Publishable executive summary

A European research team led by researchers from the CRIM laboratory of the Scuola Superiore Sant'Anna in Italy commenced work in December 2006 on the important framework-6 EC project 'NINIVE' (NMP4-CT-2006-033378). The multidisciplinary team involved in the project also includes scientists from the Center for Drug Delivery Research of the ULSOF (UK), the MPI-FKF fuer Festkoerperforschung (D), Thales research & technology (F), the Institute of Neuroscience from CNR (I) and Nanothinx S.A. (GR).

The main objective of the NINIVE project (http://www.ninive-project.org/) is the development of a non-viral vector for a safe and efficient gene transfection and Targeted drug delivery. The system proposed by NINIVE is based on use of carbon nanotubes (CNTs) which consist exclusively of carbon atoms arranged in a series of condensed benzene rings rolled-up to form single or multi-walled tubular structures. This novel nanomaterial belongs to the family of fullerenes, an allotropic form of carbon. CNTs have nanometric dimensions and unique physicochemical properties which make CNTs unique materials with several potential applications especially in the biomedical field. In the NINIVE project CNTs will act as transporters of genes in addition to being functionalized with appropriate ligands to enable specific binding to receptors over-expressed by the cells targeted for transfection. A solution containing a myriad of these coated and functionalized nano-vectors will be administered by local injection in the target tissue. These nano vectors will home on and bind to the intended target cells. In the NINIVE project, two strategies of cell transfection will be investigated: (i) cell up-take of the gene-carrying nano-vectors by endocytosis and (ii) genes from the nano-vectors will be transferred in the recipient cells via electroporation (i.e., permeabilization of cell membrane by application of short-duration electric pulses). With this mechanism, cell permeabilization will be induced by CNTs on exposure to external electromagnetic fields by exploiting the nanotransducers properties of CNTs.

Following in-vitro R & D, proof of efficacy of the NINIVE system of targeted gene delivery will require in vivo validation. For this purpose, a specific neurological disorder will be treated to confirm its therapeutic efficacy. The implementation of this ambitious but eminently realizable project requires a multidisciplinary approach and the integration of broad range of expertise in the fields of nanotechnologies, biotechnologies, communication technologies and neuroscience.

A review of the current state of the art documents the very extensive published literature on the use of carbon nanotubes in biomedicine. A key advantage of carbon nanotubes is their ability to cross plasma membranes allowing their use for the delivery of therapeutic molecules in a manner that resembles cell-penetrating peptides. CNTs thus have a distinct potential as components of efficient drug and gene delivery systems. The translocation of CNTs loaded with peptides, proteins, nucleic acids and drugs into mammalian cells has already been documented. Moderate biological effects have been reported in these studies but more importantly, these reports have confirmed that CNTs can offer advantages in terms of their pharmacological utilization.

Despite the numerous published reports, there are very few examples on the exploitation of the unique electrical, optical, thermal and spectroscopic properties of CNTS in the biomedical context despite the fact that the reported literature in the past five years demonstrates the clear interest in the exploitation of the physical properties of CNTs for cell therapy. Of course, the real breakthrough of the NINIVE project, as yet unrealized, would include the development of a new therapeutic modality based on CNT nanotechnology providing an effective and safe means of targeted in vivo gene and drug therapy.

During this first year of project, significant progress has been regarding achievement of the project goals. The two models of brain disease (Rett syndrome and stroke) selected for the experimental evaluation of the gene therapy based on the NINIVE vectors in rodents have been developed and tailored to the requirements of the project.
The various mechanisms related to the functionality of the NINIVE nanotransducers, e.g., diffusion within the target tissue, targeted cell binding, cell uptake by endocytosis and via electroporation were investigated

using both experimental in-vitro studies and finite-element modeling. Standard Operating Procedures (SOP) have been defined. These provide succinct information on the architecture of the NINIVE vectors and the Material & Methods of the project.

The idea central to the proposed NINIVE vector architecture is the "wrapping" of CNTs with a polymer able to bind the desired biomolecules (DNA, cell ligands, fluorescent molecules). The methodologies used for CNT "wrapping" are twofold: physical absorption of the polymer coating onto the CNT surface from a polymer solution and the electrochemical deposition onto the nanotube surface. The main advantage of the physical absorption is its simplicity and reliability. On the other hand, electrochemical deposition allows for precise control on the thickness of the polymer coating.
Ad hoc carbon nanotubes were produced for the project using a material of the highest possible purity and uniformity of dimensions and properties. The test sample was tested and approved for its quality and purity by partners with expertise in biomedical field. All samples used in the experiments were characterized in terms of diameter, length, number of layers, carbon purity, type and component metal particles and amorphous carbon. A detailed characterization of the nanotube morphology (structure, grapheme sheet distance, defects and chirality) was also performed in view of its relevance to the design and properties of the CNT antenna.

A careful evaluation of the cytotoxicity of the samples was performed and other issues were also considered such as the purity of the CNTs, native CNT cytotoxicity as well as the cytotoxicity of the polymer coating. Different polymers have been tested and evaluated for the coating of CNTs, e.g., Pluronic F127, chitosan, chitosan glycol, betaine-chitosan, polylysine and PEI. The results of the in vitro experiments confirmed good biocompatibility of all the samples tested. Long-term toxicity studies have also been performed (3 weeks of continuous incubation in cell culture media modified with the CNTs). In these studies the best biocompatibility was achieved with use of purer (99%) CNT samples.
A series of experiments was performed to investigate possible toxic effects of CNTs in vivo. Although many studies have been reported on the toxicity of CNTs in cell cultures few studies are available on CNT toxicity in vivo and none of them have investigated CNT toxicity to brain tissue. Furthermore all the available reported in vivo studies have used systemic treatment by injection into the bloodstream or inhalation while the effects of focal delivery of CNTs to specific region of the body, mimicking targeted administration of therapeutic CNTs by local injection to diseased tissue have not been explored. We have addressed this issue by performing stereotaxic injections of CNTs suspensions into the cerebral cortex and assessing the tissue response by histology two days post injection. For these experiments, we administered CNTs coated with a surfactant (Pluronic F127) that we had studied extensively in mixed neuronal-glial primary cultures. The results of these in-vivo studies confirmed that the CNTs injections do not cause major adverse alterations in the morphology of brain tissue or substantial necrosis. Indeed the volumetric size of the lesion in the CNT treated animals was not different from that caused by the injection of the control solution.

Some preliminary experiments have also been carried out on the dynamics of the internalization and degradation of CNTs. The findings of these studies are in agreement with the published literature, i.e., nanotube uptake by living cells occurs through an energy-independent non-endocytotic pathway. We tested different nanotube samples and we found differences in the sample uptake that could be attributed to the differences in sample physical properties (e.g., nanotube length). Specifically, the experimental data show that sub-1 μm CNTs are easily accumulated within cells.

We tested many positively charged polymers able to bind DNA to determine the optimal coating of CNTs for this purpose. The results indicate that chitosan and PEI coated CNTs are able to bind DNA. Chitosan-CNT and PEI-CNT complexed with pDNA were found to be not toxic. Finally pDNA: polymer-CNTs conjugates were tested for their ability for gene transfection. The experimental results revealed a substantial increase in the percentage of transfected cells compared to cells treated with naked DNA (negative control).

The performance of CNTs as receiver antennas was investigated as a function of CNT specifications (i.e., diameter, length and resistance) and of the transmission frequencies. Finite element modeling demonstrated the ability of a CNT to function as a receiving antenna. In parallel, a study to investigate the functioning of a nano-antenna inside the body and the influence on human tissues of radio frequency (RF) signals transmitted

to the implanted source was performed. This study was necessary to determine the limits for the wireless transmission power which should not be exceeded to avoid damage to the tissue surrounding the implanted NINIVE vectors.

The feasibility of the two methodologies proposed in NINIVE project (cell transfection via cell up-take of the vectors and gene transfer to the cells via electroporation) was investigated both from biomedical and technical viewpoints. In the case of unassisted cell uptake, the experimental results demonstrated that PEI-CNTs and chitosan-CNTs are able to bind DNA and these vectors are also effective for gene transfer by natural internalization.
Our efforts were thus devoted to demonstrate that transfection can be improved by transmitting energy to cells in the immediate presence of carbon nanotubes, thus enhancing temporarily cell permeabilization. To date permeabilization of mammalian cells via CNTs-induced electroporation had not been confirmed or reported. Our initial theoretical studies had provided direct evidence that release of local energy to living cells via carbon nanotubes increase significantly the permeability of cell membranes. This result predicted by finite element modeling was confirmed by experimental data involving assays involving different mammalian cell lines. The preliminary results demonstrated that CNT-enhanced permeabilization occurs with high efficiency and with low voltage. In contrast no permeabilization was observed in CNT-free cultures. In addition, MTT assay revealed residual high cell viability (over 90% for cells in CNT modified medium, compared to over 95% for cells in CNT- free medium).

During the first year of the project several press news have been released to European journals. The members of the Consortium have disseminated the project findings at international conferences (e.g., Cancer Nanotech, 26-28/06/2007, Paris, France; IARP - IEEE/RAS - EURON Joint Workshop on MICRO & NANO ROBOTICS, 23-24/10/2006 Paris, France; IEEE EMBC 2007, 23-26/8/2007 Lyon, France; International School on Advanced Material Science and Technology, VIII Course, Nanobiotechnologies and Nanomedicine 5-8/9/2006, Iesi, Italy) and workshops (e.g., Carbon Nanotubes for Biomedical Applications CANAPE workshop, 3/04/2007, Universitá di Tor Vergata, Rome, Italy; Nanotoxicity 2007, 26-28/06/2007, Paris, France), establishing contacts and collaborations with various groups working in the same field.

The project has generated the following major publications, patent and presentations:

V. Raffa, A. Menciassi, V. Pensabene, G. Ciofani, O. Vittorio, P. Dario, Method and device for non-invasive in vivo electroporation mediated by carbon nanotubes, PCT/IB2007/054754, November 22, 2007
L. Lacerda, S. Raffa, M. Prato, A. Bianco, K. Kostarelos, Cell-Penetrating Carbon Nanotubes in the Delivery of Therapeutics Nano Today (Elsevier) 2007, 2 (6): 38-43
M. Marchi , A. Guarda, A. Bergo, N. Landsberger, C. Kilstrup-Nielsen, G. Ratto, M. Costa, Spatio-Temporal Dynamics and Localization of MeCP2 and Pathological Mutants in Living Cells, Epigenetics 2007, 2 (3): 17965612
V. Raffa, V. Pensabene, G. Ciofani, A. Menciassi, Carbon nanotube based vectors for gene therapy, at the International School on Advanced Material Science and Technology, VIII Course, Industrial Application of Nanotechnologies", Iesi, September 2006
V. Pensabene, O. Vittorio, V. Raffa, A. Menciassi, P. Dario, Investigation of CNTs interaction with fibroblast cells, Proceedings of the 29th Annual International Conference of the IEEE EMBS Cité Internationale, Lyon, France, August 23-26, 2007: 6620-6623
V. Raffa, V. Pensabene, G. Ciofani, A. Menciassi, P. Dario, Gene therapy via CNTs-based artificial vectors, VII Convegno Nazionale Istituto Nazionale di Biostrutture e Biosistemi, Rome, October 19-20, 2006.
V. Pensabene, O. Vittorio, V. Raffa, A. Menciassi, P. Dario, Cell motion and sorting with magnetic carbon nanotubes, 6th International conference on Fine Particle Magnetism, Roma, October 9-12, 2007.
V. Pensabene, O. Vittorio, V. Raffa, A. Ziaei, A. Menciassi, P. Dario. Neuroblastoma cells displacement by magnetic carbon nanotubes IEEE Transactions on NanoBio Science, in press

NINIVE web site has been implemented. It aims at divulging, in accordance with IPR restrictions, the main innovative aspects, which evolve during the development of the project (http://www.ninive-project.org)/). In the website home pages, links to the following European organizations have been established:

http://cordis.europa.eu/ , European Commission services, http://www.esgct.org, European Society Gene and Cell Therapy (ESGCT), http://www.emea.europa.eu/, European Medicines Agency (EMEA)Link, http://www.carbio.eu/carbio, CARBIO (Multifunctional Carbon Nanotubes for Biomedical Applications), http://www.clinigene.eu/, CLINIGENE-NoE (Network of Excellence), http://www.nanowerk.com/nanotechnology/labs.html, A Nanotechnology Portal.

A brochure of the project has been uploaded on the website (http://www.ninive-project.org/Download%20Area.html): all the website visitors can download and print the leaflet for dissemination purposes. A video which describes and shows the main concepts of NINIVE project have been committed to an external company (Beglam SRL). It can be downloaded from the NINIVE website at the page http://www.ninive-project.org/Download%20Area.html.

Useful contacts:
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Project Logo:

Non Invasive Nanotransducer
for In Vivo gene thErapy,
Proposal/Contract no.: STRP 033378

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