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Review

Functionalized CNTs for delivery of therapeutics

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Abstract

Carbon nanotubes (CNTs) are high aspect ratio allotropes of carbon. Because of their unique physical and chemical characteristics, they are nominated for a vast variety of applications in the biomedical field. Their very low solubility is the only problem of them which is solved by different methods of Functionalization. After discoveries in the last few years of the capacity of CNTs to penetrate into the cells, and because of the low toxicity they display and are not immunogenic, CNT are known to have impressive potentials to be used as drug and medicine carriers and therefore they hold great potential in the field of nanobiotechnology and nanomedicine. CNTs can be functionalized with different therapeutic molecules and internalized by a variety of cell types to deliver therapeutic and diagnostic small molecules and macromolecules to cells. In this review, we will describe the potential of functionalized carbon nanotubes to deliver different types of therapeutic molecules.

Keywords: *Drug delivery, Carbon nanotube, Nanotechnology biomedical applications*

1. Introduction

A careful examination of the carbon cathode used in the arc-discharge process for the production of fullerenes by Iijima [1], resulted in the historical discovery of carbon nanotubes (CNTs), ultra-thin carbon fibers with nanometer size diameter and micrometer size length. They consist exclusively of carbon atoms arranged in a series of condensed benzene rings rolled-up into a tubular structure.

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This novel nanomaterial belongs to the family of fullerenes, the third allotropic form of carbon along with graphite and diamond. CNTs are divided into two types: (1) single-walled CNTs (SWCNTs) and (2) multi-walled CNTs Figure 1[2].

CNT have nanometric dimensions: SWNT have diameters from 0.4 to 2.0 nm and lengths in the range of 20–1000 nm, while MWNT are bigger objects with diameters in the range of 1.4–100 nm and lengths from 1 to several μm. The distance between each layer of a MWNT is about 0.34 nm. The high electron density created by their aromatic structure makes them easily observable by transmission electron microscopy.

SWNTs can be seen mainly in bundles because of the strong Van der Waals interactions, whereas MWNTs are mainly monodispersed Figure 2 [3].

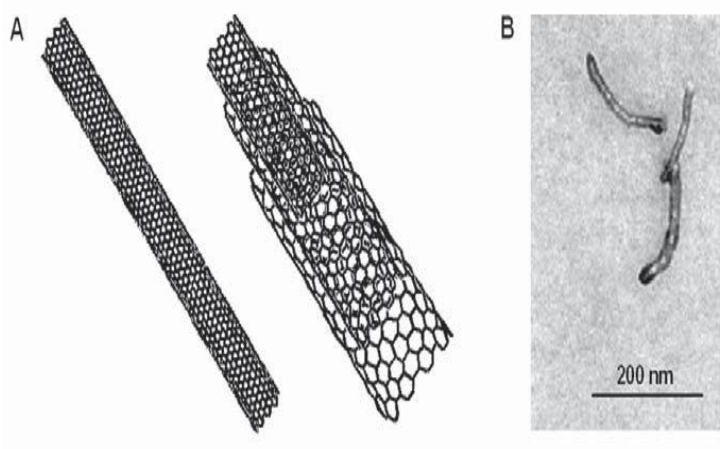

Fig.1. (A) Schematic view of a SWNT (left) and a MWNT (right), **(B)** Transmission electron micrograph of MWNT-NH_3^+.

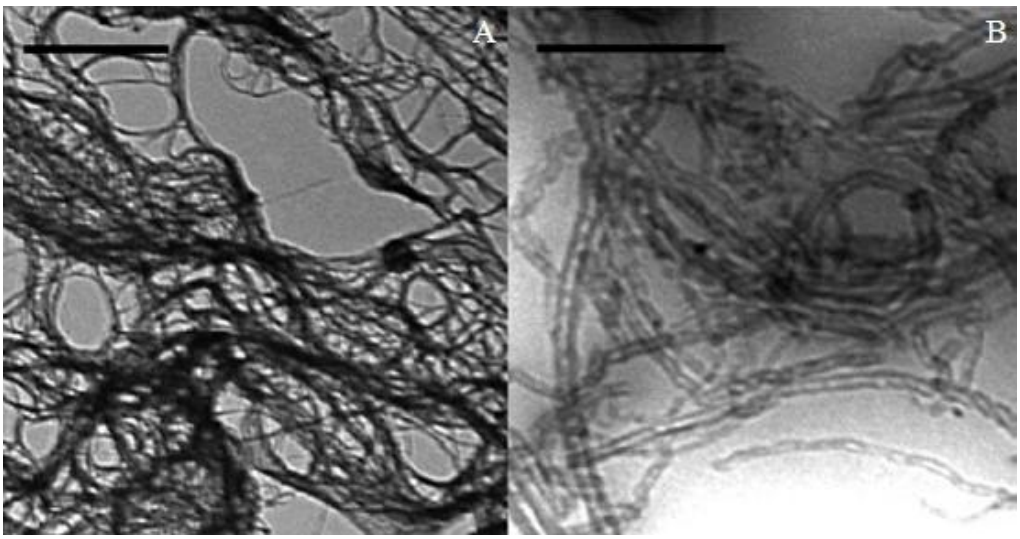

Fig.2. TEM images of single **(A)** and multi-walled **(B)** carbon nanotubes, While SWNT are present as bundles of different diameter and length, due to their strong aggregation tendency, MWNT can be instead observed as isolated entities. The scale bars correspond to 1 Am and 250 nm, respectively

CNTs walls are not reactive, but their fullerene-like tips are known to be more reactive, so end functionalization of CNTs is used relatively often to generate functional groups (e.g., –COOH, –OH, or –C O). Like in fullerenes reactivity is activated by curvature effects. Curvature in nanotubes is much smaller than in conventional fullerenes: firstly since the tube diameters are generally larger and secondly since they are curved in one direction only [4].Generally Nanomaterials enjoy exceptional properties [5] which make them appropriate for many novel applications. Herein, CNTs have very interesting physicochemical properties such as: ordered structure with high aspect ratio, Ultra light weight, high mechanical strength, high electrical conductivity, high thermal conductivity, metallic or semi-metallic behaviour and high surface area. The combinations of these characteristics make CNT a unique material with the potential for diverse applications. A wide range of applications has been envisaged for CNTs ranging from sensors for the detection of genetic or other molecular abnormalities, to substrates cellular growth for tissue regeneration and the use of CNT as substrates for neuronal growth [6,7], supports for adhesion of liposaccharides to mimic cell membrane [8], ion channel blockers [9] and delivery systems [10,11].

2. Dispersion and solubilization

CNT are materials practically insoluble, or hardly dispersed, in any kind of solvent. To integrate the nanotube technology with the biological milieu, the solubility of the tubes especially in aqueous solutions must be improved. The combination of nanotubes with proteins and other natural products including nucleic acids [12, 13] and polysaccharides have paved the way for the compatibility of such materials with biological systems. Van der Waals interactions between individual tubes often lead to significant aggregation or agglomeration. The possibility to form complexes between CNT and different types of polymers or to modify the CNT sidewalls by organic functionalization drastically increased the characteristics of solubility of CNT.

Different techniques have been used for dispersion of CNT for their use in composites and biological fields. They include solution mixing, sonication, coagulation, melt compounding, in situ mini emulsion polymerization, oxidation or chemical functionalization of the tube surface and the use of surfactants.

Functionalization of carbon nanotubes is a good way for making them soluble. Different ways of dispersion and solubilization by functionalization can be basically divided in two main approaches Figure 3 [13].

One procedure consists of CNT covalent Functionalization [14, 15]. The second methodology is based on the noncovalent functionalization of CNT with surfactants, nucleic acids, peptides, polymers and oligomers [16, 17].

In the first method, CNT are cut and oxidized to generate a certain number of carboxylic groups subsequently derivatized with different types of molecules. Derivatization reactions can be roughly divided into two categories. In the first case, the required functional groups are attached directly onto the nanotube using 1, 3-dipolar cycloaddition [18], the Birch reduction [19] and reactions with nitrenes, radicals and carbenes [20]. When taking the second approach we first build bridgeheads by oxidizing some atoms in the tube wall and then proceed using substitution reactions to change the simple (–F, –OH, –COOH) groups formed.

The introduction of moieties on the tube external surface creates repulsion between the single tubes allowing them to easily disperse into the solvent. Many approaches have been tried so far to carry out chemical functionalization of carbon nanotubes.

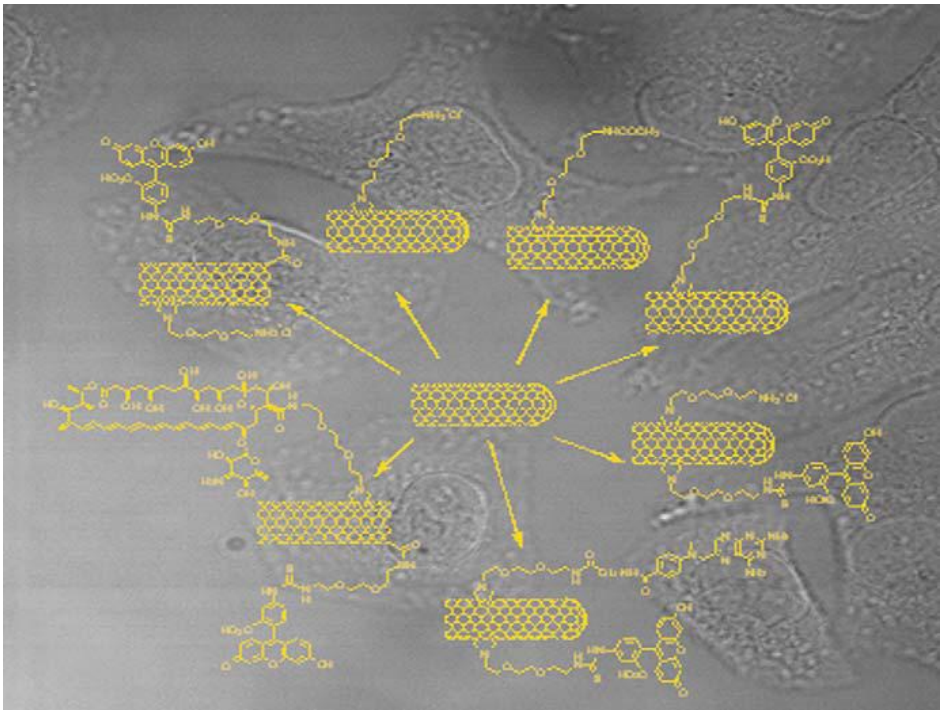

Fig.3. Several types of functionalized carbon nanotubes that can be adequately and individually dispersed in biological environments, Background image: differential interference contrast image epithelial lung carcinoma (A549) cell culture

For example, the generation of surface hydrophilic substituents such as carboxylic, hydroxyl or sulphonic acid groups by suitable chemical method is rather easy for their wide use in medical and biological applications [21] since these functional groups provide necessary sites for covalent or noncovalent coupling of SWNTs. A majority of recent reports deal with a variety of chemical treatments such as fluorination [22], alkylation [23], diazotization [24], use of organic radicals [25], or nitrenes [20] for sidewall functionalization although many methods still need greater efforts in order to improve the yield and selectivity of the products. Alternative ways to functionalize carbon nanotubes are by substitution reactions such as replacement of carbon atoms from the tube wall by boron or nitrogen [26].

In principle functionalization should also be possible from the inside of the tubes [27]. The inside of carbon cages is certainly interesting from a chemical point of view as the distribution of the carbon orbitals is quite different in comparison to their distribution on the outside. Figure 4 gives a demonstration for a (7,7) tube.

The two main sources of reactivity in SWCNTs are (i) the curvature-induced strain arising from the non-planar geometry of sp^2 carbons and (ii) the misalignment of the orbitals [28]. The most reactive places in any nanotube sample are found in the cap of the thinnest tube and least reactive are the bonds running perpendicular to the axis of the largest diameter SWCNT. However, disruption of the electronic conjugation in CNTs associated with formation of new covalent bonds in the grapheme sheet during covalent functionalization would, result in inevitable deterioration of the unique properties of CNTs.

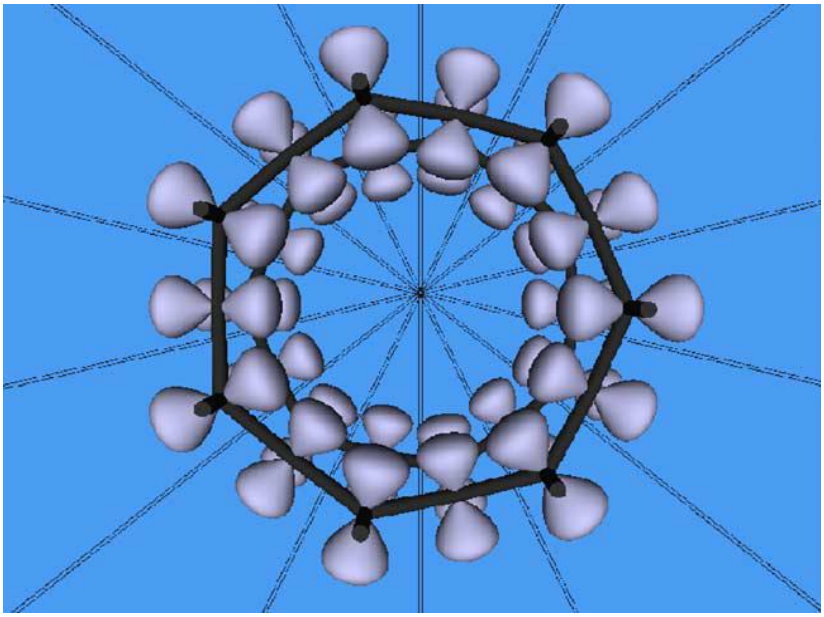

Fig.4. Schematic view of electronic orbitals inside a (7, 7) single wall carbon nanotube

On the contrary, noncovalent functionalization is particularly desirable, as it leaves the electronic structure and mechanical properties intact [29]. Hydrophobic or π–π interactions are often evoked as likely responsible for noncovalent stabilization. Nowadays, three classes of molecules are mainly used for CNT dispersion. Surfactants are used because they are easily available and low-cost. Polymers and biopolymers (nucleic acids and peptides) are also very efficient in the dispersion process. In biology, the highly selective binding between complementary sequences of deoxyribonucleic acid (DNA) plays the central role in genetic replication. This selectivity can, in principle, be used to assemble a wide range of materials, by forming adducts between DNA and the materials of interest [30].

The noncovalent absorption of low molecular weight surfactant or polymer, such as sodium dodecyl sulphate (SDS) [31, 32] and poly (ethylene oxide) (PEO) [33] on CNTs and subsequent solubilization was often adopted and silicone polymer surfactants (like siloxane polyether copolymer (PSPEO), a new amphiphilic macromolecule) are being used because of their better properties such as super-wettability and extremely low surface tension [34].

3. Drug delivery

The development of new and efficient drug delivery systems is fundamental to improve the pharmacological profiles of many classes of therapeutic molecules. A wide variety of delivery systems are currently available [35]. In the last 30 years numerous nanoscale and microscale systems have been developed in order to find efficient carrier systems for drugs, antigens and genes that will facilitate their transport into specific tissues, cell populations and intracellular compartments by minimizing deleterious side effects. The development of drug delivery systems to date has indicated that each one of the parameters described in Figure 5 can have a determinant role in the in vivo fate of any material administered, irrespective of the level of complexity or sophistication of the design features on them [36]. The same will hold for all types of novel,

'smart' nanomedicines that are currently under early stage and will soon be in preclinical development.

Recently, studies reported that CNT hold potential to becoming a viable component of delivery systems [36]. Because functionalized CNT display low toxicity and are not immunogenic, such systems hold great potential in the field of nanobiotechnology and nanomedicine. A recent study reported by Singh *et al*. revealed that water-soluble functionalized carbon nanotubes (f-CNTs) can be rapidly cleared from a systemic blood circulation through the renal excretion route [37]. The application of functionalized CNT as new nanovectors for drug delivery was apparent immediately after the first capacity demonstration of this material to penetrate into cells 3.CNT can be used to deliver their cargos to cells and organs Figure 6. Bianco *et al* demonstrated that fluorescently labelled CNT were up taken by various cell types [3]. However, the mechanism of penetration is not yet completely elucidated.

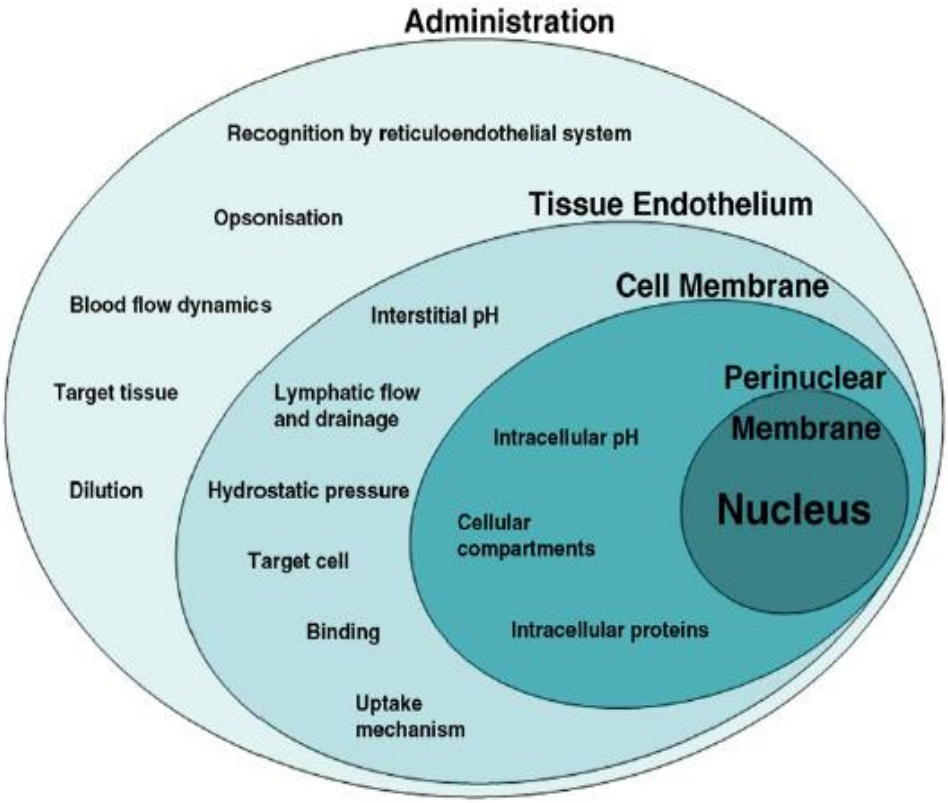

Fig.5. Some specific biomedical applications of CNTs being explored by various groups as novel delivery systems

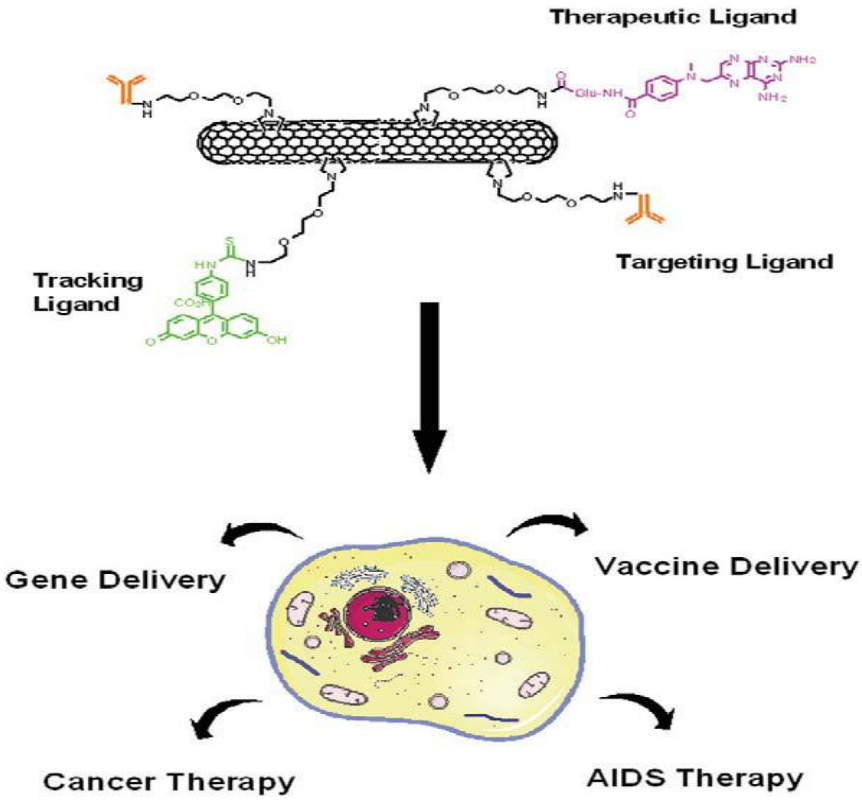

Fig.6. In vivo barriers and critical parameters affecting the fate of nanomedicines

3.1. Cell-penetrating CNTs for delivery of therapeutics

Identification of the critical factors determining CNT cell internalization will help determine the advantages they offer compared with spherical nanoparticles or any potential hazards they may entail Table.1 [2].

Two routes of internalization have been proposed. It has been found that functionalized CNT penetrate following a passive diffusion across the lipid bilayer similar to a ‘‘nanoneedle’’ able to perforate the cell membrane without causing cell death [38, 39]. Alternatively, when CNT were used to deliver proteins by adsorbing them onto their external surface, they seem to be up taken by endocytosis [40, 41]. It is highly probable that the type of molecules covalently or noncovalently attached to the external walls of the tubes play a critical role in the process of transport into the cells. Experimentally, CNTs are able to interact with plasma membranes and cross into the cytoplasm without the apparent need of engulfment into a cellular compartment to facilitate intracellular transport. In these initial studies, functionalized CNTs are able to facilitate transport of plasmid DNA (pDNA) intracellularly [39]. Interestingly, model nanotube structures have also been proposed to interact with lipid bilayers via a diffusion process directly through the bio membrane [42, 43]. Spontaneous transmembrane penetration via the flipping of membrane lipid molecules is, contrary to endocytosis, an energy-independent process, not dependent on receptor, coat, or lipid raft interactions, and is, therefore, potentially relevant to all cell types.

Table 1. Pros and cons of using CNTs for biomedical applications

Pros	Cons
Unique mechanical properties offer *in vivo* stability	Non biodegradable
Extremely large aspect ratio, offers template for development of multimodal devices	Large available surface area for protein opsonisation
Capacity to readily cross biological barriers; novel delivery systems	As-produced material insoluble in most solvents; need to surface treat preferably by covalent functionalization chemistries to confer aqueous solubility (i.e. biocompatibility)
Unique electrical and semiconducting properties; constitute advanced components for *in vivo* devices	Bundling; large structures with less than optimum biological behaviour
Hollow, fibrous, light structure with different flow dynamics properties; advantageous *in vivo* transport kinetics	Healthy tissue tolerance and accumulation; unknown parameters that require toxicological profiling of material
Mass production – low cost; attractive for drug development	Great variety of CNT types; makes standardization and toxicological evaluation cumbersome

The effect of functional group type at the surface of the functionalized CNT has also been investigated using techniques such as confocal microscopy, fluorescence-activated cell sorting (FACS), and protocols that inhibit energy-dependent internalization mechanisms (incubation at 4°C and addition of sodium azide or 2,4-dinitrophenol to the cell culture media). Interestingly, initial observations of the ability of CNTs to pierce or penetrate the plasma membrane, to a large extent by a process independent of energy, have been confirmed, regardless of cell type or characteristics (e.g. surface charge) of the functional group attached onto the CNT. In addition, very recently, the hypothesis of CNTs acting as 'nanoneedles' with regard to the plasma membrane has been experimentally reproduced for two different types of CNT: (i) block copolymer-coated noncovalently functionalized MWNT binding studies using microglia cells [44]; and (ii) oxidized, water-soluble CNTs interacting with *E. coli* under the application of microwaves [45]. The gradually accumulating work is confirming the novel mechanisms beside the classic ones.

3.2. Vaccine delivery by carbon nanotubes

The cellular uptake of free peptides and oligodeoxynucleotides is extremely poor, therefore conjugation of these molecules onto CNT surfaces may allow improvements in the delivery of such biological molecules [46].The basic concept for utilizing carbon nanotubes in vaccine delivery is to link the antigen to carbon nanotubes while retaining its conformation and thereby inducing antibody response with the right specificity. In addition, carbon nanotubes should not trigger a response by the immune system, i.e., they should not possess intrinsic immunogenicity. In particular, amino - derivatized nano tubes were covalently linked to a peptide sequence derived from the foot-and-mouth disease virus (FMDV), generating mono conjugated peptide-CNT [47]. In these initial studies, the peptide linked to the CNT displays the necessary and correct secondary conformation and shows immunological reactivity to specific polyclonal and monoclonal antibodies. In order to evaluate the antigenicity and immunogenicity properties,

as well as the influence of the number of peptides covalently linked to CNTs, mono- and bis-peptide derivatized CNTs have been prepared [21]. Specific antipeptide antibody recognition has been obtained by enzyme-linked immunosorbent assays (ELISA) and surface plasmon resonance for both conjugates. In addition, immunization of mice with these conjugates elicits higher antibody responses compared with the peptide alone and no anti-CNT antibodies have been detected, suggesting that CNTs do not have intrinsic immunogenicity properties. However, only the mono derivatized CNT conjugates induce high levels of virus neutralizing antibodies. Increasing the number of peptide units around the CNT surface enhances the immunogenicity, but does not improve the neutralizing capacity. This finding can be attributed to a reduced specificity of the antibodies generated using the bis-conjugate, likely to be the result of a conformation adopted *in vivo* by the peptide on the CNT different from the native protein. This result underlines the critical role that the carrier system may play in the presentation of the linked peptide to the immune system**.**

3.3. Delivery of genes by carbon nanotubes

One of the most promising concepts to correct genetic defects or exogenously alter the cellular genetic makeup is gene therapy. The most commonly used DNA carriers are based on viral vectors (retrovirus, lentivirus or adenovirus), liposomes, cationic lipids, polymers and nanoparticles [48, 49]. These carriers have some problems like concerns about safety of viral vectors or low gene expression efficiency of non-viral vectors. Generally, the development of a new vector for therapeutic gene transfer requires protection of DNA from degradation, good membrane penetration and low immunogenicity. In this context, CNT seem to be very promising because they do not inherently trigger an immune response [21].

Bianco *et al.* reported that functionalized carbon nanotubes (f-CNTs) can be used for presentation and delivery of antigens and for gene delivery [10]. The group prepared soluble f-CNTs using 1, 3-dipolar cycloaddition of azomethine ylides [18]. The resulting amine groups attached to the sidewall of the CNTs were linked with peptide antigens B cell epitope from the foot-and mouth disease virus (FMDV)). This was done to study their immunogenic properties and was also used to condense the pCMV-bgal plasmid DNA. In the antigenicity and immunogenicity studies, the peptide-CNT was recognized by antibodies equally well as the free peptide and immunization of mice with the peptide-CNT clearly enhanced anti-FMDV peptide antibody responses. Moreover, no immune response to CNTs was detected, which is an important issue in view of epitopic suppression when peptide antigen carriers are used. Gene expression efficiency offered by DNA-CNT was about ten times higher than that of DNA alone.

Kostas Kostarelos *et al.* [2] demonstrated CNT-mediated gene delivery and expression leading to the production of marker proteins encoded in double stranded pDNA [39]. They observe that pDNA is able to associate in a condensed globular conformation through electrostatic interactions at the surface of CNTs covalently functionalized with NH^{3+} groups. The delivery of pDNA and expression of β-galactosidase (marker gene) in Chinese hamster ovary (CHO) cells is five to ten times higher than naked pDNA alone. The concept of gene delivery systems based on CNTs has also been reported by Liu *et al.* [50] using polyethylenimine (PEI)-functionalized CNTs. They report a noncovalent association of pDNA with PEI–CNTs and have tested CNT–PEI: pDNA complexes at different charge ratios in different cell lines. The levels of expression of luciferase (marker gene) are much higher for the complexes incorporating CNTs than pDNA alone and about three times higher than PEI alone. A very different gene delivery approach has been adopted by Cai *et al.* [38], who propose the use of gene delivery systems formed by CNTs containing Ni particles enclosed in their tips and pDNA immobilized on the surface. Using a

creative magnetic ‘spearing’ technique (exposure to an external magnetic field followed by centrifugation), the researchers have shown that the CNT–pDNA conjugates enter mammalian cells and achieve gene expression in 80–100% of the cell population. Recently, CNTs have also been conjugated with siRNA and some promising initial results have been reported in siRNA-mediated gene silencing Kam *et al.* [51] have linked siRNA through disulfide bonds to polyethylene glycosylated (PEG) lipids, which coat the CNT surface. The siRNA-CNT conjugates are internalized by mammalian cells and the siRNA is delivered intracellularly, leading to gene silencing.

3.4. CNTs for cancer therapy

With more than 10 million new cases every year, cancer is one of the most devastating diseases [52].Though the current treatments of cancer by surgery, radiation and chemotherapy are successful in several cases, these curative methods also kill healthy cells and cause toxicity to the patient. Two recent papers [53, 54] detail the potential use of SWCNTs to treat several types of cancers, with minimal or no toxic effects to normal cells. Liu *et al.* [55] prepared a solution of SWCNTs wrapped in poly (ethylene glycol) (PEG) with a tumor-targeting cyclic arginine–glycine–aspartic acid peptide to the end of the PEG chains. This solution was injected into mice bearing tumors and it was observed that the targeted SWCNTs accumulated in tumors. First, they attach a tumor cell-specific peptide to the carbon nanotube Figure 7 [56].

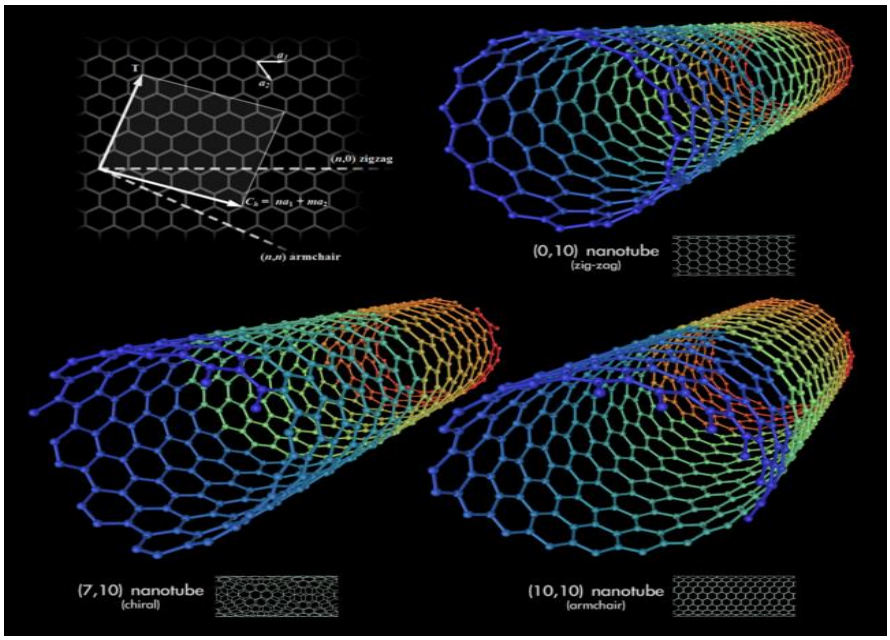

Fig.7. 3-D simulation of (top) tumor-binding peptide-poly(ethylene glycol)-functionalized carbon nanotube loaded with (right) cancer drug doxorubicin

This peptide then guides the nanotube to a tumor cell. The cell then swallows the nanotube, loaded with doxorubicin, and dies. Although the structure of the nanotube itself is hydrophobic, hydrophilic groups decorating the macromolecule create an overall amphiphilic nature which allows for passage through a cell membrane. However, the detailed molecular mechanisms remain unknown.

4. Conclusion

CNT are unique materials with exceptional chemical and electronic properties. There have been a plethora of applications proposed in the biomedical field alone. Organic functionalization has opened new horizons in the study of the biological properties of CNTs. Properly functionalized CNT seem to have a high propensity to cross cell membranes. In addition, CNT can be charged with biologically active moieties, which can then be delivered to the cell cytoplasm or nucleus.

No single mechanism can be solely or predominantly responsible for the cellular uptake of CNTs and that different or combinations of mechanisms may be contributing to their observed cellular internalization. The chemistry of CNT offers the possibility of introducing more than one function on the same tube, so that targeting molecules, contrast agents, drugs, or reporter molecules can be used at the same time. Carbon nanotubes today represent a class of emerging nanovectors that are capable to intracellularly deliver biologically functional peptides, proteins, nucleic acids and small mmolecules covalently or noncovalently attached on their surface. Future studies will determine the opportunities as well as the limitations that these novel nanovectors hold towards their clinical realization.

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