Chapter 9

Cell Biology of Carbon Nanotubes

Chang Guo, Khuloud Al-Jamal, Hanene Ali-Boucetta, and Kostas Kostarelos

Nanomedicine Lab, Centre for Drug Delivery Research
The School of Pharmacy, University of London, 29-39 Brunswick Square
London WC1N 1AX, United Kingdom
kostas.kostarelos@pharmacy.ac.uk

Carbon nanotubes (CNTs) were first specifically identified and described in 1991.[1] These nanoscale materials have since been widely used in a variety of fields due to their extraordinary properties, including high surface area, high mechanical strength, electronic properties, and excellent chemical and thermal stability. CNTs have also been developed and explored for a wide range of applications including in biomedicine, as biosensors, tissue engineering scaffolds, and drug delivery systems. The interaction between CNTs and mammalian cells was first observed by Pantarotto and co-workers in 2003.[2] Chemically functionalized single-walled CNTs were studied to report internalization by cells. Since then, more experimental techniques, materials, and cell types have been studied to identify the interaction between CNTs and cells *in vitro*. A variety of investigations are currently underway to study the interaction between biological systems and CNTs.

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9.1 Experimental Techniques Used to Study the Interaction Between Carbon Nanotubes and Cells *In Vitro*

Carbon nanotubes (CNTs) are mainly classified as single-walled (SWNTs) and multi-walled (MWNTs) according to the number of the concentric layers of graphitic sheets rolled into cylindrical structures. Both SWNTs and MWNTs have been reported to translocate into cells using several analytical techniques, including optical microscopy, micro-Raman spectroscopy, single-particle tracking (SPT), transmission electron microscopy (TEM), flow cytometry, and fluorescence microscopy. Each technique offers its own advantages and disadvantages that will be discussed separately below.

9.1.1 *Optical Microscopy*

Optical microscopy provides imaging of CNTs in live cell cultures; however, due to low resolution, normally only large amounts of uptaken CNTs can be detected in a non-quantitative manner. Although the technique is simple (no specialized instrumentation required) and readily available in most laboratories, optical microscopy can only offer qualitative results and also suffers from the incapability to differentiate cell surface adsorption from intracellular localization of the material. Optical microscopy can be proposed as a rough, pre-screening technique to study the effect of varying CNT characteristics (e.g., surface charge, charge density, aqueous dispersibility)[3] on their interaction with cultured cells before more sophisticated and time-consuming techniques are employed.

9.1.2 *Fluorescence Microscopy Techniques*

Fluorescence microscopy is widely used to study the interaction between CNTs and cells by the following: (i) detection of the intrinsic fluorescent signals of some CNT types; (ii) imaging CNTs using X-ray fluorescence microscopy (μXRF); and (iii) detection of fluorescent probes that have been linked (covalently or non-covalently) onto the CNTs.

Since pristine SWNTs exhibit unique near-infrared intrinsic fluorescence, near-infrared fluorescence microscopy has been used to observe the cellular uptake of SWNTs in live cells first described in 2004 by the Weisman group.[4] SWNTs were seen within intracellular compartments of macrophage cells understood to be uptaken by phagocytosis. However, due to its relatively low signal intensity, near-infrared fluorescence microscopy is currently limited to qualitative detection of cellular uptake. Moreover, specialized instrumentation and expertise is also required today. μXRF was also applied to image CNT localization within macrophages by Bussy and co-workers.[5] μXRF could provide information of the CNT–cell interaction by analysis of the fluorescence signal of the catalyst metal particles bound to CNTs. This technique has been shown to have enough sensitivity to detect very low concentrations of CNTs. Thus, this technique could be employed more in the future to identify the CNT–cell interactions and the effect on cells by uptaken CNTs; however, specialized instrumentation is also needed.

Fluorescence microscopy (optical or confocal laser) can only be applied to assess the cellular uptake of CNT probed with fluorescent dyes, thus offering indirect observation of their cellular uptake. With the help of confocal laser scanning microscopy (CLSM) and organic-based fluorophores (fluorescein or members of the rhodamine, cyanine, and Alexa families) methods to label cells and subcellular compartments have also been applied to access the subcellular localization of CNTs, as shown in Table 9.1. Some of the fluorescence probes in these studies are covalently bound to nanotubes, while others are non-covalently bound to CNTs. Regarding their intracellular localization, this is generally observed with the help of added intracellular compartment markers. In only a few of these studies CNTs were reported within the nucleus of the cells with reported co-localization of nuclear stains and fluorescence from the labeled CNTs. The issue of nuclear localization of CNTs is still not conclusive and is under intense investigation by various laboratories. Most studies today report fluorescence-probed CNTs in the cytoplasm and around the perinuclear regions. Taken together, it remains difficult to conclude on the final intracellular trafficking destination of CNTs mainly due to the dramatic variation in materials used (CNT types), cells, fluorescent probes, association between CNTs, and probes and

Table 9.1. Studies of CNT cellular uptake using intracellular compartment markers

	CNT type	Dispersing agent and buffer	Cell type	Duration of CNT interaction with cells	Cell fixation solution	Markers of intracellular compartments	Conclusions	Ref.
Coated (non-covalently surface-modified) CNTs	Phospholipid PEG-coated SWNT	H_2O or physiological buffers	HL60 (human promyelocytic leukemia cells), CHO (Chinese hamster ovary cells), and 3T3 (mouse embryonic fibroblast cells)	1 h	N/A	Endosomes: FM4-64	SWNTs enter cells; uptake pathway proposed is consistent with adsorption-mediated endocytosis	6, 7
	Cy3–DNA-coated SWNT	H_2O or physiological buffers	HeLa cells (human adenocarcinoma cells)	12 h	N/A	Nuclei: DRAQ5	SWNTs transport DNA cargo	8
	Protein (SA, SpA, BSA)-coated SWNT	H_2O	HeLa cells	2–3 h	N/A	Endosomes: FM4-64	Cellular uptake via energy-dependent endocytosis pathway; endocytosed species confined inside endosomes	9
	FITC–FA–chitosan-coated SWNT	PBS solution	Hep G2 cells (human hepatocellular carcinoma cells)	Up to 5 days	4% PFA	Nuclei: DAPI	SWNTs localization in the cytoplasm (not in nucleus)	10

	Phospholipid PEG-coated SWNT	DMEM (serum-free medium)	Ntera-2 cells (human teratocarcinoma cells)	3 h	4% PFA	Nuclei: Hoechst	SWNTS readily localize within small ($\sim 2\mu$m) vesicles in the cells	11
	Phospholipid PEG-coated SWNT	Folate-free RPMI	KB cells (human carcinoma cells)	2.5 h	Methanol at -20°C for 45 min	Nuclei: Hoechst	SWNT conjugates show high and specific binding to folate receptors	12
	AO-coated SWNT	Cell culture medium	HeLa cells	30 min up to 7 days	N/A	Lysosomes: LysoTracker	AO–SWNTs remain inside lysosomes for more than a week	13
Chemically functionalised CNTs	SWNT/MWNT: NH_3^+–CNT, $NHCOCH_3$–CNT, FITC–CNT, NH_3^+–CNT–FITC, FITC–CNT–MTX, AmB–CNT–FITC, NH_2–CNT–FITC	5% dextrose in H_2O or serum-free medium	A549 (human lung carcinoma), HeLa, Jurkat human (T lymphocyte), MOD-K (murine intestine-derived epithelial cells), *C. neoformans* (yeast), *E. Coli* (bacteria), *S. cerevisiae* (yeast)	1–4 h	4% PFA	Membranes: WGA; Nuclei: TO-PRO 3	• Cellular uptake of CNTs independent of functional group and cell type • Mechanism of CNT cellular uptake less than 50% due to energy-dependent mechanisms	14, 15

(*Contd.*)

Table 9.1. (*Continued*)

CNT type	Dispersing agent and buffer	Cell type	Duration of CNT interaction with cells	Cell fixation solution	Markers of intracellular compartments	Conclusions	Ref
Oxidized SWNT–FB-28	H_2O	Cardiomyocytes	N/A	4% formalin	Nuclei: PI	SWNTs localize in cellular compartments	16
SWNT–PEG–FITC	N/A	HeLa cells, U2OS (human bone osteosarcoma cells), MEF (mouse embryonic fibroblasts), HT1080 (human sarcoma cells), C33A (cervical cancer cells), HEK293	Up to 7 h	4% PFA	Mitochondria: MitoTracker; Nuclei: Hoechst or DRAQ5 or DAPI	SWNTs accumulate in the nucleus, the site of ribosomal biogenesis; highly dynamic inside the cells	17
Oxidized SWNT biotinylated by streptavidin–FITC	N/A	Human smooth muscle cells (hMSCs)	6 days	N/A	Actin: phalloidin; Nuclei: DAPI	SWNTs enter cells through the cytoplasm to nuclear localization	18
Oxidized SWNT–Qdot525–EGF	PBS	HN13 cells (human head and neck squamous carcinoma cells)	1 h	3.5% PBS–formaldehyde solution	Actin: phalloidin; Nuclei: PI	Localized within cytoplasm but not in the nucleus	19

Oxidized SWNT–BSA–FITC	Cell culture medium (pH 7.2–7.4)	HEK293 (human embryonic kidney cells)	1 h	4% PFA	Membrane: WGA; Nuclei: DAPI	SWNTs translocate into cytoplasmic vesicles but not in the nucleus	20
Oxidized SWNT–BSA–fluorescein–doxorubicin	N/A	WiDr (human colon cancer cells)	4 h	N/A	Cytoplasm: BSA–fluorescein	SWNTs observed outside the nuclei, within the cytoplasm, with no co-localization with doxorubicin after internalization	21
Oxidized SWNT–HER2 IgY	Cell culture medium	SK-BR-3 (human breast carcinoma cells)	24 h	10% neutral-buffered zinc formalin	Nuclei: DAPI	HER2 IgY–SWNT complex localize on the cell membrane of SK-BR-3 cells.	22

CNT: carbon nanotubes; PEG: polyethylene glycol; BSA: bovine serum albumin; AmB: amphotericin B; MTX: methotrexate; SA: streptavidin; SpA: Staphylococcal protein A; AO: acridine orange; FA: folate acid; PFA: paraformaldehyde; PBS: phosphate buffer solution.

experimental conditions. However, what remains consistent and reproducible throughout the studies performed today is the confirmation of the original reports that CNTs exhibit the capacity to be uptaken by cells in ways and mechanisms that do not necessarily follow established binding, internalization, and trafficking patterns known for other nanoparticles.

9.1.3 *Flow Cytometry*

Flow cytometry-based assays have been proposed to assess CNT–cell associations (both cell-bound or internalized) qualitatively by measuring the increase in the sideward scattering of cells incubated with non-fluorescent CNTs.[3] Data generated using light scattering analysis established a good correlation between the increase in sideward scattering intensity and the increase in CNT intracellular accumulation, which suggested that adsorption of the CNT onto the cell membrane will eventually lead to intracellular uptake. Qualitative measurements are based on the fact that as CNTs bind to the cells, the granularity of the cells increases, which concomitantly increases the sideward scattering intensity. It is difficult to distinguish between CNTs that are bound to the cell surface or internalized by the cells because sideward scattering intensity only offers an indication of cell surface roughness.[3] However, this technique can be combined with other techniques such as optical microscopy, CLSM, or TEM to distinguish CNT cell binding from internalization.

9.1.4 *Electron Microscopy*

TEM has been widely used to study the interaction between CNTs and biological systems. Both pristine CNTs (unpurified and purified) and different types of functionalized CNTs (f-CNT) have been studied intracellularly. TEM provides the highest possible resolution, indicating the exact intracellular localization of the CNTs. However, most TEM protocols have to be performed using fixed cells, so it is difficult to follow the trafficking pathway of the CNT translocation into cells. As evidenced from Table 9.2, the intracellular localization of CNTs can be classified into three major categories: (a) CNTs, both pristine and functionalized, observed in the perinuclear region

Table 9.2. Studies using TEM to investigate the cellular uptake of CNTs

Cellular localization of CNTs	CNT type	Dispersing agent and buffer	Cell type	Duration of CNT interaction with cells	Cell fixation protocol	Conclusions	Ref.
Imaged in at the perinuclear region and inside intracellular vacuoles or vesicles	DNA-coated SWNT	dH_2O	3T3 cells	Up to 48 h	Fixed at 4°C for 24 h	SWNTs incorporate into cytoplasmic vesicles and labeled the perinuclear region of cells, but did not enter the nuclear envelope	25
	MWNT	Cell grow medium	HEK cells (human embryonic kidney cells)	Up to 48 h	Fixed in Trump's fixative at 4°C and post-fixed in 1% OsO_4 in 0.1 M sodium pgosphate buffer	MWNTs present within cytoplamic vacuoles at all time points and induced the release of the proinflammatory cytokine IL-8 in a time-dependent manner	26
	SWNT	DMEM supplemented with 5% FBS	HeLa cells	60 h	Fixed using 2.5% glutaraldehyde in 0.1 M cacodylate buffer and post fixed with 1% OsO_4	SWNT-like material in intracellular vacuoles	27

(*Contd.*)

Table 9.2. (*Continued*)

Cellular localization of CNTs	CNT type	Dispersing agent and buffer	Cell type	Duration of CNT interaction with cells	Cell fixation protocol	Conclusions	Ref.
	Oxidized SWNT–FB-28	H_2O	Cardiomyocytes	Up to 5 days	Fixed in 2.5% phosphate-buffered glutaraldehyde, pH 7.4, for 4 h at 4°C	SWNTs localized within cellular vesicles	16
	MWNT	Ultrapure sterile H_2O (pH 5.5) with Arabic gum (0.25 wt%)	A549 cells	48 h	Fixed with 2.5% glutaraldehyde, and post-fixed with OsO_4	MWNTs localized in cytoplasm, the majority of them being surrounded by a membrane	28
	MWNT, SWNT (unpurified and purified)	N/A	Macrophages	24 h	N/A	MWNTs and both purified and raw SWNTs engulfed into vacuoles that can occupy most of the cell surface cytoplasm	5
	Water-soluble MWNT by introducing an oxygen component only	IMDM (Iscove's modified Dulbecco's medium)	Fibroblast cells	2 days	Fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 12 h, then washed and post-fixed with 1% aqueous OsO_4 for 30 min	MWNTs enter into cells and accumulate in the cytoplasm	29

	Oxidized SWNT	Cell culture medium (pH 7.2–7.4)	HEK cells	1 h	Fixed in 2.5% glutaraldehyde in 0.1 M sodium, cacodylate buffer and rinsed, and post fixed 1 h in 2% OsO_4 with 3% potassium ferriocyanide and rinsed	SWNTs reported within endosomes	20
In the cytoplasm and within the nucleus	NH_3^+–MWNT	5% dextrose	HeLa cells	1 h	Fixed with 2% solution of uranyl acetate in water overnight at 4°C	MWNTs found to cross the plasma membrane barrier and in the nucleus	30
	SWNT	THF	HMM (human monocyte-derived macrophage)	Up to 4 days	Fixed in 4% glutaraldehyde in PIPES buffer	SWNTs enter the cytoplasm and localize within the cell nucleus	23
	Oxidized SWNT	N/A	HMSC (human mesenchymal stem cells)	Up to 6 days	Fixed in 3% gluteraldehyde, dehydrated, and sectioned at −20°C	SWNTs localize in cytoplasm and also in nucleus	18

(*Contd.*)

Table 9.2. (*Continued*)

Cellular localization of CNTs	CNT type	Dispersing agent and buffer	Cell type	Duration of CNT interaction with cells	Cell fixation protocol	Conclusions	Ref.
	Oxidized SWNT–Qdot525–EGF	PBS	HN13 cells	1 h	N/A	SWNTs localize around perinuclear region	19
	MWNT–NH_2; MWNT–COOH	dH_2O	HEK cells	Up to 48 h	Fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH7.4) for 1 h at RT, and post fixed for in2% OsO_4 with 3% potassium ferrocyanide for 1 h	MWNT–COOHs and MWNT–NH_2s enter cells both through endocytosis and direct translocation	31
Non-specific intracellular regions	DNA-coated SWNT	Salt solution	Va13 (human fibroblast cells)	Overnight	Fixed in an epoxy matrix	Longer tubes on the outside of the cell membrane and shorter tubes piercing the membrane and residing in the cytosol	32
	SWNT	Serum containing (5%) medium	A549 cells	24 h	Fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 h, and post fixed in 1% OsO_4 in 0.1 M phosphate buffer for 1 h	Non-intracellular localization of SWNTs but increased number of surfactant storing lamellar bodies observed	33

FITC–FA–chitosan-coated SWNT	PBS	Hep G2 cells	1 h	Fixed in 2.5% glutaraldehyde containing 0.1 M PBS buffer for 3 h and post-fixed with 1% OsO_4 for 30 min	SWNTs located only in the cytoplasm and not in nuclei	10
SWNT and MWNT	PBS	HAEC (human aortic endothelial cells)	24 h	Fixed in Karnovsky's fixative (2.5% gluteraldehyde, 2.5% paraformaldehyde in 0.1 M sodium cacodylic buffer), post-fixed in OsO_4, mordanted in 1% tannic acid	A small number of CNTs were identified in the cytoplasm of some cells	24
80n*-MWNT	N/A	Osteoclasts	3 days	N/A	MWNTs observed inside of cells and some in the vicinity of mitochondria	34
AO-coated SWNT	Fresh culture medium containing 5% FBS	HeLa cells	30 min up to 7 days	Fixed with 2% glutaraldehyde and 1% OsO_4	AO–SWNTs remain inside lysosomes for more than a week	13

*Average diameter 80 nm; AO: acridine orange; THF: tetrahydrofuran; FA: folate acid; FBS: fetal bovine serum.

inside intracellular vacuoles or vesicles; (b) CNTs detected both in the cytoplasm and within the cell nucleus. According to these observations, CNTs were found not only to translocate across plasma membranes, but also seemed to enter the nuclear envelope. Most such studies were performed using chemically f-CNT; however, Porter *et al.*[23] imaged individual non-functionalized SWNTs within cells using low-loss energy-filtered TEM in combination with electron energy loss spectrum imaging. These techniques allowed for improved contrast between (unlabelled) SWNTs and cell organelles including the plasma membrane, vesicles, and the nucleus without staining. They showed direct evidence of the individual SWNTs crossing lipid bilayers and enter into the cytoplasm and nucleus; (c) CNTs were found into non-specific intracellular regions. An example of such study was recently reported by Simeonova *et al.*, who observed small numbers of purified (non-functionalized) SWNTs and MWNTs in the cytoplasm or along the plasma membrane of human aortic endothelial cells.[24]

9.1.5 *Micro-Raman Spectroscopy*

SWNTs show strong Raman scattering[35] evidenced by the presence of characteristic G-band peaks. CNTs uptaken within living cells could be studied by micro-Raman spectroscopy confirming their cellular uptake. This technique was first applied by Daniel *et al.* to observe the cellular uptake of CNTs in live cell cultures by comparing the Raman scattering and fluorescence spectra of SWNTs and correlating those signals to intracellular location based on area maps of the cells in comparison to optical microscopy and TEM.[25] Later, Raman spectroscopy was used to detect the cellular uptake of non-covalently surface-modified SWNTs, coated with either peptides or PEGylated lipids.[36] The advantages of micro-Raman spectroscopy are its high sensitivity and low background signal interference along with the capability for long-term detection.

9.1.6 *Intrinsic Photoluminescence (Via SPT)*

SPT is a technique used to study the diffusion of small molecules both computationally and experimentally. Although there is not high

enough resolution to allow visualization of specific cellular uptake using this technique, the interaction between SWNTs and live cells can be assessed in a dynamic fashion.[37,38] Moreover, by mapping and monitoring the trajectories of SWNTs internalized into live cells as a function of time and cell topography membrane surface adsorption and desorption, diffusion, endocytosis, and exocytosis from fibroblasts (NIH-3T3 cells) has been proposed.[37,38]

9.2 Mechanisms Involved in the Cellular Uptake of CNTs

The cellular uptake of CNTs has been reported by several laboratories employing a variety of experimental techniques as discussed in Section 9.1. Table 9.3 summarizes as comprehensively as possible the different CNT types used in various studies along with the reported conclusions offered on the mechanism(s) of intracellular uptake involved in the uniformly agreed observation of CNT intracellular localization. Below, we will attempt to summarize the main such mechanisms that have been proposed (Section 9.2.1) and discuss the critical parameters that have been implicated in determining which of those mechanisms can be deemed more predominant (Section 9.2.2).

Table 9.3. Cell biology studies and the proposed mechanisms of CNT cellular uptake

Type of CNT		Cell	Experimental technique	Mechanism of uptake proposed	Ref.
Pristine CNTs	SWNT	Macrophage-like cells	Near-infrared fluorescence microscopy	Localized in small phagosomes suggesting phagocytosis pathway	4
	SWNT, MWNT	HeLa HEK cells	TEM	Localized in cytoplamic vacuoles suggesting endocytosis pathway	26, 27
	SWNT	HMM cells	Low-loss energy-filtered TEM combined with nuclei marker	Localized in the cytoplasm and also in nucleus suggesting diffusion pathway	23

(*Contd.*)

Table 9.3. (*Continued*)

Type of CNT		Cell	Experimental technique	Mechanism of uptake proposed	Ref.
	SWNT	NIH-3T3 cells	SPT	• The association between SWTN and cells is associated with several mechanisms (see Section 9.2.1.3 for details) including membrane surface adsorption and desorption, surface diffusion and endocytosis and exocytosis The cellular uptake of SWNT is reported as size-dependent	37–39
Coated (non-covalently surface-modified) CNTs	Phospholipid–PEG-coated SWNT	HL60 cells, CHO cells and 3T3 cells	CLSM combined with endosome marker	Uptake pathway is consistent with adsorption-mediated endocytosis	6
	Poly(rU)-coated SWNT	MCF7 cells	CLSM	SWNTs could penetrate the nuclear membrane suggesting a diffusion pathway	40
	DNA-coated SWNT	3T3 cells	TEM	Localized in the cytoplasmic vesicles and the perinuclear region of the cells suggesting endocytosis pathway	25
	DNA-coated SWNT; protein-coated SWNT	HeLa cells	CLSM under endocytosis-inhibiting condition	Uptake reported via an energy-dependent endocytosis pathway and the endocytosed species are confined inside endosomes	7, 8, 41
	Peptide-coated SWNT	HeLa cells	Raman scattering	Cellular uptake reported as time- and temperature-dependent suggesting endocytosis pathway	42

Table 9.3. (*Continued*)

Type of CNT		Cell	Experimental technique	Mechanism of uptake proposed	Ref
	DNA-coated SWNT	IMR90 cells (human lung fibroblasts)	TEM and CLSM	Uptake reported as length-dependent	32
	FITC–FA–chitosan-coated SWNT	Hep G2 cells	CLSM combined with nuclei marker; TEM	Localized only in the cytoplasm and not in nuclei, suggesting endocytosis	10
Covalently modified CNT by oxidation	SWNT–PEG–FITC	HeLa , U2OS, MEF, HT1080, C33A, HEK293 cells	CLSM combined with intracellular compartment markers	Localized in the nucleus, mainly in the nucleolus suggesting diffusion	17
	Oxidized SWNT	HEK293 cells	CLSM and TEM	Localized in endosomes, suggesting uptake through an endocytosis pathway	20
	MWNT–NH_2; MWNT–COOH	HEK293 cells	TEM	Localized in endosomes and lysosomes for short term and in the nucleus at later time points, suggesting a combination of endocytosis and direct penetration	31
	Oxidized SWNT–biotin	L1210FR cells (leukemia cells)	CLSM under endocytosis-inhibiting condition	Localized inside of cells in an energy-dependent, endocytosis pathway	43
	Oxidized SWNT	BY-2 cells (Walled plant cells)	CLSM under endocytosis-inhibiting condition	• SWNTs traverse across both plant cell walls and cell membrane • SWNT/FITC is taken up by fluid-phase endocytosis	44

(*Contd.*)

Table 9.3. (*Continued*)

Type of CNT		Cell	Experimental technique	Mechanism of uptake proposed	Ref
Covalently modified CNT by 1,3 dipolar cycloaddition	SWNT–NH–FITC	Human 3T6 and murine 3T3 fibroblasts	CLSM	Localized inside of the cells by an energy-independent, passive translocation pathway	2
	MWNT–NH_3^+	HeLa, HEK293 cells	TEM	CNTs able to cross cell membrane and accumulate in cytoplasm to reach the nucleus suggested diffusion pathway	30, 45
	SWNT/MWNT: NH_3^+–CNT, $NHCOCH_3$–CNT, FITC–CNT, NH_3^+–CNT–FITC, FITC–CNT–MTX, AmB–CNT–FITC, NH_2–CNT–FITC	A549, HeLa cells, Jurkat human, MOD-K cells, C.neoformans, *E. coli*, *S. cerevisiae*	CLSM combined with intracellular compartment markers and under endocytosis-inhibiting condition		

• CNTs cellular uptake reported concentration-dependent
• CNTs cellular uptake reported independent of functional group and cell type 14, 15

9.2.1 *Trafficking Pathways in the Cellular Uptake of CNT*

The exact mechanisms involved in the cellular uptake of CNT are not yet clearly elucidated and more likely are a contribution of multiple pathways. Both energy-dependent endocytosis pathways and energy-independent translocation through the plasma membrane have been reported to play a role leading to CNT cell internalization. There are several parameters that seem to play an important role in determining the intracellular localization and trafficking of CNT, among which the most critical are type of CNT surface modification and CNT dimensions (diameter

and length). More than a single experimental technique and CNT type should be studied in combination to further understand those interactions.

9.2.1.1 Types of CNT endocytosis leading to internalization

The initial report of CNT cell internalization (using chemically f-CNT) was published by Pantarotto *et al.* in 2003 and observed the cellular uptake of fluorescent (fluorescein isothiocyanate [FITC]) probe-conjugated CNTs. This study reported the cellular uptake of f-CNTs even at low temperature (4°C) or in the presence of an endocytosis inhibitor (sodium azide). Based on such evidence f-CNTs were proposed to be able to translocate into cells (3T3 and 3T6 cells) under energy-independent pathways.[2] A following study by Dai and co-workers reported that PEGylated lipid-coated SWNTs were uptaken also in both adherent (HeLa) and non-adherent (HL60) cell cultures. Moreover, they observed that these lipid-coated SWNTs were co-localized intracellularly with an endosome marker (FM 4-64) at 37°C, while their uptake was blocked at low temperatures. Therefore, an energy-dependent endocytotic mechanism was proposed by these authors to account for the uptake of non-chemically f-CNT into cells.[9] The same group also studied shortened SWNTs (non-covalently) coated with ssDNA or protein (BSA) molecules to suggest that their cellular uptake follows a clathrin-dependent endocytosis pathway rather than a caveolae or lipid-rafts pathway.[8] The intracellular localization of CNTs was mainly observed by TEM and fluorescence microscopy, while other techniques such as SPT[37–39] have also been more recently employed to study the mechanism of cellular uptake of ssDNA-coated SWNTs in NIH-3T3 cells. Trajectories of non-photobleaching SWNTs were tracked during the interaction with NIH-3T3 cells in real time using optical microscopy. Thousands of individual trajectories allowed the analysis of the SWNTs trafficking pathway within these cells. Using image processing algorithms, it was proposed that within the 50.8% trajectories that identified different kinds of interactions between SWNTs and cellular compartments, around 12.7% seem to follow an endocytosis pathway.[37] It is becoming apparent that the type of molecules that are used to coat or wrap CNT to make them more dispersible in

aqueous and biological media plays a critical role in the interaction with cells. Whether cellular uptake of CNTs takes place through an energy-dependent endocytosis pathway and which one of the various pathways is predominant needs further investigation using a variety of different CNT types (lipid-, polymer-, DNA-coated, etc.).

9.2.1.2 Can CNTs pierce through cell membranes as "nano-needles"?

The mechanism of CNT cellular uptake using chemically functionalized CNTs in a variety of cell types was studied by Kostarelos and co-workers.[2,14] Both SWNTs and MWNTs were functionalized using identical chemical synthesis with a wide range of molecules of increasing molecular weight (ammonium, acetamido, FITC, methotrexate, amphotericin B, and their combinations) and monitored cellular uptake in several kinds of cells (including A549, fibroblasts, HeLa, CHO, HEK293, Keratinocytes, Jurkat, *E. coli*, *C. neoformans*, and *S. cervisiae*). f-CNTs cellular uptake was observed even under endocytosis-inhibiting conditions. Based on such studies it has been suggested that f-CNTs interact with cellular membranes as "nano-needles," able to pierce the plasma membrane and translocate to the intracellular compartments in a largely energy-independent, passive diffusion mechanism. Further evidence by TEM and confocal microscopy has recently been reported in support of a "nano-needle" CNT behavior.[2,14,15] Porter *et al.* observed by TEM that SWNTs could translocate across the lipid bilayers into the neighboring cytoplasm, and also be localized inside the cell nucleus.[23] By SPT and optical microscopy, Strano *et al.* suggested around 18.4% of the trajectories following surface diffusion.[38] PEGylated SWNTs have also been reported recently in the nucleus of HeLa cells observed by fluorescence microscopy.[17] The proposition from such studies that CNTs can transport across cellular membranes and through the nuclear envelope offers further support as to their capacity to pierce through membranes; however, further investigation is needed to elucidate the exact mechanisms and possible alternative pathways involved in the intracellular trafficking of these materials.

9.2.1.3 Fate of CNTs after internalization

The Strano group applied optical microscopy and SPT to explore the fate of ssDNA-coated SWNTs following cellular uptake (NIH-3T3 cells). They reported that 49.2% trajectories following a purely convective diffusion in the flow field with no cellular interaction while the remaining 50.8% trajectories followed different trafficking pathways, including 6.2% membrane surface adsorption, 18.4% surface diffusion, 12.7% endocytosis, 5.9% exocytosis, and 7.4% desorption from the membrane. That was the first published evidence indicating CNT exocytosis after cellular internalization.[37] In an alternative paradigm, recent studies by Kagan *et al.* have reported the possibility for enzymatic degradation CNTs[46]; however, this work has been carried out only chemically, in the absence of interaction with cells. Further data on the degradation mechanisms of CNTs *in vitro* and *in vivo* are very much needed. Nevertheless, information about the fate of CNTs following cellular internalization is still scarce and at very early stages, with further investigation in this area clearly needed.

9.2.2 *Parameters Involved in the Cellular Uptake of CNTs*

9.2.2.1 Surface modification of CNT: non-covalent coating versus chemical conjugation

Different approaches to modify the CNT surface result in different degrees of aqueous dispersibility, stability in cell media, and type of interaction with cellular membranes and other intracellular components. Kam *et al.* reported an energy-dependent endocytosis pathway for the cellular uptake of SWNTs coated with large molecular weight biopolymers,[7,8] while others found that energy-independent cell internalization was taking place extensively by f-CNT chemically conjugated with small molecular weight functional groups.[14] It seems that the interaction between cells and large biopolymers linked to CNT by non-covalent coating or chemical conjugation is a critical factor that favors energy-dependent endocytotic mechanisms. On the other hand, f-CNTs functionalized with small molecules are able to translocate inside the cytoplasm by

energy-independent endocytotic mechanisms that favor piercing of the plasma membrane via lipid exchange. It would be interesting and useful to determine the characteristics (e.g., molecular weight, charge, hydrophobicity) of the molecules used to surface-modify nanotubes in correlation with the cell internalization mechanisms that these will dictate.

9.2.2.2 CNT diameter and length

It is still not clear whether the diameter of CNTs (determined by the number of the concentric carbon layers) is involved in the mechanisms leading to cellular uptake, since both SWNTs and MWNTs have been reported to be able to internalize into cells. The effect of CNT length on cellular uptake has been also been studied using SWNTs. One publication has suggested length-dependent cellular uptake based on evidence that as different lengths of SWNTs (average lengths of 660 $\pm$ 40 nm, 430 $\pm$ 35 nm, 320 $\pm$ 30 nm, and 130 $\pm$ 18 nm studied) were compared, CNT of 320 $\pm$ 30 nm provided the highest cellular uptake.[39] More studies on the effect of CNT dimensions on cellular uptake are needed, even though they can be challenging since other parameters (such as aggregation in biological media, wide length, and diameter distributions among CNT samples) will exert significant impact on the studied effects; therefore, great caution is advised.

9.2.2.3 Concentration of CNT

The cellular uptake of CNTs has been reported to be dependent on their concentration interacting with cells, the higher the concentration of dispersible CNTs the higher the cellular uptake.[15,17] However, great care should be taken to make sure that cell internalization occurs at concentrations below the toxicity threshold. For example, actin cytoskeleton disruption accompanied with altered VE-cadherin localization and a concomitant diminished viability of human aortic endothelial cells has been found to be related to high concentrations of CNTs.[24] Cheng and co-workers[17] reported an interesting phenomenon of reversible accumulation of FITC-labeled PEGylated SWNTs (FITC–PEG–SWNTs) within the nucleus of

several mammalian cell lines (Table 9.1), by studying their intracellular trafficking and fate. By comparing the fluorescence intensity of intracellular CNTs and extracellular CNTs, they observed that the intranuclear distribution of SWNTs depended on the extracellular concentration of SWNTs and the translocation of CNTs in and out of cells at similar rates. Even though such results are intriguing, the underlying mechanisms of cellular internalization and exocytosis need to be verified.

9.2.2.4 Cell type

Some of the cell types that have been reported to internalize CNTs are shown in Tables 9.1 and 9.2. Our group and others have reported the cellular internalization of different f-CNTs in a wide variety of cell types, including mammalian cells including fibroblasts exhibiting deficient phagocytosis, fungi, yeast, and bacterial cells.[2,4,14] More recently, other cell types have also been reported to uptake CNTs including plant cells.[44] It seems that CNT exhibit a capacity to internalize in cells irrespective of cell type; however, more work needs to be performed to correlate the internalization of different CNTs with cell types.

9.2.2.5 Duration of CNT interaction with cells

The cellular uptake of CNTs has been reported to be dependent on incubation time; the longer the incubation with CNTs the higher the degree of cellular uptake of CNTs.[31] The incubation times between CNTs and cells in different studies are shown in Tables 9.1 and 9.2. In one report, cellular uptake in the cytoplasm and nucleus was reported after 6 days of incubation[18]; however, this parameter will greatly depend on other CNT characteristics such as the stability of the dispersion in biological media, the surface modification of the CNTs (e.g., surface charge) that may accelerate interaction with cell cultures. More systematic studies using adherent and non-adherent cell cultures with different types of CNTs are needed to elucidate the importance of this parameter.

9.3 Conclusion

The cell biology of CNTs has become an increasingly interesting area of research both at the basic biological level and also due to the variety of potential biomedical applications using CNTs. The field has experienced an exponential increase in the number of studies and laboratories using CNTs in contact with various cell types that will surely increase in the next few years. We have already learnt that the type and nature of molecules used to modify the surface of CNT play a determinant role in their initial interaction with cells and their subsequent intracellular trafficking and translocation. From the basic cell biology point of view, the now numerous reports on the capacity of CNT structures to pierce cellular membranes and translocate directly through to the cytoplasm offer a new insight into the way fabulous nanostructures interact with lipid membranes and at the same time a novel tool to transport small molecules intracellularly. This is only the beginning in a research area that promises to exploit CNTs both as a tool for basic cell biology and a useful nanodevice for the delivery of therapeutic or diagnostic agents.

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