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Smart nanotubes : A novel alternative for gene delivery

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Gene delivery, the process of introducing foreign genes into living cells, is an important technique in fields such as metabolic engineering and gene therapy. Including chemical/biological approaches (either using lipids, cationic polymers, or virus as vectors, or using conjugation) and physical approaches (heat shock, electroporation, gene gun, microinjection, and sonoporation), have been applied in bacterial, mammalian, and plant cells. However, these approaches are successful only in a few bacterial species because of the low efficiency of transformation, the complicated operation protocols, the severe damage to cells, and the high cost of complex devices. Carbon nanotubes were used to attain more than 15,000 transformants in the same situation. Therefore, the transformation method could be extended to other nanomaterials. Meanwhile, compared with the mechanism previously reported, we verified quite a different principle for the mechanism responsible for such a transformation. In sum, this unique transformation can be developed to become the third widely-used transformation method in laboratories.

Key words : Gene therapy, Carbon nanotubes

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INTRODUCTION

Gene transfer or DNA uptake refers to the process that moves a specific piece of DNA into cells. The rapid development of genetic engineering technique based on the knowledge of gene structure and function, plant breeding has been dramatically broadened. The directed desirable gene transfer from one organism to another and subsequent stable integration and expression of a foreign gene into the genome is referred as genetic transformation. The technique which are available presently rely on natural plant vector as well as vector less method which include delivery foreign DNA into plant cell.

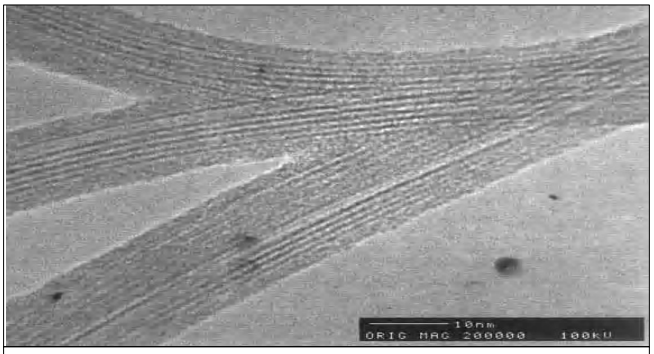

Fig. 1 : Transmission electron micrograph of fullerene carbon nanotubes

Carbon nanotube :

In 1985, Smalley and his co-workers at Rice University discovered a new form of carbon buckminster fullerene or C60 (Kroto *et al.*, 1985), and this work ultimately lead to the awarding of the 1996 Nobel Prize in chemistry. arc discharge method, and in 1991 Iijima discovered nanotubes in the products obtained from such a reactor (Fig. 1) (Iijima, 1991). These tubes are in essence rolled-up, highly ordered grapheme sheets, and they may be single walled or multiwalled. They are typically referred to in the literature as simply carbon nanotubes; however, since there are examples of nanotubes composed of more disordered forms of carbon (Fig. 2) (Miller *et al.,* 2001).

Diameters for these nanotubes range from 1 to tens of nanometers and lengths can be from microns to hundreds of microns (Ajayan, 1997). Since their discovery, there has been a massive international research effort aimed at understanding the properties of fullerene carbon nanotubes and at developing applications for these nanotubes; a number of authoritative reviews have recently appeared (Ajayan, 1997; Dai *et al.*, 1999). There have been a number of recent reports of putting biomolecules onto and into fullerene carbon nanotubes (Chen *et al.,* 2001). For example, Dai *et al.* (1999) used a simple non-

covalent route to attach a reactive molecule to the sidewalls of single-walled fullerene nanotubes. This reactive molecule could then be used to attach proteins to the walls of these nanotubes.

Lieber's group has pioneered the use of single-walled carbon nanotubes (SWNTs) as probe tips for atomic force microscopy (AFM) imaging of biomacromolecules such as DNA (Hafner *et al.,* 2001).

Since, their discovery, carbon nanotubes (CNTs) have been eminent members of the nanomaterial family. Because of their unique physical, chemical and mechanical properties, they are widely predicted and regarded as new potential materials to bring enormous benefits in cell biology studies (Bianco, 2004). Also, an increasing number of reports have studied the toxicological impact and safety profile of carbon nanomaterial on both plant (Lin and Xing, 2007) and mammalian cells, indicating that a high degree of CNT functionalization leads to a dramatic reduction in toxic effects

Table 1 : Techniques of gene transfer

Sr. No.	Name of technique	Information	Advantages	Disadvantages
1.	Agrobacterium mediated gene transfer	The appropriate gene construct is inserted within the T-region of a disarmed Ti plasmid; either a cointegrate or a binary vector is used.	- Technically simple. - Yields relatively uncomplicated insertion events - Unlimited size of foreign DNA - Efficient	-Host range is limited. -Tissue that are able to regenerate are difficult to transform.
2.	Electroporation	Passing a brief, intense electrical pulse through a suspension of (wall-less) cells in a solution of DNA results in the introduction of significant quantities of DNA into the cell.	- Specialized vectors are not needed - Useful for high-efficiency transient expression of foreign genes in plants	-It can be only used with protoplasts. -Frequency of stable -ransformation is low. -The integrated DNA is extensively rearranged this can lead to some mitotic and meiotic instability
3.	Biolistics	The introduction of DNA into cells using microprojectiles literally, DNA-coated particles are "shot" into target cells targets may be cells, tissues, whole plants.	- Theoretically unlimited "host range" (applicable to all plants) - May be used with methods that obviate the need for tissue culture/regeneration	- Does not completely eliminate the need for tractable tissue culture /regeneration systems -Lower efficiency -Instrument cost -Lack of control over the velocity of bombardment
4.	Microinjection	Microinjection is the direct mechanical introduction of DNA under microscopical control into specific target.	-Utilization of inject materials is efficient.	- Tecnicaly demanding -Costly
5.	Lipofection	Lipofection is a technique used to inject genetic material into a cell by means of liposome which are vesicles that can easily merge with the cell membrane since they are both made of a phospholipid bilayer.	-High efficiency -Its ease of use -Reproducibility -Low toxicity	-Not applicable to all cell type
6.	Calcium phosphate precipitation	In this method DNA is mixed with calcium chloride solution and isotonic phosphate buffer to form a DNA-$CaPO_4$.	-High efficiency -Inexpencive	-Reagent consistency is critical for reproducibility -small pH change can affect transformation efficiency
7.	DEAE dextran procedure	Transformation of cell with DNA complexed to hige molecular weight polymer diethyl amino ethyl(DEAE).	- used to transient expression - Inexpencive -Easy to perform	- high con. Of DEAE dextran can be toxic to cell - Transfection efficiencies will vary with cell type

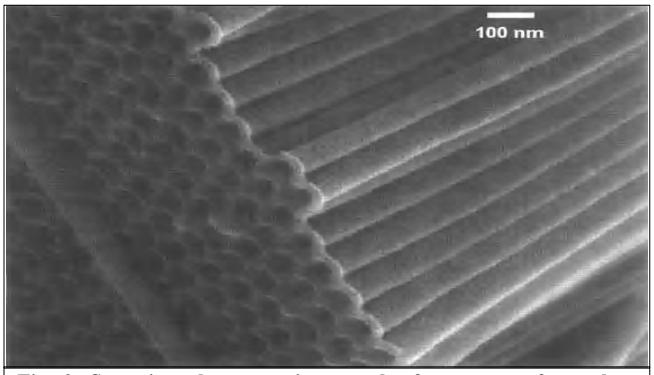

Fig. 2: Scanning electron micrograph of an array of template synthesized carbon nanotubes

(Sayer *et al.,* 2006).

Several methods of transfer of carbon nanotube :

Protoplast-based transfection method :

In protoplast-based transfection methods, the entire plant cell wall is removed to make the DNA/DNA vector accessible to cell transcription machinery. Meanwhile,the viability of protoplasts and their capability of dividing are strongly reduced by chemicals applied to disorganize the cell wall. In this experiment, we used cellulose modified Cup-stacked CNT (CSCNT-cellulase) to create nanoholes in the cell wall,through which CSCNT with adsorbed biomolecules can move intracellularly, hence circumventing complete cell wall removal.

CSCNT have lengths between 1 µm -100 µm and the mean diameter is 60~100nm. Cellulase was immobilized on fuctionalized CSCNT via a carbodiimide reaction (Fig. 3). *Arabidopsis thaliana* ecotype Columbia Col-0 and *Glycyrrhiza glabra* were used as the model plants used in this study. One mL of cell suspension was mixed with 10 µg of CSCNT-cellulase for 4h at 25^{0}C in the presence of 10 per cent OG (n-Octyl-β-Dglucopyranoside)as a paracellular permeability enhancer. They first investigated the CSCNT-cellulase system on *A.thaliana* cells. In our experimental conditions, CSCNT-cellulase was uptaken by 20 per cent of cells (Fig. 4A), while uptaken by 15 per cent of *G. glabra* cells (Fig. 4B). After 8 h, CSCNT-cellulase was localized inside the cell nucleus in 3 out of 50 cells showing internalized CSCNT-cellulase (Fig. 4C). This is the first example of plant cell transfection of dynamically enhanced CNT that have the ability to cross the plant cell wall, the cell membrane and the nuclear membrane and localize inside the cell nucleus.

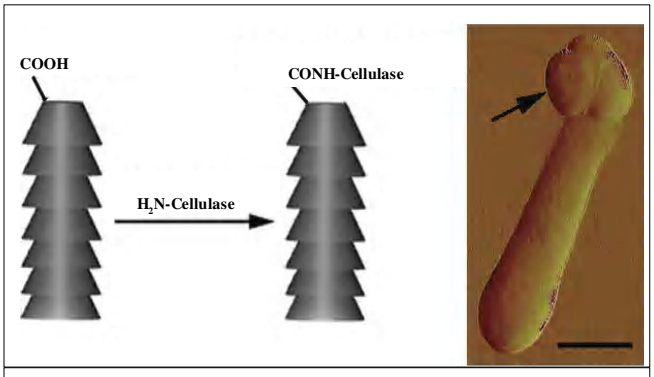

Fig. 3: AFM image of cellulose modified CSCNT (bar:100 nm)

Single-walled carbon nanotubes-mediated delivery of siRNA into antigen-presenting cells :

RNAi is mediated by small interfering RNAs (siRNAs) produced from long double-stranded RNAs (dsRNAs) of exogenous or endogenous origin by an endonuclease called dicer (Sontheimer, 2005). siRNA engages in sequence-specific interactions to inhibit gene expression by RNA degradation.

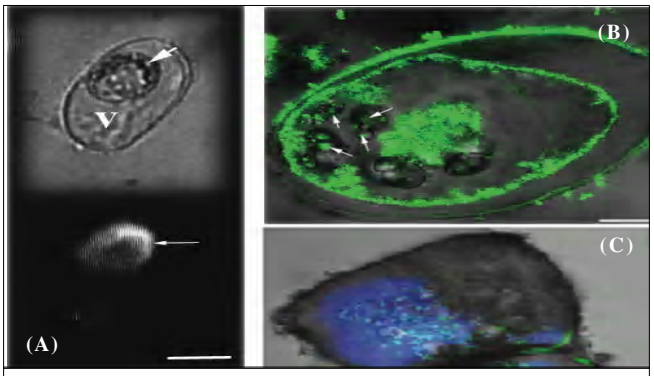

Fig. 4: (A) Detection of CSCNT fluorescence inside cell (arrow), v: vacuole, scale bar: 10µm. (B) Localization of CSCNT (arrows) inside endocytosis vesicles, scale bar: 10µm. (C) Localization of CSCNT (green spots) inside nucleus (blue; Stained by DAPI)

Although prior studies have described the penetration of functionalized SWNTs into several different cell types, their uptake by DCs has not been tested. Thus, we prepared FAM-labeled DNA-functionalized SWNTs to assess entry into DCs. The FAM-labeled DNA-functionalized SWNTs were examined by transmission electron microscopy (TEM, Fig. 5A) and atomic force microscope (AFM, Fig. 5B). The fluorescence spectra of DNA-functionalized SWNTs hybridized with complement DNA (c-DNA: 5'-TGC-ATT-TTT-AAT-GGT-ATT-TA-3'-FAM) showed stronger fluorescence at 516 nm than when incubated with non-complement DNA (n-DNA: 5'-TAA-ATA-CCA-TTA-AAA-ATI-CA-3'-FAM) (Fig. 5C), further indicating that conjugation of functionalized SWNTs with c-DNA had occurred. We also produced mouse bone marrow-derived dendritic cells (BMDCs), including immature dendritic cells (imBMDCs) and mature dendritic cells (mBMDCs), by standard procedures. Compared to imBMDCs, mBMDCs expressed high level of CD40, CD80 and CD86 co-stimulatory molecules and exhibited the typical morphology of mDCs (Fig. 5D). FAM-labeled DNA-functionalized SWNTs could enter into

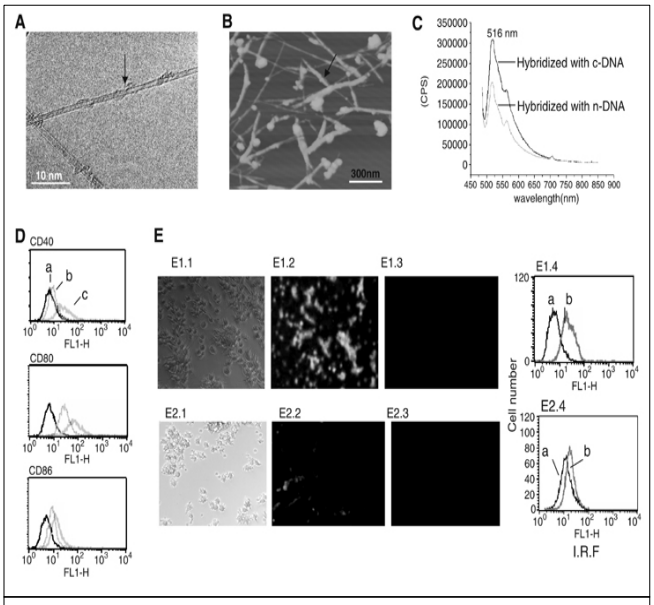

Fig. 5: Penetration of DCs by SWNTs-dsDNA-FAM

both imBMDCs and mBMDCs, although uptake by mBMDCs was significantly lower than by imBMDCs (Fig. 5E). As a control, FAM-labeled DNA fragments alone did not enter into these DCs (Fig. 5E). Consistent with other reports,16 FAM-labeled DNA-functionalized SWNTs also penetrated other mammalian cell lines.

Gene delivery to micro-organisms via an electrospray process :

An electrospray technique, formally called "electrohydrodynamic atomization", has been applied for gene delivery. Electrospray employs an electric field to disperse and accelerate liquid droplets or fine particles. An important advantage of electrospray is that the bioactive materials (*i.e.*, protein or plasmid DNA) do not lose their activity during the process. Previous proof-of-concept studies had shown the electrospray technique could deliver DNA materials into cells

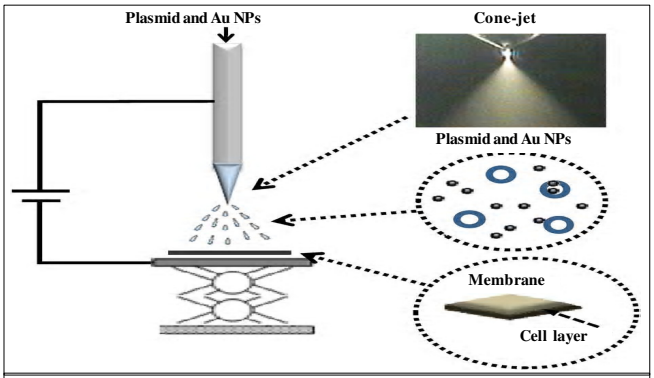

Fig. 6: Schematic diagram of an electrospray system for gene transformation (Wu *et al.*, 2010).

in two steps: 1) the plasmid was charged and accelerated by electric force during the electrospray process; and 2) the plasmid penetrated the cell membrane, thus delivered targeted genes into cells.

This novel transformation method does not require the preparation of competent cells, which is the crucial and time consuming step in traditional DNA transformation methods (Chen *et al.*, 2000; Davies *et al.*, 2005; Okubo *et al.*, 2008). Previous studies on aerosol delivery of genetic materials were qualitative rather than quantitative, and the technique was mostly targeted to transfection of mammalian cells (which lack a cell wall) and required nonviral vectors (*e.g.*, polyethylenimines) to carry DNA (Wu *et al.*, 2009). This study focused on microbial transformation of an industrial microbial host, Escherichia coli, by examining the key factors for controlling gene delivery efficiency. It has been reported that non-toxic nanoparticles (*e.g.*, gold NPs, silica NPs, and carbon nanotubes) can facilitate the macromolecules' entry into host cells (Jen *et al.*, 2004; Rojas-Chapana *et al.*, 2005; Galbraith, 2007). In particular, gold nanoparticles (Au NPs) are promising vehicles for gene delivery because these particles are readily conjugated with biomolecules at a high packing density (Mirkin *et al.*, 1996). Meanwhile, NPs (with plasmid) can increase the momentum of plasmid during electrospray and improve their penetration through cell wall. Using a newly designed electrospray device, we quantitatively tested several parameters, including NP sizes, cell growth stages, and electrospray buffer solutions, to obtain the optimal gene delivery operation conditions for *E. coli* transformation. Since the voltage in the electrospray device can be adjusted to achieve optimal momentum for the complex of Au NPs and plasmid, such gene delivery approach could be potentially used to bombard hard-to-transform microbes for achieving efficient transformation.

Plasmid transformation using nanomaterials :

In 2001, Yoshida and colleagues published a novel transformation method based on the inoculation of transforming DNA into bacteria by means of mineral nanofiber Yoshid *et al.* (2001). However, little notice has been taken of this very interesting invention because (i) the published work referred to the use of chrysotile asbestos fibers, which had carcinogenic potential and biological activity (Landrigan *et al.*, 1999); and (ii) the authors suggest the usage of a specific apparatus for optimized application of sliding friction forces, which could discourage possible users (Yoshida *et al.,* 2008). In 2010, Wilharm and colleagues improved the method based on the Yoshida effect using another mineral nanofiber (sepiolite) (Ajayan, 1997), which had been introduced by Yoshida and Sato (Wilharm *et al.,* 2008). Sepiolite-an inexpensive, resourceful, fibrous yet inoffensive mineral-makes DNA transformation rapid and simple. However, they

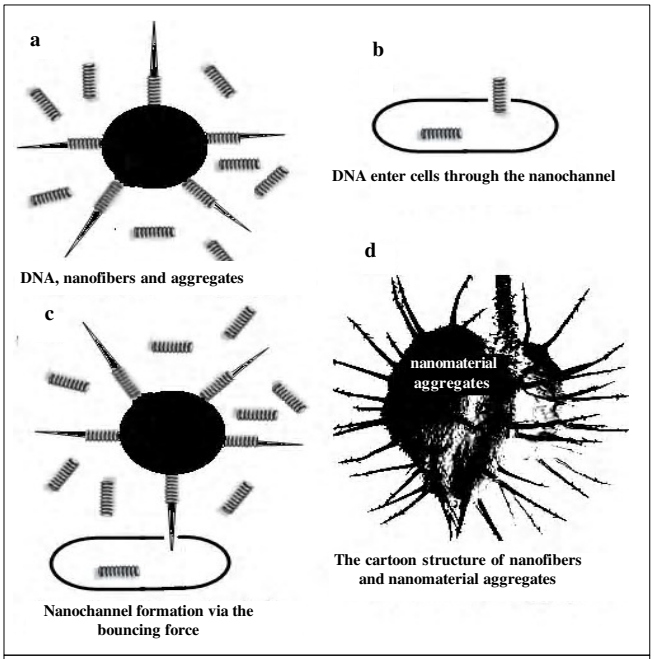

Fig. 7: The hypothesis for the mechanism of plasmid transformation by means of nanomaterials. (a–c) The model for the process of heterogeneous DNA entering a cell with the help of nanomaterials; (d) the cartoon structure of nanofiber and nanomaterial aggregates

confronted many problems using the novel method: The result seemed difficult to repeat and only a few tranformants could be obtained even when 100 ng of plasmids were used. In this case, we optimized the protocol, but it was difficult to attain more than 3,000 of transformants when 100 ng plasmid pET15b was used.

To address this problem, they changed the operating method and thereby enhanced the transformation efficiency greatly, which could match with that gained from chemical method. We guessed that such a transformation could be extended to other nanomaterials. For example, carbon nanotubes (CNTs) are widely used in most labs and are well characterized (Zhang *et al.,* 2008). CNTs have been reported as a DNA carrier during the electroporation (Rojas-Chapan *et al*., 2005); however, the results cannot point to CNTs making plasmid transformation occur, so the utilization of CNTs for DNA transformation is still unreported. Using the new method, they tried plasmid transformation based on CNTs and got more than 15,000 transformants for 100 ng pET15b. From these results and combining with those of other experiments, they found that the present mechanism could not explain our findings, so an alternative mechanism was provided here.

Application:

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 (Kostarelos and Miller, 2005; Carter and Samulski, 2008). 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 (Zheng *et al.,* 2003) .

Bianco *et al*. (2004) reported that functionalized carbon nanotubes (f-CNTs) can be used for presentation and delivery of antigens and for gene delivery (Bianco, 2004). The group prepared soluble f-CNTs using 1, 3-dipolar cycloaddition of azomethine ylides (Pantarotto *et al.,* 2003). 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.

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 (Lacerda, 2007).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 (Pantarotto *et al.,* 2003). 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 bispeptide derivatized CNTs have been prepared (Cai *et al.,* 2005). 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.

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 (Pantarotto *et al.,* 2004). 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 (Kam and Dai, 2005). Alternatively, when CNT were used to deliver proteins by adsorbing them onto their external surface, they seem to be up taken by endocytosis (Kam *et al.,* 2004). 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 (Kam and Dai, 2005). Interestingly, model nanotube structures have also been proposed to interact with lipid bilayers via a diffusion process directly through the bio membrane (Nielsen, 2004; Lopez, 2004). Spontaneous transmembrane penetration via the flipping of membrane lipid molecules is, contrary to endocytosis, an energy-independent process, not dependent onreceptor, coat, or lipid raft interactions, and is, therefore, potentially relevant to all cell types.

Biomedical application :

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 non covalently functionalized MWNT binding studies using microglia cells (Kateb, 2007); and (ii) oxidized, water-soluble CNTs interacting with *E. coli* under the application of microwaves (Chapana, 2005). The gradually accumulating work is confirming the novel mechanisms beside the classic ones.

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