculated for a given ROI using the intensity image pixel values. The DNA-SWCNT ROI was then considered to colocalize with the immunofluorescence labels if the following conditions were met. (1) The intensity-weighted centroid position overlapped with the fluorescence binary image within a 1.5-pixel radius. (2) Greater than 40% of the total ROI pixels overlapped with fluorescence binary pixels. The first condition ensured that the DNA-SWCNT intensity-
center was within a resolvable distance of the fluorescence objects,\textsuperscript{68} while the second condition essentially applied a colocalization percentage threshold.\textsuperscript{69} All colocalization analyses were performed using custom Matlab codes.

**Development of Artificial Neural Network:** An artificial neural network classification model was developed, trained, and implemented using built-in functions and models from the Matlab Statistics and Machine Learning Toolbox. The training dataset was compiled from DNA-SWCNT Raman spectra of ROIs which colocalized with early endosome, late endosome, or lysosome markers at 0h, 6h, or 24h time points, respectively. We chose to only include data from the time point with the highest degree of colocalization for each marker as a way to reduce the potential for overlap between the protein labels, therefore training the neural network with the best representative spectra for each organelle. Additionally, recycling endosome data was omitted due to a lack of observations and considerable similarities to the early endosome spectra. As a pre-processing step, principal component analysis (PCA) was applied to the input Raman spectra to reduce the spectrum dimensionality and help prevent overfitting. The final number of inputs per spectrum was reduced to 44 components, comprised of the PCA components which explained 95\% of variance within the entire training dataset. The artificial neural network was comprised of an input layer connecting to the training predictor dataset, two fully connected 25-node hidden layers activated by rectified linear unit (ReLU) functions, and a softmax layer to convert the previous layer output into a class probability distribution. Layer weights and biases were established by the training dataset, and the model was validated with a 10-fold cross-validation. The model was applied to classify the Raman spectra of all non-labeled \textsuperscript{(GT)}\textsubscript{30}-SWCNT ROI data. Each input spectrum was transformed by PCA using the same
parameters from the training dataset and input to the model for classification. The resulting output consisted of the predicted class label and the probability distribution corresponding to each class.

**Statistical Analysis:** OriginPro 2018 was used to perform all statistical analysis. All data either met assumptions of the statistical tests performed (i.e., normality, equal variances, *etc.*) or was transformed to meet assumptions before statistical analysis was carried out. Statistical significance was analyzed using two-sample two-tailed student t-test or one-way ANOVA where appropriate. Testing of multiple hypotheses was accounted for by performing one-way ANOVA with Tukey’s post hoc test. Specific information about statistical analyses can be found in figure legends.
3.6. Acknowledgements

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3.7. References


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Chapter 4

Hyperspectral Counting of Multiplexed Nanoparticle Emitters in Single Cells and Organelles

by

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4.1. Abstract

Nanomaterials are the subject of a range of biomedical, commercial, and environmental investigations involving measurements in living cells and tissues. Accurate quantification of nanomaterials, at the tissue, cell, and organelle levels, is often difficult, however, in part due to their inhomogeneity. Here, we propose a method that uses the diverse optical properties of a nanomaterial preparation in order to improve quantification at the single-cell and organelle level. We developed ‘hyperspectral counting’, which employs diffraction-limited imaging via hyperspectral microscopy of a diverse set of nanomaterial emitters, to estimate nanomaterial counts in live cells and sub-cellular structures. A mathematical model was developed, and Monte Carlo simulations were employed, to improve the accuracy of these estimates, enabling quantification with single-cell and single-endosome resolution. We applied this nanometrology technique to identify an upper-limit of the rate of uptake into cells -approximately 3,000 particles endocytosed within 30 minutes. In contrast, conventional ROI counting results in a 230% undercount. The method identified significant heterogeneity and a broad non-Gaussian distribution of carbon nanotube uptake within cells. For example, while a particular cell contained an average of 1 nanotube per endosome, the heterogenous distribution resulted in over 7 nanotubes localizing within some endosomes, substantially changing the accounting of subcellular nanoparticle concentration distributions. This work presents a method to quantify cellular and subcellular concentrations of a heterogeneous carbon nanotube reference material, with implications for nanotoxicology, drug/gene delivery, and nanosensor fields.
4.2. Introduction

In the fields of nanomedicine, nanotoxicology, and the environmental impact of nanotechnologies, characterizing the tissue and cellular uptake is of fundamental importance. The mechanism of uptake and subcellular localization of nanomaterials are typically predicted by their physicochemical properties, providing tunable parameters to control these processes. The delivered dose, i.e., the quantity of internalized particles, is particularly relevant to nanotoxicity evaluations due to potential dose-dependent adverse effects. As a result, quantitative analyses are required for nanotoxicological conclusions to be drawn, yet fundamental advances in nanotoxicology have been hindered by a lack of standardization. The complex interactions which occur in biological settings create additional variables that can directly affect material uptake, including protein corona formation, which can be modulated by even minor experimental procedures. Minimum information reporting in bio-nano experimental literature (MIRIBEL) has therefore been suggested as a ‘minimum information standard’ to advance the principles of reusability, quantification, practicality and quality in material and biological characterization and experimental protocol details. Because analytical techniques rely on the intrinsic properties of nanomaterials, appropriate methodologies will inherently vary for specific materials, and thus a single approach cannot be used. Instead, it has been suggested that the dose metric to quantify nanomaterial uptake can be standardized to enable comparison of results obtained between different studies.

The choice of an appropriate dose metric is critical for results to be relevant to their toxicological effects, yet proper determination is convoluted in the case of nanomaterials and has been subject to debate. Mass is the most widely reported metric for toxicological studies due to its linear relationship with small molecule concentration, yet it fails to
account for significant interactions resulting from nanomaterial morphology, surface area, chemistry, etc. Moreover, mass comparisons between nanomaterials with dissimilar properties such as size or density are challenging to interpret. Alternative dose metrics have been developed to more adequately describe particle quantities with respect to their observed toxicological response. Particle number concentration (PNC) is a fundamental measurement used to quantify the number of nanomaterial particles per unit volume. PNC is a standardized metric for delivered dose when quantified in terms of whole cells, subcellular compartments, or cell volume, enabling comparison of results from different studies. In contrast to mass concentration, PNC accounts for the structural and interactive components of nanomaterials using discrete, fundamental units, which can additionally be converted into estimated physical quantities such as surface area concentration. Therefore, PNC can be a useful metric to standardize the quantification of nanomaterial uptake.

Despite its analytical value, PNC measurements in biological studies can be technically challenging to obtain for certain nanomaterials as suitable experimental methods depend on individual measurable properties. The most common approach is direct imaging and counting of internalized particles using high resolution microscopy techniques. Optical microscopies such as confocal fluorescence are easily accessible and can produce 3-dimensional representations which can be useful for per-cell PNC, but particle sizes must be larger than resolution limitations. Super-resolution microscopy techniques improve the lateral resolution but still remain limited for smaller particle sizes and often require labeling with specialized fluorophores. Transmission electron microscopy (TEM) offers superior lateral resolution and additionally can distinguish
organelles, such as endosomes, to enable per-organelle PNC. However, the contrast of most non-electron-dense nanomaterials in TEM is limited when imaging in cells stained with heavy atoms. Additionally, quantification often requires a substantial number of sections to image a large enough volume, or complex techniques such as ion beam milling. Other techniques have been developed to improve upon various limitations by modeling experimental data using statistical analyses. This combined approach has demonstrated a substantial ability to investigate complex topics, including the random probability distribution of quantitative uptake and subsequent heterogeneity between endosomes. Thus, the use of mathematical modeling can further improve the accuracy of these quantitative experimental methodologies.

Conventional analytical techniques are often inadequate for characterizing many nanomaterials with unusual properties, such as single-walled carbon nanotubes (SWCNTs), which are under investigation for various uses in biomedical applications. SWCNTs exhibit intrinsic near-infrared (NIR) fluorescence emission that is photostable and environmentally-sensitive, and are produced as a mixture of species, or chiralities, which can be identified by their chiral indices \((n,m)\). These advantageous properties have been leveraged to achieve multiplexed optical imaging and sensing in addition to drug- and gene-delivery in live cells, plants, and animals. Such uses necessitate their accurate \textit{in situ} characterization in biological systems, however, the 1-dimensional structure generally makes the direct visualization of individual SWCNTs with appropriate resolution difficult. Although single-SWCNT measurement techniques have been developed, their use has generally been limited solutions, on devices, or otherwise adsorbed to a substrate.
An immediate consequence of the lack of nanometerology techniques that function in live cells is that several fundamental gaps exist in our knowledge of nano-bio interactions, for instance, is there an intrinsic limit in the number of SWCNTs that can enter a cell or a single organelle in a given amount of time? Moreover, a considerable number of factors have been shown to impact the biocompatibility and biodistribution of SWCNTs, suggesting that nanotoxicity and relevant characterizations should be assessed independently for each SWCNT formulation.

In this work, we present a ‘hyperspectral counting’ technique to report the absolute number of emissive SWCNTs within live cells and single endosomes. Using diffraction-limited hyperspectral microscopy, we acquired spatially-defined spectroscopic data of multiple carbon nanotube emission bands, from different SWCNT chiralities, within live cells. We then performed Monte-Carlo simulations to estimate SWCNT counts from the number of ROIs and number of emission peaks detected. Applying this methodology, we discovered that SWCNT uptake is rate-limited by the cell itself. During 30-minutes of incubation, endocytic uptake is limited to approximately 3,000 SWCNTs per cell. Multiple SWCNTs, loaded within single endosomes even at relatively low incubation concentrations, did not result in SWCNT self-interaction or aggregation. The method also identified significant heterogeneity in nanomaterial distribution among endosomes within a given cell. Consequently, single statistical descriptors such as the mean or median number of nanoparticles per endosome are not sufficiently accurate for describing nanotube uptake by cells, which should be considered in terms of distributions instead. This work presents a method to quantify cellular and local/subcellular concentrations of a heterogeneous
nanomaterial, with implications for nanotoxicology, drug/gene delivery, and nanosensor fields.

4.3. Results and Discussion

4.3.1. Hyperspectral characterization of carbon nanotube aggregates

Our first goal was to investigate the potential for near-infrared hyperspectral microscopy to identify aqueously-dispersed photoluminescent SWCNTs and aggregates thereof. We selected HiPco SWCNTs, non-covalently dispersed via sodium deoxycholate (SDC) as a model nanomaterial. SDC disperses SWCNTs with high efficiency, sufficiently encapsulates the SWCNT sidewall to prevent optical modulation by the chemical environment and does not alter the intrinsic chirality distribution following dispersion.\textsuperscript{50} HiPco SWCNTs were dispersed in SDC via probe-tip ultrasonication for 30 minutes. For experiments involving live cells, free SDC was removed via 100kDa Amicon filtration. The resulting SDC-SWCNT complexes were stable, with a free SDC concentration of ~2.4 mM within the critical micelle concentration (CMC) range (2-6 mM).\textsuperscript{51} We found that SDC-SWCNT complexes remained colloidally stable when diluted in 10\% serum, despite decreasing the SDC concentration below the CMC (free SDC <0.02 mM). The SWCNTs were internalized by cells via energy-dependent endocytosis, as confirmed by incubating HeLa cells with SDC-SWCNTs at 4\(^\circ\)C and 37\(^\circ\)C (Fig. S4.2). Within live cells, stable SWCNT emission was detectable at 6 and 24 hours after initial uptake (Fig. S4.3). The movement of SWCNTs in the cells was consistent with localization within lysosomes (Movies S1).\textsuperscript{52}

To obtain samples that were dispersed under identical conditions but differed in their degree of purification, we varied the centrifugation step (Fig. S4.1). One sample was
centrifuged at 15,000 x g for 5 minutes (referred to as the ‘5-minute sample’) and the other was ultra-centrifuged at 250,000 x g for 30 minutes (referred to as the ’30-minute sample’). At both these accelerations, large aggregates of SWCNTs and some carbonaceous impurities sedimented into a pellet, leaving primarily singly-dispersed SWCNTs or aqueously-dispersed nanotube bundles.50

We conducted bulk optical characterization of the material to assess the degree of aggregation. Optical absorbance spectroscopy, Raman spectroscopy, and near-infrared (NIR) photoluminescence are three widely used methods, for which documentary standards have been published.50 Optical absorbance spectra of the two samples differed, with higher background absorption and significantly lower peak-to-valley ratio for the 5-minute sample (Fig. S4.4a). This metric reflects the higher carbonaceous impurities in the 5-minute sample but can also result from aggregation. However, no noticeable wavelength shifts or broadening were detected in the E_{11} absorption peaks, which would potentially denote SWCNT-SWCNT contact/bundling (Fig. S4.4a). The radial breathing mode of the resonant Raman spectrum was identical for both samples (Fig. S4.4b) and the characteristic aggregation peak (~267 cm\(^{-1}\))\(^{53}\) was not detected for either suspension. Photoluminescence emission under 730 nm excitation was significantly higher for the 30-minute sample, consistent with the better dispersion observed in the absorption spectrum (Fig. 4.4c). Lastly, we characterized the chirality-dependent properties with a two-dimensional photoluminescence excitation-emission (PLE) map (Fig. S4.5). For the 12 chiralities observed, emission peaks were red-shifted slightly, by < 0.5 nm in the 5-minute centrifugation sample relative to the 30-minute centrifugation sample, but excitation peaks,
emission width (full-width at half maximum, FWHM) and excitation width (FWHM) did not show statistically-significant differences (Table S1).

Figure 4.1. Single-nanotube hyperspectral microscopy of surface-adsorbed SDC-SWCNTs. (a) Broadband near-infrared image of SDC-SWCNTs at 100X magnification. Scale bar = 10 µm. (b) Representative spectra from individual ROIs selected from the 5-minute centrifugation sample, arrows highlight emission peaks. (c) Hyperspectral image of SDC-SWCNTs from each sample at 100X magnification, with each region-of-interest (ROI) false-colored by the number of emission peaks detected. Scale bar = 10 µm. (d) Histogram of total intensity and Feret’s diameter from two SDC-SWCNT sample preparations. (e) Representative spectra (data points fit by Lorentzian functions) from each emission band. Center wavelengths from all SDC-SWCNTs in the 5-minute sample plotted in ascending order. (f) Scatter plot of peak emission intensity of all individual SDC-SWCNTs from the 2 preparations. Boxes represent 25-75% of the data. Statistical comparisons are unpaired t-tests with Welch’s correction.
Near-infrared photoluminescence microscopy was used to interrogate both samples at the single-ROI level. Dilute concentrations of the two preparations were adsorbed to a glass surface, rinsed, and imaged in aqueous solution at high magnification (100X) under 730 nm excitation using a NIR hyperspectral microscope. Broadband NIR photoluminescence images integrated across the emission range of 900-1500 nm, and hyperspectral cubes (spatially-resolved emission spectra of each imaged pixel) from 900-1400 nm were obtained from the same field of view (Fig. 4.1a). The full spectra were acquired for each spatial region-of-interest (ROI) in the entire field-of-view, from which we counted the number of distinct peaks in the emission spectra (Fig. 4.1b). Each ROI in the hyperspectral cube was represented by colors mapped to the number of emission peaks detected (Fig. 4.1c).

We assessed quantification of the nanotubes by several methods using the different types of acquired data. We quantified ROIs by brightness and apparent size, as well as by wavelength-defined emission bands. We measured the brightness and apparent size of photoluminescent ROIs, observed from over 2,500 ROIs in each condition (Fig. 4.1d). The median integrated emission intensity from the 30-minute (centrifugation) sample was approximately 2X higher than the 5-minute sample (19,863 ± 1,243 a.u. vs. 9,073 ± 2,052 a.u.). In contrast, the median Feret’s diameter (which models the size by fitting each ROI to an ellipse) for the 30-minute sample was ~ 30% smaller (2.83 ± 0.045 pixels vs 4.12 ± 0.064). We pooled all individual emission peaks and independently fit each with a Lorentzian function to obtain the peak intensity, center wavelength and FWHM. For the 30-minute sample, the center wavelengths clustered into 5 distinct bands (Fig. 4.1e) corresponding to chiralities [(8,3), (6,5)], [(7,5), (10,2)], [(9,4), (7,6)], [(12,1), (8,6), (11,3)]
and [(10,5), (8,7), (9,5)], respectively (Band edges and bandwidths resulting from the k-means clustering of emission bands are found in Table S2). Center wavelengths for the 5-minute sample also clustered into the same bands (Fig. S4.6).

Finally, we compared single ROIs after clustering to assess the optical properties within individual bands for the two SWCNT preparations. We measured the peak emission intensities from emission bands within individual ROIs (Fig. 4.1f). The intensity distributions of both samples were broad, in part because of intrinsic heterogeneity in SWCNT brightness due to factors including length, endohedral content, defect density, surfactant microenvironment, and oxidation state. In our experimental setup, these factors were further convolved by the unequal excitation efficiency (on-resonance, off-resonance, and k-band phonon absorption for different chiralities) due to single-wavelength excitation. Although more ROIs with significantly higher intensities were present in the 5-minute sample, no statistically significant differences between the two samples were observed (except in band 4, p < 0.05). In contrast, emission wavelengths in the 5-minute sample were red-shifted in bands 3, 4 and 5, consistent with the PLE results (Fig. S4.7, values in Table S3).

### 4.3.2. **Model to estimate number of carbon nanotubes from emission bands**

Because >12 nanotube chiralities were binned into 5 emission bands due to spectral overlap, we asked whether a priori knowledge of the emission band distribution of the nanotube sample could be used to accurately approximate the discrete probability distribution of nanotubes per ROI from the probability distribution of emissive peaks. Though we do not know the number of photoluminescent SWCNTs in an ROI, the emission from each SWCNT in that ROI must lie within one of the 5 mutually exclusive bands in
wavelength space (Fig. 4.2a). In a single band, the experimental observation is binary – zero peaks if no emitter is present, and one peak if one or more emitters are present. The full emission spectrum (900 – 1400 nm) of any ROI can therefore at most show 5 distinct peaks.

**Figure 4.2.** Monte-Carlo model to estimate emissive SWCNTs from emission peaks. (a) Schematic of model to compute the distribution of the number of SWCNTs per ROI from the distribution of the experimentally-measured number of emission peaks per ROI. (b) The population distribution histogram for the 5-minute SWCNT preparation. (c) Relative-frequency histograms representing the number of emission peaks detected for a specific number of SWCNTs for an individual ROI. (d) Heat map of the number of SWCNTs per ROI and the probability distribution of the number of detected emission peaks. Values below 0.1 are not shown, for clarity. (e) Histograms quantifying the experimentally
determined relative-frequency of emission peaks from each sample, with shadowed lines representing a quality-of-estimates check from the model output. (f) Relative-frequency histogram of the number of SWCNTs per ROI in the two preparations, as calculated by the model.

We developed a three-step computational method to approximate the number of emissive SWCNTs present in an ROI from the number of emission peaks. First, we formulated a mathematical framework to distribute ‘n’ nanotubes into ‘m’ bands of different sizes. In this extension of the classical combinatorial probability problem of ‘distributing n balls in m boxes’, the relative size of each box is the relative nanotube population present in each band and can be directly calculated from the experimentally determined chirality distribution of the population. This is an intrinsic property of a carbon nanotube preparation, convolved with the experimental detection parameters of the setup.

We determined the band size for the 5-minute sample from the total number of SWCNTs detected via hyperspectral microscopy (Fig. 4.2b). The distribution was consistent between the 5-minute and 30-minute preparation and matched the results from PLE measurements in solution (Fig. S4.8).

The probability $\rho_i$ of any carbon nanotube belonging to a specific band $B_i$ in an ROI is:

$$
\rho_i = \frac{A_i}{\sum_{i=1}^{5} A_i}
$$

Where $A_i$ correlates to the SWCNT population in $B_i$.

Next, we generated a mapping function for the number of SWCNTs in an ROI and the number of emission peaks detected by solving a two-step process: (1) If an ROI contained $N$ SWCNTs in total, where $N$ ranges from 1 to 10, how many SWCNTs on
average would belong to each band? (2) As only zero or one peak can be observed per band, how many peaks would be detected in the full emission spectrum from that ROI?

We chose 10 as the upper limit for the total number of nanotubes present within an ROI for 2 primary reasons: First, the intensity distribution from individual ROIs (Fig. 4.1f) was consistent with one broad population likely arising from one emitter, with outliers that were approximately twice as bright. Second, at the concentration range used through this work, the experimentally detected number of emission peaks per wavelength band was consistently less than two. For a system with 5 bands, this limits the number of SWCNT per ROI to ten.

In any band $B_i$, $\Phi$ is the number of emission peaks detected when $P$ SWCNTs are present:

$$(2) \quad \Phi(P) = \begin{cases} 0, & P = 0 \\ 1, & P \geq 1 \end{cases}$$

As there is no closed-form analytical solution for this system, we sought a numerical approximation using Monte Carlo simulations. For each $n$ (number of SWCNTs present, ranging from 1 to 10), we ran 5,000 simulations to obtain a histogram of the number of emission peaks detected per ROI (Fig. 4.2c, details in Supplementary Text 1). These results mapped the number of SWCNTs present in a single ROI to the probability of detecting a specific number of emission peaks (Fig. 4.2d). For example, if 4 SWCNTs were present in an ROI, the probability of detecting 2 peaks was 0.30, of 3 peaks was 0.58 and 4 peaks was 0.11.

Finally, we extended the single-ROI model to an entire population. Essentially, any system of SWCNT-containing ROIs can be characterized by either a distribution of the number of nanotubes or by a distribution of the number of emission peaks (Fig. 4.2a). For
a population of \( m \) ROI \((R_i)\), where each ROI contained \( N_m \) SWCNTs that were detected as \( P_m \) peaks, these two sets are equivalent:

\[
(3) \ f(R_i, N_i) \text{ where } i = 1 \text{ to } m \text{ and } 0 < N_i < 10
\]

\[
(4) \ g(R_i, P_i) \text{ where } i = 1 \text{ to } m \text{ and } 0 < P_i < 5
\]

A probability mass function from one variable mapped into a probability mass function from the other i.e., for a population containing ROIs with \( N_j \) SWCNTs and \( P_i \) observable peaks:

\[
(5) \sum_{i=1}^{10} a_i N_i \leftrightarrow \sum_{j=1}^{5} b_j P_j
\]

The relative probabilities associated with the number of SWCNTs \((a_i)\) and the number of peaks \((b_j)\) summed to 1:

\[
(6) \sum_{i=1}^{10} a_i = \sum_{j=1}^{5} b_j = 1
\]

The number of emission peaks per ROI (coefficients \( b_j \)) were directly calculated from hyperspectral data of the two surface-adsorbed SDC-SWCNT samples (Fig. 4.2e). Only \( \sim 70\% \) of ROIs in the 5-minute sample had one emission peak, in contrast to \( \sim 95\% \) for the 30-minute sample. Using the \( b_j \) coefficients as inputs, we solved the system of linear equations in (5) and (6) for the unknown coefficients \( a_i \) via the least-squares method to obtain a distribution of the number of SWCNTs per ROI (Fig. 4.2f). To test the quality of the solution, the coefficients \( a_i \) were used to generate a \( b_j \)’ and directly compared with the experimentally determined \( b_j \). The parameters obtained regenerate a distribution for the number of emission peaks with reasonable agreement with the experimental data (shaded lines in Fig. 4.2e, with experimental data represented by solid bars).
4.3.3. Endocytic uptake results in multiple carbon nanotubes per endosome

We introduced SWCNTs to live cells under short incubation times to investigate endosomal accumulation. Hyperspectral microscopy was used to quantify SDC-SWCNT uptake in live mammalian cells, with the primary goal of extracting quantitative parameters that could be objectively compared across multiple experiments. Our model system was defined as SWCNT uptake via a 30-minute pulse in HeLa cells, a timepoint which results in nearly complete uptake of cell-associated nanotubes but before reverse trafficking of these SWCNT-containing endosomes is initiated. The 30-minute preparation was used to ensure that the SDC-SWCNT sample itself was dispersed well with minimal aggregation. HeLa cells were incubated for 30 minutes with the 30-minute SDC-SWCNT preparation in cell media, at concentrations spanning two orders of magnitude from 0.1 – 10 mg/L. Cells were thoroughly washed to remove unbound SWCNTs and placed at 4°C for 30 minutes in fresh media to reduce movement before imaging. For each cell, a z-stack of NIR broadband fluorescence images through the entire volume and a hyperspectral cube at the central z-position were sequentially acquired. Within this acquisition time (< 2 minutes), there was minimal movement of either the cell or the ROIs. The photoluminescence images of SWCNT emission from HeLa cells were consistent with SWCNTs bound to the cell membrane, on either the outside of or just internalized into the cell (Fig. S4.9). In our experimental setup, we previously showed that the presence of relatively dim SWCNTs that were not detected is negligible, i.e. almost all ROIs with NIR emission were present in the photoluminescence image.

We quantified SWCNTs within endosomes by several methods. The total number of ROIs within each cell were counted from the maximum intensity projection image. At 30-minute incubation, these ROIs were primarily early endosomes. Most ROIs contained
just one emission peak at 0.1 mg/L, but the number of peaks ranged from one to five after incubating with 1, 5 and 10 mg/L of SWCNTs (Fig. 4.3a). These results are direct evidence of multiple SWCNTs within each ROI. Surprisingly, the photoluminescence intensity did not reflect this heterogeneity, as the emission intensity from individual ROIs at 1, 5 and 10 mg/L was not statistically different, for any of the emission bands (Fig. 4.3b). This finding indicates that emission intensity itself was an unreliable metric for quantifying carbon nanotube uptake. The emission wavelengths also did not exhibit any consistent modulation as a function of SDC-SWCNT concentration (Fig. S4.10), indicating no notable SWCNT-SWCNT interactions.
Figure 4.3. Near-infrared hyperspectral microscopy of SWCNT uptake in HeLa cells. (a) Broadband fluorescence maximum intensity projection image, computed image with each ROI false-colored by the number of emission peaks detected and representative spectra from individual ROIs, at 0.1, 1, 5 and 10 mg/L SDC-SWCNT loading concentration. Scale bar = 10 µm. (b) Intensity of individual ROIs for each loading concentration. One-way ANOVA was performed using Holm-Sidak’s multiple comparison test.

4.3.4. Saturation of nanotube uptake in cells and endosomes

Particle number concentration measurements using the combined hyperspectral and computation counting technique were performed to quantify the concentration-dependent cellular uptake and sub-cellular distribution of single-walled carbon nanotubes. The absolute count of the SWCNT-containing endosomes within a cell was experimentally
determined via high-magnification live-cell fluorescence microscopy (as shown in Fig. 4.3a). Normalized by the projected area of each cell, we assessed the number of SWCNT-containing ROIs per unit area as a function of SWCNT-loading concentration (Fig. 4.4a). The areal density of SWCNT-containing ROIs increased with the SWCNT concentration administered to the cells (Spearman correlation = 0.90 with p < 0.0001) and was accurately described by an extended Langmuir adsorption model ($R^2 = 0.996$), plateauing at ~ 0.29 ROI per µm$^2$. The data at 0.1, 1 and 5 mg/L were statistically different from each other, but no significant differences were observed between the values at 5 and 10 mg/L (gray shaded box in Fig. 4.4a).

Figure 4.4. Multiparameter characterization of SWCNT uptake in HeLa cells. (a) Density of SWCNT-containing ROIs as a function of loading concentration. Line is a fit of the Langmuir isotherm equation to the data; error bars denote SEM. Gray region did not show a statistically significant difference. (b) Average number of emission peaks per ROI, as a function of loading concentration. Line is a fit of the Langmuir isotherm equation to the data; error bars denote SEM. Gray region did not show a statistically significant difference. (c) Scatter plot of the emission peaks per ROI vs. density of SWCNT-containing ROIs. Individual cells are circles; triangles represent the mean. Errors bars denote SEM. Gray regions could not be separated via k-means clustering. (d) Mapping between the number of emission peaks detected within one ROI and the computed number of emissive SWCNTs. Dashed lines correspond to the limiting value of the number of emission peaks per ROI, determined by the fit (dashed line) in panel b. (e) Density of SWCNTs as a function of loading concentration. Line is a fit of the Langmuir isotherm equation to the
The number of SWCNTs per endosome was experimentally determined for each cell using hyperspectral microscopy. For each spatially distinct ROI, we directly counted the number of distinct emission peaks in the 900-1400 nm wavelength range. The mean number of emission peaks per ROI increased with SDC-SWCNT concentration (Spearman correlation = 0.68 with \( p < 0.001 \)) and was accurately described by an extended Langmuir adsorption isotherm (\( R^2 = 0.99, \)), plateauing at \( \sim 2.58 \) emission peaks/ROI (Fig. 4.4b). Except for the data at 0.1 mg/L SWCNT loading concentration, no statistically significant differences were observed between the data at 1, 5 and 10 mg/L (gray box in Fig. 4.4b). A scatter plot of the density of SWCNT-containing endosomes per cell and the number of distinct emission peaks per endosome (Fig. 4.4c) revealed a high degree of correlation (\( r = 0.80, \ p < 0.0001, \) paired t-test). Although the data at 0.1 and 1.0 mg/L appeared distinguishable from 5 and 10 mg/L, an unbiased k-means clustering analysis was only able to accurately separate the 0.1 mg/L data from the higher concentrations (Fig. S4.11). Cells incubated with 1 mg/L could not be identified from cells incubated with 5 and 10 mg/L SWCNT (gray region in Fig. 4.4c could not be separated). Combined, these results indicate that the density of SWCNT-containing ROI saturate above 5 mg/L loading concentration, while the number of emissive peaks per ROI plateaus by 1 mg/L loading concentration.

Using the computational model, we calculated the emissive SWCNTs within each ROI from the number of distinct emission peaks. The nanotube band distribution of the 30-minute SDC-SWCNT sample in cells, obtained using the same hyperspectral analyses used for Fig. 4.2b, was significantly different from the solution measurement (Fig. S4.12). This
result likely arose from the known chirality-dependent modulations in SWCNT emission wavelength and intensity by the intracellular environment. Following the procedure developed in a previous section for SDC-SWCNT adsorbed on a surface, we obtained an analogous heat map of the number of SWCNTs per ROI and the probability distribution of the number of detected emission peaks for SDC-SWCNTs in cells (Fig. S4.13). Our analysis generated a calibration curve between the experimentally detected number of emission peaks in an ROI and the least-squares estimate of the number of emissive SWCNTs physically present (Fig. 4.4d). The density of emissive SWCNTs per cell (Fig. 4.4e) increased with concentration (Spearman correlation = 0.81 with p < 0.0001) and plateaued at ~ 1.3 SWCNTs per µm² (Langmuir fit, R² = 0.99). No statistically significant differences were detected between the two highest concentrations. For a 30-minute incubation of SDC-SWCNTs in HeLa cells, we found the linear uptake regime to be below the 1 mg/L SDC-SWCNT concentration range in media.

We compared the number of SWCNT-containing ROIs, the total number of emission peaks and the particle number concentration to quantify nanotube uptake within a cell (Fig. 4.4f). Assuming the SWCNT count as the reference standard, counting the total number of emission peaks systematically underestimated the actual values by ~ 40%, while counting the ROIs underestimated the actual counts by ~ 70%. The mean nanotube signal per cell, as quantified by photoluminescence intensity in broadband images, was the least accurate, undercounting the SWCNT concentration by 15-fold (Fig. S4.14). At the highest loading concentration of 10 mg/L, an average cell contained 406 ± 35 ROIs, 1062 ± 198 emission peaks, and 1838 ± 509 emissive nanotubes. As approximately 1/3rd SWCNTs in
the HiPco sample are non-emissive (metallic and semi-metallic), we scaled the number of emissive SDC-SWCNTs by 1.5 to calculate the total number of SWCNTs present.

Using the particle numbers above, we obtained a quantitative description of SWCNT partitioning into individual cells and endosomes (detailed calculations in Supplementary Text 2). For an SDC-SWCNT loading concentration of 10 mg/L (~ 130 nM), approximately 3,000 SWCNTs were endocytosed per cell, with an average of 4 SWCNTs per endosome. The average HeLa cell is 3,000 µm³ in volume and the typical endosome is ~250 nm in diameter. This corresponds to a SWCNT concentration of ~2 nM within a cell, indicating an effective partitioning of 1.5% of the SWCNT concentration in solution into a cell. However, the SWCNT concentration within the endosomes is ~300 nM, which is 2.3 times the concentration in solution.

4.3.5. Quantifying intercellular and intracellular heterogeneity

We assessed inter- and intra-cellular heterogeneity of SWCNT uptake and distribution. We obtained population statistics by pooling data from individual ROIs across multiple cells. From histograms of SWCNT emission peaks per ROI, we found that over 70% of the ROIs at 1, 5 and 10 mg/L contained more than one nanotube (Fig. 4.5a). The distribution shifted to a higher number of peaks with increasing loading concentration. Analysis of individual cells revealed significant inter and intra-cellular heterogeneity, with minimal dependence of either distribution on SWCNT loading concentrations above 1 mg/L(Fig. 4.5b). Though multiple factors determine SWCNT uptake by a cell, the specific number of particles associated with each cell is random.
Figure 4.5. Inter and intra-cellular heterogeneity in SWCNT uptake by HeLa cells. (a) Relative-frequency histogram of the emission peaks detected for the entire cell population, at each SWCNT concentration. (b) Frequency histograms (absolute counts) quantifying the experimentally determined relative-frequency of emission peaks from each cell, with shadowed lines representing a quality-of-estimates check from the model output.

In contrast to single homogeneous system comprised of multiple fields-of-view of SDC-SWCNT adsorbed on a surface, the intercellular heterogeneity observed required us to consider each cell to be an independent system. Computationally, this meant solving a separate system of linear equations for each cell, where the experimentally determined distribution of emission peaks per ROI were used to obtain the least-squares estimates of the SWCNT distribution. The corresponding distribution of the number of emission peaks was subsequently calculated to the quality of fit (Fig. 4.5b, following the same procedure described in Supplementary Text 2, and used to generated Fig. 4.2e). The number of emission peaks per ROI varied broadly from 1 to 5 within a single cell, at all concentrations.
above 0.1 mg/L. Additionally, the distribution was also heterogeneous across cells at each concentration, with varying minimum, maximum, and median values. A direct consequence of this heterogeneous distribution is that statistical descriptors such as the mean or median number of emission peaks per ROI at any loading concentration only accurately describe a small fraction of the total ROI population.

4.4. Conclusions

In this work, we have developed a nanometrology technique to quantify the uptake of single emitting nanomaterials in living cells. Using NIR hyperspectral imaging, we quantified spectral bands to enable the counting of single SWCNT emitters within single endosomes. We employed experimentally-guided Monte Carlo simulations to further improve the robustness of the method. HeLa cells were determined to internalize ~3,000 SWCNTs when dosed for 30 minutes at a concentration of 10 mg/L, with an average of 4 SWCNTs per single endosome. Our analysis further determined that SWCNT uptake is rate-limited by cells with both the SWCNT-containing endosomes and number of SWCNTs per endosome plateauing at less than 5 mg/L concentration of SWCNTs in media, with a linear uptake regime below 1 mg/L. Moreover, both the intracellular and intercellular distribution of SWCNTs per endosome are highly heterogeneous. The tails of such distributions are significant, as several mechanisms of nanoparticle-induced toxicity result in signaling from individual organelles or cells. Even if the average endosome per cell has between 1-2 nanotubes, the presence of larger quantities in a single endosome could induce localized toxic effects, in addition to generating systematic errors in sensing, imaging and delivery applications. For future applications, calculations of cellular and
endosomal nanotube concentrations need to be considered in terms of distributions instead of single statistical descriptors.

With multiple families of fluorophores under development, and hyperspectral microscopy becoming increasingly available in research labs, the framework developed in this work has broad applicability for various nanomaterials. Moreover, the ability to compare uptake of SWCNTs with other types of nanomaterials is possible using particle number concentration (PNC) as the dose metric, promoting advancements in our understanding of complex interactions vital to nanomedicine.

4.5. Materials and Methods

**Preparation of Single-Walled Carbon Nanotube Suspensions:** Single-walled carbon nanotubes produced by the HiPco process (Unidym) were suspended by probe-tip ultrasonication (Sonics & Materials, Inc.) of 20 mg sodium deoxycholate (SDC) with 1 mg of ‘raw’ SWCNTs in 1 mL of deionized water for 30 minutes at 40% of the maximum amplitude (~ 9 Watts). Following sonication, the dispersions were ultracentrifuged (Sorvall Discovery 90SE) for 30 minutes at 280,000 x g. The top ¾ of the resulting supernatant was collected. Concentration was determined with a UV/Vis/NIR spectrophotometer (Jasco) using the extinction coefficient $A_{910} = 0.02554 \text{L-mg}^{-1}\cdot\text{cm}^{-1}$. To remove free SDC, 100 kDa Amicon centrifuge filters (Millipore) were used to concentrate the nanotube dispersions and re-suspend via mixing by pipette. Nanotubes were prepared immediately before addition to cell media. A photoluminescence excitation-emission contour plot was constructed to identify the nanotube chiralities present in the sample using a custom-built instrument.$^{59,60}$
**Cell Culture:** HeLa CCL-2 cells (ATCC) were grown under standard conditions at 37°C and 5% CO₂ in sterile-filtered DMEM with 10% heat-inactivated FBS, 2.5% HEPES, 1% glutamine, and 1% penicillin/streptomycin (all Gibco). Cells were plated onto T-75 flasks at 20% confluence and passaged every 3 days. For imaging experiments, cells were plated onto glass-bottom petri dishes (MatTek) and used at 70-80% confluence.

**Hyperspectral Imaging:** As described previously, an instrument to conduct NIR fluorescence hyperspectral microscopy was used to obtain spectrally-resolved images of emissive nanotubes in HeLa cells (Photon etc.). Briefly, a continuous wave (CW) 730 nm diode laser (with output of 230 mW, measured at the sample) was injected into a multimode fiber to produce the excitation source for photoluminescence experiments. A long pass dichroic mirror with a cut-on wavelength of 880 nm was aligned to reflect the laser into an Olympus IX-71 inverted microscope (with internal optics modified for near infrared transmission) equipped with a 100X (UAPON100XOTIRF, NA=1.49) oil-immersion objective (Olympus). Emission from the nanotubes was spatially and spectrally resolved by passing through a volume Bragg grating and into a thermo-electrically cooled 256 x 320 pixel InGaAs array detector. A continuous stack (hyperspectral cube) of 126 spectrally-defined images was obtained between 900 to 1400 nm, collected in 4 nm steps. The data was processed to produce a near-infrared spectrum for every pixel of the image. To quantify the absolute number of nanotubes per cell, z-stacks were constructed from HeLa cells incubated with varying concentrations of SDC-HiPco for 30 minutes, washed with fresh media, and then placed in 4°C for 15 minutes prior to imaging (to temporarily halt endosomal movement). The number of distinct nanotube-containing endosomes was determined by manually counting the z-stack images.
Two-dimensional excitation/emission photoluminescence plots: Photoluminescence (PL) plots were acquired using a home-built 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 to tune the output from 500 – 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) where it passed through a 20x NIR objective (Olympus) and illuminated a 200 µL nanotube sample in a 96-well plate (Greiner). Emission from the nanotube sample was collected through the 20x objective and passed through a dichroic mirror (875 nm, Semrock). The light was f/# matched to the spectrometer using several lenses and injected into an Isoplane spectrograph (Princeton Instruments) with a slit width of 410 µm which dispersed the emission using an 86 g/mm grating with 950 nm blaze wavelength. The light was collected by a PIoNIR InGaAs 640 x 512 pixel array (Princeton Instruments).

Excitation, emission, and wavelength corrections and calibrations were performed as follows. The power at each excitation wavelength was measured at the objective with a PM100D power meter (Thorlabs) from which a power spectrum was constructed and used to correct the emission intensities for non-uniform excitation. A HL-3-CAL-EXT halogen calibration light source (Ocean Optics) was used to correct for wavelength-dependent features in the emission intensity arising from the spectrometer, detector, and other optics. A Hg/Ne pencil style calibration lamp (Newport) was used to calibrate spectrometer wavelength.
Acquisition was conducted in automated fashion controlled by Labview code which iteratively increased the excitation laser source from 500 – 824 nm in steps of 3 nm, acquired data with an exposure time of 0.3 seconds for a nanotube concentration of 1 mg/L SDC-HiPco, and saved the data in ASCII format. The spectral range was 930 – 1369 nm with a resolution of ~0.7 nm. Background subtraction was conducted using a well filled with DI H₂O. Following acquisition, the data was processed with MATLAB code which applied the aforementioned spectral corrections, created the contours with a Gaussian smoothing function, and constructed figures to be used for manual assignment of nanotube chiralities from the two-dimensional peaks.

**Solution Raman Spectroscopy:** All Raman scans and measurements were performed with a Renishaw InVia Raman microscope (Renishaw, Hoffman Estates, IL) equipped with a 785 nm diode laser (300 mW cm⁻²) and a 1 in. charge-coupled device detector with a spectral resolution of 1.07 cm⁻¹. Raman spectra were acquired through a 5× objective (Leica, Buffalo Grove, IL), where laser output at the objective was measured to be 100 mW cm⁻² using a hand-held laser power meter (Edmund Optics, Inc., Barrington, NJ), as previously described. Data analysis of the spectral images was performed in MATLAB (R2014b) and PLS Toolbox v.8.0 (Eigenvector Research, Inc., Wenatchee, WA). For displayed SERS intensities, baseline subtraction was performed on the collected spectra using a Whittaker filter with λ = 200 cm⁻¹.
4.6. Acknowledgements

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4.7. References


Chapter 5

Aggregation Reduces Subcellular Accumulation and Cytotoxicity of Single-Walled Carbon Nanotubes

by

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5.1. Abstract

The non-covalent biomolecular functionalization of fluorescent single-walled carbon nanotubes (SWCNTs) has resulted in numerous \textit{in vitro} and \textit{in vivo} sensing and imaging applications due to many desirable optical properties. In these applications, it is generally presumed that pristine, singly-dispersed SWCNTs interact with and enter live cells at the so-called nano-bio interface, \textit{e.g.} cell membrane. Despite numerous fundamental studies published on this presumption, it is known that nanomaterials have the propensity to aggregate in protein-containing environments before ever contacting the nano-bio interface. Here, using DNA-functionalized SWCNTs with defined degrees of aggregation as well as near-infrared hyperspectral microscopy and toxicological assays, we show that despite equal rates of internalization, initially-aggregated SWCNTs do not further accumulate within individual subcellular locations. In addition to subcellular accumulations, SWCNTs initially with a low degree of aggregation can induce significant deleterious effects in various long-term cytotoxicity and real-time proliferation assays, which are markedly different when compared to SWCNTs that are initially aggregated. These findings suggest the importance of aggregation state as a critical component related to intracellular processing and toxicological response of engineered nanomaterials.

5.2. Introduction

Bionanotechnology is an important field in biomedical research that encompasses the use of various nanoparticles and nano-structured materials for applications in drug delivery,\textsuperscript{1} biosensing,\textsuperscript{2} and bioimaging.\textsuperscript{3,4} Nanoparticles are uniquely suited for biological sensing and imaging due to their nano-scale size, emergent optical,\textsuperscript{3} electronic,\textsuperscript{5} and magnetic properties,\textsuperscript{6} and ability to exhibit enhanced biocompatibility upon
One carbon-based nanoparticle whose fundamental properties have been highly investigated is the single-walled carbon nanotube (SWCNT). SWCNTs are desirable for use as biological sensors due to their intrinsic fluorescence which is photostable, environmentally responsive, and emits in the near infrared (NIR) wavelengths of light. Additionally, SWCNTs are intriguing for use in the field of intracellular biosensing and imaging due to their 1-dimensional structure and diversity of species known as chiralities. Unprocessed SWCNTs are intrinsically hydrophobic and must first be complexed with either surfactants or amphiphilic molecules to be dispersed in aqueous solutions and increase colloidal stability. In biomedical applications, single-stranded DNA has been shown to solubilize SWCNTs via π-stacking and hydrophobic interactions, producing DNA-SWCNT hybrid nanomaterials with substantially enhanced biocompatibility. These DNA-SWCNT complexes have been incorporated into biomaterials, introduced to biological fluids, and immobilized in high-resolution imaging substrates as biosensors to detect various physiological processes. Moreover, DNA-SWCNTs can be internalized by cells via active endocytosis, where their optical properties can be employed to investigate specific cellular processes and detect intracellular biomolecules.

Despite significant recent advances within the field of bionanotechnology, there are still underlying issues regarding the physical states of the nanomaterials that must be addressed. Aggregation is often overlooked when considering how SWCNTs interact with their surroundings upon introduction to complex biological environments. It is well established that a biomolecular corona can form around DNA-SWCNTs in the presence of proteins, lipids, carbohydrates, etc., in cell media and biological fluids however the
subsequent aggregation between SWCNT-biomolecule complexes can create much larger particles than their initial, singly dispersed form.\textsuperscript{24, 37} These larger-sized aggregates can differentially interact with cells and tissues when compared to singly-dispersed SWCNTs in pristine laboratory experiments,\textsuperscript{38} and as a result, their presence can influence experimental outcomes. In the case of DNA-SWCNT biosensors, which rely on an optical response upon environmental interactions,\textsuperscript{27, 39, 40} aggregation inherently alters these dynamics by reducing analyte accessibility to the SWCNT surface. Moreover, direct SWCNT aggregation can reduce or completely quench the fluorescence signal\textsuperscript{27, 41} in addition to modulating emission-wavelength,\textsuperscript{27} creating convoluted noise in a signal designed to detect specific analytes.\textsuperscript{42}

Another issue that must be addressed concerning nanoparticle usage for biomedical sensing are the possible cytotoxic effects of the nanoparticle, and if aggregation state has any effect on the cytotoxicity. Generally, an inverse relationship between particle size and cytotoxicity has been observed for a range of nanoparticles,\textsuperscript{43} partially explained by the increase in surface area as particle size decreases. Consequently, cells interact much differently with small nanoparticles (< 100 nanometers) than larger nanoscale or even micron-sized particles.\textsuperscript{44-46} There have been many studies which have determined the effects of nanoparticle size, shape, and charge on cell-nanoparticle interactions,\textsuperscript{46} however aggregation will inherently alter these characteristics, and has been largely ignored in the past. Previous research has shown that larger agglomerations of SWCNTs are less cytotoxic when compared to an equal weight of single-dispersed SWCNTs due to their reduced surface area-to-volume ratios.\textsuperscript{47} It was concluded that the SWCNT aggregates displayed a reduced amount of hydrophobic surfaces, when normalized by total SWCNT
weight, which has been attributed to deleterious and toxic effects in cells. Additionally, we have shown that intracellular trafficking processes can induce aggregation of singly dispersed DNA-SWNTs within the lysosomes of cells. In the context of translating these novel sensing and imaging technologies towards clinical use, it therefore becomes important to investigate how an initially aggregated SWCNT sample is processed within various types of cells.

Here, we investigate the differential uptake, optical modulations as a result of intracellular processing, and resultant cytotoxicity of DNA-SWNTs as a function of aggregation state and cell type. Three cell lines were chosen representing, RAW 264.7 murine macrophages, HeLa human cervical cancer cells, and A549 adenocarcinoma human alveolar cells, representing a diverse range of potential interactions in addition to application-based relevance. We define an aggregated sample as one in which a significant proportion of the nanotubes are irreversibly bound to each other in solution due to hydrophobic interactions. We find that when dosing cells with equal concentrations of either singly-dispersed or initially-aggregated DNA-SWNTs, the difference in average uptake per cell area is insignificant between cells, across all three cell lines. Interestingly, the in vitro fluorescence intensity of the singly-dispersed DNA-SWCNT sample was significantly higher than the fluorescence intensity of the initially-aggregated sample across each cell line. Moreover, the initially-aggregated sample was shown to have reduced intracellular accumulation. Finally, we observed elevated cytotoxicity in HeLa cervical cancer cells incubated with singly-dispersed DNA-SWCNTs as compared to the initially-aggregated sample, yet insignificant differences in both RAW 264.7 macrophages and A549 lung cancer cells. These results have significant implications
for the rational design of novel nanosensors and imaging agents that function robustly in live-cells and animals with limited toxicities.

5.3. Results and Discussion

5.3.1. Characterization of DNA-SWCNT suspensions

DNA-wrapped SWCNTs were prepared by probe-tip sonication and subsequent centrifugation of an aqueous mixture of single-stranded (GT)$_{15}$ oligonucleotides and HiPco SWCNTs. The sample quality, i.e., amount and degree of nanotube aggregation, was controlled during this process to obtain two distinct samples from the same starting components. A high-quality, essentially monodisperse sample, and lesser-quality, heterogeneously aggregated sample (hereby referred to as 250k-SWCNT and 1k-SWCNT, respectively) were created by altering the sonication and centrifugation parameters (Fig. S5.1). Absorbance spectroscopy was used to confirm differential suspension of the two samples (Fig. 5.1a), which exhibited clear differences in the peak-to-valley ratios in both the near-infrared (NIR) and visible regions of the spectra. The fluorescence spectra of both samples were obtained via excitation with a 730 nm laser (Fig. 5.1b). Multiple emission peaks could be observed in the NIR region, however the integrated fluorescence intensity of 1k-SWCNTs was nearly 4 times lower (21.2%) than 250k-SWCNTs despite the same solution concentration (20 mg-L$^{-1}$). The radial breathing mode (RBM) region of the Raman spectrum of each suspension was acquired with a 1.58 eV (785 nm) excitation (Fig. 5.1c), revealing a clear increase in the 1k-SWCNT (10,2) peak intensity at 267 cm$^{-1}$ relative to 250k-SWCNT. To summarize, the increased optical absorbance and fluorescence intensities of 250k-SWCNTs suggest a higher degree of singly-dispersed nanotubes compared to 1k-SWCNTs.$^{48}$ Furthermore, given the 785 nm laser excitation, the intensity
of the (10,2) RBM peak can be directly attributed to the degree of aggregation within a sample, indicating the presence of aggregated nanotubes within the 1k-SWCNT sample.

To directly visualize the two DNA-SWCNT samples, we employed a spin-coated hydrogel platform to interrogate the NIR fluorescence with single-SWCNT resolution. Briefly, a thin layer of agarose was spin-coated onto a glass substrate and the 250k-SWCNTs or 1k-SWCNTs were embedded between a second agarose layer, resulting in a single focal plane of nanotubes with preserved optical properties. Broadband NIR fluorescence (900 – 1600 nm) images were obtained of both samples at 100x magnification (Fig. 5.1d). Generally, larger particles could be identified in the 1k-SWCNT sample, while the majority of fluorescent regions appeared smaller in the 250k-SWCNT fluorescent micrographs. To quantify these differences, the images were processed and a global intensity threshold was applied to obtain regions of interest (ROIs) corresponding to individual groups of fluorescent pixels. Feret’s diameter, defined as the largest distance between two points in a group of pixels, was calculated for each ROI and histograms were constructed (Fig. 5.1e), revealing a higher frequency of larger diameter ROIs present in the 1k-SWCNT sample. Due to the optical diffraction limit of our system (~500 nm) and the pixel size of our detector (~150 nm), emission from a single SWCNT is blurred across a minimum of 3 × 3 pixels in NIR fluorescence images, and thus small bundles and aggregates of SWCNTs could not be resolved from the single nanotube population using Feret’s diameter measurements. Therefore, we employed a spectral counting method to investigate the degree of aggregation for each sample. Hyperspectral cubes of the spin-coated DNA-SWCNTs were acquired and again split into fluorescent ROIs. The fluorescence spectrum from each ROI was manually observed and the number of distinct
fluorescence emission peaks was quantified (Fig. S5.2), providing a metric to spectrally resolve multiple nanotubes in diffraction limited regions. Histograms were constructed to show the relative frequency of emission peaks per ROI (Fig. 5.1f). While the majority of ROIs (i.e. median) from both samples contained only one fluorescence emission peak, the 1k-SWCNT population exhibited a marked increase in the amount of ROIs containing more than one emissive nanotube, suggesting elevated amounts of nanotube aggregates. Although the spectral counting method is limited to semiconducting SWCNTs that are excitable by the laser source, clear differences between the peak per ROI distributions, Feret’s diameter histograms, and characteristic optical spectra confirmed increased amounts of nanotube bundles and aggregates in the 1k-SWCNT sample relative to the singly-dispersed 250k-SWCNT solution.
Figure 5.1. Optical characterization of DNA-SWCNT samples. (a) Absorbance spectra, normalized at 910 nm, (b) NIR fluorescence spectra for equal concentrations of SWCNT (20 mg·L\(^{-1}\)), and (c) normalized Raman spectra of 250k-SWCNTs and 1k-SWCNTs in solution. (d) Broadband NIR fluorescence images of the two DNA-SWCNT preparations in a spin-coated hydrogel platform. (e) Histograms of the Feret’s diameter of DNA-SWCNT ROIs from NIR fluorescence images. (f) Histograms of the number of nanotube emission peaks per fluorescent ROI obtained from the two DNA-SWCNT preparations.
5.3.2. **Intracellular fluorescence intensity is cell-type dependent**

The two DNA-SWCNT samples were introduced to live mammalian cells in culture to investigate how the sample quality influences their interactions with biological systems. We chose three distinct cell types to represent a range of potential interactions which could be encountered by *in vitro* cell lines: RAW 264.7 murine macrophages; HeLa human cervical adenocarcinoma epithelial cells; and A549 human lung carcinoma epithelial cells. The interactions between macrophages and nanoparticles, especially biologically aggregated and protein-adsorbed nanoparticles, are important to investigate because macrophages are among the first cells to process nanoparticles in systemic circulation. HeLa cells were investigated due their widespread usage for *in vitro* modeling of nanoparticle uptake and cytotoxicity. Finally, A549 lung cancer cells were chosen due to an increasing interest in delivering drug-loaded nanoparticles directly into the lungs to treat disease, creating a relevance to investigate how lung cells interact with aggregated nanoparticles. Each cell type was dosed with 1 mg-L⁻¹ 250k-SWCNT or 1k-SWCNT for 30 minutes, rinsed, and allowed to incubate for an additional 30 minutes or 6 hours, to promote cell uptake and localization of the DNA-SWCNTs to the early endosomes or late endosomes/lysosomes, respectively, after which broadband NIR fluorescence images were acquired from the live cells. Figure 5.2a shows fluorescence images from each condition at the 6 hour time point. In general, cells dosed with the 250k-SWCNTs exhibited increased intracellular fluorescence intensities compared to those dosed with the 1k-SWCNTs, while RAW 264.7 cell fluorescence appeared to be the brightest among the three cell lines. To quantify these differences, we first segmented the cells from each image into individual ROIs using the transmitted light images obtained in parallel (Fig. S5.3). The integrated NIR fluorescence intensity was then calculated for each cell ROI and divided by
the total cell area, producing intensity measurements that normalized for the substantial differences in cell type sizes. Box plots were constructed from all single-cell measurements to compare each condition (Fig. 5.2b). Clear differences in the cell intensity distributions were identified between cells dosed with 250k-SWCNTs and 1k-SWCNTs, the former of which was significantly more intense regardless of cell type and incubation time. Additionally, fluorescence from RAW 264.7 cells was significantly more intense than the other cell lines (Fig. S5.4), an expected result which is directly related to the phagocytic nature of macrophages.

Two potential factors could explain why cells dosed with 1k-SWCNTS were substantially less intense than those dosed with 250k-SWCNTs: (1) DNA-SWCNT uptake is higher for monodisperse nanotubes and therefore the fluorescence intensity is also higher, or (2) uptake is the same and the fluorescence of internalized nanotubes is decreased by the presence of bundles and aggregates. To account for the intrinsic fluorescence intensity differences observed between the two DNA-SWCNT preparations (Fig. 5.1c), we determined a quenching coefficient from a series of control experiments (Fig. S5.5). Next, we applied this coefficient to the intracellular 1k-SWCNT data to account for these intrinsic differences and compared the corrected intensity values with those from the 250k-SWCNT dosed cells (Fig. S5.6). Interestingly, the correction factor resulted in near-identical values for the 250k-SWCNT and 1k-SWCNT dosed cells at the 30 minute time point, suggesting the latter case could be viable. The trend was relatively similar at the 6 hour time point (Fig S5b).
Figure 5.2. Cell type-dependent intracellular fluorescence of DNA-SWCNTs. (a) Transmitted light images overlayed with broadband NIR fluorescence micrographs of RAW 264.7, HeLa, or A549 cells dosed with 250k- or 1k-SWCNTs for 30 minutes and incubated for an additional 6 hours. (b) The broadband fluorescence intensity per total cell area from individual cells as a function of DNA-SWCNT sample and incubation time for each cell line. Minimum of n ≥ 96 cells per condition. Boxes represent 25 – 75% of the data, white squares represent means, horizontal lines represent medians, and whiskers represent mean ± s.d. One way ANOVA with Tukey post hoc analysis was performed (* * * * p < 1e-4).

5.3.3. Cell uptake is independent of DNA-SWCNT dispersion quality

We employed confocal Raman microscopy to quantitatively probe the cell type dependent uptake of each DNA-SWCNT preparation. Cells were again dosed with 1 mg-L⁻¹ of 250k-SWCNTs or 1k-SWCNTs for 30 minutes, washed, and placed into fresh media.
for an additional 6 hours, at which time the samples were fixed and prepared for confocal Raman microscopy. Hyperspectral Raman images were acquired from individual cells at 100x magnification to investigate the G-band (Fig. S5.7), a Raman feature of SWCNTs which linearly scales with SWCNT concentration. G-band intensity images were constructed and overlayed with transmitted light images for cells in each condition (Fig. 5.3a), and the uptake was quantified as the integrated G-band intensity per cell area (Fig. 5.3b). The SWCNT uptake was highest into RAW 264.7 macrophages, which internalized at least 78% more DNA-SWCNTs than HeLa and A549 regardless of the dosed DNA-SWCNT sample. This finding confirmed that higher nanotube uptake contributed to the increased fluorescence intensities seen previously in the macrophages relative to HeLa and A549 cells. In contrast, the difference in uptake between the 250k-SWCNTs and the 1k-SWCNTs was insignificant in any particular cell line (p > 0.05 according to two-sample two-tailed student t-test for each cell line), further supporting the hypothesis that variations in intrinsic brightness of the samples (see Fig. 5.1b) contributed to the significantly different fluorescence intensity values.

**Figure 5.3.** DNA-SWCNT uptake via confocal Raman microscopy. (a) Transmitted light images of RAW 264.7, HeLa, or A549 cells overlayed with G-band intensity maps of intracellular 250k-SWCNTs and 1k-SWCNTs after 6 hours. (b) Bar graph of intracellular G-band intensity per cell area for each cell line. Bars represent the average, lines represent
the median, and whiskers represent mean ± s.d. for each condition (n ≥ 4 cells per condition). One way ANOVA with Tukey post hoc analysis was performed between cell lines dosed with the same DNA-SWCNT sample (**p < 0.01, *p < 0.05).

5.3.4. Dispersion heterogeneity reduces intracellular nanotube accumulation

Next, we sought to investigate how the intracellular processing of DNA-SWCNTs can vary depending on the dispersion quality. As a quantitative measure of this processing, the endosomal loading of DNA-SWCNTs was interrogated using the same hyperspectral counting approach previously described within single intracellular ROIs of the three cell lines. Hyperspectral cubes were acquired at 100x magnification for each cell type following a 30 minute pulse of 250k-SWCNTs or 1k-SWCNTs and an additional 30 minutes of incubation in fresh media. Subsequent spectral counting analysis and spectral image overlays revealed that the number of emission peaks ranged from one to five for all conditions (Fig. 5.4a). For ROI populations from both DNA-SWCNT dispersions (Fig. 5.4b), RAW 264.7 cells were observed to contain the highest total fraction of ROIs with ≥ 4 emission peaks relative to the ROI distributions from HeLa and A549 cells. Nanotube dispersion quality was also a major contributing factor to the subcellular localization of DNA-SWCNTs. Regardless of cell line, intracellular ROIs were more likely to contain more emissive nanotubes when dosed with the 250k-SWCNTs versus the lesser-quality 1k-SWCNTs. To explain this anomaly, we hypothesized that despite equal total nanotube uptake, cells were more likely to intracellularly combine the cargo from internalized vesicles when single SWCNTs or small quantities were encapsulated. In contrast, the intracellular ROI peak distribution of 1k-SWCNTs in HeLa and A549 more closely resembled the starting sample shown in Figure 5.1f compared to the 250k-SWCNT distributions. These results could indicate the downregulation of a particular endosomal maturation pathway if the initial load of nanotubes is above a certain threshold. In this
scenario, the subcellular localization and accumulation, i.e., processing into regions of high local SWCNT density, could be controlled by modulating the initial DNA-SWCNT dispersion quality.

Figure 5.4. Spectral counting quantifies endosomal nanotube loading. (a) Transmitted light images overlayed with ROI maps and false colored by the number of emission peaks detected for cells dosed with 250k-SWCNTs (top) or 1k-SWCNTs (bottom). (b) Histograms of the SWCNT emission peaks per ROI for all cells (n > 150 ROIs per condition) with median values (M) indicated.

5.3.5. Proliferation and cytotoxicity

To quantify potential deleterious effects to cell health as a function of dispersion quality, we utilized two diverse methods to assess the response of each cell line to a constant dose of DNA-SWCNTs. First, real-time cell proliferation was monitored using an xCELLigence system, which measures electrical impedance across interdigitated micro-electrodes embedded in the bottom of specialized tissue culture E-Plates. The impedance
measurement, represented as a cell index value, can then be directly correlated to various cellular characteristics including cell number, viability, and morphology. Cells were seeded in the tissue culture E-Plates and incubated for 24 hours, after which a dose of 250k-SWCNTs or 1k-SWCNTs was introduced into the culture media and allowed to incubate for the remainder of the experiment. To compare relative proliferations between the different conditions, the cell index was normalized to the final measurement taken before nanotube dosing (Time 0). HeLa cells exhibited a clear response to the addition of both types of DNA-SWCNTs and at standard (1 mg-L\(^{-1}\)) and elevated (10 mg-L\(^{-1}\)) concentrations relative to a no SWCNT control (Fig. 5.5a). Interestingly, RAW 264.7 and A549 did not show the same trend (Fig. S5.8-9), and in certain cases, responded to the dose by increasing the rate of proliferation relative to control cells. We calculated the average area under the curve (AUC) as a quantitative measure to compare the total growth following nanotube exposure (Fig. 5.5b). The proliferation relative to control was significantly reduced in the continuous presence of DNA-SWCNTs, however 250k-SWCNTs appeared to have a greater negative impact that scaled with dosing concentration when compared to the 1k-SWCNTs. Next, an apoptosis-necrosis assay was performed on HeLa cells using the same incubation conditions from the proliferation experiment to directly assess the potential for cytotoxicity. When incubating the HeLa cells with 1 mg-L\(^{-1}\) of SWCNTs continuously for 24 hours, a slight increase in the apoptotic cell population was apparent from both of the nanotube dispersions relative to the control cells (Fig. 5.5c). At 10 mg-L\(^{-1}\), a shift in the cell population was evident for both samples of DNA-SWCNTs (Fig. 5.5c), inducing over 10% of cells to become apoptotic over a 24 hour period due to nanotube exposure.
Figure 5.5. Proliferation and viability to identify adverse responses. (a) Real time monitoring of HeLa proliferation after addition of indicated DNA-SWCNTs via xCELLigence impedance measurements. Line represents the mean cell index ($n = 4$) and error bars represent the mean ± s.d. Data were normalized to the final measurement before nanotube exposure. For clarity, error bars are shown on 1 out of every 20 data points. (b) Bar graph representing total proliferation using the mean integrated area under the curve from the data shown in (a). One way ANOVA with Tukey post hoc analysis was performed ($****p < 1e^{-4}$). Stars directly above the bars represent significance versus control. (c) Scatter plots depicting apoptosis data for control cells (left) and cells dosed with 1 mg-L$^{-1}$ or 10 mg-L$^{-1}$ of either 250k-SWCNTs (center) or 1k-SWCNTs (right). Viable, apoptotic, and necrotic cells are found in the bottom left, bottom right, and top right quadrants, respectively.
5.4. Conclusions

The goal of this study was to systematically investigate nanoscale aggregation as a fundamental material property and characterize the resultant cellular interactions. Two DNA-SWCNT samples were differentially fabricated from the same starting material to produce a monodisperse sample and a lesser quality dispersion with defined levels of aggregation. We found that, when equally dosed, the rate of uptake was independent of the starting sample quality in any given cell line. The number of nanotubes within individual subcellular regions was quantified upon internalization using a hyperspectral counting method. Higher quantities of nanotubes were consistently found in subcellular regions when dosed with a monodisperse sample, while the intracellular distribution of aggregates appeared to match the stock sample population. This sample-dependence could indicate that single SWCNTs can be selectively accumulated in single vesicles upon internalization, and thus the degree of aggregation state could influence certain intracellular pathways. Cell proliferation and viability were also found to be lower in cells dosed with monodisperse nanotubes, further indicating the aggregation state modulates internalization and processing mechanisms. Therefore, the effects of aggregation must be acknowledged when characterizing nanomaterials in biological settings.

5.5. Materials and Methods

DNA-SWCNT Sample Preparation: Raw single-walled carbon nanotubes produced by the HiPco process (Nanointegris) were used throughout this study. For each dispersion, 2 mg of (GT)_{15} oligonucleotide (Integrated DNA Technologies) was added to 1 mg of raw nanotubes in 1 mL of 0.1M NaCl (Sigma-Aldrich) in deionized water. The
singly-dispersed sample (250k-SWCNT) was ultrasonicated using a 1/8” tapered microtip for 30 min at 40% amplitude (Sonics Vibracell VCX-130; Sonics and Materials). The resultant suspension was ultra-centrifuged (Sorvall Discovery M120 SE) for 30 min at 250,000 × g and the top ~80% of the supernatant was collected. The initially-aggregated sample (1k-SWCNT) was ultrasonicated using a 1/8” tapered microtip for 5 min at 40% amplitude (Sonics Vibracell VCX-130; Sonics and Materials). The resultant suspension was benchtop-centrifuged (Eppendorf Centrifuge 5430 R) for 5 min at 1,000 × g and the top ~25% of the supernatant was collected. Concentrations were determined using a UV/vis/NIR spectrophotometer (Jasco, Tokyo, Japan) and the extinction coefficient of $A_{910} = 0.02554 \text{ L mg}^{-1} \text{ cm}^{-1}$.\textsuperscript{60}

**Near-Infrared Fluorescence Microscopy:** A near-infrared hyperspectral fluorescence microscope, comparable to a previously detailed system,\textsuperscript{60} was used to obtain all hyperspectral fluorescence data. In short, a 730 nm excitation laser source was reflected on the sample stage of an Olympus IX-73 inverted microscope equipped with either a UApo N 100× /1.49 oil immersion IR objective (Olympus, USA) or a LCPlan N, 20× /0.45 IR objective (Olympus, U.S.A.), as indicated in the figure captions. Resultant fluorescence emission was passed through a volume Bragg Grating and collected with a 2D InGaAs array detector (Photon Etc.) to generate spectral image stacks. Live-cell samples were mounted on a stage top incubator unit (Okolab) to maintain 37 °C and 5% CO$_2$ culture conditions throughout the imaging procedure. All hyperspectral cubes, broadband fluorescence images, and transmitted light images were corrected and processed in Matlab.

**Diffraction-Limited Fluorescence Imaging of DNA-SWCNTs:** DNA-SWCNT samples were prepared for direct 100× NIR fluorescence imaging using a spin-coating
technique to embed a single focal plane of SWCNTs between thin hydrogel layers. Briefly, a thin film of 2% agarose was deposited on a glass coverslip by spin-coating 450 μL at 1400 rpm for 30 seconds. The first layer was allowed to solidify before 200 μL of 5 mg/L 250k-SWCNTs or 1k-SWCNTs were subsequently spin-coated onto the agarose surface at 1000 rpm for 30 seconds. A final agarose layer was cast on top of the spin-coated films and allowed to gel for at least 5 minutes. The hydrogel samples were mounted on a NIR hyperspectral fluorescence microscope with excess DI water during fluorescence data acquisition.

**Cell Culture:** RAW 264.7 TIB-71, HeLa CCL-2, and A549 CCL-185 cell lines (ATCC, Manassas, VA, USA) were cultured under standard incubation conditions at 37 °C and 5% CO₂. D-10 cell culture medium containing sterile filtered high-glucose DMEM with 10% heat-inactivated FBS, 2.5% HEPES, 1% L-glutamine, 1% penicillin/streptomycin, and 0.2% amphotericin B (all acquired from Gibco) was used for all cell lines.

**In Vitro Sample Preparation for Optical Microscopies:** For all *in vitro* 20× NIR fluorescence imaging experiments, cells were seeded into 24-well tissue culture plates (CELLTREAT) to concentrations of 5.26 x 10⁴ (RAW) or 2.11 x 10⁴ cells/cm² (HeLa/A549) and allowed to culture overnight. To dose the cells, the media was removed from each culture dish, replaced with 1 mg/L of either 250k-SWCNTs or 1k-SWCNTs diluted in media, and incubated for 30 minutes to allow internalization into the cells. The SWCNT-containing media was subsequently removed, and the cells were rinsed twice with sterile phosphate buffered saline (PBS, Gibco) before fresh media was replenished. All time points were defined with respect to this step, *i.e.*, 30 minutes, 6 hours, or 24 hours
after the SWCNT-media was removed from the cells. For all 100× NIR fluorescence imaging and 20× confocal Raman microscopy experiments, the cells were seeded into 35mm glass bottom microwell dishes (MatTek) to the same concentrations as previously listed and allowed to culture overnight. The same SWCNT-dosing procedure was followed, however cells were fixed at each time point. Fixation was performed with 4% paraformaldehyde in PBS for 10 minutes, after which cells were rinsed 3 times and covered with PBS to retain an aqueous environment during imaging.

**Confocal Raman Microscopy:** All Raman data was acquired using an inverted WiTec Alpha300 R confocal-Raman microscope (WiTec, Germany) equipped with an Olympus LCPlan N, 20× /0.45 IR objective, a 785 nm laser (35 mW output measured at the sample), and a UHTS 300 spectrograph (300 lines/mm grating) coupled with an Andor DR32400 CCD detector (-61 °C, 1650 x 200 pixels). Small cellular areas were scanned, and spectra were obtained in 1 × 1 μm intervals using 0.8 s integration time per spectrum to construct hyperspectral images of individual cells. Global background subtraction and cosmic-ray removal were performed on each scan using Witec Project 5.2 software. Hyperspectral data was extracted and processed using custom codes written with Matlab software.

**Hyperspectral Counting:** We utilized hyperspectral counting, a recently developed quantitative technique, to estimate the number of SWCNTs contained in diffraction-limited regions of interest (ROIs). Briefly, hyperspectral images were acquired at 100× magnification, initially processed to correct for background intensities, and split into individual ROIs corresponding to localized fluorescence from one or more nanotubes. ROIs were determined manually in FIJI for all DNA-SWCNT characterization data, while
a previously developed segmentation pipeline was globally applied to all cell data to identify ROIs based on pixel intensities and morphological structures. Analysis was then carried out individually on each ROI, from which the average fluorescence spectrum was extracted, labeled, and compiled into one of two datasets corresponding to either direct SWCNT imaging or intracellular fluorescence data. Once all data was pooled appropriately, each spectrum was manually viewed to assess the number of identifiable emission peaks from each ROI. Additionally, a custom Matlab app was designed to facilitate the blind analysis of > 1,000 intracellular ROI spectra acquired for all conditions.

**Label-Free Cell Proliferation and Adherence Monitoring:** Proliferation and adherence were measured using an xCELLigence Real Time Cell Analysis (RTCA) instrument (Agilent). To measure the baseline impedance of the wells, 140 µL of cell media were added to each of the RTCA E-Plates wells. Next, 50 µL of cells diluted in media were added to each well to reach final concentrations of 20000 cells/well (RAW), 2500 cells/well (HeLa), or 4000 cells/well (A549). The cells were allowed to adhere to the plates for 30 min in a cell culture hood to reduce convection currents, allowing for an evenly distributed initial seeding of cells over the electrodes. After 30 min, the E-Plates were placed into the xCELLigence instrument and data acquisition occurred every 15 min. After 24 hours each plate was spiked with 10 µL of either 250k-SWCNTs or 1k-SWCNTs diluted in media to reach final concentrations of 1 mg/L or 10 mg/L. Data acquisition occurred every 1 min for 60 min, then every 5 min for 12 h, and finally every 15 min for the remainder of the experiment. Data was normalized to the last measured data point before SWCNT addition, and \( n = 4 \) wells were used for each experimental data set.
Cell Viability Assay Image Cytometry Assay: Cells were plated on 35mm glass bottom microwell dishes (MatTek) and were allowed to culture overnight. Initial plating cell concentrations were $5.26 \times 10^4$ cells/cm$^2$ (RAW), $1.59 \times 10^4$ cells/cm$^2$ (HeLa), and $2.11 \times 10^4$ cells/cm$^2$ (A549). The following day the media was replaced with 1 mg/L of either singly-dispersed or initially-aggregated (GT)$_{15}$-SWCNT diluted in media and incubated for 24 hours. After 24 hours the cells were collected from the dishes and stained with Annexin V and propidium iodide (Dead Cell Apoptosis Kit V13242, Invitrogen) following the manufacturer’s protocol. Fluorescence images of the stained cells were acquired by using a Cellometer Vision CBA Image cytometer (Nexcelom Bioscience), and images were analyzed by using ImageJ and custom MATLAB codes. For each cell condition a control dish was plated without SWCNT addition to create the gates on the Annexin V and propidium iodide axes of the histograms.

Statistical Analysis: OriginPro 2018 was used to perform all statistical analysis. All data either met assumptions of the statistical tests performed (i.e., normality, equal variances, etc.) or was transformed to meet assumptions before statistical analysis was carried out. Statistical significance was analyzed using two-sample two-tailed student t-test or one-way ANOVA where appropriate. Testing of multiple hypotheses was accounted for by performing one-way ANOVA with Tukey’s post hoc test. Specific information about statistical analyses can be found in figure legends.
5.6. Acknowledgements

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5.7. References


Chapter 6
A Wearable Optical Microfibrous Biomaterial with Encapsulated Nanosensors Enables Wireless Monitoring of Oxidative Stress

by

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6.1. Abstract

In an effort to facilitate personalized medical approaches, the continuous and noninvasive monitoring of biochemical information using wearable technologies can enable a detailed understanding of an individual’s physiology. Reactive oxygen species (ROS) are a class of oxygen-containing free radicals which function in a wide range of biological processes. In wound healing applications, the continuous monitoring of ROS through a wearable diagnostics platform is essential for the prevention of chronicity and pathogenic infection. Here, a versatile one-step procedure was utilized to fabricate optical core-shell microfibrous textiles incorporating single-walled carbon nanotubes (SWCNTs) for the real-time optical monitoring of hydrogen peroxide concentrations in in vitro wounds. The environmentally sensitive and non-photobleachable fluorescence of SWCNTs enabled continuous analyte monitoring without a decay in signal over time. The existence of multiple chiralities of SWCNTs emitting near-infrared fluorescence with narrow bandwidths allowed a ratiometric signal readout invariant to the excitation source distance and exposure time. The individual fibers encapsulated the SWCNT nanosensors for at least 21 days without apparent loss in structural integrity. Moreover, the microfibrous textiles were utilized to spatially resolve peroxide concentrations using a camera and further integrated into commercial wound bandages without significant degradation in their optical properties.

6.2. Introduction

Reactive oxygen species (ROS) are continuously generated and consumed in all eukaryotic and prokaryotic cells as a consequence of aerobic life. In this biological context, ROS primarily function to preferentially react with specific atoms of biomolecules
involved in a wide range of physiological processes.\textsuperscript{1} ROS play a crucial role in biological signaling including the inhibition or activation of proteins, subsequent promotion or suppression of inflammation, immunity, and carcinogenesis.\textsuperscript{1,3-6} Oxidative stress can occur if the ROS-induced signal is too strong, if it persists for too long, or if it occurs at the wrong time or place.\textsuperscript{1,3,5,7} As a key example, wound healing is one of the most dynamic biological processes involving ROS-linked cellular signaling throughout the entire mechanism.\textsuperscript{8-11} Additionally, basal concentrations of ROS aid in the fight against invading microorganisms into open wounds.\textsuperscript{4,11,12} The excessive and uncontrolled production of ROS contributes to the sustaining and deregulation of inflammation processes, which play a central role in the pathogenesis of chronic non-healing wounds.\textsuperscript{13-17} Physiologically, hydrogen peroxide (referred herein as peroxide) and superoxide function as intracellular ROS messengers stimulating key phases of wound healing including cell recruitment, production of cytokines, and angiogenesis.\textsuperscript{16,18,19} Of note, peroxide acts as the principal secondary messenger in wound healing and is present at low concentrations (100–250 µm) in normal wounds.\textsuperscript{8,11,19,20} Increased peroxide concentration is a biomarker for inflammation and chronicity in which biofilm-forming pathogens can grow significantly faster than acute wounds.\textsuperscript{15,17,20} Interestingly, strictly controlling the ROS levels through antioxidants has recently been shown to improve inflammatory skin conditions and wound healing process in diabetic and hypoxic environments.\textsuperscript{21}

Due to the short half-lives of ROS, the direct detection and quantification of their concentrations are often difficult in the laboratory and in patients.\textsuperscript{20} Although multiple classes of sensors and spectrophotometric assays have been developed to monitor various types of ROS, the current methods are limited in their capabilities. Spectrophotometric
methods, such as total antioxidant capacity assays (TAC) and gel electrophoresis have been utilized to indirectly determine the oxidation products of lipids, proteins and DNA, but these are not capable of real-time monitoring in the wound site. Various label-free electrochemical biosensors have been developed to accurately quantify the ROS concentrations by immediately converting the chemical information to an electrical signal. The main drawback of electrochemical techniques is the requirement to incorporate electrodes into different biomaterials and wireless platforms. Moreover, the need to utilize an electrochemical signal transducer restricts the application of the current sensors on wounds in different organs of the body. Fluorescent nanoparticles and genetically encoded fluorescent molecules highly selective for peroxide have been created to study the redox events in mice, zebrafish, and cells, but these assays cannot be utilized for real-time monitoring in clinical applications. Therefore, developing a point-of-care diagnostic technology for the real-time monitoring of ROS concentrations in wound sites is essential to prevent chronicity and infection, and to deliver accurate amounts of antioxidants and antibiotics to the wounds.

Single-walled carbon nanotubes (SWCNTs) with engineered wrappings have recently been developed and utilized in various disparate fields ranging from additives that strengthen material composites to biomedical applications including near-infrared (NIR) optical biosensing, and biological imaging. The electronic band gap energies of SWCNTs are dependent on their chiral identity, denoted by integers (n,m), and vary based on diameter and rollup angle, resulting in various semiconducting species which exhibit a distinct narrow-bandwidth photoluminescence in the second NIR window. The SWCNT photoluminescence responds to their local environment, resulting in shifts in
emission wavelengths\textsuperscript{40-43} and/or variations in intensity.\textsuperscript{44, 45} Certain amphiphilic polymers such as short single-stranded deoxyribonucleic acids (ssDNA),\textsuperscript{41} Phospholipid-Polyethylene glycol (PL-PEG),\textsuperscript{46} and synthetic polymers\textsuperscript{34} have all been shown to effectively solubilize SWCNTs, imparting enhanced biocompatibility and desirable fluorescence properties. The resultant hybrid nanomaterials have been optimized for the detection of a wide range of analytes \textit{in vivo} and \textit{in vitro} including neurotransmitters,\textsuperscript{45} lipids,\textsuperscript{35} and proteins.\textsuperscript{43} SWCNT-based optical nanosensors have also recently been developed for real-time spatial and temporal monitoring of ROS in various plant species as a biomarker for plant health.\textsuperscript{44, 47} Moreover, ratiometric SWCNT-based optical sensors have enabled the real-time monitoring of ROS in plants, allowing an absolute calibration independent of overall intensity.\textsuperscript{48} The current ratiometric sensing approaches based on SWCNTs require separation of at least two highly pure single chiralities, wrapped in two different polymers, where one polymer-chirality pair is sensitive to the local environment and the other pair does not spectrally respond to the variations in the local environment and acts as a reference.\textsuperscript{48}

Although the ssDNA- and polymer-wrapped SWCNT nanosensors have attracted significant interest in the past decade for biosensing applications \textit{in vivo} and \textit{in vitro}, the integration of these biosensors into other bulk biomaterial platforms has been a challenge as their NIR fluorescence is remarkably sensitive to the chemistry of their local environment and can be suppressed by other components in the biomaterial preparation processes.\textsuperscript{34, 40} Moreover, due to the hydrophilicity of these nanosensors, it is unfavorable to engage them in any process involving organic solvents as they form bulk aggregates in hydrophobic environments.
With revolutionary advances in nanotechnology and biomaterials in recent years, an extensive range of smart wound care biomaterials have been developed that enable localized delivery of drugs on the wound site\textsuperscript{49, 50} and real-time monitoring of the wound microenvironment.\textsuperscript{51, 52} Electrospun microfibers are one of the novel classes of wound dressings as they mimic the chemical and mechanical environment of the 3D extracellular matrix.\textsuperscript{53, 54} Microfiber-based wound dressings have been designed to enhance cell migration,\textsuperscript{55} prevent inflammation and infection,\textsuperscript{56} and inhibit scar formation on wounds.\textsuperscript{54} Herein, we utilized a one-step co-axial electrospinning process to fabricate wearable microfibrous textiles incorporating peroxide-sensing SWCNTs. The electrospun fibers feature a core-shell morphology in which the SWCNTs are encapsulated inside of a polymer shell that is soluble in an organic solvent. We chose polycaprolactone (PCL) as the shell material as it is an FDA-approved polymer which has been extensively studied for tissue engineering and wound healing applications.\textsuperscript{57-59} Utilizing confocal Raman microscopy, we found that the SWCNT nanosensors stay encapsulated within the individual fibers for up to at least 21 days, indicating that the long-term identity of the nanosensing platform is maintained. Our wearable optical platform was able to wirelessly and reversibly detect peroxide in a physiologically-relevant range for wounds (1-250 µm), without the requirement to embed any electronics or power sources within the textile itself. The ratiometric characteristic of the NIR fluorescence sensor facilitates \textit{in vivo} and clinical applications as it transduces an absolute signal that is not dependent on excitation source distance nor exposure time. Moreover, our optical textiles were able to detect peroxide in real-time in presence of RAW macrophages, demonstrating the capability of the platform for ROS monitoring in complex biological environments. We also indicated the potential
of our optical textiles for spatially resolving the peroxide concentration on the surface of a wound using hyperspectral fluorescence microscopy. Finally, we attached our microfibrous platform to a conventional wound bandage and demonstrated the feasibility of \textit{in situ} measurements of peroxide in wounds.

6.3. Results and Discussion

6.3.1. Preparation and Characterization of Optical Microfibers

We prepared aqueously-dispersed ssDNA-SWCNT nanosensors by probe-tip sonicating HiPco SWCNTs in the presence of single-stranded (GT)$_{15}$ DNA (Figure 6.1a). The (GT)$_{15}$ sequence was selected as SWCNT-based nanosensors with this sequence of DNA have been utilized in live cells and plants for the real-time and selective monitoring of peroxide, in contrast to other signaling molecules including nitric oxide (NO), superoxide ([O$_2$]•), singlet oxygen (1O$_2$) and hydroxyl radical ([OH]•).[27, 44, 48] Following sonication, the sample was ultracentrifuged to remove bundles of undispersed SWCNTs as well as residual catalyst particles to produce an ink-like solution with strong NIR absorbance and fluorescence spectra (Figure S6.1). With colloidally stable nanosensors, we employed a core-shell electrospinning procedure to encapsulate the hydrophilic ssDNA-SWCNTs along with poly(ethylene oxide) (PEO) into the polymer polycaprolactone (PCL), that is soluble in an organic solvent (Figure 6.1b). Briefly, the shell and core are extruded from a two-compartment spinneret, and once injected, form a core-shell pendant droplet as the result of surface tension.[54] A high voltage is applied to the droplet that produces a two-compartment Taylor cone as well as a constant electric field between the spinneret and a metal grounded collector.[54] The electrical force significantly elongates the two components of the Taylor cone until they turn into microfibers.[54] After
rapid solvent evaporation, the immiscibility of the core and shell causes complete encapsulation of the core within the shell.\[60\] In this process, the hydrophilic ssDNA-SWCNT nanosensors are protected against a prolonged interaction with an organic solvent. Additionally, the intrinsic NIR fluorescence of the nanosensors is maintained as the process does not introduce any other chemicals such as crosslinking agents. There are a number of physical parameters involved in the electrospinning process which control the reproducibility and homogeneity of the final samples. We have optimized the flow rates of the polymers, rotation rate of the collector, and the distance between the needle and collector to achieve a stable electrospinning jet (data not shown).

**Figure 6.1.** Fabrication of optical microfibers. (a) Nanosensor preparation by probe-tip sonicating SWCNTs in the presence of ssDNA followed by ultracentrifugation of the resultant dispersion. (b) Core-shell electrospinning setup for fabrication of the optical microfibrous textiles. The two syringes containing the core and shell polymer solutions are connected to the inlets of a custom core-shell needle. Once the polymer solutions are injected out, a core-shell pendant droplet is formed. A high-voltage supply is connected to
the tip of the needle and electrifies the droplet, forms a Taylor cone, and eventually elongates the cone until microfibers are created. The resultant fibers are collected onto a rotating metal grounded collector.

To optimize the morphology of the fibers and aggregation state of the nanosensors, we have tuned the applied voltage during the electrospinning process. Figure 6.2 demonstrates NIR broadband fluorescence images (900-1400 nm) and scanning electron microscopy (SEM) images of fibers produced with three different voltages. The applied voltage of 12 kV did not provide a high enough rate of elongation, and as a result, SWCNT aggregates appeared in the NIR fluorescence images (Figure 6.2a and S2). When the applied voltage was 16 kV, occasional aggregates again emerged along the fibers (Figure 6.2c and S4), presumably due to incomplete formation of the Taylor cone. An applied voltage of 14 kV produced a homogenous fiber morphology with no significant spatially localized aggregations (Figure 6.2b and S3). The SEM images of the fibers produced with the three voltages revealed two subsets of fibers with diameters of more than ~1 µm or less than ~100 nm (Figure 6.2d-f, S5). By visually comparing the SEM and NIR fluorescence images, we observe that the diameter range of the NIR optical fibers is identical to the micron-size fibers in the SEM images (Figure S6.2-5). Thus, although we have produced a matrix of micro- and nanofibers, we acknowledge the fact that the optically-active fibers have sizes of more than 1 µm.
Figure 6.2. NIR broadband fluorescence images of microfibers produced with the applied voltages of (a) 12 kV, (b) 14 kV, and (c) 16 kV. The microfibers were illuminated with a 730 nm laser and images were acquired with a 2D InGaAs array detector in the wavelength range 900-1400 nm. SEM images of the fibers fabricated with applied voltages of (d) 12 kV, (e) 14 kV, and (f) 16 kV.

We employed confocal Raman microscopy to confirm the core-shell morphology and assess the complete encapsulation of nanosensors within the individual fibers. Confocal Raman microscopy is a powerful technique used to analyze multicomponent material samples, in which the unique Raman spectrum of each component can be identified and spatially resolved. SWCNTs exhibit distinct Raman signatures such as the G-band (1589 cm\(^{-1}\)), which proportionately scales with increasing amounts of graphitic carbon (i.e. SWCNT concentration)[61,62] and the radial breathing mode (150 - 350 cm\(^{-1}\)), which can identify the chiral composition of a SWCNT mixture.[63,64] Figure 6.3a displays
the brightfield and G-band intensity overlay of a single as-produced fiber. A k-means clustering analysis was applied to the entire dataset, in which the spectrum from each pixel was partitioned into one of 4 clusters ($k = 4$) based on the location and intensity of individual peaks, creating 4 average spectra which best represented all regions, including the background, from the Raman area scan (Figure 6.3b and c).\cite{65} Based on the clustering analysis, each individual fiber was categorized to three areas: core, intermediate, and shell (background constituted the fourth cluster). The average Raman spectrum of the core area corroborated previous reports of SWCNT Raman spectra (Figure 6.3c).\cite{32, 41} The average Raman spectrum of the shell area predominantly matched with the spectrum from the PCL polymer (Figure S6.6a) with two additional peaks at $\sim$1589 and 240 cm$^{-1}$, which can be correlated to small quantities of SWCNTs. However, it is worth noting that SWCNTs benefit from signal enhancement due to resonance Raman scattering,\cite{66} and thus the intensity of their peaks in the shell clusters could over-represent their actual quantity with respect to PCL. Interestingly, the average Raman spectrum from the intermediate area features spectral characteristics from both SWCNTs and PCL polymer, indicating an area of heterogeneity at the core-shell interface. These results demonstrate that the highest density of the nanosensors reside in the core area of the fibers, while their Raman signal diminishes in the outward radial direction and the main component becomes the polymer shell. Although the spectral characteristics of the PEO polymer are not apparent in the Raman spectra of the three areas, they are presumably dominated by the enhanced Raman signal of the SWCNTs. Moreover, the Raman spectra of the PCL and PEO polymers show minimal overlap with the G-band and RBM peaks from SWCNTs (Figure S6.6a and b),
indicating that the identified SWCNT signal contains no contribution from the other nanofiber components.

Figure 6.3. Confocal Raman microscopy of dry fibers and fibers soaked in PBS for 7 days. (a) and (d) The representative overlay of G-band intensity and brightfield images of dry fibers and fibers soaked in PBS for 7 days, respectively. (b) and (e) k-means clustering analysis of all spectra in each area scan, where $k = 4$ clusters (background cluster omitted from figure). (c) and (f) The average Raman spectra obtained from each cluster of (b) and (e).

To assess the ability of individual fibers to preserve the nanosensors over time, we soaked the fiber samples in phosphate buffered saline (PBS) solution and performed confocal Raman microscopy at different timepoints. Figure 6.3d and S7 indicate that the SWCNTs remain encapsulated within individual fibers after 7 days, without forming noticeable aggregates or any deformation in the fiber structure. Moreover, k-means clustering analysis identified the same spectral characteristics for the three identified areas.
(core, intermediate and shell) at different time points, further demonstrating that the fibers retain their entire structural integrity in an aqueous environment (Figure 6.3e, f, and S7b, c, e, f). We acknowledge that the ratios between the peaks in Raman spectra of particularly the shell vary among different time points, but this can be attributed to the slight heterogeneity among individual fibers as we have not imaged the same fiber at different time points.

To quantify the amount of the released nanosensors from a bulk fibrous matrix, the microfibrous textiles with thickness of ~0.7 mm were cut to 1 square inch pieces and soaked in PBS. We collected the PBS solutions over time for up to 21 days and acquired their Raman spectra (Figure S6.8). Comparing the Raman spectra of the released nanosensors with that of three standard samples with known concentrations (0.01, 0.1, 1 mg L\(^{-1}\)), the lack of G-band signal indicates that the released nanosensors fall within the noise range of our Raman spectrometer. We conclude that over the course of at least 21 days, a negligible amount of the nanosensors are released from the fibers.

6.3.2 Ratiometric Peroxide Detection Using Optical Microfibrous Textiles

The HiPco SWCNTs contain multiple chiralities emitting NIR fluorescence in the range of 900-1400 nm (Figure S6.1b).\(^3\) As both chirality and DNA sequence determine the spectral responses of SWCNTs to their local environment,\(^4\) we hypothesized that certain chiralities of SWCNTs within the HiPco sample would differentially respond to hydrogen peroxide due to their differing bandgaps energies.\(^6\) We first acquired NIR hyperspectral fluorescence images of the fluorescent fibers containing (GT)\(_{15}\)-SWCNTs (Movie S1) using the optical setup illustrated in Figure 6.4a. Figure 6.4c-e indicate the fluorescence intensity of three different chiralities in the same fiber sample, i.e. the (9,4),
(8,6), and (8,7)-SWCNT chiralities.[41] To test the environmental sensitivity of the optical fibers to peroxide, we exposed the bulk microfibrous samples (area: 0.5 mm$^2$, thickness: \( \sim 0.7 \) mm) to various concentrations of peroxide and acquired fluorescence spectra after 24 hours, utilizing a probe NIR fluorescence spectrometer (Figure 6.4b), which enabled the resolution of the three mentioned chiralities. In agreement with previous studies,[44, 47, 48] Figure 6.4f and S9 reveal that the three chiralities quench upon exposure to peroxide, however, the extent of quenching varies significantly among the chiralities. By normalizing each plot by its maximum intensity (i.e. the intensity of the (9,4) chirality), we observe that the normalized (8,6) and (8,7) peaks monotonically intensify with increasing peroxide concentration, illustrating that a ratiometric signal can be obtained for peroxide detection (Figure 6.4g). We selected the (8,7) / (9,4) intensity ratio to further calibrate a biosensor for peroxide detection as it appears to be more sensitive to peroxide concentrations compared to the (8,6) / (9,4) ratio.
Figure 6.4. (a) The schematic of the optical setup utilized to obtain the hyperspectral NIR fluorescence intensity images from the microfibers. (b) The schematic of the probe NIR fluorescence spectrometer utilized to obtain the fluorescence spectra from bulk microfibrous samples. (c) (9,4), (d) (8,6) and (e) (8,7) chirality intensity images, obtained using the system shown in part a. (f) The fluorescence spectra of the microfibrous samples exposed to various peroxide concentrations, obtained from the system shown in part b. (g) The fluorescence spectra shown in part d were normalized by their max intensity.
6.3.3. Calibrating the Microfibrous Textiles for Peroxide Detection

To obtain a calibration curve for aqueous peroxide detection, we exposed the initially dry microfibrous samples to various concentrations of peroxide ranging from 1 µm to 5 mm and monitored the samples over time for up to 72 hours. Figure 6.5a demonstrates that all samples produced the same initial ratiometric signal, clarifying the reproducibility of our method for fabricating bulk samples of optical fibers encapsulating nanosensors. After 24 hours, a concentration-dependent ratiometric signal was obtained from the samples. While 0 to 5 µm peroxide gave no significant change in the ratiometric signal, it monotonically increased with peroxide concentration in the range of 5 µm to 5 mm and could be fit to a linear function with $R^2 = 0.99$ on a log-log scale of signal vs. concentration (Figure 6.5a and Table S1). When examined at 48 and 72 hours after the addition of peroxide, the ratiometric signal systematically increases while maintaining the trend in the calibration curve. The data can still be fit to a linear function in the range of 5 µm to 5 mm. To explain the time dependency of the calibration curves, we propose that the noncovalently wrapped DNA adopts more compact conformations on the SWCNT surfaces over time as they progressively interact with ions, mainly sodium, in the PBS. This rearrangement alters the fluorescence intensity in a chirality-dependent fashion, and thus the ratio of the peaks over time, regardless of peroxide concentration. Moreover, the diffusion of the peroxide molecules through the pores in the 3D matrix and through the free spaces in between the polymer chains on the shell is a time-dependent process, so it will result in further fluorescence quenching over time. These two phenomena governing the temporal dependence of the peroxide calibration curve are depicted in Figure 6.5b.
Figure 6.5. (a) Calibration curve showing the ratiometric signal, i.e. (8,7) intensity divided by (9,4) intensity, as a function of peroxide concentration at three different time points. Mean values were obtained by adding each peroxide concentration to three different samples (n = 3), and error bars represent the standard deviation. (b) Two phenomena governing the temporal dependence of the peroxide calibration curve; (I) The diffusion of peroxide molecules through the 3D matrix of fibers and through the shell to reach the ssDNA-SWCNTs in the core of the individual fibers. (II) Due to the ionic strength of the surrounding environment, the ssDNA on the surface of nanotubes undergoes a conformational change over time and forms a more compact wrapping around the nanotubes. As a result of these two phenomena, the NIR fluorescence of SWCNTs and the ratiometric signal are altered over time.

Therefore, in order to design a wearable fibrous device for continuous peroxide monitoring, we propose a two-dimensional calibration curve where the ratiometric signal is a function of both analyte concentration and time. The heatmap in Figure 6.6a demonstrates the ratiometric signal as a function of the tested concentrations of peroxide and time points. We plotted the ratiometric signal as a function of time for all concentrations (Figure 6.6b). Interestingly, the data for all examined peroxide concentrations could be fit to single exponential association functions (Equation 1 and Figure S6.10 and S11) where the offset ($R_0$) and time constant ($\tau$) of the single exponential showed a narrow distribution with small standard errors of the means (Figure S6.12 and Table S2). The lack of dependence on peroxide concentration found within the fitted time
constants, including the dataset of zero added peroxide, suggests the dominant physical process being modeled is that of the DNA rearrangement on the SWCNT surface. In contrast, the pre-exponential factors ($A$) displayed a concentration-dependent trend which could be fit to a power function (Equation 2, Figure 6.6c and S13, Table S3). By incorporating Equation 2 into Equation 1, we obtained a two-input calibration function where the ratiometric signal could be expressed as a function of both peroxide concentration and time (Equation 3, Figure 6.6d). To further examine the real-time response of the textiles to peroxide upon concentration fluctuations, we exposed the samples to two different concentrations of peroxide (500 µm and 5 mm), and acquired the fluorescence spectra every 1 minute (Figure S6.14). As apparent from the Figure S6.14a and b, there was an abrupt concentration-dependent decrease in the fluorescence intensity immediately following the addition of peroxide at 5 minutes. Moreover, the ratiometric signal deviated from the control in a concentration-dependent manner, confirming the fast response time of the textiles to varying peroxide concentrations (Figure S6.14c). Finally, to examine the reversibility of the platform, we exposed a sample to peroxide (200 µm) and then washed it with PBS. As shown in Figure S6.15, we observed that the ratiometric signal increased 10 minutes after exposure to the peroxide and then completely reverts back to the original signal 50 minutes after removing it.

(1) $\begin{align*}
R &= R_0 + A \left( 1 - e^{-t/\tau} \right); \\
R_0 &= 0.383 \pm 9.28E-4, \; \tau = (29.305 \pm 0.615) \text{hour} \\
A &= (C + C_0)^p - A_0
\end{align*}$

(2) $A = (C + C_0)^p - A_0$

(3) $C_0 = (10.796 \pm 0.596) \mu m, \; p = 0.035 \pm 2.52E-4, \; A_0 = 0.972 \pm 3.34E-3$

$R = R_0 + [(C + C_0)^p - A_0] \left( 1 - e^{-t/\tau} \right)$
Figure 6.6. Calibrating the optical microfibrous textiles for continuous peroxide detection. (a) Two-dimensional heat-map illustrating the ratiometric signal as a function of both concentration and time. (b) Ratiometric signal as a function of time for each peroxide concentration. The dashed lines indicate single exponential association fits. (c) Pre-exponential factors extracted from the single exponential association fits and plotted as a function of peroxide concentration. The dashed line indicates a power function fit. (d) Contour plot demonstrating a two-dimensional calibration curve where the fitted ratiometric signal is a function of both time and concentration. Mean values were obtained by adding each peroxide concentration to three different samples (n = 3), and error bars represent the standard deviation.

In the presence of biological fluids, it is known that nanoparticles will interact with proteins and other biomolecules to spontaneously and rapidly form a “protein corona” on their surface. This corona can trigger an immune response and result in variations in the intrinsic characteristics of the nanoparticles. In the case of the functionalized SWCNTs, this can alter their fluorescence emission and sensitivity to the chemistry of their
local environment. To elucidate the functionality of the optical textiles in a complex biological environment containing proteins, carbohydrates and salts, we seeded RAW 264.7 macrophages on the surface of the textiles for 24 hours and utilized phorbol 12-myristate 13-acetate (PMA) in order to stimulate them to produce and export peroxide to their surrounding media (Figure S6.16a and b). Macrophages are the immune system’s first line of defense, whether as a primary response to a wound or to engulf foreign substances such as nanoparticles that enter the bloodstream. By stimulating the macrophages to produce approximately 1000 µm of peroxide, an abrupt reduction in the fluorescence intensity and increase in the ratiometric signal was observed in less than 30 minutes, confirming our results from the solutions of peroxide (Figure S6.16c, d and e). We acknowledge that the overall fluorescence intensity is reduced in the biological environment compared to the textiles exposed to PBS, for all three nanotube chiralities, and this can be attributed to the formation of a biomolecular corona on the SWCNTs.

In contrast to an average concentration reported by a single readout, the ability to spatially resolve peroxide concentrations will enable an end-user the ability to map peroxide on the surface of a wound. We exposed the microfibrous samples to different concentrations of peroxide and acquired hyperspectral fluorescence images from the surface of the samples under 5X magnification using the setup shown in Figure 6.4a. By dividing the maximum intensity images of the (8,7)-SWCNT by the (9,4)-SWCNT, we created maps where each pixel represented a ratiometric signal (Figure 6.7 and S17). At time zero, the maps for all concentrations of peroxide were dominated by blue pixels (low ratiometric signal) (Figure S6.17). After 24-hour exposure to peroxide, the pixel colors among the maps diverge and are dominated by yellow color at low concentrations and red
color at high concentrations. This demonstrates the potential of employing our optical fibrous platform to quantitatively image the surface of a wound in a label-free manner utilizing a NIR camera along with the appropriate bandpass filters. This can be performed either after a bandage has been removed from the wound by correlating to spatial position from the wound if the bandage is carefully removed and its location documented or while the bandage is in place on the wound.

**Figure 6.7.** Spatially detecting peroxide. The maps were created by acquiring NIR fluorescence hyperspectral images and dividing the max intensity image of the (8,7)-SWCNT by the max intensity image of the (9,4) chirality SWCNT. Since the data were acquired using a 5X objective, the individual fibers cannot be observed.

To illustrate the potential of our optical fibrous platform as a smart wound dressing for *in situ* monitoring of peroxide, we attached the fibrous samples onto a commercial wound bandage (**Figure 6.8a**). By attaching the samples to the complete bandage (adhesive material + adsorbent pad) or only to the adhesive material of the bandage, the fluorescence of SWCNTs was still detectable by our probe spectrometer through the bandage and with a high signal to noise ratio for each peak (**Figure 6.8b** and S18). The drop in the signal compared to the control is presumably due to the polymers in the bandage that absorb a portion of the excitation light and/or emitted fluorescence from the SWCNTs. Additionally, the combination of sample plus the adhesive material did not significantly alter the ratiometric signal even after 7 days of soaking in PBS whereas the optical attenuation in
presence of the complete bandage appears to have a dependence on wavelength, and thus it alters the ratiometric signal and increases the heterogeneity from the readout as indicated from the larger error bars (Figure 6.8c). We believe this arises from variations in the thickness, porosity, and/or local heterogeneities in the absorbent pad itself.

Finally, Figure 6.8d and Movie S2 demonstrate the feasibility of a real-time wireless wound screening utilizing our flexible optical fibrous platform attached onto the commercial bandage. Transferring this technology from the bench to the bedside is undoubtedly contingent upon designing portable and wearable NIR fluorescence spectrometers. The recent advances in optoelectronics has enabled fabrication of miniaturized thermoelectrically cooled (TEC) InGaAs photodiodes.\textsuperscript{77-79} As a future direction for this work, we aim to construct portable and wearable NIR fluorescence detectors, capable of transducing the data to a smart phone,\textsuperscript{80} by assembling a TEC InGaAs photodiode, an LED light source and bandpass filters into portable and wearable platforms to enable point-of-care wound diagnostics. Furthermore, it is worth mentioning that hydrogen peroxide has been traditionally utilized for wound cleaning. We acknowledge that the exogenously applied peroxide will undoubtedly disrupt initial sensor readouts; however, alternative wound cleaning strategies have been recently developed due to severe side effects of the peroxide including tissue damage and preventing angiogenesis in long-term.\textsuperscript{81-83}
Figure 6.8. (a) Integrating the optical fibrous samples into a commercial wound bandage. (b) Comparison of the fluorescence spectra of microfibers alone, through adhesive bandage material, or through both adhesive material plus an adsorbent pad (complete bandage). (c) Comparison of the ratiometric signals of the three conditions mentioned in part b, after being soaked in PBS over time. Mean values were obtained by acquiring the fluorescence spectra from three different samples (n = 3) per each condition. The error bars represent standard deviation. Two-sample t-tests were performed on the data (*, p < 0.05, **, p < 0.01). (d) Real-time wireless fluorescence spectra readout utilizing the flexible optical microfibers attached onto a commercial bandage.
6.4. Conclusions

Multi-compartment smart wound dressings have attracted a substantial interest in the past few years due to their potential for enabling simultaneous wound monitoring and healing.\cite{51, 52} A smart wound dressing usually integrates multiple layers into a single flexible biomaterial platform.\cite{84} A therapeutic layer can be designed to enable wound healing by incorporating antibiotics, growth factors, etc., into a 3D biocompatible scaffold such as hydrogels and microfibers. Moreover, a sensing layer would enable continuous monitoring of multiple biomarkers in the wound environment. In this work, we employed a one-step coaxial electrospinning process to encapsulate ssDNA-SWCNT nanosensors into individual microfibers to fabricate wearable optical microfibrous textiles, as a sensing layer, for monitoring oxidative stress in wounds. Utilizing confocal Raman microscopy of individual fibers over time, we uncovered that the SWCNT nanosensors are preserved inside of the fibers over time and that a negligible quantity of the nanosensors are released from a 3D fibrous matrix after 21 days. As multiple nanotube chiralities in the HiPco sample respond differentially to peroxide molecules, we designed an optical wearable platform to ratiometrically detect peroxide at physiologically relevant concentrations. Utilizing this flexible optical microfibrous material, the wireless detection of peroxide was demonstrated in cellular environment. In addition to a single real-time readout, we demonstrated the potential of our platform for spatially resolving the peroxide concentrations on a wound surface using an InGaAs camera, without the need to use any additional marker. We finally integrated our microfibrous textiles onto a commercial wound bandage and indicated the compatibility of this platform with existing wound dressings. In its current form, we have utilized a benchtop NIR probe spectrometer for signal acquisition from the samples, however, in our future work we aim to miniaturize the
external unit to create portable and wearable versions of the detection platform. Furthermore, we see potential for developing wearable technologies capable of detecting a wide range of other biomolecules including proteins, hormones and carbohydrates, based on our wearable platform, in wounds and other biological fluids such as sweat.

6.5. Materials and Methods

**ssDNA-SWCNT Nanosensor Preparation:** Raw single-walled carbon nanotubes produced by the HiPco process (1 mg, Nanointegris) were added to desalted (GT) oligonucleotide (2 mg, Integrated DNA Technologies) in a microcentrifuge tube with NaCl solution (1 mL, 0.1 m, Sigma-Aldrich). The mixture was then ultrasonicated using a 1/8” tapered microtip (Sonics Vibracell; Sonics & Materials) for 30 min at 40% amplitude, with an average power output of 8 W, in a 0 °C temperature-controlled microcentrifuge tube holder. After sonication, the dispersion was ultracentrifuged twice (Sorvall Discovery M120 SE) for 30 min at 250 000×g, and the top 80% of the supernatant was extracted. The resultant dispersion was filtered using 100 kDa Amicon centrifuge filters (Millipore) to remove free ssDNA. A UV/vis/NIR spectrophotometer (Jasco, Tokyo, Japan) was utilized to determine the concentration using the extinction coefficient of $A_{910} = 0.02554 \ \text{L mg}^{-1} \ \text{cm}^{-1}$.\[32, 33\]

**Preparation of Core and Shell Polymer Solutions:** A 4 wt.% poly(ethylene oxide) (PEO, $M_v = 900,000 \ \text{g mol}^{-1}$, Sigma-Aldrich) solution was prepared by dissolving PEO in DI water and stirring the solution overnight on a hotplate set to 48 °C. A concentrated ssDNA-SWCNT dispersion (~400-500 mg L$^{-1}$) was prepared by filtering out the as-prepared ssDNA-SWCNT dispersion using an Amicon filter (100 kDa) and resuspending it in a lower volume of NaCl solution (0.1 m). The concentrated dispersion
was then diluted in the resultant PEO solution to obtain a homogenous nanotube concentration of 10 mg L\(^{-1}\). Because of the high ssDNA-SWCNT concentration, the final concentration of the PEO solution was not significantly altered by adding ssDNA-SWCNT dispersion to it. Polycaprolactone (PCL, \(M_w = 70,000\) g mol\(^{-1}\), Scientific Polymer Products, Inc.) was dissolved in a mixture of chloroform and dimethylformamide (DMF) with the volume ratio of 80:20, by stirring the solution for 6 hours at room temperature, to obtain a final PCL concentration of 13 wt.%.

**Fabrication of Electrospun Optical Microfibrous Textiles:** A one-step co-axial electrospinning process was used to produce core-shell fibers. Figure 6.1b illustrates the schematic of the experimental setup. A customized core-shell needle (Rame-hart Instrument co.) with two separate inlets was built by placing a 24 Gauge needle inside of a 15 Gauge needle. The inlets of the needle were connected to two syringes filled with the polymer solutions and placed on a syringe pump capable of controlling the flow rates separately. The flow rates of the core and shell solutions were set to 0.3 and 2 mL h\(^{-1}\), respectively. A high voltage supply was connected to the tip of the needle and the rotating collector was grounded. The working distance between the needle and collector was set to 12 cm. To fabricate bulk fibrous textiles with a thickness of \(~0.7\) mm, the fibers were continuously collected on the metal collector for 7 hours. To prepare samples for NIR and confocal Raman microscopy, microscope coverslips were taped to the surface of the collector and a thin layer the fibers were collected on the coverslips for 10 minutes.

**Near-Infrared Fluorescence Microscopy of the Fibers:** As described previously,\(^{[41, 85]}\) a near-infrared hyperspectral fluorescence microscope was used to acquire fluorescence images and hyperspectral cubes from a thin layer of fibers collected
on a microscope coverslip. In short, a continuous 730 nm diode laser with 2.5 W output power was injected into a multimode fiber to produce an excitation source, which was reflected on the sample stage of an Olympus IX-73 inverted microscope equipped with a 20X LCPlan N, 20x/0.45 IR objective (Olympus, U.S.A.). To generate spectral image stacks (cubes), the emission was passed through a volume Bragg grating and collected with a 2D InGaAs array detector (Photon Etc.) with a spectral resolution of 4 nm. A background subtraction was performed using a custom MATLAB code. The background-subtracted images and hyperspectral cubes were processed and extracted using the Fiji software.

**Scanning Electron Microscopy of the Fibers:** The samples were sputtered with gold prior to imaging. The SEM images were taken using a Zeiss Sigma VP field emission scanning electron microscope (FE-SEM), with an InLens detector and an accelerating voltage of 3.00 kV.

**Confocal-Raman Microscopy:** A thin layer of fibers collected on a microscope coverslip was imaged with a WiTec Alpha300 R confocal-Raman microscope (WiTec, Germany) equipped with a Zeiss EC Epiplan-Neofluar 100×/0.9 air objective, a 785 nm laser source set to 35 mW sample power, and collected with a UHTS 300 spectrograph (300 lines/mm grating) coupled with an Andor DR32400 CCD detector (-61 °C, 1650 × 200 pixels). 10 × 40 μm areas were scanned, and spectra were obtained in 0.25 × 0.25 μm intervals with 0.4 s integration time to construct hyperspectral images of individual fibers. Global background subtraction, cosmic-ray removal, and k-means cluster analysis were performed on each scan using WiTec Project 5.2 software. G-band intensity images were constructed by integrating the spectrum of each SWCNT-containing pixel from 1575 to 1605 cm⁻¹ using custom Matlab codes.
Quantifying the Amount of the Released Nanosensor Using Solution-Based Raman Spectroscopy: 1 square inch pieces of the bulk fibrous samples with thickness of ~0.7 mm were soaked in 3 mL of PBS (1X) over time. The PBS was collected at different time points (1 hour, 24 hours, 48 hours, 72 hours, 7 Days, 14 Days and 21 Days) and replaced with 3 mL of fresh PBS. The collected samples were placed into glass vials and Raman spectra were obtained using an inverted WiTec Alpha300 R confocal Raman microscope (WiTec, Germany) equipped with a Zeiss Epiplan-Neofluar 10×/0.25 objective, a 785 nm laser source set to 50 mW sample power, and collected with a UHTS 300 spectrograph (300 lines/mm grating) coupled with an Andor DR32400 CCD detector (1650 × 200 pixels). Each spectrum was averaged 5 times with 5 s integration time. Background subtraction was performed on all data using Witec Project 5.2 software.

Real-Time Near-Infrared Fluorescence Spectroscopy of Bulk Microfibrous Samples: Bulk fibrous samples with thickness of ~0.7 mm were placed into plastic Petri dishes and 3 mL of the peroxide solution diluted to different concentrations in PBS (1X) was added to the samples. The NIR fluorescence spectra was acquired from each sample at 24, 48 and 72 hours. Individual NIR fluorescence spectra from the bulk samples were obtained using a custom-built preclinical fiberoptic probe spectroscopy system described in previous studies. In summary, a continuous-wave 1320-mW 730-nm laser (CNI lasers) was injected into a bifurcated fiber optic reflection probe bundle. The bundle consisted of a 200-mm, 0.22 numerical aperture (NA) fiber optic cable for sample excitation located in the center of six 200-mm, 0.22 NA fibers for collection. Long-pass filters were used to block emission below 1100 nm. The light was focused into a 193-mm focal length Czerny-Turner spectrograph (Kymera 193i, Andor) with the slit width set at
410 mm. Light was dispersed by an 1501/mm grating with blaze wavelength of 1250 nm and collected with an iDus InGaAs camera (Andor). The distance between the laser probe tip and the sample was set to 2.2 cm. A custom MATLAB code was used to perform background subtraction on the acquired fluorescence spectra.

**Cell Culture:** RAW 264.7 TIB-71 cells (ATCC, Manassas, VA, U.S.A.) were cultured under standard incubation conditions at 37 °C and 5% CO2 in cell culture medium containing sterile filtered high-glucose DMEM with 10% heat-inactivated FBS, 2.5% HEPES, 1% L-glutamine, 1% penicillin/streptomycin, and 0.2% amphotericin B (all acquired from Gibco). For the cell studies, cells were allowed to grow until 90% confluency and used up to the 20th passage. To investigate the ability of the optical textiles for peroxide monitoring in a biological environment, the cells were seeded onto the microfibrous textiles in 35 mm² Petri dishes (2 mL, 20 × 10⁵ cells mL⁻¹) for 24 hours. To stimulate the peroxide release, the cells were thoroughly washed with PBS (1X) and 400 ng mL⁻¹ of phorbol 12-myristate 13-acetate (PMA, ∼99%, Sigma) in PBS (1X) was added to the cells. After 30 minutes, the real-time fluorescence spectra of the textiles were acquired over time, utilizing the probe fluorescence spectrometer.

**Statistical Analysis:** All curve fittings, statistical measurements and analyses were performed in OriginPro 2016. The two-sample t-tests were performed under the null hypothesis.
6.6. Acknowledgements

This work was supported by National Science Foundation CAREER Award #1844536, the RI-INBRE Early Career Development Award Grant P20GM103430 from the National Institute of General Medical Sciences of the National Institutes of Health, the Rhode Island Foundation–Medical Research Fund, and the URI College of Engineering. The confocal Raman data and SEM images were acquired at the RI Consortium for Nanoscience and Nanotechnology, a URI College of Engineering core facility partially funded by the National Science Foundation EPSCoR, Cooperative Agreement #OIA-1655221. We acknowledge I. Andreu for the help in taking SEM images. We acknowledge P. V. Jena for designing, building and installing the custom preclinical fiberoptic probe spectroscopy system.
6.7. References


11. Dunnill, C.; Patton, T.; Brennan, J.; Barrett, J.; Dryden, M.; Cooke, J.; Leaper, D.; Georgopoulos, N. T., Reactive oxygen species (ROS) and wound healing: the


77. Hansen, M. P.; Malchow, D. S. In Overview of SWIR detectors, cameras, and applications, Thermosense Xxx, International Society for Optics and Photonics: 2008; p 69390I.


Chapter 7

The research projects described throughout this dissertation have been enabled by the robust optical properties of DNA-wrapped single-walled carbon nanotubes and their functionality in biological environments. We first focused on characterizing the intracellular stability of SWCNTs as a function of their DNA-wrapping length to gain a fundamental understanding of how the DNA-SWCNTs behave upon internalization. This was followed up with a more comprehensive approach to understand the exact mechanisms of intracellular trafficking and precisely quantify endosomal loading. Finally, we developed a next-generation smart bandage integrated with DNA-SWCNTs for wearable biomarker monitoring.

The idea to use DNA sequence length as a variable for intracellular stability came from a previous study within our lab group. Utilizing a high throughput experimental framework, it was found that DNA-SWCNT stability in solution was strongly correlated with DNA sequence length, but not necessarily the density of DNA surface coverage. Therefore, we questioned whether the same would be true in a dynamic biological environment with a much higher degree of complexity. We used multiple imaging platforms and methodologies to first test whether sequence length correlated to intracellular stability. Optical instability, i.e., modulation of fluorescence emission, was found to occur only in the shortest DNA sequence ((GT)_6). This was followed up by testing the hybrid stability using a custom fluorescence assay, revealing the intracellular environment could rapidly displace the DNA strands from a short sequence but not a long DNA sequence ((GT)_{30}), corroborating the previous findings regarding length-dependent stability. Additional experiments utilized confocal Raman microscopy to quantify the weight of
DNA-SWCNTs internalized by cells and after 24 hours as a function of DNA length. Our results found that short DNA enabled greater SWCNT uptake compared to long DNA in addition to greater exocytosis. This unexpected finding suggested that less stable DNA-SWCNTs may be recognized by the cell upon DNA displacement, causing upregulation of lysosomal exocytosis and thus eliminating internalized DNA-SWCNTs. Overall, this study highlighted biocompatibility as a property which can be compromised by instability.

The insight gained from the intracellular stability study raised additional questions about the consequences of perturbing the lysosomal pathway in addition to the mechanisms of instability. We therefore developed an entirely new approach to characterize intracellular trafficking and utilize DNA-SWCNTs as both the material being processed and the environmental reporter in response to these events. We were able to colocalize subcellular regions containing DNA-SWCNTs to simultaneously extract and analyze fluorescence and Raman spectra. This revealed relationships between local DNA-SWCNT concentration, fluorescence modulation, and aggregate formation, while a significant increase in the formation of aggregates was found to occur with additional incubation time. This was translated to specific subcellular localization by developing a second combined approach, enabling immunofluorescence markers of endosomal vesicle to be colocalized with Raman spectra from DNA-SWCNTs. This technique revealed that DNA-SWCNTs localize to lysosomes within 6 hours of internalization, where they aggregate due to the catabolic environmental conditions. We additionally were able to develop a machine learning model to predict the progression of endosomal maturation based solely on the Raman spectrum from the subcellular DNA-SWCNTs. Each method presented in this study
could be adapted to study additional biological process, while the ability to utilize machine learning as a classification tool was shown to be powerful for multivariate data analyses.

Another type of intracellular analysis was also developed to quantify numbers of DNA-SWCNTs within cells. Unlike confocal Raman microscopy, which can quantify uptake in terms of weight, the ability to quantify numbers of SWCNTs creates a standardized metrology which can be compared with other types of nanomaterials. This method utilized hyperspectral fluorescence microscopy to count the number of emissive SWCNT peaks within individual endosomes. The potential for non-emissive chiralities such as metallic SWCNTs was also accounted for by applying a mathematical model, further improving the accuracy. This method was able to identify an intrinsic upper-limit to the rate of SWCNT uptake regardless of dosing concentration. Substantial heterogeneity was also observed both between cells and within subcellular regions, corroborating results from the intracellular trafficking study and highlighting the dynamic environments encountered.

As a follow up, we asked whether the presence of aggregates in the initial DNA-SWCNT sample could influence uptake and endosomal loading. To systematically investigate this question, we varied our dispersion process to produce a second DNA-SWCNT sample with a defined degree of aggregation which could be compared against a standard, monodisperse DNA-SWCNT suspension. We found that, when equally dosed in 3 different cell lines, the rate of uptake was independent of the starting sample quality, further confirming the rate-limited uptake of DNA-SWCNTs. In contrast, endosomal loading was consistently found to be higher when cells were incubated with singly-dispersed DNA-SWCNTs regardless of cell type, suggesting that single SWCNTs were
selectively accumulated in single vesicles upon internalization. Cell proliferation and viability were also found to be lower in cells dosed with single DNA-SWCNTs, further indicating differential processing mechanisms could also induce cytotoxicity. This study points to aggregation as a property which can not be ignored when designing and characterizing nanomaterials.

Although the majority of this dissertation focuses on interactions with cells, we also developed a wearable sensor to monitor wound healing. By incorporating DNA-SWCNTs into a polymeric nanofiber material, peroxide concentrations were able to be detected via fluorescence measurements directly from the fiber. We envision this technology could be developed for point-of-care diagnostic applications which require real-time monitoring of biochemical processes. Furthermore, the near-infrared fluorescence readout is easily obtained through biological tissues which may be implanted, opening the door for countless future applications.
Appendix I

*Supplementary Information for Chapter 2*

**Biomolecular Functionalization of a Nanomaterial to Control Stability and Retention within Live Cells**

by

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Kingston, Rhode Island 02881, United States
Table S2.1. Table of physical properties for the shortest and longest sequences ((GT)$_6$- and (GT)$_{30}$- respectively) of DNA-SWCNTs used

<table>
<thead>
<tr>
<th>Property</th>
<th>DNA-SWCNT</th>
<th>Reference</th>
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<td></td>
<td>(GT)$_6$</td>
<td>(GT)$_{30}$</td>
</tr>
<tr>
<td>Average SWCNT Length</td>
<td>186.6 ± 7.86 nm</td>
<td>180.6 ± 14.25 nm</td>
</tr>
<tr>
<td>Total Wrapped DNA per SWCNT Weight Ratio</td>
<td>0.696 ± 0.048 mg/mg</td>
<td>0.472 ± 0.047 mg/mg</td>
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<tr>
<td>SWCNT Diameter Range</td>
<td>0.8 – 1.2 nm</td>
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Figure S2.1. Solution-based optical characterizations of DNA-SWCNTs. (a) Fluorescence spectra for all (GT)$_n$-SWCNTs diluted to 1 mg/L in PBS and normalized to area under the curve. (b) Absorbance spectra for all (GT)$_n$-SWCNTs in PBS normalized to the absorbance at 910 nm.
Figure S2.2. Fluorescence intensities of DNA-SWCNTs in solution. Average fluorescent intensity as a function of DNA length diluted to 1 mg/L in (a) PBS and (b) cell culture media. Data is represented as mean ± s.d, with Pearson correlation coefficient ($r_p$), $n = 4$. (**p < 0.001, according to two-tailed two-sample t-test).
**Figure S2.3.** Time-dependent intracellular fluorescence of DNA-SWCNTs. (a) NIR fluorescence images of macrophages after 30-minute pulse of (GT)$_n$-SWCNTs. Image intensities were normalized to the initial intensity for each DNA-SWCNT. Scale bar = 30µm. (b) The percent of initial average fluorescent intensity for each DNA-SWCNT. Experiments were performed in triplicate and are represented as mean ± s.d. Significance is with respect to initial intensities (*$p < 0.05$, **$p < 0.01$, according to two-tailed two-sample t-test).
Figure S2.4. Fluorescence spectroscopy of DNA-SWCNTs. (a) Average fluorescence spectra of each (GT)$_n$-SWCNT diluted to 1 mg/L in cell culture media and (b-f) intracellular fluorescence spectra 0- and 24-hours after internalization for each (GT)$_n$-SWCNT. The intensity of each spectrum was normalized to the area under the curve. All experiments were performed in triplicate.
Figure S2.5. pH modulation of fluorescence spectra. (a-e) Average fluorescence spectra of each (GT)ₙ-SWCNT in cell culture media at pH of 7.4 or 4.5. The intensity of each spectra was normalized to the area under the curve. (f) Heat map representing the average change in SWCNT emission energy after decreasing the pH to 4.5 as a function of DNA sequence and chirality. (8,7)-SWCNTs were excluded due to poor Gaussian fits. All experiments were performed in triplicate.
Figure S2.6. Confocal Raman concentration-intensity calibration. (a) Raman spectrum of (GT)$_6$-SWCNTs diluted to 50 mg/L in PBS. (b) Example of average Raman spectrum from all (GT)$_6$-SWCNT-containing pixels in one cellular ROI. Linear fits of G-band intensity versus known (c) (GT)$_6$-SWCNT and (d) (GT)$_{30}$-SWCNT concentrations. (e) Example of cell ROI CCD count intensity scale converted to concentration using linear fit equation. Scale bar = 5 µm.
Table S2.2. Table of calculated peak emission energy shift for all examined (GT)_n-SWCNTs. Each peak was fit to a Gaussian function to obtain a center energy and subtracted from corresponding controls in cell culture media. n = 3.

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<th>6h</th>
<th>± s.e.</th>
<th>24h</th>
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<td>Average (meV)</td>
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Appendix II

Supplementary Information for Chapter 3

Multispectral Fingerprinting Resolves Dynamics of Nanomaterial Trafficking in Primary Endothelial Cells

by

Mitchell Gravely$^1$ and Daniel Roxbury$^1$

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Kingston, Rhode Island 02881, United States
Figure S3.1. Solution-based optical characterization of DNA-SWCNTs. (a) Absorbance spectrum of DNA-SWCNTs in PBS, normalized to the absorbance at 910 nm. (b) Fluorescence spectrum of DNA-SWCNTs in cell culture media using a 730 nm excitation source, normalized to the total integrated intensity. Raman spectrum of (c) (GT)$_6$-SWCNTs or (d) (GT)$_{30}$-SWCNTs in cell culture media acquired using a 785 nm excitation source. Inset shows a close-up of the radial breathing mode (RBM) region.
Figure S3.2. Fluorescence intensity and local concentration of DNA-SWCNTs are co-dependent within single cells. (a) Transmitted light, (b) broadband NIR fluorescence (950-1350 nm), and (c) G-band Raman intensity micrographs of individual cells dosed with 1 mg-L\(^{-1}\) (GT)\(_6\)-SWCNTs for 1h and incubated in fresh media for indicated times. (d) Fluorescence intensity and (e) G-band intensity histograms of SWCNT-containing pixels from all examined cells at each time point. The distributions are fitted to log-normal curves and the widths are estimated by the log standard deviation parameter (\(\sigma\)). (f) Fold change of average fluorescence and G-band intensities with respect to 0h averages. Error bars represent mean ± s.d. with \(n \geq 4\) cells per condition. Five pointed stars between columns represent significance between fluorescence and G-band intensities and six pointed stars above columns represent significance versus 0h values. (*\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\) according to two-tailed two-sample t-test).
Figure S3.3. RBM of (a) (GT)$_6$-SWCNTs or (b) (GT)$_{30}$-SWCNTs in solution or intentionally aggregated and precipitated out of solution.
Figure S3.4. Temporal resolution of DNA-SWCNT spectral features indicates aggregation within subcellular regions. (a) Average fluorescence spectrum of (GT)_6-SWCNTs in single cells after variable lengths of intracellular processing, normalized to the total integrated intensity of each spectrum. Fluorescence bands are indicated by shaded regions. (b) Average normalized fluorescence band intensities from (GT)_6-SWCNTs in single cells after variable lengths of intracellular processing. Each spectrum was normalized by the total cell intensity, and average normalized band intensities are reported. (c) Ratiometric intensity of fluorescence band 4 divided by band 1 as a function of time. (d) RBM region of the average Raman spectrum of (GT)_6-SWCNTs in single cells after variable lengths of intracellular processing, normalized to the total integrated intensity of each spectrum. RBM bands are indicated by shaded regions. (e) Average normalized RBM band intensities from (GT)_6-SWCNTs in single cells after variable lengths of intracellular processing. Each spectrum was normalized by the total cell RBM intensity, and average normalized band intensities are reported. (f) Ratiometric intensity of RBM band 2 divided by band 1, with linear fit, as a function of time. Error bars represent mean ± s.d. for all, with n ≥ 4 cells per condition. (*p < 0.05, **p < 0.01, ***p < 0.001 according to two-tailed two-sample t-test).
Figure S3.5. Spectral response to inhibition of endosomal progression. (a) Fold change of G-band and (b) fluorescence intensities, with respect to 0h controls, from intracellular (GT)$_6$-SWCNTs after 6h of incubation with Nocodazole (NOC, 10 μg-mL$^{-1}$) or Chloroquine (CQ, 100 μM). Averages from untreated cells at 0h or 6h are shown as blue or red lines, respectively. (c) Ratiometric intensity of fluorescence band 4 divided by band 1 and (d) RBM band 2 divided by band 1 from inhibitor-treated cells after 6h. Error bars represent mean ± s.d. for all, with $n \geq 4$ cells per condition. Stars above error bars represent significance versus 6h untreated cells. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ according to two-tailed two-sample t-test). (e) Average intracellular fluorescence and (f) RBM spectra from inhibitor-treated cells after 6h compared with spectra from untreated cells at indicated times. Each spectrum was normalized to the total integrated intensity.
Figure S3.6. Fluorescence modulation from DNA-SWCNTs within concentrated subcellular regions. (a) (GT)$_6$-SWCNT fluorescence intensity as a function of G-band intensity from all intracellular ROIs, with linear fits, at indicated time points. Pearson correlation coefficients, displayed in parentheses, were calculated from scatter data at each time point. Transmitted light images, (9,4)-SWCNT emission maps, and G-band intensity maps of individual cells at (b) 0h or (c) 6h time points. Color scale range encompasses 20 – 80% of values from each ROI map. (d) G-band intensity as a function of (9,4)-SWCNT emission wavelength from all 0h or (e) 6h intracellular ROIs. Average values from 0h data, represented as dashed lines, were used to compute the percent of ROIs in each quadrant.
Figure S3.7. G-band intensity of (GT)$_{30}$-SWCNTs as a function of (9,4)-SWCNT emission wavelength after treatment with pharmacological inhibitors. All ROIs from cells treated with (a) 10 μg·mL$^{-1}$ Nocodazole or (b) 100 μM Chloroquine for 6h after initial DNA-SWCNT exposure. Average values from untreated 0h cells, represented as dashed lines, were used to compute the percent of ROIs in each quadrant.
Figure S3.8. G-band intensity of (GT)$_6$-SWCNTs as a function of (9,4)-SWCNT emission wavelength after treatment with pharmacological inhibitors. All ROIs from cells treated with (a) 10 µg-mL$^{-1}$ Nocodazole or (b) 100 µM Chloroquine for 6h after initial DNA-SWCNT exposure. Average values from untreated 0h cells, represented as dashed lines, were used to compute the percent of ROIs in each quadrant.
Figure S3.9. G-band intensity of (GT)$_{30}$-SWCNTs as a function of (8,6)-SWCNT emission wavelength from all (a) 0h or (b) 6h intracellular ROIs, and from cells treated with (c) 10 μg·mL$^{-1}$ Nocodazole or (d) 100 μM Chloroquine for 6h after initial DNA-SWCNT exposure. Average values from untreated 0h cells, represented as dashed lines, were used to compute the percent of ROIs in each quadrant.
Figure S3.10. G-band intensity of (GT)$_6$-SWCNTs as a function of (8,6)-SWCNT emission wavelength from all (a) 0h or (b) 6h intracellular ROIs, and from cells treated with (c) 10 μg·mL$^{-1}$ Nocodazole or (d) 100 μM Chloroquine for 6h after initial DNA-SWCNT exposure. Average values from untreated 0h cells, represented as dashed lines, were used to compute the percent of ROIs in each quadrant.
Figure S3.11. Spectral deconvolution of the RBM Raman spectrum of (GT)$_6$-SWCNTs (a) in solution or (b) aggregated and precipitated out of solution and (GT)$_{30}$-SWCNTs (c) in solution or (d) aggregated and precipitated from solution.
**Figure S3.12.** (a) Bar graph showing the relative change of RBM intensities when DNA-SWCNTs are precipitated out of solution as a function of sequence and chirality (left) with the theoretical $E_{22}$ of each chirality in surfactant dispersion overlaid (right) [1]. Each chirality intensity was normalized by the total RBM intensity from each replicate, and average intensity changes are reported. (b) The ratio of $(10,2)/(10,5)$ RBM intensities from controls in solution or aggregated and precipitated out of solution. Error bars represent mean ± s.d. for all, with $n \geq 50$ spectra for each condition. (**p < 1e-4 according to two-tailed two-sample t-test).
**Figure S3.13.** Quenched fluorescence of DNA-SWCNTs. (a) G-band intensity and (b) NIR fluorescence intensity micrographs of a single cell dosed with (GT)$_{30}$-SWCNTs and incubated for 24h. Outlined regions signify ROIs which contain DNA-SWCNTs with quenched fluorescence. (c) Fluorescence and (d) RBM spectra from the ROIs identified in (a) and (b).
Figure S3.14. Intracellular aggregate formation is time dependent. (a) The RBM peak intensity of a single SWCNT depends on its transition energy ($E_{22}$) and the excitation energy ($E_{laser}$). Aggregation shifts the optical transition to lower energies ($\Delta E_{22}$), resulting in selective intensity enhancement for chiralities brought into resonance ($E_{22, soln} \geq E_{laser}$) and intensity reduction for chiralities brought out of resonance ($E_{22, soln} \leq E_{laser}$) with the excitation. (b) Heat map representing the change of $(GT)_6$-SWCNT RBM intensities from solution as a function of chirality and time within the cells. Control intensities of intentionally aggregated $(GT)_6$-SWCNTs are displayed as a reference. The chirality intensities from each ROI or control replicate were normalized by the total RBM intensity and average values are reported. (c) The ratio of $(10,2)/(10,5)$ RBM intensities of all intracellular ROIs as a function of time. Boxes represent 25-75% of the data, small white squares represent the mean, trend lines connect medians, and dashed lines indicate values from aggregated or solution controls. One-way ANOVA with Tukey post hoc analysis was performed (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 1e-4$). The ratio of $(10,2)/(10,5)$ RBM intensities as a function of $(9,4)$-SWCNT emission wavelength of all (d) 0h, (e) 6h, or (f) 24h ROIs. Boxed column scatter plots on the right-hand side depict RBM ratio values from ROIs with poorly fitting or quenched fluorescence. Median values from 0h data, represented as dashed lines, were used to compute the percent of ROIs in each quadrant. Shaded regions indicate the $(10,2)/(10,5)$ RBM intensity threshold identified from aggregated controls.
Figure S3.15. Laser power differentiates fluorescence and Raman signals. (a) Transmitted light image of a single cell incubated with (GT)$_{30}$-SWCNTs. Immunofluorescence staining was not performed on the sample. The outlined region was scanned three times using (b) low power (10µW) 532nm excitation, (c) high power (10mW) 532nm excitation, and (d) 35mW 785nm excitation. Scale bars = 5µm.
**Figure S3.16.** Image generation from hyperspectral datasets. (a) Representation of a hyperspectral fluorescence cube acquired from a cell stained with immunofluorescence markers. (b) Fluorescence spectrum of the goat anti-rabbit IgG Alexa Fluor 532 secondary antibody used for all fluorophore labeling. Shaded area depicts the region integrated to produce fluorescence images. (c) Representation of a hyperspectral Raman cube acquired from a cell incubated with DNA-SWCNTs. (d) Raman spectrum of (GT)$_{30}$-SWCNTs in solution. Shaded area depicts the region integrated to produce DNA-SWCNT Raman images.
Figure S3.17. Colocalization of fluorescence and Raman images. (a) Fluorescence intensity image, RBM intensity image, and the overlay (top row) along with the corresponding binary image representations (bottom row). (b) The RBM intensity image with ROIs outlined in green. Middle panel shows magnified view of the outlined region. The corresponding labeled ROI mask is shown in the right panel, color coded by ROI label. (c) Image overlay from (a) and the resulting colocalization profile determined for the cell.
Figure S3.18. G-band intensity from endosomal vesicles. The G-band intensity of all immunofluorescence-labeled ROIs as a function of (a) endosomal vesicle and (b) incubation time. Boxes represent 25-75% of the data, small white squares represent the mean, black line represents the median, and whiskers represent mean ± s.d. One-way ANOVA with Tukey post hoc analysis was performed (*p < 0.05, **p < 0.01).
Figure S3.19. Low-intensity Raman modes of colocalized DNA-SWCNTs. (a) The Average Raman spectrum of (GT)$_{30}$-SWCNTs colocalized with endosomal markers and (b-e) magnified views of various regions. Data in the spectra shown were smoothed to reduce the level of noise in the low-intensity regions.
Figure S3.20. Neural network validation results. (a) Confusion matrix of the 10-fold cross validation results for the trained neural network classification model. Values are shown as the percent of input data from each individual class. (b) Receiver operating characteristic (ROC) curves and area under the curve (AUC) values for each data class.
**Figure S3.21.** Effect of pH on (GT)$_{30}$-SWCNT spectra. (a) NIR fluorescence spectra of (GT)$_{30}$-SWCNTs incubated in solutions titrated with HCl. Each spectrum was normalized by the total spectrum intensity. Inset magnifies the outlined region and reference line shows the emission center wavelength of (9,4)-SWCNTs at pH = 5.98. (b) Raman spectra of (GT)$_{30}$-SWCNTs incubated in solutions titrated with HCl. Each spectrum was normalized by the total spectrum intensity. Inset shows the RBM spectra normalized by the total intensity from only the RBM range.
Figure S3.22. Effect of biological components on (GT)$_{30}$-SWCNT spectra. (a) NIR fluorescence spectra of (GT)$_{30}$-SWCNTs incubated in solutions of deionized water (DI), fetal bovine serum (FBS), and DI containing 0.1M indicated salts. Each spectrum was normalized by the total spectrum intensity. Inset magnifies the outlined region, normalized by the max intensity of each spectrum, and reference line shows the emission center wavelength of (9,4)-SWCNTs in DI. (b) Raman spectra of (GT)$_{30}$-SWCNTs in the same solutions from (a). Each spectrum was normalized by the total spectrum intensity. Inset shows the RBM spectra normalized by the total intensity from only the RBM range.
Figure S3.23. Summary of control fluorescence spectra. (a) Heat maps summarizing intensity differences and (b) emission wavelength shifts shown in Figures S21 and S22. Values shown were normalized to 0.1M NaCl (Na$^+$), the solution used to prepare and store DNA-SWCNTs, to represent change relative to initial conditions.
Table S3.1. Estimate of the optical properties of DNA-SWCNT chiralities identified in the fluorescence spectrum when excited by a 730 nm laser.

<table>
<thead>
<tr>
<th>Band</th>
<th>Range (nm)</th>
<th>SWCNT Chiralities</th>
<th>( \lambda_{11} ) (nm)[^{[1]}]</th>
<th>( E_{11} ) (eV)[^{[1]}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>976 - 1024</td>
<td>(8,3)</td>
<td>968.78</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>(6,5)</td>
<td>982.36</td>
<td>1.262</td>
</tr>
<tr>
<td>2</td>
<td>1044 - 1092</td>
<td>(7,5)</td>
<td>1042.29</td>
<td>1.190</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10,2)</td>
<td>1074.94</td>
<td>1.154</td>
</tr>
<tr>
<td>3</td>
<td>1112 - 1160</td>
<td>(8,4)</td>
<td>1123.04</td>
<td>1.104</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(9,4)</td>
<td>1125.14</td>
<td>1.102</td>
</tr>
<tr>
<td>4</td>
<td>1176 - 1224</td>
<td>(8,6)</td>
<td>1193.74</td>
<td>1.039</td>
</tr>
<tr>
<td>5</td>
<td>1264 - 1312</td>
<td>(10,5)</td>
<td>1275.28</td>
<td>0.972</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(8,7)</td>
<td>1280.43</td>
<td>0.968</td>
</tr>
</tbody>
</table>

Table S3.2. Estimate of the optical properties of DNA-SWCNT chiralities identified in the RBM region of the Raman spectrum when excited by a 1.58 eV laser.

<table>
<thead>
<tr>
<th>Band</th>
<th>Range (cm(^{-1}))</th>
<th>SWCNT Chiralities</th>
<th>RBM (cm(^{-1}))[^{[2]}]</th>
<th>( E_{22} ) (eV)[^{[2]}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>217 - 242</td>
<td>(10,5)</td>
<td>225.3</td>
<td>1.577</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(11,3)</td>
<td>233</td>
<td>1.565</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(12,1)</td>
<td>237.2</td>
<td>1.556</td>
</tr>
<tr>
<td>2</td>
<td>250 - 275</td>
<td>(9,4)</td>
<td>256.6</td>
<td>1.722</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10,2)</td>
<td>265.3</td>
<td>1.689</td>
</tr>
</tbody>
</table>

Table S3.3. Performance statistics of the neural network classification model.

<table>
<thead>
<tr>
<th>Class</th>
<th>Accuracy</th>
<th>Precision</th>
<th>Recall</th>
<th>Specificity</th>
<th>F1-Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early Endosome</td>
<td>0.91</td>
<td>0.91</td>
<td>0.87</td>
<td>0.94</td>
<td>0.89</td>
</tr>
<tr>
<td>Late Endosome</td>
<td>0.85</td>
<td>0.70</td>
<td>0.72</td>
<td>0.89</td>
<td>0.71</td>
</tr>
<tr>
<td>Lysosome</td>
<td>0.84</td>
<td>0.74</td>
<td>0.77</td>
<td>0.87</td>
<td>0.75</td>
</tr>
</tbody>
</table>
References:


Appendix III

*Supplementary Information for Chapter 4*

**Hyperspectral Counting of Multiplexed Nanoparticle Emitters in Single Cells and Organelles**

by

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³Weill Cornell Medical College, New York, New York 10065, United States
Figure S4.1. Scheme for preparing single-walled carbon nanotube (SWCNT) samples with differing degrees of dispersion. The “5-minute sample” and the “30-minute sample” were used for cell-free assays. Unbound sodium deoxycholate (SDC) was removed from the 30-minute sample immediately prior to experiments in live cells.
Figure S4.2. Uptake study of SDC-SWCNT complexes by HeLa cells at 4°C and 37°C. Overlay of transmitted light and near-infrared (NIR) photoluminescence images taken 30 minutes after incubating HeLa cells with 1 mg/L of the SDC-SWCNTs (stock solution for cellular assays) in cell media at 4°C and 37°C. Scale bar is 10 µm.
Figure S4.3. Photostability of SDC-SWCNT complexes in HeLa cells at 6 hours and 24 hours after uptake. Overlay of transmitted light and NIR photoluminescence images of HeLa cells, taken 6 hours and 24 hours after incubation for 30 minutes with 1 mg/L SDC-SWCNTs. Scale bar is 10 μm
Figure S4.4. Ensemble optical measurements of the two SDC-SWCNT preparations. (a) Absorption spectra of the 5-minute and 30-minute samples, normalized at 910 nm (corresponding to 1 mg/L effective concentration). (b) Raman spectra of the two samples (1 mg/L) under 785 nm excitation. (c) Photoluminescence emission spectra of the two samples at 1 mg/L concentration under excitation at 730 nm.
Figure S4.5. Two-dimensional photoluminescence excitation emission plots of the 5-minute sample and the 30-minute sample. Intensity was independently normalized for each plot.
Figure S4.6. Clustering of emission center wavelength of individual surface-adsorbed ROIs from both samples. The shaded boxes indicate five emission bands. \( n = 272 \) for the 5-minute sample and \( n = 170 \) for the 30-minute sample.
**Figure S4.7.** Emission center wavelength of surface-adsorbed SDC-SWCNT complexes in each band from both samples. Scatter plot of emission center wavelength of all individual ROIs emitting in each band from the two preparations. Boxes represent 25-75% of the data. Statistical comparisons are unpaired t-tests with Welch’s correction. ** indicates < 0.01, **** indicates p < 0.0001.
Figure S4.8. Population distributions of 5-minute and 30-minute samples. Solution population distribution quantified using the intensity of emission peaks corresponding to each band in the spectra obtained via hyperspectral microscopy of surface-adsorbed SWCNTs, as detailed in Fig. 4.2b.
Figure S4.9. Photoluminescence imaging study of SDC-SWCNT distribution in HeLa cells. Transmitted light and NIR broadband images focusing on the bottom, 1/3rd, 2/3rd and top of 3 typical HeLa cells incubated with 1 mg/L SDC-SWCNTs for 30 minutes. Scale bar is 10 µm.
**Figure S4.10.** Emission wavelengths of SDC-SWCNT complexes in HeLa cells at 3 SDC-SWCNT loading concentrations. Center wavelength of individual ROIs in bands 1 to 5 (corresponding to the emission ranges defined in Table S4) for HeLa cells incubated with 1, 5 and 10 mg/L of SDC-SWCNTs for 30 minutes. One-way ANOVA was performed using Holm-Sidak’s multiple comparison test. * = p < 0.5, ** = p < 0.01, **** = p < 0.001.
**Figure S4.11.** $k$-means analysis of concentration-dependent peaks per ROI vs. ROIs per $\mu$m$^2$ for single cells. (a) Experimental values of peaks per ROI vs ROI per area for cells incubated with 0.1, 1, 5 and 10 mg/L SDC-SWNT for 30-minutes (stock solution for cellular assays). (b) Clusters identified from the same set of data as in panel a using an unbiased $k$-means algorithm. (c) Clusters identified from the same data set as in panel a by a $k$-means algorithm using mean positions from the references data in panel a as starting seed locations.
Figure S4.12. Population distribution of SDC-SWCNTs in solution and in HeLa cells. Solution population distribution of the 30-minute sample was measured using the intensity of emission peaks corresponding to each band in the emission spectra. The distribution in cells was obtained via hyperspectral microscopy of HeLa cells incubated with the 30-minute SDC-SWCNT sample (stock solution for cellular assays).
Figure S4.13. Heat map of the number of SWCNTs per ROI and the probability distribution of the number of emission peaks detected per ROI. This mapping relation is for HeLa cells incubated with the 30-minute SDC-SWCNT sample (stock solution for cellular assays), with photoluminescence emission acquired under 730 nm excitation. Values below 0.05 are not shown, for clarity.
Figure S4.14. Comparison of four different metrics to quantify SWCNTs in cells. Each measurand is divided by its value at 0.1 mg/L SDC-SWCNT concentration to in order to compare the particle number counts (ROIs per cell, peaks per cell, photoluminescent SWCNTs per cell) with intensity. Photoluminescent intensity was calculated as the mean emission intensity over the area of each cell.
### Table S5.1. Optical Parameters from Photoluminescence Excitation Emission (PLE) Plots

<table>
<thead>
<tr>
<th>Chirality</th>
<th>Intensity – 5 min (a.u.)</th>
<th>Intensity – 30 min (a.u.)</th>
<th>% Difference (a.u)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(8,3)</td>
<td>0.066423 ± 0.000102</td>
<td>0.068873 ± 0.000545</td>
<td>****, 3.7% increase</td>
</tr>
<tr>
<td>(6,5)</td>
<td>0.12217 ± 0.000139</td>
<td>0.12445 ± 0.000568</td>
<td>****, 1.8% increase</td>
</tr>
<tr>
<td>(7,5)</td>
<td>0.10175 ± 0.000113</td>
<td>0.10447 ± 0.000273</td>
<td>****, 2.6% increase</td>
</tr>
<tr>
<td>(10,2)</td>
<td>0.078863 ± 0.000168</td>
<td>0.08011 ± 0.000411</td>
<td>***, 1.6% increase</td>
</tr>
<tr>
<td>(9,4)</td>
<td>0.12188 ± 0.000224</td>
<td>0.12109 ± 0.000049</td>
<td>ns</td>
</tr>
<tr>
<td>(8,4)</td>
<td>0.090087 ± 0.000182</td>
<td>0.089973 ± 0.000152</td>
<td>ns</td>
</tr>
<tr>
<td>(7,6)</td>
<td>0.14597 ± 0.0000586</td>
<td>0.14799 ± 0.000107</td>
<td>****, 1.4% increase</td>
</tr>
<tr>
<td>(8,6)</td>
<td>0.09706 ± 0.000201</td>
<td>0.094167 ± 0.000298</td>
<td>****, 2.9% decrease</td>
</tr>
<tr>
<td>(9,5)</td>
<td>0.056377 ± 0.000258</td>
<td>0.054717 ± 0.000026</td>
<td>***, 2.9% decrease</td>
</tr>
<tr>
<td>(10,5)</td>
<td>0.042943 ± 0.000219</td>
<td>0.040873 ± 0.000026</td>
<td>****, 4.8% decrease</td>
</tr>
<tr>
<td>(8,7)</td>
<td>0.05368 ± 0.000111</td>
<td>0.051987 ± 0.000166</td>
<td>***, 3.2% decrease</td>
</tr>
<tr>
<td>(9,7)</td>
<td>0.022777 ± 0.0000524</td>
<td>0.021307 ± 0.000142</td>
<td>***, 6.5% decrease</td>
</tr>
<tr>
<td>Chirality</td>
<td>Excitation – 5 min (nm)</td>
<td>Excitation – 30 min (nm)</td>
<td>Difference (nm)</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------------------</td>
<td>--------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>(8,3)</td>
<td>668.47 ± 0.0248</td>
<td>668.21 ± 0.179</td>
<td>*, 0.25 nm blue-shift</td>
</tr>
<tr>
<td>(6,5)</td>
<td>570.66 ± 0.0323</td>
<td>570.62 ± 0.0328</td>
<td>ns</td>
</tr>
<tr>
<td>(7,5)</td>
<td>650.2 ± 0.026</td>
<td>650.16 ± 0.0442</td>
<td>ns</td>
</tr>
<tr>
<td>(10,2)</td>
<td>743.02 ± 0.0221</td>
<td>742.95 ± 0.00509</td>
<td>ns</td>
</tr>
<tr>
<td>(9,4)</td>
<td>728.09 ± 0.0146</td>
<td>728.08 ± 0.0492</td>
<td>ns</td>
</tr>
<tr>
<td>(8,4)</td>
<td>590.79 ± 0.0435</td>
<td>590.74 ± 0.044</td>
<td>ns</td>
</tr>
<tr>
<td>(7,6)</td>
<td>652.97 ± 0.0246</td>
<td>652.99 ± 0.00959</td>
<td>ns</td>
</tr>
<tr>
<td>(8,6)</td>
<td>723.82 ± 0.0237</td>
<td>723.72 ± 0.0176</td>
<td>ns</td>
</tr>
<tr>
<td>(9,5)</td>
<td>675.35 ± 0.0126</td>
<td>675.24 ± 0.0225</td>
<td>ns</td>
</tr>
<tr>
<td>(10,5)</td>
<td>789.37 ± 0.067</td>
<td>789.01 ± 0.051</td>
<td>***, 0.36 nm blue-shift</td>
</tr>
<tr>
<td>(8,7)</td>
<td>734.19 ± 0.15</td>
<td>734.13 ± 0.0282</td>
<td>ns</td>
</tr>
<tr>
<td>(9,7)</td>
<td>793.66 ± 0.0398</td>
<td>793.23 ± 0.0786</td>
<td>****, 0.42 nm blue-shift</td>
</tr>
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</table>
**Table S4.3. PLE Analyses – Emission Maximum**

<table>
<thead>
<tr>
<th>Chirality</th>
<th>Emission – 5 min (nm)</th>
<th>Emission – 30 min (nm)</th>
<th>Difference (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(8,3)</td>
<td>965.3 ± 0.00769</td>
<td>965.15 ± 0.0298</td>
<td>****, 0.15 nm blue-shift</td>
</tr>
<tr>
<td>(6,5)</td>
<td>983.54 ± 0.02</td>
<td>983.4 ± 0.0366</td>
<td>****, 0.14 nm blue-shift</td>
</tr>
<tr>
<td>(7,5)</td>
<td>1032.9 ± 0.00791</td>
<td>1032.8 ± 0.0058</td>
<td>****, 0.14 nm blue-shift</td>
</tr>
<tr>
<td>(10,2)</td>
<td>1060.4 ± 0.0162</td>
<td>1060.5 ± 0.0152</td>
<td>****, 0.12 nm red-shift</td>
</tr>
<tr>
<td>(9,4)</td>
<td>1113 ± 0.00321</td>
<td>1112.8 ± 0.016</td>
<td>****, 0.20 nm blue-shift</td>
</tr>
<tr>
<td>(8,4)</td>
<td>1122 ± 0.00657</td>
<td>1121.8 ± 0.0116</td>
<td>****, 0.25 nm blue-shift</td>
</tr>
<tr>
<td>(7,6)</td>
<td>1129.8 ± 0.00591</td>
<td>1129.6 ± 0.00953</td>
<td>****, 0.22 nm blue-shift</td>
</tr>
<tr>
<td>(8,6)</td>
<td>1184.1 ± 0.00865</td>
<td>1183.8 ± 0.017</td>
<td>****, 0.34 nm blue-shift</td>
</tr>
<tr>
<td>(9,5)</td>
<td>1258.5 ± 0.0123</td>
<td>1258.2 ± 0.0128</td>
<td>****, 0.28 nm blue-shift</td>
</tr>
<tr>
<td>(10,5)</td>
<td>1264 ± 0.00662</td>
<td>1263.7 ± 0.0173</td>
<td>****, 0.37 nm blue-shift</td>
</tr>
<tr>
<td>(8,7)</td>
<td>1275.5 ± 0.0157</td>
<td>1275.1 ± 0.0267</td>
<td>****, 0.36 nm blue-shift</td>
</tr>
<tr>
<td>(9,7)</td>
<td>1335 ± 0.0213</td>
<td>1334.6 ± 0.0453</td>
<td>****, 0.41 nm blue-shift</td>
</tr>
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</table>
Table S4.4. PLE Analyses – Emission Full-Width at Half-Maximum

<table>
<thead>
<tr>
<th>Chirality</th>
<th>FWHM – Emission (nm) 5 min</th>
<th>FWHM – Emission (nm) 30 min</th>
<th>Difference (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(8,3)</td>
<td>40.954 ± 0.0735</td>
<td>39.938 ± 0.623</td>
<td>ns</td>
</tr>
<tr>
<td>(6,5)</td>
<td>37.318 ± 0.0343</td>
<td>36.622 ± 0.167</td>
<td>ns</td>
</tr>
<tr>
<td>(7,5)</td>
<td>35.014 ± 0.0715</td>
<td>34.142 ± 0.154</td>
<td>ns</td>
</tr>
<tr>
<td>(10,2)</td>
<td>38.232 ± 0.229</td>
<td>36.985 ± 0.2</td>
<td>ns</td>
</tr>
<tr>
<td>(9,4)</td>
<td>39.683 ± 0.112</td>
<td>38.979 ± 0.0672</td>
<td>ns</td>
</tr>
<tr>
<td>(8,4)</td>
<td>75.518 ± 0.369</td>
<td>82.641 ± 2.3</td>
<td>****, 7.1 nm increase</td>
</tr>
<tr>
<td>(7,6)</td>
<td>33.345 ± 0.0175</td>
<td>34.381 ± 0.127</td>
<td>ns</td>
</tr>
<tr>
<td>(8,6)</td>
<td>38.635 ± 0.0834</td>
<td>38.762 ± 0.139</td>
<td>ns</td>
</tr>
<tr>
<td>(9,5)</td>
<td>59.23 ± 1.05</td>
<td>48.148 ± 0.544</td>
<td>****, 11 nm decrease</td>
</tr>
<tr>
<td>(10,5)</td>
<td>72.055 ± 2.14</td>
<td>71.697 ± 0.402</td>
<td>ns</td>
</tr>
<tr>
<td>(8,7)</td>
<td>28.379 ± 0.284</td>
<td>27.856 ± 0.266</td>
<td>ns</td>
</tr>
<tr>
<td>(9,7)</td>
<td>31.688 ± 1.82</td>
<td>36.802 ± 2.16</td>
<td>**, 5.1 nm increase</td>
</tr>
</tbody>
</table>
**Table S4.5. PLE Analyses – Excitation Full-Width at Half-Maximum**

<table>
<thead>
<tr>
<th>Chirality</th>
<th>FWHM – Excitation (nm)</th>
<th>FWHM – Excitation (nm)</th>
<th>Difference (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
<td>30 min</td>
<td></td>
</tr>
<tr>
<td>(8,3)</td>
<td>102.54 ± 0.554</td>
<td>109.09 ± 0.838</td>
<td>ns</td>
</tr>
<tr>
<td>(6,5)</td>
<td>204.09 ± 8.17</td>
<td>213.07 ± 4.37</td>
<td>ns</td>
</tr>
<tr>
<td>(7,5)</td>
<td>126.88 ± 1.38</td>
<td>125.74 ± 0.799</td>
<td>ns</td>
</tr>
<tr>
<td>(10,2)</td>
<td>89.575 ± 0.751</td>
<td>85.981 ± 0.547</td>
<td>ns</td>
</tr>
<tr>
<td>(9,4)</td>
<td>95.657 ± 0.247</td>
<td>92.415 ± 0.416</td>
<td>ns</td>
</tr>
<tr>
<td>(8,4)</td>
<td>130.77 ± 1.38</td>
<td>131.65 ± 2.18</td>
<td>ns</td>
</tr>
<tr>
<td>(7,6)</td>
<td>160.24 ± 1.39</td>
<td>158.31 ± 1.64</td>
<td>ns</td>
</tr>
<tr>
<td>(8,6)</td>
<td>136.16 ± 1.79</td>
<td>135.89 ± 0.615</td>
<td>ns</td>
</tr>
<tr>
<td>(9,5)</td>
<td>122.95 ± 3.66</td>
<td>137.41 ± 6.43</td>
<td>ns</td>
</tr>
<tr>
<td>(10,5)</td>
<td>96.735 ± 5.37</td>
<td>106.07 ± 0.663</td>
<td>ns</td>
</tr>
<tr>
<td>(8,7)</td>
<td>52.348 ± 35.9</td>
<td>56.93 ± 0.0686</td>
<td>ns</td>
</tr>
<tr>
<td>(9,7)</td>
<td>64.274 ± 0.838</td>
<td>67.949 ± 4.11</td>
<td>ns</td>
</tr>
</tbody>
</table>
Table S4.6. Band edges and widths from k-means clustering of emission bands for SDC-SWCNT adsorbed on a surface

<table>
<thead>
<tr>
<th>Band</th>
<th>Starting Wavelength (nm)</th>
<th>Ending Wavelength (nm)</th>
<th>Band Size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>956</td>
<td>1020</td>
<td>64</td>
</tr>
<tr>
<td>2</td>
<td>1024</td>
<td>1092</td>
<td>68</td>
</tr>
<tr>
<td>3</td>
<td>1104</td>
<td>1160</td>
<td>56</td>
</tr>
<tr>
<td>4</td>
<td>1172</td>
<td>1232</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>1260</td>
<td>1312</td>
<td>52</td>
</tr>
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</table>
Table S4.7. Optical parameters of SDC-SWCNT bands on surface

<table>
<thead>
<tr>
<th>Band</th>
<th>Wavelength (nm) – 5-minute sample</th>
<th>Wavelength (nm) – 30-minute sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>CI [25,75]</td>
</tr>
<tr>
<td>1</td>
<td>984.9 ± 2.519</td>
<td>[979.7, 994.4]</td>
</tr>
<tr>
<td>2</td>
<td>1065 ± 1.575</td>
<td>[1060, 1073]</td>
</tr>
<tr>
<td>3</td>
<td>1128 ± 0.7657</td>
<td>[1124, 1134]</td>
</tr>
<tr>
<td>4</td>
<td>1194 ± 1.073</td>
<td>[1187, 1200]</td>
</tr>
<tr>
<td>5</td>
<td>1282 ± 1.079</td>
<td>[1276, 1286]</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Band</th>
<th>Intensity (a.u.) – 5-minute sample</th>
<th>Intensity (a.u.) – 30-minute sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>CI [25,75]</td>
</tr>
<tr>
<td>1</td>
<td>2247 ± 234.9</td>
<td>[1535, 3032]</td>
</tr>
<tr>
<td>2</td>
<td>2388 ± 185.3</td>
<td>[1310, 3032]</td>
</tr>
<tr>
<td>3</td>
<td>2688 ± 205.3</td>
<td>[1381, 3301]</td>
</tr>
<tr>
<td>4</td>
<td>2420 ± 224.2</td>
<td>[1241, 2875]</td>
</tr>
<tr>
<td>5</td>
<td>2605 ± 228.3</td>
<td>[1835, 3129]</td>
</tr>
</tbody>
</table>
Appendix IV

Supplementary Information for Chapter 5

Aggregation Reduces Subcellular Accumulation and Cytotoxicity of Single-Walled Carbon Nanotubes

by

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Kingston, Rhode Island 02881, United States
**Figure S5.1.** Schematic depicting preparation of two DNA-SWCNT samples with varying degrees of aggregation.
Figure S5.2. Sample spectra from spectral counting analysis. (a-d) Fluorescence spectra of DNA-SWCNT ROIs imaged with hyperspectral microscopy in a spin-coated hydrogel platform containing 1, 2, 3, or 4 emission peaks.
Figure S5.3. Sample single cell ROI analysis. (a) Transmitted light image and (b) broadband NIR fluorescence image of HeLa cells dosed with 1 mg-L$^{-1}$ 250k-SWCNTs. Images were acquired 30 minutes after dosing. Right panels overlay manually-determined cell outlines from the transmitted light image.
Figure S5.4. Comparison of intracellular fluorescence as a function of cell type and DNA-SWCNT dispersion quality. Average intracellular fluorescence intensity per cell area for single cells dosed with 250k-SWCNTs or 1k-SWCNTs after **(a)** 30 minutes or **(b)** 6 hours of additional incubation time. Error bars represent mean ± s.d. One way ANOVA with Tukey post hoc analysis was performed between cell lines dosed with the same DNA-SWCNT sample (****p < 1e-4)
**Figure S5.5.** Fluorescence intensity from stock solutions of DNA-SWCNTs. (a) Average broadband fluorescence intensity of 250k-SWCNTs and 1k-SWCNTs diluted to 20 mg/L. Error bars represent mean ± s.d. A two-tailed two-sample t-test was performed (**p < 1e-4). n = 4 fluorescence images.
**Figure S5.6.** Intracellular fluorescence intensities corrected for intrinsic sample brightness obtained from solution values. The average integrated fluorescence intensity per intracellular area from individual cells as a function of DNA-SWCNT sample after (a) 30 minute or (b) 6 hour incubation time. A correction factor for intrinsic sample brightness (i.e. the 1k-SWCNTs are 46% as bright as 250k-SWCNTs) was applied to 1k-SWCNT data, represented here as 1k-SWCNT_{corrected}. Error bars represent mean ± s.d. One way ANOVA was performed for each condition (****p < 1e-4, *p < 0.05).
Figure S5.7. Full-range Raman spectrum of 250k-SWCNTs and 1k-SWCNTs, with the radial breathing mode (RBM, 150 – 350 cm\(^{-1}\)) and G-Band (~1589 cm\(^{-1}\)) spectral features indicated.
Figure S5.8. Proliferation and viability to identify adverse responses in RAW 264.7 cells. (a) Real time monitoring of RAW 264.7 proliferation after addition of DNA-SWCNTs via xCELLigence impedance measurements. Line represents the mean cell index ($n = 4$) and error bars represent the mean ± s.d. Data were normalized to the final measurement before nanotube exposure. For clarity, error bars are shown on 1 out of every 20 data points. (b) Bar graph representing total proliferation using the mean integrated area under the curve from the data shown in (a). One way ANOVA with Tukey post hoc analysis was performed ($**p < 1e-4$, $**p < 0.01$). Stars directly above the bars represent significance versus control. (c) Scatter plots depicting apoptosis data for control cells (left) and cells dosed with 1 mg-L$^{-1}$ or 10 mg-L$^{-1}$ of either 250k-SWCNTs (center) or 1k-SWCNTs (right). Viable and apoptotic cells are found in the bottom left or bottom right quadrants, respectively.
Figure S5.9. Proliferation and viability to identify adverse responses in A549 cells. (a) Real time monitoring of A549 proliferation after addition of indicated DNA-SWCNTs via xCELLigence impedance measurements. Line represents the mean cell index (n = 4) and error bars represent the mean ± s.d. Data were normalized to the final measurement before nanotube exposure. For clarity, error bars are shown on 1 out of every 20 data points. (b) Bar graph representing total proliferation using the mean integrated area under the curve from the data shown in (a). One way ANOVA with Tukey post hoc analysis was performed (****p < 1e-4, **p < 0.01). Stars directly above the bars represent significance versus control. (c) Scatter plots depicting apoptosis data for control cells (left) and cells dosed with 1 mg-L⁻¹ or 10 mg-L⁻¹ of either 250k-SWCNTs (center) or 1k-SWCNTs (right). Viable and apoptotic cells are found in the bottom left or bottom right quadrants, respectively.
Appendix V

Supplementary Information for Chapter 6

A Wearable Optical Microfibrous Biomaterial with Encapsulated Nanosensors Enables Wireless Monitoring of Oxidative Stress

by

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1 Department of Chemical Engineering, University of Rhode Island, Kingston, Rhode Island 02881, United States
Figure S6.1. (a) Absorbance spectra, and (b) Fluorescence spectra of (GT)$_{15}$-SWCNTs at 730 nm excitation.
Figure S6.2. NIR broadband fluorescence images of the fibers produced with the applied voltage of 12 kV acquired at different regions.
Figure S6.3. NIR broadband fluorescence images of the fibers produced with the applied voltage of 14 kV acquired at different regions.
Figure S6.4. NIR broadband fluorescence images of the fibers produced with the applied voltage of 16 kV acquired at different regions.
Figure S6.5. SEM images of the micro- and nanofibers fabricated with three different voltages of 12, 14 or 16 kV.
Figure S6. Raman spectra of (a) polycaprolactone (PCL) polymer and (b) poly(ethylene oxide) (PEO) polymer.
Figure S6.7. Confocal Raman microscopy of fibers soaked in PBS for 5 minutes and 24 hours. (a) and (d) The representative overlay of G-band intensity and brightfield images of fibers soaked in PBS for 5 minutes and 24 hours, respectively. (b) and (e) \( k \)-means clustering analyses of all spectra in each area scan, where \( k = 4 \) clusters (background clusters omitted from figure). (c) and (f) The average Raman spectra obtained from each cluster of (b) and (e).
Figure S6.8. Comparing the Raman spectra of the collected PBS samples over time with that of three standard samples with known SWCNT concentrations.
Figure S6.9. The fluorescence spectra of the microfibrous samples exposed to various peroxide concentrations. Each peroxide concentration was added to three different samples to confirm the reproducibility ($n = 3$).
Table S6.1. Linear fits to the ratiometric signal versus peroxide concentration, when plotted on a log-log scale, in the range of 5 µM – 5 mM for three different time points: \( R = a + bC \). Values are mean with standard error of mean (SE), from three samples for each peroxide concentration.

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Intercept: ( a \pm SE )</th>
<th>Slope: ( b \pm SE )</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>-0.38715 ± 0.00413</td>
<td>0.045 ± 0.00139</td>
<td>0.99241</td>
</tr>
<tr>
<td>48 hours</td>
<td>-0.35896 ± 0.00563</td>
<td>0.04996 ± 0.0017</td>
<td>0.99086</td>
</tr>
<tr>
<td>72 hours</td>
<td>-0.35609 ± 0.00508</td>
<td>0.05651 ± 0.00221</td>
<td>0.98796</td>
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</table>
**Figure S6.10.** Ratiometric signal as a function of time for each peroxide concentration (0-50 µM). The dashed lines indicate single exponential association fits. Each peroxide concentration was added to three different samples (n = 3).
Figure S6.11. Ratiometric signal as a function of time for each peroxide concentration (100-5000 µM). The dashed lines indicate single exponential association fits. Each peroxide concentration was added to three different samples ($n = 3$).
Figure S6.12. Histograms indicating the distribution of (a) Offset and (b) Time constant extracted from the single exponential association fits of the ratiometric signal versus time, for all examined concentrations of peroxide.
Table S6.2. Single exponential association fits to the ratiometric signal versus time for each peroxide concentration: \( R = R_0 + A \left( 1 - e^{-t/\tau} \right) \). Values are mean with standard error of mean (SE), from three samples for each peroxide concentration.

<table>
<thead>
<tr>
<th>Concentration [µM]</th>
<th>Offset: ( R_0 \pm SE )</th>
<th>Pre-Exponential Factor: ( A \pm SE )</th>
<th>Time Constant: ( \tau [s] \pm SE )</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.3887 ± 1.41E-04</td>
<td>0.11629 ± 0.00454</td>
<td>33.39577 ± 0.12185</td>
<td>0.99365</td>
</tr>
<tr>
<td>1</td>
<td>0.39137 ± 0.00204</td>
<td>0.10605 ± 0.00498</td>
<td>35.87988 ± 1.9239</td>
<td>0.99477</td>
</tr>
<tr>
<td>5</td>
<td>0.38787 ± 0.00201</td>
<td>0.12837 ± 0.00342</td>
<td>32.9493 ± 1.61385</td>
<td>0.99214</td>
</tr>
<tr>
<td>10</td>
<td>0.38456 ± 0.00184</td>
<td>0.13064 ± 0.00398</td>
<td>29.39384 ± 1.29415</td>
<td>0.99826</td>
</tr>
<tr>
<td>20</td>
<td>0.38661 ± 0.00118</td>
<td>0.1504 ± 0.00228</td>
<td>30.07832 ± 1.24041</td>
<td>0.99739</td>
</tr>
<tr>
<td>50</td>
<td>0.3848 ± 0.00202</td>
<td>0.183 ± 0.00281</td>
<td>28.94404 ± 0.86832</td>
<td>0.99827</td>
</tr>
<tr>
<td>100</td>
<td>0.38052 ± 0.00158</td>
<td>0.20274 ± 0.00258</td>
<td>25.86262 ± 0.74715</td>
<td>0.99279</td>
</tr>
<tr>
<td>200</td>
<td>0.38713 ± 8.67E-04</td>
<td>0.2268 ± 7.08E-04</td>
<td>30.33552 ± 0.45051</td>
<td>0.99862</td>
</tr>
<tr>
<td>500</td>
<td>0.37924 ± 0.00139</td>
<td>0.27885 ± 0.00903</td>
<td>26.99243 ± 0.356</td>
<td>0.99615</td>
</tr>
<tr>
<td>1000</td>
<td>0.3743 ± 0.00163</td>
<td>0.26379 ± 0.00418</td>
<td>23.58728 ± 0.76148</td>
<td>0.98822</td>
</tr>
<tr>
<td>2000</td>
<td>0.37977 ± 7.78E-04</td>
<td>0.32639 ± 0.00155</td>
<td>27.53061 ± 0.21989</td>
<td>0.99676</td>
</tr>
<tr>
<td>5000</td>
<td>0.37714 ± 0.00234</td>
<td>0.36604 ± 8.48E-04</td>
<td>26.72116 ± 0.70784</td>
<td>0.99657</td>
</tr>
<tr>
<td>Avg = 0.383 ± 9.28E-4</td>
<td>Avg = 29.305 ± 0.615</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
Figure S6.13. The pre-exponential factor extracted from the single exponential association fits and plotted as a function of peroxide concentration. The dashed lines indicate the power fits. Each peroxide concentration was added to three different samples \( n = 3 \).
Table S6.3. Power fits to pre-exponential factor versus peroxide concentration:

\[ A = (C + C_0)^p - A_0 \]

<table>
<thead>
<tr>
<th>$C_0$ [μM]</th>
<th>$p$</th>
<th>$A_0$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.69924</td>
<td>0.03495</td>
<td>0.97934</td>
</tr>
<tr>
<td>2</td>
<td>9.81501</td>
<td>0.03408</td>
<td>0.96994</td>
</tr>
<tr>
<td>3</td>
<td>11.87454</td>
<td>0.03458</td>
<td>0.96881</td>
</tr>
<tr>
<td>Avg</td>
<td>10.796 ± 0.596</td>
<td>0.035 ± 2.52E-4</td>
<td>0.972 ± 3.34E-3</td>
</tr>
</tbody>
</table>

Avg ± SD
**Figure S6.14.** Real-time peroxide detection. (a) The fluorescence spectra of the microfibrous samples exposed to 500 μM peroxide and monitored over 30 minutes, with 1 minute intervals (the peroxide was injected into the solution at 5 minutes). (b) The normalized peak fluorescence intensities of the (9,4) and (8,7) SWCNT chiralities, while peroxide was injected into the solution after 5 minutes, to a final concentration of either 500 μM or 5 mM. (c) The ratiometric signal monitored over time for the conditions mentioned in part b.
Figure S6. Reversible peroxide detection using the optical microfibrous textiles. (a) The NIR fluorescence spectra of the samples right before adding 200 µM peroxide (0 min), 10 minutes after peroxide addition and 50 minutes after removing the peroxide (the samples were washed with PBS after 10 minutes of exposure to peroxide). (b) The ratiometric signal at the three time points mentioned in part a. Mean values were obtained by repeating each condition three times ($n = 3$), and the error bars represent the standard deviation.
Figure S6.16. Real-time peroxide simulation and detection in RAW 264.7 macrophages. Transmitted light images of (a) The cells in PBS (control) and (b) The cells induced by PMA for peroxide production. (c) The fluorescence spectra of the microfibrous samples in presence of the cells producing peroxide over time. (d) The fluorescence spectra shown in part d were normalized by their max intensity. (e) The ratiometric signal extracted from the c and plotted versus of time.
The maps were created by acquiring NIR fluorescence hyperspectral images and dividing the max intensity image of the (8,7)-SWCNT by the max intensity image of the (9,4) chirality SWCNT. Since the data were acquired using a 5X objective, the individual fibers cannot be observed.
Figure S6.18. Comparison of the fluorescence spectra of microfibers alone, through adhesive bandage material, or through both adhesive material plus an adsorbent pad (complete bandage). The fluorescence spectra were acquired from three different samples per each condition (n = 3).