Chemically Functionalized Carbon Nanotubes: Emerging Vectors for Cell Therapy

Gianni Ciofani* and Vittoria Raffa

Scuola Superiore Sant'Anna, Piazza Martiri della Libertà, 33 – 56127 Pisa, Italy

Abstract: Carbon nanotubes are widely used for biomedical applications as intracellular transporters of biomolecules, due to their high propensity to cross cell membranes. In this review we summarize the recent advancement in the functionalization of carbon nanotubes, focusing particular attention on the chemistry involved in the preparation of nanovectors for drug delivery systems, and on the physical and chemical properties that affect cellular up-take and biodistribution phenomena.

Key Words: Carbon nanotubes, nanovectors, biomedical applications, functionalization, drug delivery, gene delivery, cellular up-take, biodistribution.

1. INTRODUCTION

Carbon nanotubes (CNTs) are molecular-scale tubes of graphitic carbon with outstanding properties. They are among the stiffest and strongest fibres known, and have remarkable electronic properties and many other unique characteristics [1]. For these reasons, they have attracted huge academic and industrial interest, with thousands of papers on nanotubes being published each year. The current interest in CNTs is a direct consequence of the discovery that carbon could form stable and ordered structures, different than graphite and diamond [2]. A new impetus was given to the this research, when it was shown that fullerenes can be easily produced in a simple arc-evaporation apparatus readily available in all laboratories. It was using such an evaporator that the Japanese scientist Sumio Iijima discovered fullerene-related carbon nanotubes in 1991 [3].

Applications of CNTs in the field of biotechnology have recently started to emerge, raising great hopes. Concerning the biological applications, their use is becoming relevant, for example, in neuroscience research [4] and tissue engineering [5]. They have been developed as scaffolds for neuronal and ligamentous tissue growth for regenerative interventions of the central nervous system (e.g., brain, spinal cord) and of orthopaedic sites. CNTs have also been used as new platforms to detect antibodies associated with human autoimmune diseases with high specificity [6]. These findings pave the way to the development of CNT-based diagnostic devices for the discrimination and identification of different proteins from serum samples, and in the fabrication of microarray devices for proteomic analyses [7]. In a similar context, CNTs covalently modified at their open ends with DNA have led to innovative systems for hybridization of complementary DNA strands, allowing for ultrasensitive DNA detection [8]. CNTs have also emerged as a new alternative and efficient tool for transporting and translocating therapeutic molecules. The development of new and efficient drug delivery systems is of paramount importance to improve the pharmacological profiles of many classes of therapeutic molecules [9]. CNTs can be functionalised with bioactive peptides, proteins, nucleic acids and drugs, and used to deliver their cargos to cells, tissues, and organs.

With the prospect of gene therapy, cancer treatments, and innovative therapies, the science of nanomedicine has become an ever-growing field that has an incredible ability to bypass biological barriers previously thought unavoidable [10].

The properties and characteristics of CNTs are still being heavily researched and scientists have barely begun to investigate the potential of these structures. However, in some cases, CNTs have already proven to serve as safer and more effective alternatives to traditional drug delivery methods [11]. They can serve as ideal vehicles, carrying therapeutic drugs, vaccines, and nucleic acids deep into the cell to previously unreachable targets, responding to static and dynamic energetic fields [12]. The discovery of CNTs has the potential of revolutionizing the biomedical research, as they can show superior performance over other nanoparticles. The advantage lies in a unique, unprecedented combination of electrical, magnetic, optical and chemical properties, which is greatly promising for the development of a new class of CNT-based drugs and therapies [13,14].

The arrows of Fig. (**1**) connect CNT properties with their current exploitation in biomedicine, and the next future hold the promise to discover and prove many new connections.

In this review a brief summary of the CNT properties and how they can serve to this purpose will be provided, followed by an overview of the current state of the art and of the influence of these properties on the interactions with cells and tissues. The discussion will be focused on CNTs as targeted nanotransducers, with particular attention to the exploited chemistry.

*Address correspondence to this author at the CRIM Lab - Scuola Superiore Sant'Anna, Viale Rinaldo Piaggio, 34, 56025 Pisa, Italy;
Tel: +39050883019; Fax: +39050883497; E-mail: g.ciofani@sssup.it

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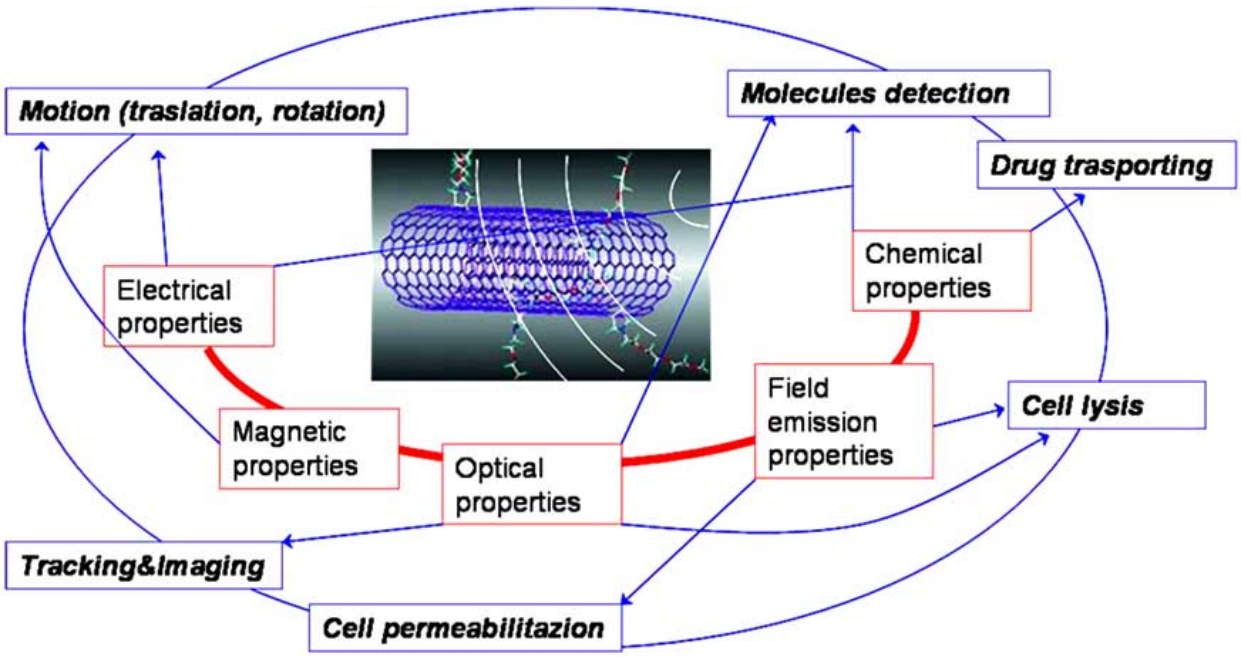

Fig. (1). CNT properties and their applications in the biomedical field.

2. FUNCTIONALIZED CNTs AS DRUG DELIVERY SYSTEMS

The search for new and effective drug delivery systems is a research field in rapid expansion. Many different methodologies have been developed according to the different classes of bioactive molecules to be delivered (e.g., peptides, proteins, nucleic acids and small organic molecules) and the characteristics of the target tissues. Liposomes, emulsions, polymers, micro- and nanoparticles are the most commonly studied vehicles [15]. A drug delivery system is generally designed to improve the pharmacological and therapeutic profile of a drug molecule. Problems associated with the administration of free drugs, such as limited solubility, poor biodistribution, lack of selectivity, unfavourable pharmacokinetics, healthy tissue damage, can be overcome and/or ameliorated by the use of a drug delivery system. Recently, new approaches are emerging, mainly due to significant advances in nanotechnology and nanofabrication [16].

The ability of f-CNT (functionalized CNTs) to penetrate into the cells offers the potential of using f-CNTs as vehicles for the delivery of small drug molecules. However, the use of f-CNTs for the delivery of anticancer, antibacterial or antiviral agents has not yet been fully ascertained. The development of delivery systems able to carry one or more therapeutic agents with recognition capacity, optical signals for imaging and/or specific targeting is of fundamental advantage, for example in the treatment of cancer and of different types of infectious diseases.

Pantarotto *et al.* [17] have shown that a peptide responsible for the G protein function, when covalently linked to single walled CNTs (SWCNTs), penetrates into the cell. For the conjugation of the peptide onto CNTs, they have used a selective chemical ligation approach: 1,3-dipolar cycloaddition of azomethine ylides can produce highly water soluble derivatives on CNTs, ready for further modifications [18]. The chosen peptide was the α subunit of the Gs protein, a peptide that was shown to block β-adrenergic activation of adenyl cyclase in permeable cells and to mimic the effect of Gs to increase agonist affinity for the β-adrenergic receptor. The peptide sequence corresponds to K(FITC)QRMHLRQ YELLC, which is characterized by the insertion of a cysteine at the C-terminus for the conjugation to the maleimido group of the wires, and by the N-terminal fluorescence moiety coupled to the side chain of a lysine.

CNT based systems can help to solve transport problems for pharmacologically relevant compounds that need to be internalised, and may have potential therapeutic applications including vaccine delivery [19]. In an important new development, the work by Bianco, Prato, and collaborators demonstrated the potential use of carbon nanotubes in vaccine delivery. The basic concept for using CNTs 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, CNTs should not trigger a response by the immune system, i.e., they should not possess intrinsic immunogenicity [20]. In this work, after the chemical functionalization introduced in the previous example, authors conjugated the peptide onto CNTs using chemoselective ligation. They have chosen a B-cell epitope from the foot-and-mouth disease virus (FMDV), corresponding to the 141-159 region of the viral envelope protein VP1. The free amino groups of SWCNTs were first derivatized using N-succinimidyl 3-maleimidopropionate; thereafter the N-terminal acetylated FMDV peptide, bearing a cysteine at position 1, was linked to the maleimido moiety of the wires, obtaining the peptide-SWCNT. In preliminary experiments, the FMDV peptide-CNT elicited strong antipeptide antibody responses after intraperitoneal immunization of mice. This emphasizes the effectiveness of CNT-supports to present peptides to the immune system [21].

The development of functionalised carbon nanotubes to target and to be up-taken by specific cell populations without collateral consequences for healthy tissues would be of paramount importance also in cancer treatment. The molecular targeting of CNT-based delivery systems derivatised with a therapeutic agent is possible if an active recognition moiety is simultaneously present at the surface of the nanocarrier. In addition, attachment of a fluorescent molecule would provide optical signals for imaging and localisation of the CNT–drug conjugates. Therefore, multiple functionalization of CNTs is of particular interest for multimodal delivery of anticancer agents.

Pastorin *et al.* [22] described a straightforward methodology for the introduction of two orthogonally protected amino groups on the sidewalls of CNT, subsequently derivatised with fluoresceinisothiocyanate (FITC) and methotrexate (MTX). MTX is a drug widely used against cancer, however, it suffers from low cellular up-take. Its conjugation to CNTs represents a promising approach to overcome its limited cellular up-take by enhancing its internalisation *via* the f-CNT. Again, the 1,3-dipolar cycloaddition of azomethine ylides was exploited. To prove the feasibility of double functionalization of carbon nanotubes, authors first removed monophthalimide (Pht) and labelled the multi-walled CNTs (MWCNTs) with fluorescein isothiocyanate. Then, MWCNTs were deprotected from tert-butyloxycarbonyl (Boc) group and carboxylic-activated MTX coupled to the free amino groups of the MWCNTs. The ability of the bifunctional MWCNTs to penetrate into the cells was subsequently evaluated by confocal microscopy on Human Jurkat T lymphocytes.

A similar approach was followed by Wu *et al.* [23], that also performed multiple functionalization of CNTs with different types of molecules. A fluorescent probe (fluorescein) for tracking the cellular up-take of the material, and an antibiotic moiety (amphotericin B) as the active molecule were covalently linked to the MWCNT.

Other researches have shown alternative mechanisms of interaction between CNTs and peptides. Various proteins, in fact, spontaneously adsorb on the sidewalls of acid-oxidized CNTs. This simple non-specific binding scheme can be used to afford non-covalent protein-nanotube conjugates [24]. The combined treatment of refluxing and sonication in nitric and/or sulfuric acid is known to produce short individual (50-500 nm) or small bundles of CNTs with carboxylic groups (e.g., -COOH) along the sidewalls and at the ends of the tubes. These functional groups impart hydrophilicity to the nanotubes and make them stable in aqueous solutions.

In the work proposed by Shi Kam and Dai [24], it was found that simple mixing of oxidized SWCNTs with protein solutions led to non-specific binding of proteins to the nanotubes as it can be deduced from AFM data, due either to electrostatic forces between functional groups on SWCNTs and positively charged domains on proteins and/or to hydrophobic interactions. Bovine serum albumin (BSA), protein A, and human IgG, all of them labelled with a fluorescent probe, and finally cytocrome *c* (cyt-*c,* an apoptosis inducer) were *in vitro* tested. For NIH-3T3 cells incubated with cyt-*c* alone and cyt-*c*-SWCNT conjugates, the degree of annexin V-FITC (an apoptosis marker) staining was analysed, and significantly higher percentages of apoptotic cells were obtained in cultures incubated with cyt-*c*-SWCNT conjugates than in those ones incubated with cyt-*c* alone.

Biomedical applications of CNTs is most promising also because the particular physical properties of these nanovectors. A study of Shi Kam *et al.*, [25], for example, shows that while biological systems are transparent to 700–1100 nm near-infrared (NIR) light, the strong absorbance of SWCNTs in this window (that originates from electronic transitions between the first or second van Hove singularities [26-28]) can be used for optical stimulation of nanotubes inside living cells, to afford various useful functions. Oligonucleotides transported inside cells by nanotubes, for example, can translocate into cell nucleus upon endosomal rupture triggered by NIR laser pulses. Continuous NIR radiation can cause cell death because of excessive local heating: selective cell destruction can be therefore achieved by functionalization of SWCNTs with a folate moiety, selective internalization of SWCNTs inside cells overexpressing folate receptor (FR), and NIR-triggered cell death, without harming receptor-free normal cells. Folic acid was conjugated to PL-PEG-NH_2 (2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino (PEG)2000]) with 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide as activator. The polymer was thereafter used for a non-covalent wrapping of CNTs.

A recent study made use of the unique properties of carbon nanotubes also for electroporation [29]. Rojas-Chapana *et al.* used the "lightening rod effect" of CNTs mixed within cell suspensions to create localized high field regions at the tips, creating pores in the membrane, allowing the up-take of extra-cellular substances. When placed in an electric field, the CNTs strongly enhance the electric field at their ends by a factor of 10-100, making them ideal for localized electroporation. It has been shown that the transport of gold nanoparticles across the cell wall following microwave irradiation is much more enhanced in the presence of CNTs, without affecting the cell viability. CNTs were dispersed in aqueous solution following oxidation with the aid of sulfuric/nitric acid treatment.

Concerning cancer therapy, it is worth to mention an example of *in vivo* application reported by Liu *et al.* [30]. In this study, the authors showed SWCNT drug delivery for tumor suppression in mice. They conjugated paclitaxel (PTX), a widely used cancer chemotherapy drug, to branched polyethylene glycol chains on SWCNTs, to obtain a water-soluble SWCNT-PTX conjugate. SWCNTs were dispersed *via* sonication in a solution of DSPE-PEG5000-4-arm-(PEG-amine). PTX was thereafter modified by succinic anhydride adding a carboxyl acid group on the molecule and SWCNTs with branched PEG-NH_2 non-covalent functionalization were reacted with the modified PTX in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS). SWCNT-PTX affords higher efficacy in suppressing tumor growth than clinical Taxol in a murine 4T1 breast cancer model, owing to prolonged blood circulation and 10-fold higher tumor PTX up-take. Drug molecules carried into the reticuloendothelial system are released from SWCNTs and ex-

creted *via* biliary pathway without causing toxic effects to normal organs.

Ou and colleagues [31] also proposed a chemical functionalized vector based on CNTs for cancer therapy. In this study, a novel functional SWCNT based on an integrin $\alpha_v\beta_3$ monoclonal antibody was developed and was used for cancer cell targeting *in vitro*. SWCNTs were non-covalently wrapped by phospholipid-bearing polyethylene glycol (PL–PEG). Following activation by EDC/NHS to afford SWCNT–PEG–NHS, SWCNT–PEG–protein A was prepared by incubating protein A with SWCNT–PEG–NHS. The mixture of the solution of integrin $\alpha_v\beta_3$ mAb–FITC and the solution of SWCNT–PEG–protein A was further incubated to produce SWCNT–PEG–protein A–integrin $\alpha_v\beta_3$ monoclonal antibody–FITC (abbreviated as SWCNT–PEG–mAb). *In vitro* study revealed that SWCNT–PEG–mAb presented a high targeting efficiency on integrin $\alpha_v\beta_3$-positive U87MG cells with low cellular toxicity.

Heister *et al.* [32] presented a method for a triple functionalization of oxidised SWCNTs with the anti-cancer drug doxorubicin, a monoclonal antibody, and a fluorescent marker at non-competing binding sites. Oxidised SWCNTs were dispersed in a doxorubicin solution and allowed to non-covalently complex. After purification, they were reacted with fluorescein-labeled BSA with EDC/NHS to achieve carbamide binding between the carboxylic group of CNTs and the amine groups of BSA. A similar approach was exploited to bind the CEA (carcinoembryonic antigen) antibodies to the carboxylic groups of the BSA. The proposed methodology allowed for the targeted delivery of the anti-cancer drug to cancer cells and the visualisation of the cellular uptake of SWCNTs by confocal microscopy. The complex resulted efficiently taken up by cancer cells with subsequent intracellular release of doxorubicin.

A very recent work of Bhirde and collaborators also exploited multiple SWCNT functionalization [33]. Oxidised CNTs were covalently decorated *via* EDC with quantum dots, cysplatin and EGF as cancer targeting ligand, showing interesting results in terms of targeting and tumor treatment even *in vivo*.

Covalent functionalization of CNTs has the great disadvantage to produce an irreversible chemical modification of nanotubes, thus altering their mechanical, chemical and electric properties, that we believe to play a relevant role in the CNT applications. Among the non-covalent approaches, of which we have just considered some interesting examples, our group has recently proposed a non-covalent dispersion of multi-walled carbon nanotube based on a lipidic mixture, that can guarantee high concentration and high stability as well as high cytocompatibility [34]. Moreover, CNTs wrapped with a lipid membrane were functionalised with a dodecapeptide, that specifically recognizes activated platelets, without chemical modification of the nanotube itself. Two kinds of lipids mixtures were investigated. The first one, indicated as "lipidic cationic mixture" (CL), was composed by DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine), 1,5-hexadecyl N-lysyl-L-glutamate (Lys-Glu2C16), cholesterol and PEG-DSPE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[monomethoxy poly(ethylene glycol) (5,000)]). The second one, called "lipidic anionic mixture" (AL), was composed by DPPC, cholesterol, DHSG (1, 5-dihexadecyl-succcinic anhydrate) and PEG-DSPE. Nanotubes were then dispersed *via* ultrasonication in the mixture solutions. The mixture for the preparation of functionalized lipid-wrapped CNTs (L-CNT) was essentially the AL mixture with the addition of PEG modified with a maleimide group (ALM mixture). The maleimide group is needed to link the cysteine residue of the dodecapeptide (CHHLGGA KQAGDV), a synthetic peptide based on the C-terminal side of γ-chain of GPIIb/IIIa, kinds of blood coagulation system-related protein. Dodecapeptide, together with other blood factors, contribute to the platelet aggregation. This approach produces CNT dispersions owing an excellent stability, excellent cytocompatibility and high CNT concentration. Specific platelet aggregation assays showed the effectiveness of these functionalized CNTs to induce secondary aggregation, thus demonstrating the retention of biochemical properties of immobilized peptides. Moreover, good results have been achieved exploiting cationic L-CNTs as DNA carriers (Obata, Y.; Ciofani, C.; Raffa, V.; Cuschieri, A; Menciassi, A.; Dario, P.; Takeoka, S. Evaluation of lipid coated multiwalled carbon nanotubes for plasmid DNA delivery to neuronal cells. To be published).

We have showed that a lot of CNT functionalizations have been successfully achieved, but, notwithstanding, no applications for drug delivery has been approved or entered in the market yet, favouring an increasing skepticism toward any bio-application of CNTs [35]. At the moment, traditional nanoplatforms such as liposomes, or polymer based carriers are still preferred because of their assessed biosafety *in vivo* and *in vitro*. Notwithstanding, we believe that CNTs should be much moor deeply investigated for their potential impact in nanomedicine, in particular combining their impressive physical properties to the chemical approach. It is of paramount importance to conjugate the concept of CNT-mediated drug delivery to the exploitation of their ability to be manipulated with external magnetic, electric or optical means.

3. FUNCTIONALIZED CNTs AS CARRIERS FOR NUCLEIC ACIDS

The use of f-CNT as a new carrier system for nucleic acids is another area of research currently under investigation. The most commonly used DNA carriers are based on viral vectors (retrovirus, lentivirus or adenovirus), liposomes, cationic lipids, polymers and nanoparticles. Although viral vectors are very efficient in gene expression, their use is limited because of the concerns about their safety. Non-viral vectors offer various alternatives regarding the size and type of vectors, they are chemically controllable, and display a reduced immunogenicity. However, they often present low gene expression efficiency rates because of their poor capability in reaching and crossing the nuclear membrane. In this context, CNTs seem to be very promising because they do not inherently trigger an immune response [36].

Singh *et al.* [37] have explored the potential of f-CNTs as delivery vehicles of nucleic acids. To optimize f-CNTs as gene delivery vehicles, it is essential to characterize their interactions with DNA. The authors studied the interactions

of three types of f-CNTs, ammonium-functionalized single-walled and multi-walled carbon nanotubes (SWCNT-NH_3^+; MWCNT-NH_3^+), and lysine-functionalized single-walled carbon nanotubes (SWCNT-Lys-NH_3^+), with plasmid DNA. Nanotube-DNA complexes were analyzed by scanning electron microscopy, surface plasmon resonance, PicoGreen dye exclusion, and agarose gel shift assay. The results indicated that all three types of cationic CNTs are able to condense DNA. All three different f-CNT types in this study exhibited up-regulation of marker gene expression over naked DNA using a human cell line.

Dwyer *et al.* [38] proposed an alternative functionalization method for binding CNTs with DNA. Their study represents a step toward the DNA-guided assembly of carbon nanotubes, by demonstrating that the well-known chemical pathway already discovered for attaching amino-terminal compounds to carbon nanotubes is also compatible with DNA functionalization. The single-walled carbon nanotube material, as formed by a laser ablation method, is first oxidized in a mixture of sulphuric/nitric acid. The product of this purification is a solution of open-ended nanotubes with terminal carboxylic acid groups. Linking DNA strands *via* carbamide bound to the these nanotube requires afterwards amino-terminated DNA strands.

Non covalent binding DNA-CNTs was investigated by Liu *et al.* [39]. Polyethylenimine (PEI) is a type of polymer with a high density of amines, thus negative charged DNA may be immobilized securely onto the surface of MWCNTs that have been functionalized with PEI through strong electrostatic interactions. Hence, they have adopted a grafting-form approach to prepare polyethylenimine-grafted multi-walled carbon nanotubes (PEI-g-MWCNTs). PEI was grafted onto the surface of MWCNTs by performing a cationic polymerization of aziridine in the presence of amine-functionalized MWCNTs (NH_2–MWCNTs). NH_2–MWCNTs were obtained by introducing carboxylic acid groups onto the surface of MWCNTs by heating during reflux in nitric acid. The carboxylic acid groups were then transformed into acyl chloride groups by treatment with thionyl chloride followed by treatment with ethylenediamine. DNA was securely immobilized onto the surface of PEI-g-MWCNTs as demonstrated by the total inhibition of the migration of DNA in gel electrophoresis, and PEI-g-MWCNTs showed transfection efficiency similar to or even several times higher than that one of PEI alone, and several orders of magnitude higher than that one of naked DNA.

An interesting study of Gao *et al.* [40] demonstrated that not only covalent and non-covalent binding between DNA and CNTs can be exploited in order to deliver the nucleic acids. Their molecular dynamics simulations showed that a DNA molecule could be spontaneously inserted into a carbon nanotube in a aqueous environment. The Van der Waals and hydrophobic forces were found to be important for the insertion process, with the former playing a more dominant role in the DNA-CNT interaction. This study suggested that the encapsulated CNT-DNA molecular complex can be further exploited for applications such as DNA modulated molecular electronics, molecular sensors, electronic DNA sequencing, and nanotechnology of gene delivery systems. DNA could be therefore encapsulated inside carbon nanotubes in a water solute environment *via* an extremely rapid dynamic interaction process, provided that the tube size exceeds certain critical values.

To explore the possibilities of SWCNTs as transporters for gene delivery and transfection, for the first time Lu *et al.* examined the translocation of RNA, polymer poly(rU), into breast cancer cells (MCF7). The non-specific binding mechanism for SWCNTs and poly(rU) employed in this study is opposed to the covalent binding scheme and may offer different options for the release of the load carried by SWCNTs upon delivery [41]. The binding affinity between SWCNTs and poly(rU) hybrids was evident when, upon binding, the Raman G band was broadened and the radial breathing mode was suppressed for an isolated SWCNT. This non-specific binding scheme results in the exposure of the charged phosphate backbone of poly(rU), which aids in the solubilisation of SWCNTs. The confocal fluorescence images of MCF7 breast cancer cells incubated with the SWCNT-poly(rU) hybrids (labelled with propidium iodide) showed translocation into the cytoplasm without cell damage or cytotoxicity.

A frontier in the field of gene and protein therapy is RNA interference (RNAi) for gene silencing by short interfering RNA (siRNA) delivered to mammalian cells. Efficient intracellular transport and delivery of siRNA are critical to RNAi potency. As an application of cleavable functionalization of SWCNTs, Shi Kam *et al.* showed transport, release, and delivery of siRNA in mammalian cells by SWCNT carriers, and achieved highly efficient lamin A/C gene silencing compared to existing transfection agents [42]. This functionalization approach involves first making stable aqueous suspensions of short SWCNTs by non-covalent adsorption of phospholipid molecules (PL) with poly(ethylene glycol) chains and terminal amine or maleimide groups (PL-PEG-NH_2 or PL-PEG-maleimide). The PL-PEG binds strongly to SWCNTs *via* Van der Waals and hydrophobic interactions between the PL alkyl chains and the SWCNT sidewall, with the PEG chain extending into the aqueous phase to impart solubility in water. The amine or maleimide terminal on the PL-PEG immobilized on SWCNT can then be used to conjugate a wide range of biological molecules. For incorporation of a disulfide bond, the authors exploited a heterobifunctional cross-linker sulfosuccinimidyl 6-(3c-[2-pyridyldithio] propionamido)hexanoate (sulfo-LC-SPDP) for any thiol-containing biomolecule (X) to afford SWCNT-PL-PEG-SS-X. Transporting biological molecules inside mammalian cells *via* SCWNTs was explored and enzymatic cleavage of the disulfide bonds in lysosomal compartments for releasing and delivering molecules to the cytosol exploited. siRNA known to silence the gene encoding lamin A/C protein present inside the nuclear lamina of cells was used. Confocal imaging of HeLa cells incubated with CNT-siRNA revealed significant reduction in lamin A/C protein expression by CNT-siRNA relative to untreated control cells. CNT-siRNA exhibits a 2-fold advantage over transfection by lipofectamine, a widely used transfection agent. This could be attributed to the high surface area of SWCNTs for efficient siRNA cargo loading, high intracellular transporting ability of SWCNTs, and high degree of endosome/lysosome escape owing to the disulfide approach.

Recently, Cai *et al*. have designed an alternative physical method of gene transfer, appropriate only for *in vitro* and *ex vivo* applications, called nanotube "spearing", capable of inducing cell internalization of plasmid DNA [43]. Nanotubes grown from plasma-enhanced chemical vapour deposition contain nickel particle catalysts entrapped into their tips, allowing them to respond to a magnetic field. The tubes were functionalized with a DNA strain containing the sequence coding for the enhanced green fluorescent protein (pEGFP-c1). Oxidized CNTs were mixed to the plasmid solution in the presence of EDC for the conjugation between primary amine groups of DNA and carboxylic groups on CNTs. Dividing and non dividing cells like Bal17, B-lymphoma, *ex vivo* splenic B cells and primary neurons were incubated with magnetic pDNA/CNTs. A rotating magnetic field first drove the nanotubes to mechanically spear the cells. In a subsequent step, a static magnetic field pulled the tubes into the cells. The cells were efficiently transfected as confirmed by fluorescent microscopy measurements and it was demonstrated that both spearing steps are necessary for an efficient transduction.

Several biomedical applications of CNTs have been proposed in the last few years. Concerning their exploitation in the delivery of nucleic acids, they are versatile platforms because of their high surface area, facile surface functionalization, and their ability to cross the cell membranes [44]. Notwithstanding, the development of CNTs as delivery systems for nucleic acids is still in its nascent stages, and many more studies are needed to determine the advantages and limitations offered by these novel materials. Again, they will reach a valuable success only when, in authors' opinion, they will be used not just as simple carriers, but their physical properties will be fully exploited to achieve an actual targeted, controlled, and triggered release.

4. BIODISTRIBUTION OF FUNCTIONALIZED CNTs

The biodistribution and pharmacokinetics of nanoparticles rely to a large extent on their physicochemical characteristics such as size, shape, aggregation, chemical composition, surface functionalizations and solubility [45]. To our knowledge, only few studies have been reported so far concerning the biodistribution of CNTs, but none of them report toxic side effects or mortality. However, except some important recent works, the long-term fate of nanotubes intravenously injected into animals *in vivo* is currently almost unknown, a critical issue to potential clinical applications of these materials.

Yang *et al.* analysed the stealth character of PEGylated single-walled carbon nanotubes (PEG-SWCNTs) under blood circulation and biodistribution [46]. The ^{13}C enriched oxidised SWCNTs were covalently functionalized with diamine-terminated PEG oligomers and the resulting PEG-SWCNTs were utilized for *in vivo* experiments. The concentrations of PEG-SWCNTs in blood were measured by isotope mass spectroscopy, observing a low hepatic up-take and demonstrating the distinct stealth character of PEG-SWCNTs.

A study of Liu and collaborators exploited intrinsic Raman spectroscopic signatures of SWCNTs, in order to measure the blood circulation of intravenously injected SWCNTs and to detect SWCNTs in various organs and tissues of mice *ex vivo* over a period of three months [47]. Non-covalent functionalization of SWCNTs by branched PEG chains was developed, enabling a SWCNT blood circulation up to 1 day, relatively low up-take in the reticuloendothelial system (RES), and near-complete clearance from the main organs in almost 2 months. Raman spectroscopy detected SWCNTs in the intestine, feces, kidney, and bladder of mice, suggesting excretion and clearance of SWCNTs *via* the biliary and renal pathways. No toxic side effect of SWCNTs to mice was observed in necropsy, histology, and blood chemistry measurements.

Wang *et al.* [48] used ^{125}Iodine-labeled multiple hydroxylated SWCNT (^{125}I-SWCNT-OH), functionalised by oxidation, and radiotraced their distribution in mice after administration by intraperitoneal (*i.p.*) administration. Other routes of administration were compared to *i.p.* such as subcutaneous, oral (by stomach intubation) and intravenous. CNT biodistribution did not result significantly influenced by the administration route, and the ^{125}I-SWCNT-OH distributed quickly throughout the whole body. The preferred organs for accumulation were the stomach, kidneys and bone. Most importantly, from the safety point of view, 94% of the nanotubes were excreted into the urine and 6% in the feces.

Another study was performed by Singh *et al.*, [49], focusing on the intravenous route of administration and using functionalized SWCNTs and MWCNTs following the 1,3-dipolar cycloaddition reaction. The CNTs were functionalised with the chelating molecule diethylen-triamine-pentaacetic acid (DTPA) and radiolabeled with ^{111}Indium ([^{111}In] DTPA-CNT). The biodistribution profiles showed an affinity for kidneys, muscle, skin, bone and blood 30 min after administration. However, all types of nanotubes were found to be rapidly cleared from all tissues and a maximum blood circulation half-life of 3.5 h was determined. DTPA-CNTs, both SWCNTs and MWCNTs, were found to be excreted through the renal route into the bladder and urine following intravenous administration. Moreover, both types of DTPA-CNTs were observed intact in the excreted urine by transmission electron microscopy.

The study carried out by Cherukuri *et al.* uses individualized, pristine SWCNTs non-covalently suspended in a non toxic surfactant, Pluronic F-108 [50]. In this study, CNTs have been intravenously administered to rabbits and monitored through their characteristic near-infrared fluorescence. The nanotube concentration in the blood serum decreased exponentially with a half-life of about 1 h. No adverse effects from low-level nanotube exposure could be detected from behaviour or pathological examination. At 24 h after *i.v.* administration, significant concentrations of nanotubes were found only in the liver.

Liu *et al.* investigated the biodistribution of radiolabelled SWCNTs (with ^{64}Cu chelated by (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), DOTA, linked to the polymeric wrapping) in mice by *in vivo* positron emission tomography (PET) and *ex vivo* by Raman spectroscopy [51]. It was found that SWCNTs non-covalently functionalized with phospholipids bearing polyethylene-glycol (PL-

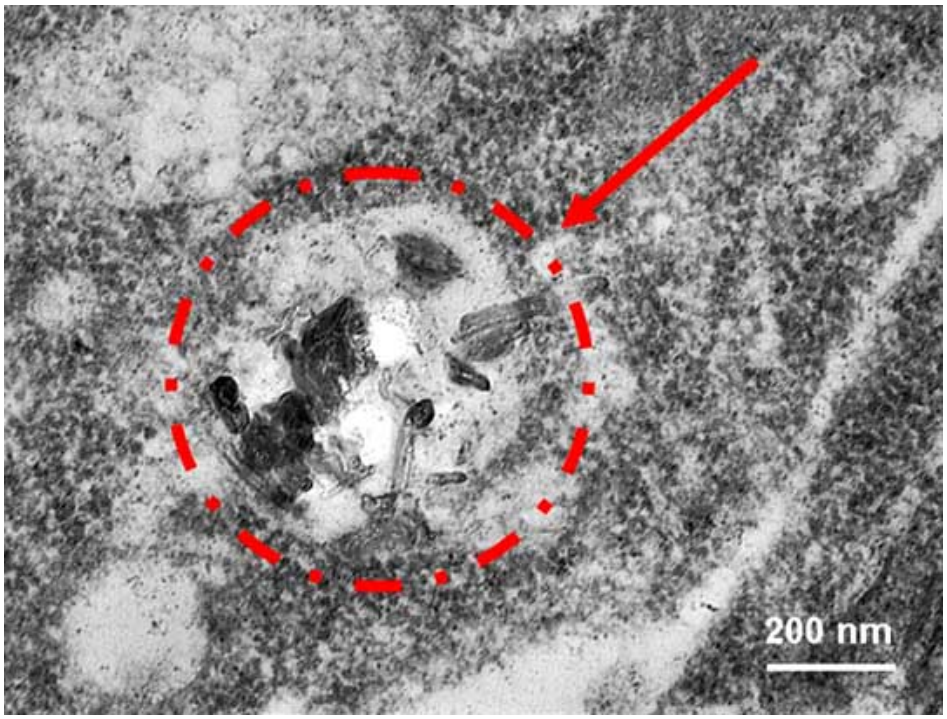

Fig. (2). Sub-1 μm CNTs internalized by immortalised mouse hippocampal HN9.10e cells. Adapted from [57] with permission by Elsevier.

PEG) are surprisingly stable *in vivo*. Effectively PEGylated SWCNTs exhibit relatively long blood circulation times and low up-take by the reticuloendothelial system (RES). Efficient targeting of integrin positive tumour in mice was achieved with SWCNTs coated with PEG chains linked to an arginine–glycine–aspartic acid (RGD) peptide. A high tumour accumulation is attributed to the multivalent effect of the SWCNTs.

Combined ^{3}He and proton magnetic resonance (MRI) were used by Al Faraj *et al.* in a rat model to evaluate the biodistribution and biological impact of raw SWCNTs (raw-SWCNTs) and super-purified SWCNTs (SP-SWCNTs) [52]. The susceptibility effects induced by metal impurity in the intrapulmonary instilled raw-SWCNT samples were large enough to induce a significant drop in magnetic field homogeneity detected in ^{3}He MR image acquired under spontaneous breathing conditions using a multiecho radial sequence. No MRI susceptibility variation was observed with SP-SWCNT exposition even though histological analysis confirmed their presence in instilled lungs. Proton MRI allowed detection of intravenously injected raw-SWCNTs in spleen and kidneys using gradient echo sequence sensitive to changes of relaxation time values. Again, no signal modifications were observed in the SP-SWCNT injected group, indicating that only the presence of SWCNTs with associated metal impurities can be detected *in vivo* by non-invasive MR techniques.

Once again, we see as the functionalization of CNTs play a crucial role in the behaviour of these vectors at systemic level. The half-life circulation of the CNTs is strictly dependent on their chemical treatment. A polymeric/lipid wrapping dramatically increases the circulation time of CNTs, potentially allowing more prolonged therapeutic effects. In any case, the choice of the approach to prepare CNT solutions should take in account the intended use of these carriers, i.e., if a short, medium, or long-time effect is desired, in order to optimize their pharmacokinetics properties, and not only their carrying and targeting abilities.

5. CELLULAR UP-TAKE OF FUNCTIONALIZED CNTs

The interaction between CNTs and tissue at cellular level has been deeply analyzed in the literature. An important feature of functionalized carbon nanotubes, as more times highlighted, is their high propensity to cross cell membranes. The mechanism of up-take of f-CNTs appears to be passive and endocytosis-independent. Incubation with cells in the presence of endocytosis inhibitors did not influence the cell penetration ability of f-CNT [53]. Furthermore, f-CNT showed similar behaviour when incubation was carried out at low temperatures.

Cellular up-take was confirmed by Shi Kam and colleagues [54], who in later studies used oxidised CNTs to covalently link fluorescein or biotin, allowing for a biotin–avidin complex formation with fluorescent streptavidin. In this case, protein–CNT conjugates were found in endosomes, suggesting an up-take pathway *via* endocytosis. To investigate the fate of protein-SWCNT conjugates *in vitro,* Shi-Kam *et al.* [25] used proteins fluorescently labeled by Alexafluor 488. The observed internalization of the non-covalently bound proteins *via* oxidized SWCNT transporters was similar to that of streptavidin transported by biotinylated SWCNTs, for which endocytosis was identified as the internalization pathway.

Also in another study [55] Shi Kam *et al.* confirmed the endocytosis pathway as f-CNT internalization mechanism.

Table 1. Summary of the Relevant Examples Approached in this Review. Where Available, CNT Average Sizes or Size Ranges are Given

CNT Type	Cargo Type	Functionalization Approach	Type of Testing	Reference
SWCNT (diameter 1 nm; length 300–1000 nm)	α subunit of the Gs protein	Maleimido group grafted on CNT functionalized by 1,3-dipolar cycloaddition of azomethine ylides linked to a Cys residue of the protein	*In vitro* internalization assay	[17]
SWCNT (final products as bundle of 8-53 nm)	B-cell epitope from the FMDV	Maleimido group grafted on CNT with the approach of [17] linked to a Cys residue of the protein	Antibody response	[21]
MWCNT	Methotrexate	Orthogonal bifunctionalization with 1,3-dipolar cycloaddition of azomethine ylides	*In vitro* internalization assay	[22]
MWCNT (length 180-940 nm)	Amphotericin B	Amphotericin grafted on CNT functionalized by 1,3-dipolar cycloaddition; FITC labeling exploiting carboxylic group of oxidised CNTs	Cell internalization, antifungal activity	[23]
SWCNT (diameter 1.5 nm; length 10-100nm; or small bundles, up to 5 nm in diameter)	BSA; protein A; IgG; cytocrome c	Cargos non-covalently and non-specifically bound on oxidized CNTs	*In vitro* internalization assay, apoptosis induction	[24]
SWCNT (diameter 1.2 nm; length 150 nm)	DNA; targeting with FA	Non-covalent wrapping with PL-PEG, conjugated with different cargos (FITC, FA)	Selective cancer cell destruction	[25]
MWCNT	N/A	Water dispersion following sulfuric/nitric acid treatment	Electroporation	[29]
SWCNT (length 20-300 nm)	Paclitaxel	Non-covalent wrapping with PL-PEG, conjugated with Paclitaxel	Biodistribution, *in vivo* tumor treatment	[30]
SWCNT (diameter 1–4 nm; length 500-1000 nm)	Integrin $\alpha_v\beta_3$ mAB	Non-covalent wrapping with PL-PEG, conjugated with the antibody	*In vitro* targeting	[31]
SWCNT (diameter 1 nm; length 200-1000 nm)	Doxorubicin, anti CEA mAb, and fluorescent BSA	Non-covalent absorption of doxorubicin; carbamide bound between oxidised CNTs and aminogroups of BSA; carbamide bound between carboxylic groups of BSA and amine groups of mAB	*In vitro* targeting, tracking and cancer cell treatment	[32]
MWCNT (bundles of 300-400 nm)	H12 dodecapeptide	Lipidic wrapping, conjugated to the H12 peptide with a tioether bound	Platelet aggregation	[34]
SWCNT (aggregates of 1-4 um) and MWCNT (> 4 um)	DNA	Ionic interactions between DNA and CNTs functionalized with NH_3 or Lys with the same technique of [17]	DNA transfection	[35]
SWCNT	DNA	Carbamide bound between oxidised CNTs and amino-terminated DNA strands	DNA binding assay	[38]
MWCNT	DNA	Ionic interactions between DNA and PEI-g-CNTs obtained by polymerization of aziridine in the presence of amine-functionalized CNTs	DNA transfection	[39]
SWCNT (diameter 1.36; length 2.95 nm)	DNA	Spontaneous insertion	Molecular simulation	[40]
SWCNT (diameter 1.4 nm; length 400 nm)	RNA	Non covalent binding between RNA and CNT	Intracellular tracking	[41]
SWCNT (length 50-300 nm)	DNA, siRNA	Non-covalent wrapping with PL-PEG-maleimide, than conjugated with the cargos with a disulfide bound	Gene expression silencing	[42]
SWCNT (length < 2 um; aspect ratio 2.9)	DNA	Oxidized CNTs conjugated with primary amine groups of DNA	DNA transfection via " spearing"	[43]
SWCNT (length 300-1000 nm)	N/A	Covalent PEGylation with PEG-NH_2 on oxidised CNTs	Biodistribution	[46]

(Table 1. Contd….)

CNT Type	Cargo Type	Functionalization Approach	Type of Testing	Reference
SWCNT (diameter 2 nm; length 100 nm)	N/A	Non-covalent PL-PEG wrapping	Biodistribution	[47]
SWCNT (diameter 1.4 nm; length 280-450 nm)	^{125}I for radiotracing	Ionidation of -OH functionalized CNTs	Biodistribution	[48]
SWCNT (diameter 1 nm; length 300-1000 nm) MWCNT (diameter 20-30 nm; length 0.5 - 2 um)	^{111}In for radiotracing, chelated by DTPA	^{111}In chelation with DTPA grafted to the CNTs by 1,3-dipolar cycloaddition	Biodistribution	[49]
SWCNT (diameter 1 nm; length 300 nm)	N/A	Non-covalent wrapping with surfactant PF-108	Biodistribution	[50]
SWCNT (diameter 1–5 nm; length 100–300 nm)	c(RGDyK) oligopeptide; ^{64}Cu for radiotracing, chelated by DOTA	Non-covalent wrapping with PL-PEG, conjugated with different cargos (DOTA, c(RGDyK) oligopeptide)	Biodistribution, *in vivo* tumor targeting	[51]
SWCNT (diameter 0.8-1.2 nm; length 100-1000 nm)	N/A	Non-covalent absorption of bovine serum albumin	Biodistribution	[52]
SWCNT (diameter 1-5 nm; length 100-1000 nm)	Biotin/streptavidine	Covalent binding of biotin on oxidised CNTs	*In vitro* internalization assay	[54]
SWCNT (diameter 1-5 nm; length 50–200 nm); bundle (diameter 3-15 nm; length 200-2000 nm)	BSA; streptavidin; DNA	Non-covalent adsorption of proteins and DNA	*In vitro* internalization assay	[55]
MWCNT (diameter 20-40 nm; length 300-600 nm)	N/A	Non-covalent wrapping with surfactant PF-127	*In vitro* internalization assay	[57]

Cellular incubations with SWCNT-DNA or fluorescent streptavidine conjugates (both non-covalently complexed) were carried out at 4 °C or with pretreatment with NaN_3. Treatment with NaN_3 is known to disturb the production of ATP in cells, thus hindering the endocytotic pathway. Indeed, the fluorescence levels observed with confocal microscopy from cells after incubation in SWCNT conjugates at 4°C or with NaN_3 were much lower than the controls. This therefore indicates endocytosis as the internalization mechanism for the up-take of SWCNT conjugates at 37 °C.

Results of Shi Kam *et al.* clearly differ from the suggestion by Pantorotto *et al.* [17] that nanotube up-take by living cells, through an energy-independent non-endocytotic pathway, involves insertion and diffusion of nanotubes across cell membranes. It has to be noticed, however, that there are several differences in both the nanotube material and experimental procedures that were used between the two studies. Pantorotto *et al.* employed materials that contained positively charged peptide-functionalized bundles and aggregates of SWCNTs, which were microns in length and submicron in bundle size. The presence of nanotubes inside the cells was observed after incubation at 4°C: this led to the suggested non-blocking of nanotube up-take at 4°C and thus to the non-endocytotic up-take. However, further investigations have to been addressed in order to clarify discrepancy in up-take mechanisms for nanotubes, that is, endocytosis found by Shi Kam *et al.*, insertion and diffusion by Pantarotto and Bianco, and also phagocytosis proposed by Cherukiri *et al.* [56].

A recent paper of our group [57] demonstrated that the cellular up-take of as-produced MWCNTs depends also on their length. At this aim, different kinds of MWCNTs were produced and fully characterized, and their cellular up-take was evaluated by using standard fluorescent dyes and TEM imaging (Fig. (**2**)). The CNTs investigated in this study were simply dispersed in a biocompatible solution of the surfactant Pluronic F-127 *via* a non-covalent approach [58,59]. Experimental results showed that sub-1 μm MWCNTs are easily accumulated within cell through a non-endocytotic pathway (same results achieved in presence of NaN_3 or at 4°C), while we did not evidence significant up-take of longer Pluronic coated CNTs. The results clearly indicated that the length play a most important role as important as the chemical properties, being shorter nanotubes much more easily internalized by living cells.

As mentioned, many studies in the literature describe the high propensity of f-CNTs to cross cell membrane, but different up-take mechanisms have been proposed. These apparently conflicting results could be attributed not only to their chemical differences [60], but also to the physical properties, and nanotube length could be one of these properties.

CONCLUSION

The introduction and delivery of DNA, proteins, or drug molecules into living cells is an important challenge of the biomedical research. Inorganic nanomaterials, including nanocrystals, nanotubes, and nanowires, exhibit advanced physical properties promising for various biological applications, including new molecular transporters [61]. Carbon nanotubes are cylindrical structures with diameter varying from a few nanometres to tens of nanometres and length ranging from less than one micron to centimetres. They have excellent mechanical strength, electrical conductivity and thermal stability, so that many potential applications have been proposed: e.g., CNT based sensors, probes, nanoelectronic devices, and drug delivery systems for biomedical applications [62].

In this review we have summarized the most significant and recent advancements in the nanomedicine applications of CNTs, with a deep analysis of the chemistry involved in the preparation of biocompatible and effective nanovectors (Table **1**). We have showed that the choice of the chemical groups exploited for the functionalization of CNTs influence not only the assembling with the associated drug, but also the interactions with the living cells, the tissues and the whole organism. It is therefore of paramount importance to define "design criteria" to develop promising CNT-based carrier systems, in order to choice the best solution for the intended purpose.

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