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Imaging and Biomedical Application of Magnetic Carbon Nanotubes

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1. Introduction

The concepts of 'nano-technology' was introduced by the physicist Richard Feynman in his talk "There's Plenty of Room at the Bottom" at an American Physical Society meeting at Caltech on December 29, 1959. Feynman described a process by which individual atoms and molecules might be manipulate, using a set of precise tools to build and operate another smaller scale set of tools, scaling down to the nano-scale. In the course of this, he noted, scaling issues would arise from the changing magnitude of various physical phenomena: gravity would become less important, surface tension and Van Der Waals attraction would become increasingly more significant, etc. The term "nanotechnology" was defined by Professor Norio Taniguchi of Tokyo Science University in a 1974 paper in which he states "'Nano-technology' mainly consists of the processing of, separation, consolidation, and deformation of materials by one atom or by one molecule." Almost four decades later, nanotechnology has had an impact on all sectors of human life including electronics, computers and mobile phones; food and agriculture industries; composite materials, textiles, paints and cosmetics; and, of course, healthcare. In today's market there are thousands of products based on nanotechnology, produced by hundreds of companies worldwide. Essentially, nanotechnology entails the manufacturing and manipulation of matter at a scale ranging from a single atom to micron-sized objects. In biology, nanomaterials are a comparable size to many biological functional molecules such as proteins and are often small enough to fit inside a cell. Being at the same microscopic scale as biological functions allow nanoparticles to interact with many biological processes, this potentially can have an impact in many aspects of the healthcare. The new field of 'Nanomedicine' (Allhoff, 2009) will not meets its immense potential until the safety of nanomaterials is fully demonstrated. It is prudent to investigate any potential adverse effects on health or the environment of nanomaterials. However with suitable safe guards and internationally agreed standards emanating from these nano-safety and toxicity studies, there seems little reason to doubt the overriding benefit that nanomedicines will provide in the 21st Century (Jain et al., 2008).

Nanomedicine has been defined as the application of nanotechnology in healthcare. Their size and shape confers them with unique electrical, thermal, optical and magnetic properties (Emerich et al., 2007). They have large surface area to volume ratio and if chemically modified increases their application. In principle, it is possible to fabricate nanoparticles that can be used in the early detection and prevention of diseases, to improve diagnosis, treatment and follow-up. It has already enabled miniaturization of many current devices resulting in faster operation or enhanced integration of several operations. There are many biomedical nanoparticles on the market, with different physical and chemical properties. In the following discussion we will be investigating the potential use of carbon nanotubes in biomedical applications. This will include safety issue, and an overview of the current and the future perspectives of the exploitation of their magnetic properties for imaging and therapies.

2. Carbon nanotubes

Carbon nanotubes are either single-wall (SWCNTs) consisting of a single graphite lattice rolled into a perfect cylinder or multi-wall (MWCNTs) made up of several concentric cylindrical graphite shells (Russian doll configuration). CNTs are usually produced by catalytic chemical vapour deposition and contain metals, mainly Fe at their closed ends (Kim et al., 2005). For this reason they are paramagnetic – a valuable property for certain biomedical applications. CNTs vary in diameter (from a few nm to 100 nm) and widely in length (up to several mm). Their molecular structure accounts for their unique properties: high tensile strength, high electrical conductivity, heat resistance and efficient thermal conduction and relative chemical inactivity (constituent atoms not easily displaced). The exact structure of CNT especially their n – m chirality determines their electric properties. When n-m is a multiple of 3 (armchair type), the CNTs have static dielectric properties, i.e. exhibit a metallic longitudinal and an insulator transverse response. In practice all multiwalled CNTs behave in this way. By virtue of their nano-scale, electron transport in CNTs occurs through quantum effects and thus only propagates uni-dimensionally along the axis of the tube. One of the problems which have impeded the use of CNTs for biomedical applications which has since been resolved is their insolubility in aqueous solution. This is an essential requirement for biological interactions and biocompatibility. Coating their surface with covalent and non-covalent polymer improves their solubility, enabling in-vitro cell viability assays and in-vivo studies on biocompatibility (Kagan et al., 2010; Dutta et al., 2007). Carbon nanotubes will interact with cells and their 'needle-like' shape helps them cross cell membranes. By virtue of this characteristic, they can be used as carriers for drug and DNA delivery. Several groups have demonstrated that both SWCNTs and MWCNTs can be internalized by a variety of cell types with no external agent required facilitating the delivery of therapeutic and diagnostic small molecules (Kostarelos et al., 2007; Shi Kam et al., 2005).

2.1 Open issue: CNTs toxicity

In recent years, conflicting data have been reported concerning safety and biocompatibility of these nanotubes. In a study on the cytotoxicity of unrefined SWCNT to immortalized human epidermal keratinocytes (HaCaT), Shvedova et al showed accelerated oxidative stress, loss in cell viability and morphological alterations of cellular structures. They concluded that those effects were the result of high concentration of residual iron catalyst

(30%) present in the unrefined SWCNTs (Shvedova et al, 2003). Other groups confirmed these toxic effects, e.g., induction of intracellular reactive oxygen species (ROS) (Pulskamp et al., 2007), DNA damage (Zhu et al., 2007) and cell apoptosis (Bottini et al., 2007). Recently, Poland et al demonstrated that exposure of the mesothelial lining of the peritoneal cavity of mice to long (10-15mm) multi-walled carbon nanotubes results in asbestos-like, length-dependent, pathological inflammation and the formation of giant cell granulomas (Poland et al., 2008). In sharp contrast, other reports have demonstrated that CNTs do not induce toxic effects on cells. Huczko et al showed that CNTs exhibited negligible risk of skin irritation and allergy (Huczko et al., 2001). Cherukuri et al observed that macrophages phagocytosed SWCNTs at the rate of approximately one SWCNT per second without any apparent cytotoxicity (Cherukuri et al, 2004). Kam et al reported no cytotoxicity for the pristine SWCNTs and Pantarotto et al concluded that CNTs coated DNA provided a useful vector for safe gene delivery (Shi Kam et al., 2004; Pantarotto et al., 2004).

The exact fate of carbon nanotubes inside the cells and in animals remains controversial. In vitro studies showed that SWCNTs can be degraded by living cells which cause their complete biodegradation (Kagan et al., 2010); however the degradation of MWCNTs has not been demonstrated. Only a few studies have been published on the bio-distribution of carbon nanotubes in vivo. Singh et al. studied CNT bio-distribution following intraperitoneal administration in mice and they observed rapid blood clearance from systemic blood circulation through renal excretion. Moreover, urinary excretion studies using both f-SWNT and functionalized MWCNT followed by electron microscopy analysis of urine samples revealed that nanotubes were excreted as intact nanotubes after three hours (Singh et al., 2006). More work is needed to understand the clearance of nanotubes and more fully characterise what influences their biodistribution, degradation and excretation. A major source of toxicity is the presence of impurities, so it is important that CNT should be fully characterised reporting their length, purity, metal content and carbon soot. However, the research in this field is not conclusive for two reasons: 1) the absence of detailed data about the characterization of the nanomaterials confuses the interpretation of toxicity data; 2) the reaction that CNTs have with certain reagents in the in vitro cell viability studies invalidates the results of these assays.. Wörle-Knirsch et al. demonstrates interferences of carbon nanotubes with the MTT proliferation assay (Wörle-Knirsch et al., 2006). For this reason, the use of different and independent assays to study toxicological effects of nanoparticles has been proposed and generally accepted. In order to define valid guidelines for toxicological studies on nanomaterials, the EU has recently established the Nano-safety Cluster – a network of researchers and toxicologists. They will consider the somewhat conflicting results and develop appropriate guidelines. In addition, there is an urgent need for the definition of 'medical grade' CNT in terms of length, purity and metal content.

2.2 CNTs properties and their biomedical applications: The future of nanomedicine

In the last ten years, several groups have shown that SWCNTs and MWCNTs can be used as excellent intracellular transporters to deliver therapeutic and diagnostic small molecules and macromolecules to cells. Different uptake mechanisms (phagocytosis, diffusion and endocytosis) have been reported in the literature. Some physico-chemical characteristics of the carbon nanotubes (e.g., the nanotube dispersion, the formation of supramolecular complexes, and the nanotube length) drive the uptake pathway (Raffa et al., 2010). Both SWCNTs and MWCNTs can be internalized by a variety of cell types and thus used to

deliver therapeutic and diagnostic molecules. Conjugation of CNTs with different molecules can be used for new vaccine production, novel therapies against retrovirus infection and tumor cell proliferation. Pantarotto et al reported that VP-1 protein of the foot-and-mouth disease virus (FMDV) covalently linked to SWCNT induced a specific anti-body response in vivo without any cross reactivity (Pantarotto et al., 2003). Liu et al transfected human T cells and peripheral blood mononuclear cells with siRNA molecules conjugated to CNTs to abrogate the expression of cell-surface receptors CD4 and co-receptors CXCR4 necessary for HIV entry and T cells infection (Liu et al., 2007). Additionally McDevitt et al constructed a specific CNT conjugated antibody to target the CD20 epitope on Human Burkitt lymphoma cells and simultaneously deliver a radionuclide (McDevitt et al 2007). The physical and chemical properties of MWCNTs (e.g., high strength, electrical conductivity, flexibility, functionalization with biomolecules) make them attractive as nano-vectors for enhanced cell and tissue growth on scaffolds in vitro (Balani et al., 2007) and for the development of complex neural prosthetic implants (Khabashesku et al 2005).

CNTs also possess intriguing magnetic properties which derive from the metal catalyst impurities entrapped at the CNT extremities during their manufacture, these magnetic fields will interact with external magnetic fields. This property has been utilised by Cai et al. to develop a physical technique for in-vitro and ex-vivo gene transfer known as 'nanotube spearing', capable of effective cell transfection with plasmid DNA (Cai et al., 2005). Similarly, we have recently demonstrated that MWCNTs are able to interact with cells and, when exposed to a magnetic field, induce their migration towards the magnetic source (Vittorio et al 2010). This control of cell movement has important medical applications both in cell therapy for regeneration, cell transplantation and in cancer therapy (anti-metastasis). Moreover, we recently have demonstrated that MWCNTs with low metal impurities (2.57 % iron) can be used as MRI (magnetic resonance imaging) contrast agents even at concentrations of tens of µg/ml, as the MWCNTs have a significant effect on the observed ^1H transverse ($1/T_2$) relaxation rate of water. Consequently, cells labelled with MWCNTs exhibit a reduced image intensity in T_2-weighted MR images compared to cells without internalized MWCNTs. The 3D MRI cellular study suggests that it should be possible to track stem cells injected in vivo by labeling cells with these low Fe MWCNTs. It is possible that MRI could be used in the studies of biodistribution and the fate of carbon nanotubes *in vivo* (Vittorio et al 2011). Another important property of CNTs is their strong absorbance of near infra red (NIR) light and subsequent release of this heat that can be utilised in the destruction of cells – nanohyperthermic ablation of tumours. Shi Kam and colleagues achieved selective cancer cell destruction in vitro by using folate functionalized nanotubes heating and continuous NIR radiation (Shi Kam et al 2005 a). Additionally CNTs acquire and release heat on application of radio-frequency waves (58). Gannon et al. induced efficient heating of aqueous suspensions of SWNTs by applying RF waves. In particular they produced a selective and SWNT concentration dependent thermal destruction in vitro of human cancer cells that contained internalized SWNTs. Moreover they observed that intratumoral in vivo injection of SWNTs in the liver followed by immediate exposure to RF waves induced necrosis in the tumor mass with no apparent adverse effects on the healthy tissues (Gannon et al., 2007). A recent discovery (and patented by the NINIVE consortium) is the ability of CNTs to act as a dipole antenna and acquire a charge on exposure to electromagnetic radiation in the microwave range. This is the basis of an exciting new technology for remote wireless low voltage electro-chemotherapy and gene transfection and novel forms of electro-stimulation therapies for neurodegenerative disease (advanced

Parkinson's disease unresponsive to medication), electrostimulation for skeletal and visceral muscle palsies and for cardiac arrhythmias.

Several groups have investigated the potential of using nanoparticles in neurological applications. As carbon nanotubes/fibers have excellent electrical conductivity, strong mechanical properties and similar nano-scale dimensions to neurites, researcher have been explored their ability to guide axonal regeneration and to improve neural activity by acting as biomimetic scaffolds at sites of nerve injury. Mattson et al were the first group to demonstrate neurons grow on MWCNTs (Mattson et al., 2000). Moreover they reported an increase of over 200% in total neurite length and approximately 300% increase in the number of branches and neurites on MWCNTs coated with 4-hydroxynonenal compared to uncoated MWCNTs. Hu et al reported that positively charged MWCNTs significantly increased the number of growth cones and neurite branches compared to negatively charged MWCNTs (Hu et al., 2004). Gheith et al investigated the biocompatibility of a free-standing positively charged single wall carbon nanotubes (SWCNT)/polymer thin-film membrane prepared by layer-by-layer assembly (Gheith et al., 2005). They observed a 94-98% viability of neurons on the SWCNT/polymer films after 10 days of incubation and this induced neuronal cell differentiation, guided neuron extension and directed more elaborate branches than controls. Moreover, Lovat et al demonstrated that purified MWCNTs have the potential to boost electrical signal transfer of neuronal networks (Lovat et al., 2005). Recently Cellot et al investigated the nature of CNT-neuron interactions and proposes a mechanism in which carbon nanotubes (CNTs) can boost neuronal activity by providing a shortcut for electrical coupling between somatic and dendritic neuronal compartments (Cellot et al., 2009).

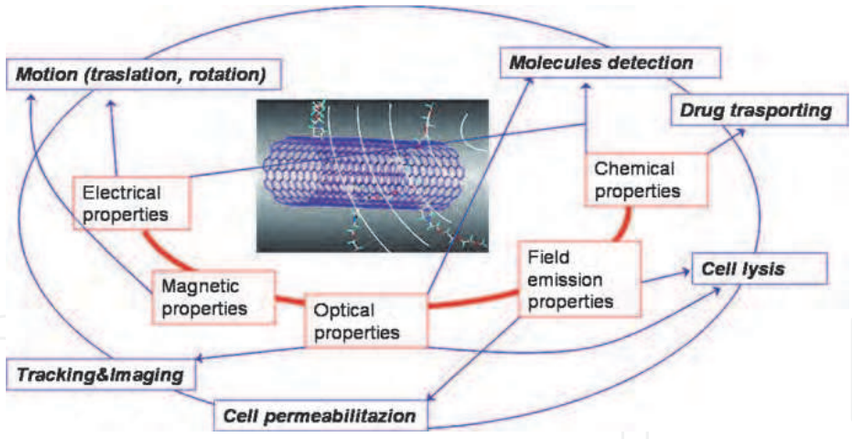

Fig. 1. CNT properties and their application in the biomedical field (Ciofani, G., Raffa, V., 2009).

2.3 Bio-medical imaging of CNTs

One of the goals of nano-medicinal research is to produce bespoke multifunctional "nano-devices" that can act as diagnostic devices, tissue-specific drug delivery systems or vehicles for gene therapy, ideally combining this with multimodal imaging capabilities (Hong et al., 2009; Kostarelos et al,. 2009; Lui et al., 2009; Lu et al., 2009; Liu et al., 2010). Carbon nanotubes have many properties that make them particularly useful as biomedical devices (Hong et al., 2009; Kostarelos et al,. 2009; Lui et al., 2009; Lu et al., 2009; Liu et al., 2010).

Their microscopic morphology confers them with unique electrical, thermal, optical and magnetic properties. Coating the surface of CNT improves their solubility and their 'needle-like' shape allows internalized by cells. By functionalizing the surface with biological active compounds, it is possible to use CNTs as 'nano-vehicles' delivering compounds into cells such as small molecule drugs, proteins, vaccines or in the case of gene therapy delivering siRNA. Thus CNTs have proved to be very effective carriers for gene delivery, with the advantage of avoiding the viral vectors used by other methods (Kostarelos et al., 2009). In addition, there are a number of CNTs strategies being developed to target CNTs to a region of interest, either by exploiting their magnetic properties or attaching targeting-ligands, such as antibodies or peptides, to their surface. With the exciting research published in this area, one should not forget that this technology is still in a developmental stage. Much work is still required in for example testing: (i) the effectiveness of CNT atfor targeting disease tissues; (ii) the stability efficacy of using CNTs to carry drugs to a region of interest and the profile of drug release; (iii) the precise mechanism of cell internalization as well as , and observing the sub-cellular compartments in which the CNTs accumulate; and also (iv) efficiency of using CNT as a heating methods for hyperthermia treatments. Undoubtedly there is still a lot to be done assessing the efficacy of the different CNT therapies as well as understanding the safety issues associated with CNTs.

Bio-medical imaging has a vital role to play in testing both the efficacy of these new therapies and investigating their biodistribution. To date, the majority of imaging of CNTs has involved optical methods, such as fluorescence and bioluminescence microscopy (Hang et al., 2009). Optical microscopy has high spatial resolution, however it requires optically transparent samples. This is less of an issue in near-infrared photoluminescence imaging, Raman imaging, or photo acoustic tomography. THowever, there is a reduction in spatial resolution and sensitivity with these methods, and they still have tissue depth limitations. Attaching radionuclide labels onto CNTs allows three dimensional (3D) whole body, in vivo molecular imaging such aseg position emission tomography (PET) and single photon emission computed tomography (SPECT). These techniques have high sensitivity, moderate resolution and have the advantage of being well established medical techniques, but they do involve ionising radiation.

Magnetic resonance imaging (MRI) is an extremely versatile imaging modality, and particularly suitable for testing novel medical therapies as it is available as pre-clinical and clinical platforms, allowing a seamless transition from the laboratory into the clinic. MRI produces high resolution 3D images, non-invasively, from within optically opaque samples. The MRI signal originates from protons in water and lipid, and as a result the images contain impressive anatomical and pathological information. Furthermore, magnetic resonance (MR) can be used to interrogate tissue morphology, physiology, function, vascularity and metabolism. The versatility of MRI originates from the fact that signal intensity is dependent upon a number of physical parameters including the density of protons in the liquid state, 1H longitudinal (T1) and transverse (T2) relaxation times, and mass transport processes such as diffusion or flow (Modo et al 2007). In a simple 90o-180o-acquire spin echo imaging sequence, the intensity of the MR magnetisation (Mt) observed in the spin echo can be expressed as:

$$Mt = Mo \cdot \exp(-TE/T2) \cdot [1 - \exp(-TR/T1)] \quad (1)$$

if diffusion and flow are negligible, and when Mo is magnetisation at time zero, TE is echo time between excitation and detection of magnetisation, and TR is repetition time between successive imaging pulse sequences.

A limitation of MRI is that it is an inherently insensitive technique; this is due to its quantum physics and the Boltzmann's distribution in particular. Its low sensitivity and low signal-to-noise ratios are the reason most MR images map the location of water and lipids, as these molecules are found in high concentrations. It is also the reason that the intrinsic MRI spatial resolution is rarely below the order of 10's of microns. Consequently, that it is not possible to detect a MRI signal directly from solid CNTs with low proton concentrations. Second, even if CNTs did produce a MRI detectable signal, they are so small that it would not be possible to see the shape of an an individual nanoparticle in an MRI image due to insufficient spatial resolution. However it is possible to use MRI to observe the location of CNTs indirectly, because magnetic CNTs can act asare MRI contrast agents and this can modify the appearance of an image. There are two classes of MRI contrast agents (CAs): T1 CAs reduces the 1H T1 relaxation times of the water molecules in their vicinity, whilst T2 CAs reduces 1H T2 relaxation times. T1 MRI CAs produces hyper-intensity in T1-weighted images thus appears white. They typically contain lanthanide ions; gadolinium is the most potent in this class with seven unpaired f-electrons. There are several Gd-chelated contrast agents with FDA approval including Omniscan, Multihance and Magnevist. T2 MRI contrast agents produce hypo-intensity in T2-weighted images thus appears black; this can be difficult to interpret if there are other black features in the image. Super-paramagnetic iron oxide nanoparticles are particularly potent T2 MRI CAs and formulations such as Feridex and Resovist have FDA approval for clinical use. The main purpose of contrast agents in the clinic is to improve disease detection and increase diagnostic confidence.

The effectiveness of MRI contrast agents are evaluated by determining their longitudinal or transverse relaxivity, r1 or r2 respectively. Relaxivity is defined as the change in the relaxation rate of water protons per molar concentration of the contrast agent with units of s-1mM-1 and is expressed as:

$$ri\,[CA] = 1/Ti - 1/Tio \quad (2)$$

where ri is relaxivity; i=1 or 2; [CA] is the concentration of contrast agent; 1/Ti is the longitudinal or transverse relaxation rate in the presence of contrast agent [CA]; and 1/Tio is the relaxation rate of the medium in the absence of contrast agent. To assist comparison of MRI data from different laboratories, it would be useful if new CNTs were tested against a set of 'standard'standardized conditions such as using 1% agarose gels as a phantom and also using similar cell types.

Choi et al attached superparamagnetic iron oxide nanoparticles onto SWCNTs to demonstrate the potential of CNTs as T2 MRI contrast agent. They imaged murine machrophage cells that had been incubated with these CNTs. The image contrast is generated by localised magnetic inhomogeneities induced by the magnetic CNTs, which reduces the T2 relaxation time of nearby water protons. Mesenchymal stem cells containing MWCNTs produced regions of hypo-intensity compared to untreated cells in T2-weighted RARE images (Vittorio et al. 2011) (Figure). Ananta et al measured the r2 relaxivity of three different types of SWCNTs. Interestingly, the r2 of pristine, low Fe content CNTs was twice as large as the relaxivity of raw, unpurified SWCNT; even though pristine SWCNT had three times less iron. Similarly, the relaxivity r2 of MWCNT with low iron content (2.57%) measured by Vittorio et al was 564 ± 41 s-1mM-1, (Vittorio et al. 2011), which was over three times higher than the relaxivity of commercial contrast agent Feridex measured under similar conditions (Chen et al. 2010). They attributed the relaxivity r2 to the presence of iron oxide impurities in the CNTs, which originate from the fabrication process, and to the

carbon MWCNT structure itself. Although the equation shown in Eq 2 is conventionally used to determine relaxivity, in this situation to measure relaxivity relative to iron concentration alone is slightly misrepresentative. A study using magnetic iron oxide nanoworms neatly demonstrated that the r2 relaxivity was dependent on the intrinsic shape of the nanoparticles as well as to the presence of iron oxide impurities (Park et al., 2008). These results suggest that these nanoparticles are members of a new class of T2 relaxation agents where shape of the particle also contributes to relaxivity r2, this warrants further investigations.

CNT T1 contrast agents have been produced by loading Gd3+ ions onto carbon nanotubes;. Sitharaman et al used ultra-short SWCNTs (Sitharaman et al., 2005), whilst Richard et al used MWCNTs (Richard et al., 2008). Superparamagnetic ultra-short single walled carbon nanotubes called 'gadonanotubes' have been produced (Sitharaman et al., 2010) with r1 and r2 relaxivity of 170 s-1mM-1 and 578 s-1mM-1 respectively. They are substantially more potent than the paramagnetic Gd chelates currently in clinical use. These contrast agents have the advantage that they can be used as either T1 positive CAs or T2 negative CAs. An in vivo study with rats using gadonanotubes generated negative hypo-intensity (Sitharaman et al., 2010). There are investigations into using these gadonanotubes as pH-probes since the T1 relaxation is sensitive to pH (Hartman et al., 2008).

In vivo MRI has been utilised in detecting the bio-distribution and potential impact of CNTs and potential impact. CNTs are extremely stable and not easily bio-degraded and v. Very little is known of about how their long term they behaviour in vivo or their pharmacological profile. Faraj et al have used MRI to evaluated the bio-distribution of SWCNT in animals. A Their longitudinal, time course, in vivo MRI study observed the accumulation of carbon nanotubes in the spleen and liver of rats (Al Faraj et al., 2011) after intravenous injection, although no acute toxicological effect on the liver's metabolic profile was observed. They also assessed the effects on the lungs of rats after inhaling SWCNTs using hyperpolarized 3-He MRI (Al Faraj et al., 2009). The results from a 3 month follow-up study showed that granulomatous and inflammatory reactions were produced over time in a dose dependent manner (Al Faraj et al., 2010).

In summary, the objective of the majority of MRI studies involving CNTs, to-date, have been evaluating the potential of CNTs as MRI contrast agents. The development of these bio-nanomaterials is still in its early stage,P and consequently the fabrication, functionalisation and characterisation of novel CNTs is an important and fruitful area of research. Producing CNTs that are potent and stable MRI contrast agents is particularly essential when using this technique to track their movement in vivo the behaviour of CNTs in vivo. Obvious applications of this would be the tracking of stem cells and guiding their delivery. MRI also has a crucial role to play in assessing the behaviour of CNTs in vivo. Multinuclear MRI studies have been published following CNTs bio-distribution in animal models after intravenous injection and inhalation. The non-invasive nature of MRI makes it particularly suitable for such longitudinal time course, follow-up studies. However, it is our belief that the most important role of MRI in carbon nanotubes research will be evaluating the effectiveness of the various bio-medical applications and therapies associated with CNTs both in animal models and in the clinic. In hospitals, MRI is a work-horse imaging modality for the diagnosis of a broad range of diseases. It contributes to almost every aspect of disease management from diagnosis and staging a disease, to selecting and assessing therapies, and in follow-up and to detecting recurrence. It is theits ability of MRI to produce high resolution images with excellent anatomical and pathological contrast in animal

models of diseases as well as in the clinic, which will be needed when evaluating these new nanomaterials in targeted chemotherapy, focussed electrotherapy and gene therapy etc.

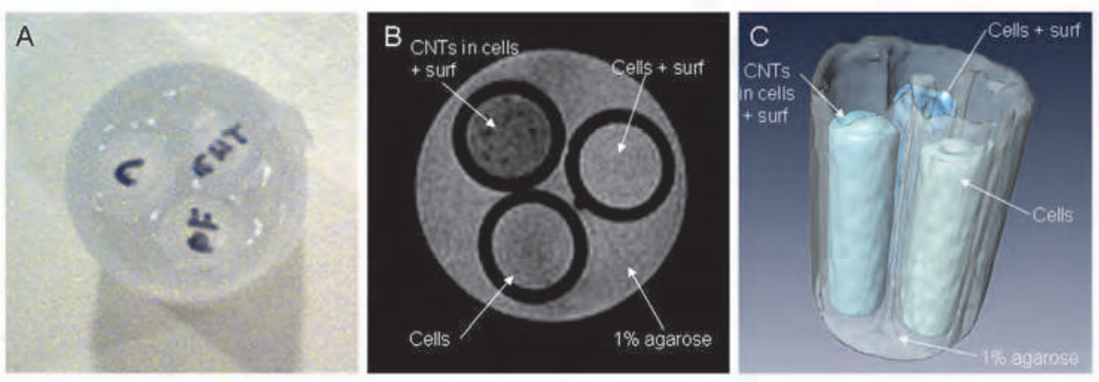

Fig. 2. MWCNTs MRI study of mesenchymal stem cells (MSCs). Three vials were embedded in container of 1 % agarose: vial 1 (test) contains 97% MWCNTs in MSCs treated with surfactants held in agarose gels (cnt); vial 2 (control i) contains MSCs treated with surfactants held in agarose gels (pf), and vial 3 (control ii) contains MSCs cells held in agarose gels (c). (A) Photo of the dorsal view of the sample; (B) T2-weighted RARE-4 axial image of sample from the 128 by 128 by 128 RARE-4 (TR/TE=250/40 ms) data set; (C) 3D surface reconstruction of the sample from the same MRI data set as (B). MRI measurements were completed at 7.1 T, at 19^{o}C, with field of view is 30 mm, and voxel spatial resolution of 0.234 mm/pixel. (Vittorio et al., 2011 b)

2.4 Magnetic properties of MWCNTs: Application for cell displacement *in vitro*

The magnetic properties of MWCNTs can be exploited for many biomedical applications. An interesting application is represented by the possibility of labelling the cells with the magnetic nanoparticels and guide their localization by an external magnetic field. It is easy to image the exploitation of this methodology to move and collect metastatic cells, or to guide the cell transpalatation towards the target organ. We recently used the same approach to guide and improve the nerve regeneration after the axon treatment with magnetic nanoparticles (www.marvene-project.org). The magnetic interactions of MWCNTs can be attributed to the metal particles encapsulated in the graphene sheet (Zhang et al., 2001; Glenis et al., 2004). After dispersion treatment, the tubes become water dispersible and as a result are shorter in length. These effects are propitious for the interaction between cells and CNTs: the reduced length and the reduced degree of agglomeration should limit toxicity response and at the same time could favour passive endocytosis (Wick et al., 2007). We have experience in the study of interactions between nanomaterials and different cell lines. We spent much efforts in finding the best conditions to label cells with MWCNTs and to move them by magnetic field without compromising their physiological conditions.

Under the effect of a permanent dipole magnet, cells have been seeded for three days with the CNT-modified medium: a progressive displacement of cells toward the more intensive magnetic fields was visible in the dishes where the CNTs have been added in culture, while in the control dishes (without CNTs) there was negligible translations of cells during the same period (Pensabene et al.,2008). Our results suggest that cell displacement occurs

during cell duplications. However, the displacement mechanism is still not clear, but the present result is a starting point to further investigation of the interaction between CNTs and cells and the controlled displacement of cells for selective cancer therapy. Concerning these issues, there are currently contrasting opinions and the mechanisms of interaction are still not clear. Two mechanisms can be supposed: the uptaking of the tubes (by endocytosis or pinocytosis) or their attachment to the cell membrane (Monch et al., 2005). The evidence of Figure 3 leads us to affirm that the binding of MWCNTs to cells is strong and the CNTs are able to drag along the cells, following the traction force generated by the permanent magnet. This effect could be used also to study how a mechanical stimulation of cells by CNTs can influence the cell activity (Cartmell et al., 2002).

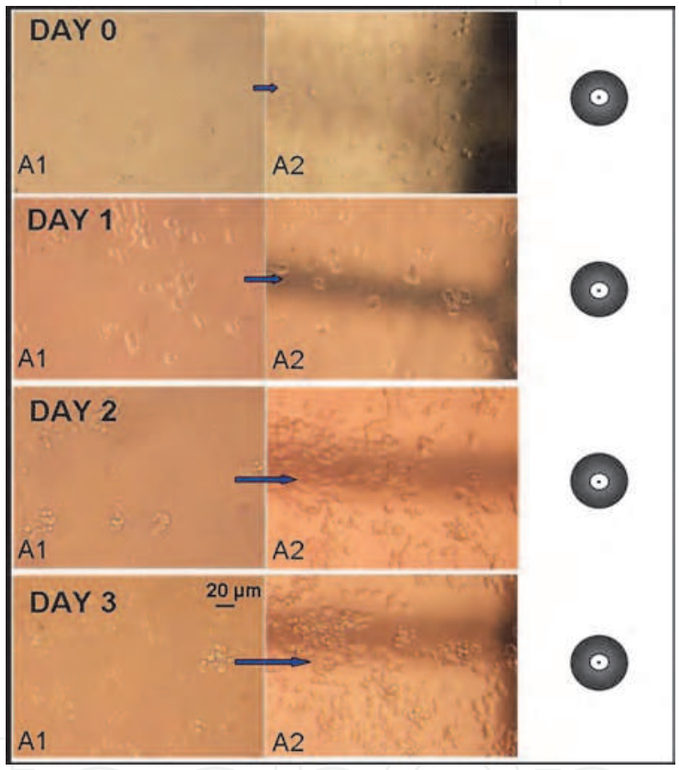

Fig. 3. Neuroblastoma cells displacement after three days in culture with MWNTs-modified medium. Control sample not showed (with Nikon TE2000U inverted optical microscope, magnifications 20X). (Pensabene et al., 2008)

We also studied the interaction of magnetic carbon nanotubes with PC12 cells which represent a valid in vitro model to study the effect of nanoparticles in neuronal cells and the effect on differentiation in neurons (Vittorio et al 2011c). We demonstrate that when PC12 cells were cultured in a MWCNT-containing medium, the nanotubes interact with the cells without compromising the cell's viability and their ability to differentiate into neurons following exposure to NGF. As a result of this interaction, CNTs in the presence of a magnetic field are able to translocate cells, in response to a permanent magnetic field. In contrast, no displacement was observed in control samples when cells were cultured with a CNT-free cell culture medium. These results confirm the ability of MWCNTs to shepherd PC-12 cells in response to the force generated by a permanent magnet. We believe that

recent advances in nanoscience and nanotechnology may provide new therapeutic options as alternative/supplemental therapies to established surgical repair techniques for the treatment of peripheral nerve injuries. We studied the interaction between neuronal cell line and magnetic carbon nanotubes, because this is an essential step to investigate the exploitation of magnetic nanoparticles to enhance/accelerate nerve regeneration and to provide guidance for the regenerating axons.

2.5 Magnetic properties of MWCNTs: Application for stem cell displacement *in vitro* and to drive the homing of mesenchymal stem cells after their transplantation *in vivo*

Next, we decided to try to move stem cells after their incubation with MWCNTs. With the aim of finding the best protocol to guide the cell homing towards the target organs and improve stem cell transplantation strategy. Many experts in stem cells transplatation report the lack of efficient technology to inject stem cells in vivo in target sites and avoiding their dispersion.

The experimental data obtained in our study confirm that magnetic fields can be used to control the movement and location of MSCs cultured with carbon nanotubes (Vittorio et al., 2011 a). The cell distribution in control cultures MSCs in the Petri dish was homogeneous (350-400 cells/mm^2), whereas the cell density was more heterogeneous in the test culture. Specifically, in the test culture the MSCs density ranged from 200 cells/ mm^2 to 800 cells/mm^2, correlating respectively with distances from the magnetic pole of 9-14 mm and 5 mm (Figure 4) . This cell streaming behaviour was closely related to the intensity of the flux density within the Petri dish which ranged from a very low value (<0.1 T) when the distance from the magnetic pole was >9 mm to about 0.5 T at a distance of 5 mm. Mathematically, an MSC interacting with CNTs is subjected to a translational force in the presence of a gradient field of:

$$F_m = \frac{1}{\mu_0} \cdot \chi_{rp} \cdot V \cdot B \cdot \frac{dB}{dr} \tag{3}$$

where μ_0 is the magnetic permeability of free space, X_{rp} and V are respectively the magnetic susceptibility and the total volume of magnetic nanotubes attached to the cell. Eq. 3 is a usefull mathematical tool to design the manipulation of MSCs cultured with carbon nanotubes by a magnetic flux gradient. The migration dynamics of a single cell shows that the cell moves towards the magnetic source with a speed of approximating 30 μm/h. Cell proliferation assays were performed to identify if there was any adverse effect of the nanotubes and/or the magnetic field on the viability, proliferation and functionality of MSCs. The results showed that the PF127 surfactant marginally decreased the cell viability but the cell viability reduction was negligible with PF127-CNTs. This can be explained by the action of the surfactant which wraps the nanotubes surface and reduces the amount of free PF127 in the medium (Vittorio et al 2009). Additionally, the data on the cell growth assays showed that the MSCs doubling time was not significantly influenced by the presence of either the nanotubes or the magnetic field and no apoptotic cells were observed in any of the samples studied.

The in vitro studies also confirmed that cells treated with CNTs for up to 5 days maintain their ability to differentiate under specific conditions just as well as the untreated cells. The lack of adverse effects on cell viability and proliferation by MWCNTs observed in the present study needs to be compared with the reported findings by Mooney et al. In this study, hMSCs were treated for 24 h with both COOH-functionalised SWCNTs and OH-functionalised MWCNTs, at various concentrations. The authors demonstrated that COOH-functionalised SWCNT, at

concentration up to 32 μg/ml, did not affect cell viability, proliferation, differentiation and metabolic activity; but at higher concentrations, SWCNTs exerted detrimental effects on the cells. In contrast, OH-functionalised MWCNTs were found toxic at all concentrations (Mooney et al., 2008). In the present study, the MWCNTs were not functionalised but simply dispersed in an anionic surfactant and used at concentration of 10 μg/ml. Finally, the staining of the actin filaments suggested clearly that the treatment with the nanotubes and the exposure to the magnetic field did not alter cell morphology.

The results of the *in vivo* experiments provide a proof of concept that MSCs cultured with CNTs can be shepherded by means of an external magnetic source towards a specific organ; specifically, we demonstrated that the application of an external magnetic field alters the biodistribution of CNT-labelled MSCs after intravenous injection into rats, increasing the accumulation of cells into the target organ. This observation assumes importance in view of the widespread interest in MSC-based cell therapy for tissue engineering, regeneration/ repair of damaged organs and allogeneic transplantation. As MSCs also exhibit immuno-modulatory and anti-inflammatory effects they may play a role in the in vivo induction of tolerance. It was observed that MSCs reduce the incidence and severity of Graft-Versus-Host-Disease (GVHD) (Le Blanc et al., 2007), as well as prolong skin graft survival.

The clinical applications of MSCs require the administration of cells by the intravenous route, but their subsequent dispersion in many tissue and organs (Allers et al., 2004) reduces the number of cells which colonize the intended target organ. There is therefore a need to increase targeted stem cell localization and homing to the diseased site. The approach proposed in this paper has the potential to achieve the objective of site-specific localization of the CNT-labelled MSCs, by reducing their colonisation of other sites with consequent adverse effects resulting from their proliferation and differentiation in ectopic sites. In the animal model studied we were able to achieve a 3-fold increment of MSC localization in the target organ (liver), and a corresponding decrease of MSC localization in the lung and kidney, which represent a natural filter for stem cells (Figure 5). In a recent work, Gao demonstrated that intravenous infusion of sodium nitroprusside, a vasodilator, administered prior to the cells infusion, reduced by 15% the number of cells present in the lungs and increased by 10% the cells in the liver (Gao et al., 2001). Based on this result we are currently investigating the combined action of a vasodilator and the magnetic guidance to enhance the targeted homing of cells. Additionally, we are optimising the protocol of CNT-labelled MSC transplantation and we are designing a magnetic applicator device which would allow a more accurate and controlled configuration of the magnetic field gradient applied to the target site. Further improvements could be the design of the magnetic field by finite element modelling (FEM), the development of a wearable device and the use of electromagnets for switching the magnetic field.

In summary, we have demonstrated that when mesenchymal stem cells are cultured in a CNT- containing medium, the nanotubes interact with the cells without compromising the cell's viability, proliferation rate, cell phenotype and cytoskeletal conformation. Moreover we confirmed that magnetically labelled cells maintained the ability to differentiate in adipocytes and osteocytes. As result of the cells interaction with CNTs, the application of a magnetic field, enables shepherding of MSCs to the desired location in vitro. Moreover, in the experimental model used we were able to increase significantly the localization of mesenchymal stem cells within the liver, with a reduction of their migration to other organs. This paves the way for the development of a new methodology for shepherding cells in a target tissue/organ. The application of such technology would significantly improve both

the range and efficacy of therapies based on transplanted cells, included totipotent, pluripotent multipotent stem cells, reprogrammable adult cells, induced pluripotent stem cells (iPSC) and embryonic stem cells. Compared to the existing methodologies, CNT-labelled MSCs maintain their magnetization for a period over 24 hours and up to 3 weeks. The long-time magnetization should allow for an efficient cell manipulation via magnetic fields and a reliable cell tracking via magnetic resonance imaging. The application of such technology could significantly improve the range and efficacy of the current and future cell therapies. Our findings pave the way for the exploitation of the magnetic properties of biocompatible carbon nanotubes to localize the stem cells in a target organ, after their transplantation. A controlled and localized stem cell transplantation represent the future of regenerative medicine.

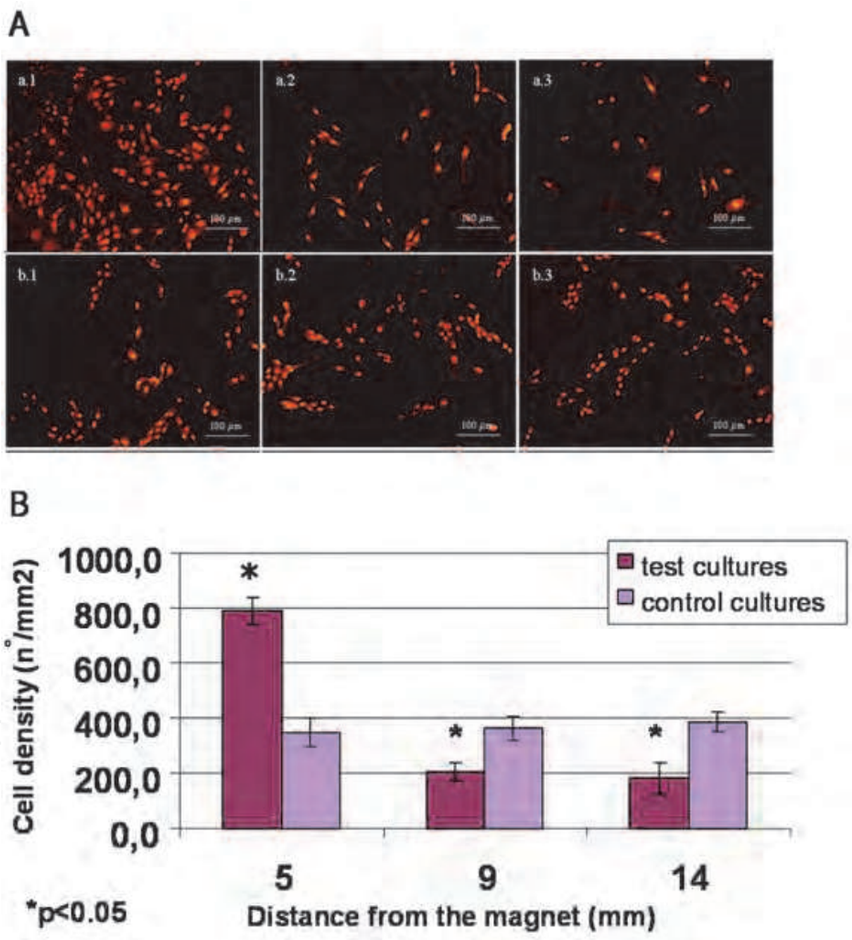

Fig. 4. Imaging of MSCs shepherding: (A). MSC-CNT-cultures (a) MSC-control (b). Cultures 72 h after magnet placement. Images taken at different distances from the magnet pole (1, 2 and 3 correspond respectively to 5, 9 mm and 14 mm from the magnetic pole). Cell is stained with Syto 82 fluorescent dye. (B). MSC density for test and control cultures after 72 h from the magnet placement at different distances from the magnet pole. Assays performed in quadruple and results are the mean±S.E.M. (vertical bars)
(Vittorio et al 2011 a)

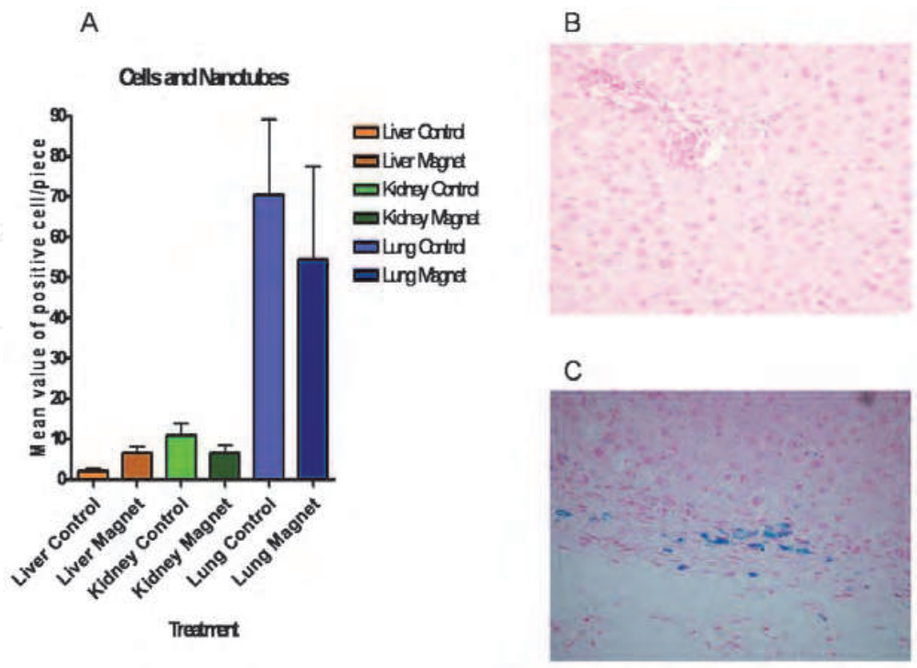

Fig. 5. Histochemical analysis: (A) Mean and S.E.M. values of positive cells for Perls staining in each type of organ;(B) Liver's section of control sample; (C) Liver's section with Perls labelled mesenchymal stem cells with magnetic nanotubes (Vittorio et al., 2011 a)

2.6 Exploitation of magnetic CNTs in gene therapy

The magnetic properties of CNTs derive from metal catalyst impurities entrapped at their extremities during their manufacture or from filling the nanotubes with tailored materials in which the active content is encapsulated by a protecting carbon shell (Weissker et al., 2010).

One medical application of ferromagnetic material is the so-called "magnetic fluid hyperthermia" (MFH). MFH is based on a controled transfer of power to magnetic nanoparticles by an alternating magnetic field which results in local generation of heat. Depending on the equilibrium temperature of the tumour tissue, this heat may either destroy the tumor cells directly (thermoablation) or result in a synergic reinforcement of radiation efficacy (hyperthermia) (Latorre et al., 2009). Much of the current research focuses on iron oxide nanoparticles which have proven their feasibility in animal experiments (Johannsen et al., 2005; Matsuoka et al., 2004)] and are now under clinical trials (Johannsen et al., 2007). The protective carbon shell of the magnetic CNT may avoid the toxicity problem associated of iron oxidation. Degradation of the filling materials is avoided and potential toxicity and adverse effects suppressed, so that CNT become effective carrier system on the nanometer scale. Magnetic studies on the feasibility of iron-containing carbon nanotubes for magnetic hyperthermia have been shown (Krupskaya et al., 2009). The authors presented a detailed magnetic study of iron containing carbon nanotubes (Fe-CNT), and highlighted their potential for contactless magnetic heating in hyperthermia cancer treatment. DC magnetization studies showed a different magnetic response of Fe-CNT powder compared to Fe-CNT dispersed in aqueous solution. The ferromagnetic Fe-CNT in powder did not show any hysteresis when dispersed in a liquid.

Such magnetic behaviour implies the rotation of the magnetic moments following the magnetic field. The fact that in the frozen dispersion they observed a ferromagnetic-like hysteresis, clearly indicates that the rotation of the magnetic moments in liquid is associated with the motion of the whole nanotube, but not of the moments within each particle. AC susceptibility measurements showed an increase of the susceptibility of Fe-CNT in a liquid dispersion compared to the powder. Again, this can be explained by the motion of Fe-CNT in the AC magnetic field. AC inductive heating experiments showed a substantial temperature increase of Fe-CNT dispersions in AC magnetic fields, which was dependent on the magnetic field. In summary, these results showed the feasibility of Fe-containing CNT in magnetic hyperthermia. Another intriguing application of magnetic CNTs is the so-called magnetofection.

Magnetic nanoparticle-based transfection methods are based on the principles developed in the late 1970s by Widder and others for magnetically targeted drug delivery. Transfection using of magnetic microparticles was first demonstrated in 2000 by Cathryn Mah, Barry Byrne and others at the University of Florida, both in vitro with C12S cells and in vivo in mice using an adenoassociated virus (AAV) linked to magnetic microspheres via heparin (Ma et al., 2002). Since these initial studies, the efficiency of this technique has been demonstrated in a variety of cells (Dobson, 2006). The technique is based on the coupling of genetic material to magnetic nanoparticles. In the case of in vitro magnetic nanoparticle based transfection, the particle/DNA complex (normally in suspension) is introduced into the cell culture where the field gradient produced by rare earth magnets (or electromagnets) placed below the cell culture. They increase sedimentation of the complex and increase the speed of transfection. In vivo applications apply magnetic fields focused over the target site have the potential to not only enhance transfection but also target the therapeutic gene to a specific organ or site within the body. Generally, particles carrying the therapeutic gene are injected intravenously and strong, magnets gradients are applied to capture the particles as they flow through the blood-stream. Once captured by the field, the particles are held at the target, where they are taken up by the tissue. The therapeutic genes can be released either via enzymatic cleavage of the cross-linking molecules, charge interactions, or degradation of the polymer matrix. Alternatively, if the DNA is embedded within the matrix, such as with hydrogels, alternating fields may be applied to heat the particles and release the genes from the magnetic carrier. An application of magnetically-driven drug-carrying CNTs in cells was suggested (Cai et al., 2005).

Cai et al have designed an alternative physical method of *in vitro* and *ex vivo* gene transfer, called nanotube ''spearing'': CNTs 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 covalently functionalized with a DNA strain containing the sequence coding for the enhanced green fluorescent protein (pEGFP-c1). Dividing and non dividing cells like Bal17, B-lymphoma, *ex vivo* splenic B cells and primary neurons were grown on a substrate and incubated with magnetic pDNA/CNT. First a rotating magnetic field drives the nanotubes to mechanically spear the cells. In a subsequent step, a static magnetic field pulled the tubes into the cells. The spearing set-up and procedure are illustrated in Figure 6. The cells were efficiently transfected as confirmed by fluorescent microscopy measurements. They demonstrated that both spearing steps are necessary for efficient transduction. The efficiency was equal to viral approaches even for non-dividing cells, such as primary B cells and neurons which are generally more difficult to transfect.

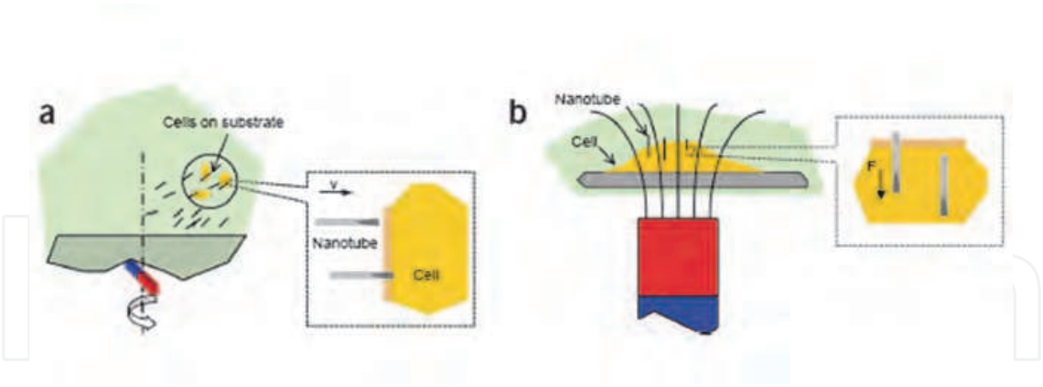

Fig. 6. Two step procedure for nanotube spearing: a) a rotating magnetic field drives nanotubes to spear the cells on a substratum (surface) and b) a static field pulls nanotubes into the cells. (Cai et al.,2005).

The same researchers investigated the compatibility of the nanospearing with primary *ex vivo* cultures of B lymphocytes (Cai et al., 2007). They reported that, by applying the original 2-step procedure of the nanospearing, they noted by phase contrast microscopy B-cell blasts and cellular aggregation, suggesting non-specific B-cell activation (i.e., increased cell size, activation of signal transduction pathways, increased protein and RNA content, normally associated with stimulation by extrinsic growth factors). They conducted a comprehensive characterization of the potential effects of exposing *ex vivo* primary B cells to CNTs with respect to cellular activation, survival, and signal transduction. The original multistep nanospearing protocol was modifield to a single step process (cells were speared by positioning the cover slips directly over a permanent magnet) and by using non-covalent DNA immobilization strategy. The results indicate that CNT-mediated nanospearing of primary B cells does not result in non-specific activation of naıve B lymphocytes but facilitate efficient delivery of nucleic acids into B cells (Figure 7).

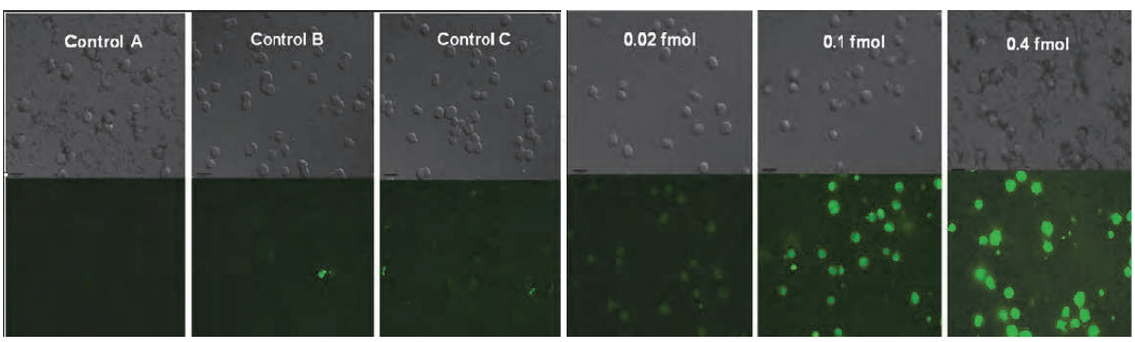

Fig. 7. CNT were covalently functionalised with poly(L-lysine) (PLL-CNT) and incubated with FITC-oligos in order to achieve FITC-oligo–PLL–CNTs. The pictures show the delivery of FITC-oligo–PLL–CNTs into B lymphocytes following nanospearing. Bal17 cells were speared with 0.4 fmol non-FITC-oligo–PLL–CNT complexes (Control A) or with 0.02, 0.1, or 0.4 fmol FITC-oligo PLL–CNTs. More control results are shown as 'Control B' (FITC-oligo alone) and 'Control C' (FITC-oligo-PLL) with the corresponding reagent treatments to cell following the spearing procedures (Cai et al., 2007).

Recently, Cai and colleagues showed that CNT–cell complexes form in the presence of a magnetic field (Cai et al., 2008). The complexes were analyzed by flow cytometry as a quantitative method for monitoring the physical interactions between CNTs and cells. They observed an increase in side scattering signals, where the amplitude was proportional to the amount of CNTs that are associated with cells. Even after the formation of CNT–cell complexes, cell viability was not significantly decreased. The association between CNTs and cells was strong enough to be used for manipulating the complexes and thereby conducting cell separation with magnetic force.

2.7 Summary and concluding

The emergence of CNTs in the biomedical field have raising great hopes. CNTs have been proposed as components for DNA and protein biosensors, ion channel blockers and as bioseparators and biocatalysts. Their use is becoming relevant in many fields such as neuroscience research and tissue engineering. CNTs 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 orthopaedic sites They have also been used as new platforms to detect antibodies associated with human autoimmune diseases with high specificity. This 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. Plus CNTs covalently modified with DNA and PNA (peptide nucleic acid) have led to innovative systems for hybridization of complementary DNA strands allowing ultrasensitive DNA detection. Furthermore, CNTs have also emerged as a new efficient, alternative tool for transporting and translocating therapeutic molecules. The development of new and efficient drug delivery systems is of fundamental importance in improving the pharmacological profiles of many classes of therapeutic molecules. CNTs can be functionalised with bioactive peptides, proteins, nucleic acids and drugs, and used to deliver their cargos to cells and organs.

With the prospect of gene therapy, cancer treatments, and innovative therapies, the science of nanomedicine has become an fast-growing field that has an incredible ability to bypass barriers previously thought unavoidable. The properties and characteristics of CNTs are still being explored and scientists have barely begun to mine the potential of these structures. CNTs have already proven to serve as safer and more effective alternatives to previous drug delivery methods. They can serve as nano-vehicles, carrying therapeutic drugs, vaccines, and nucleic acids deep into the cell to targets previously unreachable in response to static and dynamic energetic fields.

Carbon nanotubes have the potential of revolutionizing bio-medical research. They frequently show superior performance over other nanoparti-cles. The advantage lies in their unique combination of electrical, magnetic, optical and chemical properties which can be exploited in the development of new classes of CNT-based drugs and therapy.

In this chapter we showed the extraordinary physical properties of carbon nanotubes and how such properties can play an important role in their biomedical application. In particular we discussed to the magnetic properties of CNTs and considered how they can improve the imaging, the cell labelling, the guided homing of stem cell and the selected delivery of drug towards tumour cells.

The magnetic properties of nanotubes can be also exploited to study in vivo biodistribution by using magnetic resonance. Al Faraj and colleagues performed a non-invasive follow-up in vivo study to evaluate the biodistribution of CNTs and effect of nanotube deposition after

exposure (Al Faraj et al., 2009). They used both helium-3 and proton magnetic resonance (MRI) to evaluate the biodistribution and biological impact of raw single-wall CNTs (raw- SWNTs) and superpurified SWNTs (SP-SWNTs) in a rat model. Superpurified SWNTs, thanks to low content of metal residuals, represent the best samples in terms of biocompatibility, but it is not possible with only MRI to fully understand their biodistribution in vivo.

In a series of other experiments, the effectiveness of cellular imaging as T1 and T2 contrast agent was investigated.

Cai et al. have designed an alternative physical method of in vitro and ex vivo gene transfer, called nanotube ''spearing''. The tubes were functionalized with DNA plasmids immobilized onto the CNTs and subsequently speared into target cells via external magnetic fields.

Moreover, Cai and colleagues showed that CNT–cell complexes are formed in the presence of a magnetic field (Cai et al., 2008). Even after the formation of CNT–cell complexes, cell viability was not significantly decreased. The association between CNTs and cells was strong enough to be used for manipulating the complexes and thereby con-ducting cell separation with magnetic force.

We demonstrated that, by combining the magnetic response with the ability to interact with cells (Vittorio et al., 2011a), CNTs can also be used for cell manipulation. Our data showed that cells treated with nanotubes with ~3% iron content are able to internalize this nanoparticle and to move towards a magnetic source.

In cancer therapy, there is potential to prevent cell migration in metastasis. For example, CNTs could be functionalized so they bind selectively to cancer cells, by applying external magnetic field in order to constrain cancer cell migration. In addition, cancer cells treated with CNTs could act as intracellular transporters of chemotherapies or as heater probes. Moreover magnetic fields could be used to control movement and location of MSCs cultured with carbon nanotubes to shepherd MSCs towards the magnetic source, increasing the accumulation of cells into the target organ (liver). Undoubtedly, MWCNTs hold a distinct potential for use as nano-devices to improve therapeutic protocols for transplantation and homing of stem cells in vivo.

We believe Nanomedicine will revolutionize the future of medical approaches.

3. Acknowledgment

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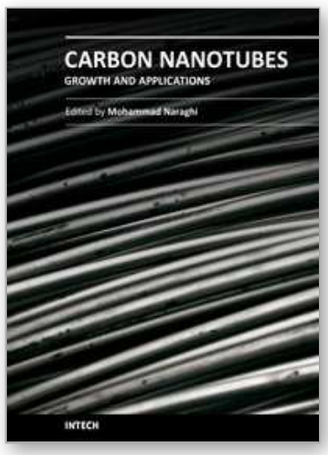

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Carbon Nanotubes are among the strongest, toughest, and most stiff materials found on earth. Moreover, they have remarkable electrical and thermal properties, which make them suitable for many applications including nanocomposites, electronics, and chemical detection devices. This book is the effort of many scientists and researchers all over the world to bring an anthology of recent developments in the field of nanotechnology and more specifically CNTs. In this book you will find:

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