# Mechanism of CNTs Uptake by Cells and Spheroids

Subjects: Cell & Tissue Engineering Contributor: Roman A. Anisimov, Dmitry A. Gorin, Anatolii A. Abalymov

Cell spheroids (CSs) are three-dimensional models in vitro that have a microenvironment similar to tissues. Such threedimensional cellular structures are of great interest in the field of nano biomedical research, as they can simulate information about the characteristics of nanoparticles (NPs) by avoiding the use of laboratory animals.

Keywords: cell spheroids ; carbon nanotubes ; CNTs ; nanomedicines ; penetration

## 1. Introduction

The field of nanomedicine offers great opportunities for the development of new materials that can improve the therapy of various diseases  $^{[1][2]}$ . Carbon nanotubes (CNTs) are fairly new nanomaterials that have unique properties and potential in various fields  $^{[3][4]}$ . In particular, CNTs can broaden the horizon of biomedical research due to their important chemical, thermal, electrical, mechanical, and structural properties, which are currently of great interest. CNTs have a high modulus of elasticity and possess the properties of metallic, semiconducting, and superconducting materials  $^{[5]}$ . Also, CNTs have a nanoarchitecture that allows both encapsulation of molecules inside and conjugation to the surface  $^{[6]}$ . It has been shown that CNTs can be used in many applications, including biosensors  $^{[Z][8]}$ , nanofluidic systems  $^{[9]}$ , biopharmaceutical applications  $^{[10]}$ , and diagnostic tools and devices in radiation oncology  $^{[11]}$ . Unfortunately, CNTs still have no direct application in clinical settings due to the poor understanding of their biological properties and behavior in living objects  $^{[12]}$ . In addition, in large-scale production, CNTs must also have well-characterized biological, environmental, and safety profiles. CNTs can vary significantly in size, morphology, structure, and purity depending on the method of preparation, purification, and functionalization used for their synthesis. Therefore, the interaction of CNTs with the biological environment is very complex and sometimes unpredictable, which requires an additional study on complex living systems  $^{[13]}$ .

### 2. Properties, Modifications, and Application of CNTs

The nanoparticles made completely of carbon are known as carbon nanomaterials (CNMs). CNMs can be divided into 0D-CNMs (i.e., fullerenes, particulate diamonds, and carbon dots), 1D-CNMs (i.e., CNTs, carbon nanofibers (CNFs), and diamond nanorods), 2D-CNMs (i.e., graphene, graphite sheets, and diamond nanoplatelets), and 3D-CNMs. All decreased dimensionalities, including fullerenes, contain CNMs made completely of sp2-bonded graphitic carbon. All of the materials presented above can also be used for nano-biomedical applications, as evidenced by already existing scientific work <sup>[14][15][16][17]</sup>.

Carbon nanotubes are an allotropic form of carbon. CNTs are well-ordered, high-aspect-ratio hollow graphite rods that were identified by lijima in 1991 <sup>[18]</sup>. Since then, CNTs have been widely used in many areas, including electrode materials <sup>[19]</sup>, nanoelectronics components <sup>[20]</sup>, biosensors <sup>[21]</sup>, strengthening of materials <sup>[22]</sup>, and as components of biomaterials for drug delivery or other types of therapy <sup>[23][24][25][26]</sup>. The synthesis of CNTs is a broad topic and will not be described here in detail; however, it should be noted that the most used methods are electric-arc discharge <sup>[27]</sup>, laser ablation <sup>[28]</sup>, and the wide family of catalytic chemical vapor deposition (CCVD) methods <sup>[29]</sup>. A CNTs can be described as a rolled layer of graphene that can be opened and closed at the ends with fullerene caps <sup>[30]</sup>.

One of the most important parameters of the CNTs is the number of concentric walls (**Figure 1**). The number of walls primarily determines the diameter of the CNTs. For example, single-walled carbon nanotubes (SWCNTs) have a small diameter (usually 1–2 nm), while multi-walled carbon nanotubes (MWCNTs) reach a diameter of up to 100 nm. Ref. <sup>[31]</sup> However, an increase in the number of walls also increases the number of defects, thus facilitating their modification and functionalization. Double-walled carbon nanotubes (DWCNTs) are located in the middle and are also quite promising since the diameter is still quite small, mechanical properties and electrical conductivity remain high due to the inner layer, but their surface modification is also possible due to the second wall <sup>[32]</sup>.



Figure 1. Carbon nanotube properties are important for interaction with cells.

The difference in the number of walls in CNTs can also affect cell viability in different ways <sup>[33]</sup>. For example, it has already been described that the difference in the cytotoxic effect on cells between single-walled and multi-walled CNTs is quite large <sup>[34]</sup>. It is hypothesized that MWCNTs lead to the production of reactive oxygen species (ROS), causing inflammation, while SWCNTs increase oxidative stress through damage to mitochondria <sup>[35]</sup>. Additionally, the difference in toxicity between multi-wall and single-wall CNTs is associated with their hydrophobic–hydrophobic interaction of MWCNTs with the cell membrane and following hole formation and loss of the plasma membrane integrity <sup>[36]</sup>.

When synthesizing CNTs, parameters such as the diameter and length of the CNTs can be tuned; for example, it can be tuned by different flow rates and flow duration of the carbon precursor gas (C2H2) on the growth of CNTs by a thermal CVD method <sup>[37]</sup>. By changing the length of the carbon tubes, researchers change the specific area, which can be a very important parameter when using CNTs to load and deliver molecules <sup>[38]</sup>. However, the length and diameter of the CNTs also influence the degree of toxicity of the CNTs in vivo and in vitro <sup>[39]</sup>. It is proven that with an increase in the length of CNTs, the toxic effect also increases. This is because macrophages can more easily envelop CNTs with a shorter length <sup>[40][41]</sup>.

Another key parameter, both for the physicochemical properties of CNTs and biocompatibility, is the surface chemistry of CNTs. Surface chemistry determines properties such as charge, hydrophobicity <sup>[42]</sup>, photocatalytic activity <sup>[43]</sup>, and the ability to bind to various biological molecules (one of the most important factors for the formation of a protein crown and connection with cells) <sup>[44]</sup>. Each of these factors can affect both in vitro co-localization and in vivo biodistribution <sup>[45][46]</sup>. One of the methods for functionalizing the surface of CNTs with groups is plasma treatment <sup>[47]</sup>. The advantage of plasma treatment is that it does not pollute the environment and provides a wide range of functional groups depending on the plasma parameters. Fine-tuning of the surface is achieved by changing the plasma processing parameters such as power, gases used, processing time, and gas pressure <sup>[48]</sup>. Surface functionalization of CNTs can provide good targeting to the desired cell type, such as surface functionalization with antibodies that selectively bind to the desired receptors (e.g., EGFR) on cancer cells. Such functionalization technologies are widely used in radioactivity and drug-delivery systems <sup>[49]</sup>.

Depending on the properties of CNTs, they find various applications for biomedical purposes. Some of the most obvious applications of CNTs are molecule delivery <sup>[50]</sup>, photothermal therapy <sup>[51]</sup>, use as biosensors <sup>[52]</sup>, and as a component for the synthesis of hybrid materials for tissue engineering <sup>[53]</sup>. The choice of a molecule for delivery and its loading/conjugation primarily depends on the purpose of delivery. It can be peptides, nucleic acids, therapeutic molecules, etc.

Peptide delivery has already been demonstrated using the foot-and-mouth disease virus (FMDV) B-cell epitope, which was covalently bound to amino groups on the surface of CNTs. After conjugation, the peptides adopt a suitable secondary structure and can be recognized by specific monoclonal and polyclonal antibodies. Immunization of mice with FMDV peptide-nanotube conjugates induced a high humoral response compared to the free peptide. Similar results indicate the possibility of using carbon nanotubes as components for vaccines <sup>[54]</sup>. Delivery of nucleic acids using CNTs is also possible. This direction is extremely promising. For example, by functionalizing the surface with ammonium, nucleic acids bind to the surface of the CNT via electrostatic interaction <sup>[55]</sup>. The search for new and effective delivery systems for therapeutic agents also suggests the possibility of using CNTs as a carrier. Anti-cancer drugs such as doxorubicin (DOX)

successfully bind to the surface of CNTs via  $\pi$ - $\pi$  stacking, making the CNTs–DOX conjugation the basis of CNT-based drug delivery systems for the delivery of DOX to cancer cells <sup>[56]</sup>.

CNTs can also be used for photothermal therapy, as they have excellent optical absorption in the visible and near-infrared sectors. When irradiated with near-infrared light, the local temperature of the tissues in which the CNTs are located rises to 40–45 °C and kills the cells that are within the heating radius <sup>[51]</sup>. Induction of high temperature for sufficient time causes physical damage such as protein denaturation and membrane lysis and can increase oxidative stress, eventually causing coagulative necrosis or apoptosis. The wide electromagnetic absorption spectrum of CNTs creates exceptional properties compared to other plasmon-heated nanomaterials (e.g., gold nanoshells and nanorods), which depend on the size and shape of CNTs <sup>[57]</sup>. Studies show that CNTs can achieve thermal destruction using tenfold-lower doses in solution and using threefold-lower laser power than that required for gold nanorods, and these also indicate that MWCNTs are more potent than bulk single-walled nanotubes in transferring the NIR light into heat.

Currently, the scientific community has identified three possible mechanisms of CNT cell toxicity. The first is based on irreparable mechanical damage to the membrane (cellular or nuclear) [58]. It is very likely that endocytosis, phagocytosis, or nanopermeasurement, which are the main ways in which the nanomaterial interacts with the lipid membrane, are strictly dependent on the geometry of the CNTs, especially their length <sup>[59]</sup>. The next putative mechanism of toxicity is oxidative stress, resulting from an increase in reactive oxygen species (ROS) and leading to numerous side effects in the cell, such as apoptosis, necrosis, cytochrome c release, oxidative DNA damage, reduced proliferation, inhibition of cell growth, etc. [58]. The last mechanism, the mechanism of genotoxicity, is in one way or another associated with DNA damage, characterized by a wide spectrum: CNTs interaction with proteins involved in chromosome aberration; CNTs effect on the mitotic spindle, micronuclei formation, indirect DNA oxidation, DNA breakage, etc. Although the toxic mechanisms of CNTs have been studied from several perspectives, there is still a strong correlation between triggered or inhibited molecular pathways and cell types [60]. Despite the described complexity of the processes occurring inside the cells targeted by CNTs, some scientific works suggest ways to overcome the toxic effects of CNTs by modifying the material surface with functionalizing groups, coating with metal oxides, or protein attachment. For example, coating with recombinant C1q, which is a protein that activates the classical pathway of the complement system involved in the innate immune system, is a promising approach to regulating inflammation. In addition, several theoretical studies on modeling a possible cellular response to CNTs demonstrate the mechanical interaction of nanotubes with the lipid layer or with proteins, suggesting a safer geometry of CNTs, which furthers the understanding of the action of CNTs on cells [61].

#### 3. Properties, Fabrication, and Application of CSs

The use of cell cultures is the first step in biomaterial development, research, and clinical activities. There are a huge number of methods that are used to determine the cellular condition and behavior, for example, when exposed to cytostatics or on biomaterials surface <sup>[62]</sup>. However, in most scientific and research works for such tests, 2D cultures are used, i.e., cells located on the surface of the culture plastic and forming a monolayer <sup>[63]</sup>. However, we are in a three-dimensional world and consist of tissues, which in turn are also three-dimensional. Due to this three-dimensionality, tissues in the body have a large number of gradients, which can be mechanical <sup>[64]</sup>, chemical <sup>[65]</sup>, electrical <sup>[66]</sup>, etc. <sup>[67]</sup>. Such gradients are practically impossible to obtain in 2D cultures, which makes them suitable but extremely distant from real biological objects. In this regard, the direction of studying 3D CSs has been actively developed in recent years <sup>[68]</sup>. Such 3D CSs can have properties much closer to real tissues and successfully fill the gap between cell culture and laboratory animals, which makes their use significant for biological research <sup>[69]</sup>.

One important purpose of using an in vitro cell testing system is to replicate the cell microenvironment (**Figure 2**) <sup>[Z0]</sup>. For example, cells within a tissue are surrounded by neighboring cells and an extracellular matrix, which constantly provide the cells with biochemical and mechanical signals <sup>[Z1]</sup>. This 3D network of cell–cell and cell–ECM interactions maintains the specificity and homeostasis of a particular tissue <sup>[Z2]</sup>. As a result, testing and detection of interactions between NPs and 2D cultures cannot be called reliable since the tissue-specific properties characteristic of these cells in a 3D environment are lost. The complex 3D network of the tissue microenvironment influences not only the penetration and distribution of CNTs but also the function of many physiological factors <sup>[Z3]</sup>. In addition, conventional animal testing often fails to predict the actual efficacy of a therapeutic agent in humans because the cells, microenvironment, and physiology of animals differ from those of humans. This species gap can be bridged by culturing human cells in 3D. It is also necessary to understand that tissues have their specific properties, such as the size of the intercellular space <sup>[Z4]</sup>, tissue stiffness <sup>[Z5]</sup>, cell density <sup>[Z6]</sup>, phagocytic function <sup>[Z2]</sup>, and cell morphology <sup>[68]</sup>. As in solid tumors, cells in spheroids form layers; the outer layer consists of proliferating cells, followed by a layer of senescent cells. In the very center is the necrotic core. This gradient in cell survival and proliferation depends on the availability of nutrients and oxygen <sup>[78]</sup>.



Figure 2. Schematic representation of the main characteristics of 3D spheroids that are crucial.

Intercellular contacts inside spheroids are much more complicated than in 2D cultures. Cells deposit ECM components such as collagen, laminin, fibronectin, proteoglycans, tenascin, etc. There are also a large number of intercellular compounds; for example,  $\alpha$ 5- and  $\beta$ 1-integrin, E-cadherins are a barrier to cytostatic molecules <sup>[79]</sup>.

The spheroids can be formed from one or more cell types, such as breast cancer cells and fibroblasts, endothelial cells, and immune cells. In this way, cellular heterogeneity, which is present in normal and oncological tissues, can be achieved [80][81].

Optimizing spheroids for nanoparticle testing, in particular CNTs, is one of the important aspects of working with 3D cultures [82]. The cells used in the experiments should be in culture from 1 to 20 passages. The cells should be kept in a humidified incubator at 37 °C and 5% CO2. Standard culture medium should be used for cultivation. Cells should have a 70-80% fill rate. Cultures should be transplanted with trypsin/EDTA solution (0.05% (wt/vol) trypsin and 0.02% (wt/vol) EDTA). There are several methods for creating cell spheroids, the hanging drop method [83], ultra non-adhesive well plates [83], magnetic nanoparticles [84], incubation in hydrogels [85], and the use of bioreactors. For CNTs testing, the first three methods are the most optimal since they are the easiest to use in all laboratories and have the smallest variation in the size of the spheroids. To create a spheroid 400 µm in diameter on the fourth day of formation, the desired concentration and cell proliferation rate must be determined. For this purpose, spheroids are formed from different numbers of cells (from 250 to 3000 cells/spheroid in the case of ultra non-adhesive plates, magnetic nanoparticles, and suspended droplet method). When creating spheroids, it is recommended to use a multichannel pipette, which will reduce the standard deviation among the spheroid diameters to 5% in one plate and 10% in different experiments. Cells form a spheroid within 96 h in a CO<sub>2</sub> incubator at 37 °C. A phase-contrast microscope with 5x and 10x lenses is used to determine the size of the spheroid. The microscope is used to assess the integrity, diameter, volume and roundness of the spheroid. Once the optimal number of cells has been determined, it is possible to proceed with CNTs testing. This requires titration of CNTs and making 2x solutions of the substances tested. After that, 50 µL of the medium must be removed from the plate where the spheroids are formed, and 50 µL of the test solution must be added, thus making a concentration of CNTs of the desired concentration. The spheroids can then be incubated with the CNTs for the desired time.

### 4. Mechanism of CNTs Uptake by Cells and Spheroids

The ECM that surrounds the cells serves as a good barrier to the penetration of therapeutic agents and NPs, including CNTs <sup>[86]</sup>. There are two types of transport of molecules and NPs into the spheroid: transcellular and diffusion through the extracellular matrix <sup>[87]</sup>. In the first case, the cells must absorb the carriers and pass them on to each other until the carrier reaches the cells of the necrotic nucleus. In the second case, CNTs must pass into the extracellular space, which usually has a size of 25–500 nm (**Figure 3**) <sup>[88]</sup>. In both cases, it depends on two parameters: the type of tissue and the properties

of CNTs. It is worth paying attention not only to the properties of CNTs but also to the properties of other models NPs that have already been studied for penetration into tumor spheroids. The main properties of nano- and micro-sized objects that can be absorbed by cells are their size, shape, charge, surface chemistry, and rigidity <sup>[89]</sup>.

Carbon

Figure 3. Overview of the primary mechanisms of uptake CNTs into cellular spheroid and cell.

There is now agreement in the literature that smaller particles penetrate spheroids faster. This has been tested with particles and spheroids of various types. CNTs are highly anisotropic objects with diameters ranging from ~0.4 to ~100 nm and lengths from ~0.14 nm to ~55 cm, so it is difficult to compare them with existing models. However, it is known that when 50 and 100 nm gold NPs penetrate for 24 h, 50 nm NPs penetrate deeper <sup>[90]</sup>. Similar results depending on the size could be obtained when the spheroids were immobilized in the "tumor-on-chip" system. This system made it possible to analyze the penetration of NPs in combination with real-time observation of the accumulation of NPs. Small spherical PEG-coated NPs (40 and 70 nm) rapidly accumulated in MDA-MB-435 spheroids and accumulated in the interstitial space, while larger NPs (110 and 150 nm) were more and more rejected from accumulation in the tumor <sup>[91]</sup>. Although the rule is clear that smaller particles have better penetration, no clear upper limit has been reported so far that would lead to the complete exclusion of particles from spheroid models, although there are indications that penetration becomes low after sizes larger than 1000 nm.

The next important feature is the shape of the particles. As mentioned earlier, CNTs are highly anisotropic particles. However, it has been previously repeatedly demonstrated that elongated small particles enter 2D cell culture much better than spheres. The results of the study show that the rate of internalization increases as the aspect ratio increases. If an equal number of particles are added per cell, then the total volume of internalized particles increases with the volume of individual particles <sup>[92]</sup>. However, as said above, a 2D system is very different from a 3D. Jiacheng Zhao et al. in their work describe particles from poly(1-O-methacryloyl- $\beta$ -d-fructopyranose)-b-poly(methyl methacrylate) having the shape of spheres (diameter 30 nm), rods (diameter 30, length 120), and carriers (hollow sphere 160 nm in diameter). The study showed that there is no difference between the passage of spheres and rods into the spheroids, and both types of particles enter the spheroid at the same speed, unlike carriers <sup>[93]</sup>.

CNTs can be internalized both by the outer layers of cells and by cells that are closer to the center of the spheroid. When NPs are ingested by cells, including CNTs, there are several types of internalization: active (energy-dependent), passive (energy-independent), and diffusion. The active pathway of CNTs' internalization through the cell membrane occurs by endocytosis. In the case of endocytosis, CNTs enter cells inside vesicles (endosomes), and then they are gradually transported to the perinucleolar space, becoming lysosomes <sup>[94]</sup>. Studies related to the selective inhibition of endocytosis pathways showed that CNTs internalization includes several pathways, such as macropinocytosis, caveolae-mediated endocytosis, and clathrin-dependent endocytosis <sup>[95]</sup>. The results show that macropinocytosis is the main mechanism of internalization of SWCNTs, while clathrin-mediated endocytosis is length-dependent and relatively important for the

shortest CNTs. Phagocytosis allows the uptake of CNTs longer than 1  $\mu$ m and conglomerates, as well as microsized composite particles with CNTs embedded in their structure <sup>[96][97]</sup>. When cells were incubated with CNTs at 4 °C, the internalization of particles was strongly reduced because low-temperature blocks all types of endocytosis. It is also known that the contact of CNTs with the cell membrane occurs from the tip <sup>[98]</sup>. For nanotubes with end caps or a carbon sheath at the ends, the uptake process involves tip recognition via receptor binding, rotation induced by asymmetric elastic deformation at the tube-bilayer interface, and finally, penetration into the cell in a nearly vertical direction. For nanotubes without caps and sheaths on their ends, the needle entry mode is not realized.

Passive diffusion of CNTs is not dependent on temperature or endocytosis, as the particles simply penetrate through the lipid bilayer <sup>[99]</sup>. It is already known that CNTs functionalized with amino acids can easily penetrate the cell without entering the lysosome. Removal of CNTs involves processes of exocytosis and enzymatic degradation. It has been reported that CNTs are displaced from cells by exocytosis several hours or days after internalization <sup>[99]</sup>.

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