NanoInnovation 2016 — Rome, 20-23 September — Conference & Exhibition

Evidence for electro-chemically mediated interactions between Carbon Nanotubes and the biological environment

Silvana Fiorito

National Research Council (CNR), Rome

silvana.fiorito@ift.cnr.it

SAPIENZA UNIVERSITÀ DI ROMA

CNRS — UNIVERSITE D'ORLEANS

UNIVERSITÉ TOULOUSE III PAUL SABATIER

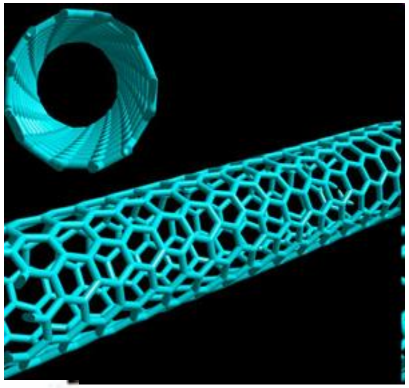
Consiglio Nazionale delle Ricerche

- **CNTs** are composed of graphene sheets rolled up one on the other . Carbon atoms are linked in hexagonal shapes, with each carbon atom covalently bonded to three other carbon atoms.

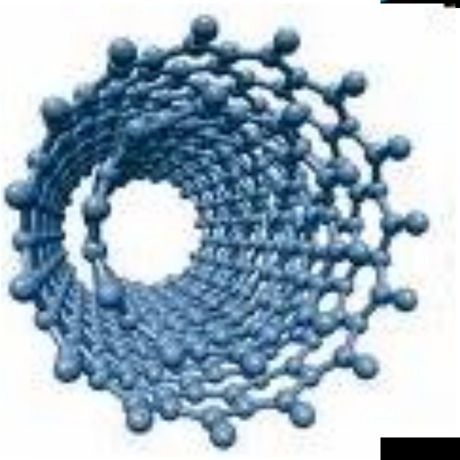
- **possess remarkable electrical, chemical, mechanical and structural properties that make them extremely attractive for the task of biomedical applications**

- **However, only a few practical applications have materialized due to several issues concerning their potential toxicity.**

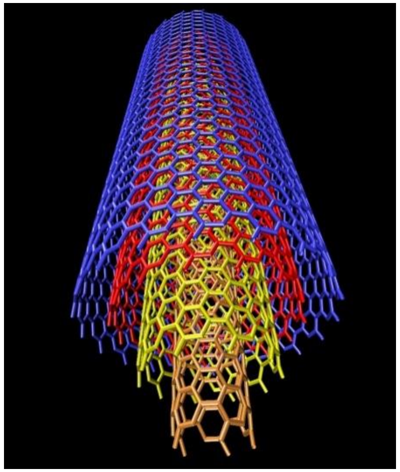
- **The main issue associated with CNT biocompatibility and/or toxicity is represented by their heterogeneity: each single sample is different from each other due to the huge variety of carbon-based and inorganic compound-based (i.e., residual metal catalyst) impurities, to different electrochemical properties, to different shape, lenght, aggregation state, surface functionalities etc.**



SWCNTs



DWCNTs



MWCNTs

Electrical properties

- **CNTs behave electrically as metal or semiconductor depending on their structure,namely their diameter and helicity**

- **CNTs have the ability to mediate electron transfer reactions with electroactive species in solution**
- *Nugent, J. M. Et al., Nano Lett. 2001, 1, 87.*

- **Residual catalyst metallic impurities (Co, Mo, Fe) are responsible for the electrocatalytic activity of CNTs toward the reduction or oxidation of important compounds (hydrogen peroxide, halothane, amino acids, peptides, glucose, cytochrome c, catecholamines, neurotransmitters, or ascorbic acid)**

- *Pumera M. and Iwai H.., J. Phys. Chem. C 2009, 113, 4401*
- *Ambrosi, A. et al., Chem.sEur. J. 2010, 16, 1786.*
- *Batchelor-McAuley, C. Et al., Sens. Actuators, B 2008, 132, 356.*
- *Sˇ ljukic´, B. Et al., Nano Lett. 2006, 6, 1556*

Carbon nanotube biomedical applications

- CNTs are under active investigation for their potential use in several biomedical applications in which their electrical properties can be exploited
- Many of these studies have focused on the potential of these nanomaterials to interact with electro-sensitive cells, mostly neural and bone cells, due to their electrical properties
- Their electrical properties have been shown to make them perfect candidates for various prosthetic devices, including bone and joint repair or useful biocompatible materials for helping neuronal cells to proliferate

CNT-based structures have been explored for the purpose of healing neurological and brain-related injuries

- **CNTs stimulate isolated neurons (electro-sensitive cells) in culture**
- **Stimulate nerve cells functions (e.g., electrical regenerative properties and synaptic activity) and nerve cell morphological features (e.g., adhesion, growing and neurite/dendrite extension abilities)**
- **The conductivity of CNTs can help neuron growth on nanotubes,**
- **Substrates prepared from MWCNTs and SWCNTs are biocompatible platforms for neuronal growth and differentiation**
- **CNTs can directly stimulate brain circuit activity**

- *Supronowicz PR, et al., J Biomed Mater Res A 2002; 59: 499–506.*
- *Mattson MP, et al., J Mol Neurosci 2000;14: 175–82.*
- *Hu H, et al., Nano Lett 2004; 4:507–11.*
- *Lovat V. et al. Nano Lett 2005;* ;5(6):1107-10
- *Mazzatenta A. et al. J Neurosci 2007; 27(26): 6931-6936*
- *Sucapane, A. et al. J. Nanoneurosci. 2009, 1, 10–16;*
- *Fabbro A. et al. PlosOne 2013, 8 (8), e73621*
- *Monaco AM and Giugliano M. Beilstein J. Nanotechnol. 2014, 5, 1849–1863*

Effects of CNTs on electro-sensitive cells

- **Thin layers of DWNTs can serve as effective substrates for neural cell culture**
  Béduer A. et al. Langmuir 2012, 28,17363

- **Short MWCNTs Promote Neuronal Differentiation of PC12 Cells via up- regulation of the Neurotrophin Signaling Pathway**
  Meng L. et al. Small 2012 Nov 7. doi: 10.1002/smll.201201388

- **Carbon nanotubes: artificial nanomaterials to engineer single neurons and neuronal networks.**
  Fabbro A. et al. ACS Chem Neurosci 2012, 3, 611

- **Carbon nanotube rope with electrical stimulation promotes the differentiation and maturity of neural stem cells**
  Huang YJ et al. Small 2012, 8, 2869

- **Neural differentiation of mouse embryonic stem cells on conductive nanofiber scaffolds**
  Kabiri M. et al. Biotechnol Lett 2012, 34, 1357

- **Carbon nanotubes and graphene as emerging candidates in neuroregeneration and neuro-drug delivery**
  Agnes Aruna J. et al.Int J Nanomed 2015:10 4267–4277

The deep understanding at a molecular level of such electrical coupling is very poor and the studies exploring the mechanisms governing the electrochemical interactions between CNTs and cell membranes are lacking

We aimed to explore the mechanisms through which CNTs interact with different cell types, in order to verify if their effects on cell responses could be mediated by electro-chemical interactions between CNTs and cell membrane electrical properties.

CARBON 47 (2009) 2789–2804

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/carbon

Evidence for electro-chemical interactions between multi-walled carbon nanotubes and human macrophages

Silvana Fiorito[a,*], Marc Monthioux[b], Rossana Psaila[a], Pasquale Pierimarchi[a], Manuela Zonfrillo[a], Enrico D'Emilia[a], Settimio Grimaldi[a], Antonella Lisi[a], Francois Béguin[c], Robert Almairac[d], Laure Noé[b], Annalucia Serafino[a]

[a]Institute of Neurobiology and Molecular Medicine, National Research Council, via Fosso del Cavaliere 100, 00133 Rome, Italy
[b]CEMES, UPR 8011 CNRS, 29 Rue Jeanne Marvig, 31055 Toulouse, France
[c]CRMD, CNRS-University, 1b rue de la Férollerie, 45071 Orléans, France
[d]LCVN-Université Montpellier II-CNRS, Place E. Bataillon, 34095 Montpellier, France

BASIC SCIENCE

ELSEVIER

Nanomedicine: Nanotechnology, Biology, and Medicine
8 (2012) 299–307

nanomedicine
Nanotechnology, Biology, and Medicine

nanomedjournal.com

Research Article

Highly electroconductive multiwalled carbon nanotubes as potentially useful tools for modulating calcium balancing in biological environments

Annalucia Serafino, PhD[a], Anna Rita Togna, PhD[b], Giuseppina I. Togna, PhD[b], Antonella Lisi, PhD[a], Mario Ledda, PhD[a], Settimio Grimaldi, PhD[a], Julie Russier, PhD[b], Federica Andreola, BS[a], Marc Monthioux, PhD[c], Francois Béguin, PhD[d], Massimo Marcaccio, PhD[e], Stefania Rapino, PhD[e], Francesco Paolucci, PhD[e], Silvana Fiorito, MD[a,*]

[a]*Institute of Translational Pharmacology, Consiglio Nazionale delle Ricerche (CNR), Rome, Italy*
[b]*Department of Physiology and Pharmacology, University La Sapienza, Rome, Italy*
[c]*Centre d'Elaboration des Matériaux et d'Etudes Structurales, Toulouse, France*
[d]*Centre de Recherche sur la Matiére Divisée, Centre National de la Recherche Scientifique-University, Orléans, France*
[e]*Department of Chemistry "G. Ciamician," Bologna University, Bologna, Italy*

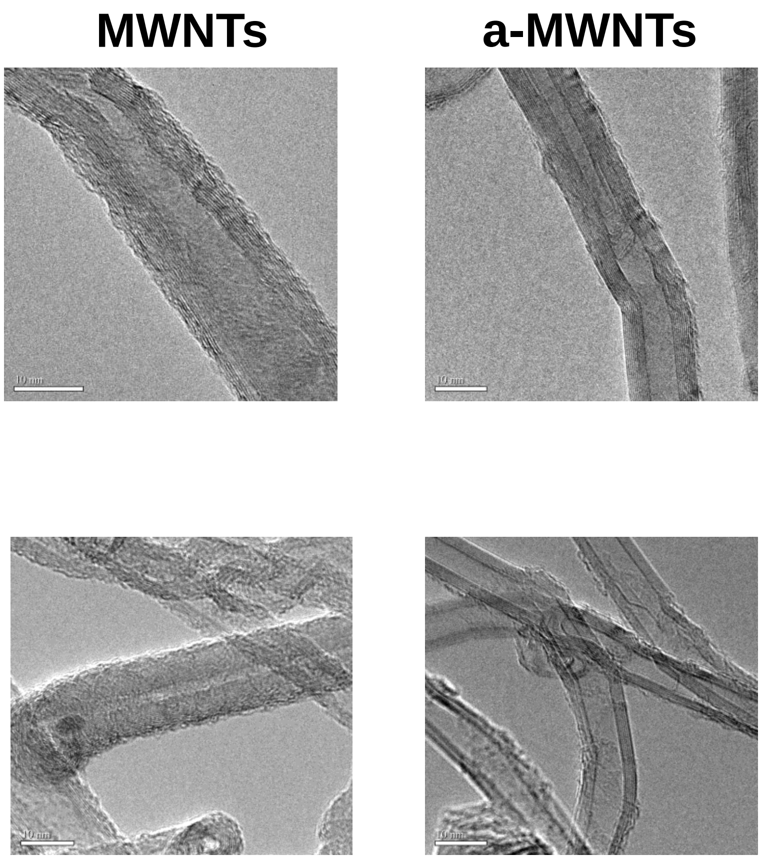
Received 8 November 2010; accepted 20 June 2011

MWNTs (CRMD, CNRS-Université d'Orléans, France)

- ❑ **As prepared and purified before annealing MWNTs (MWNTs) synthesized by CVD**

- ❑ **Annealed MWNTs (a-MWNTs) synthesized by CVD**

- ➢ **Purification by annealing at 2400ºC** made the nanotube surface get rid of most of the amorphous carbon coating, and thereby exhibit a smooth surface roughness resulting in:
  - **better nanotexture quality**
  - **much cleaner surface**
  - **much higher electrical conductivity**

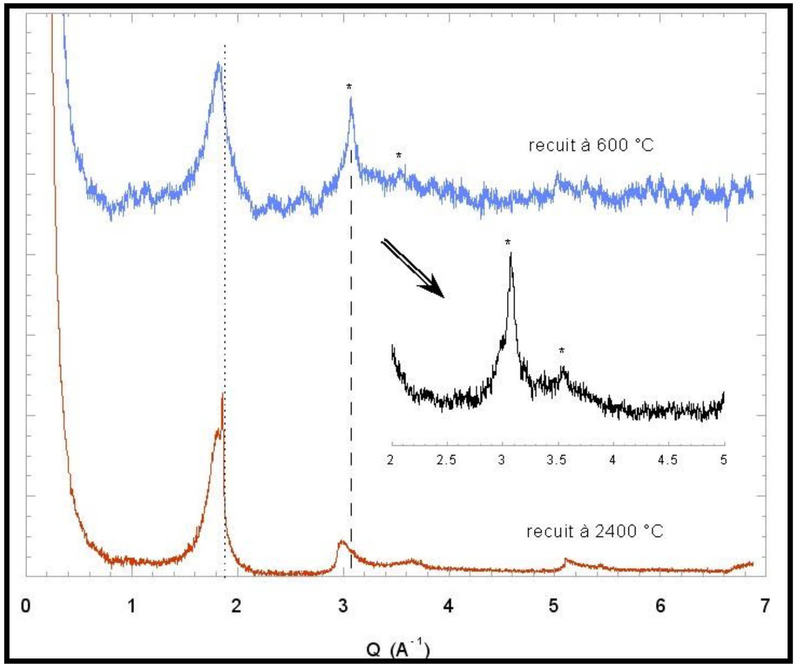


TEM

Serafino et al. Nanomedicine: NBM 2012;8:299-307

MWCNTs

X-Ray diffractography



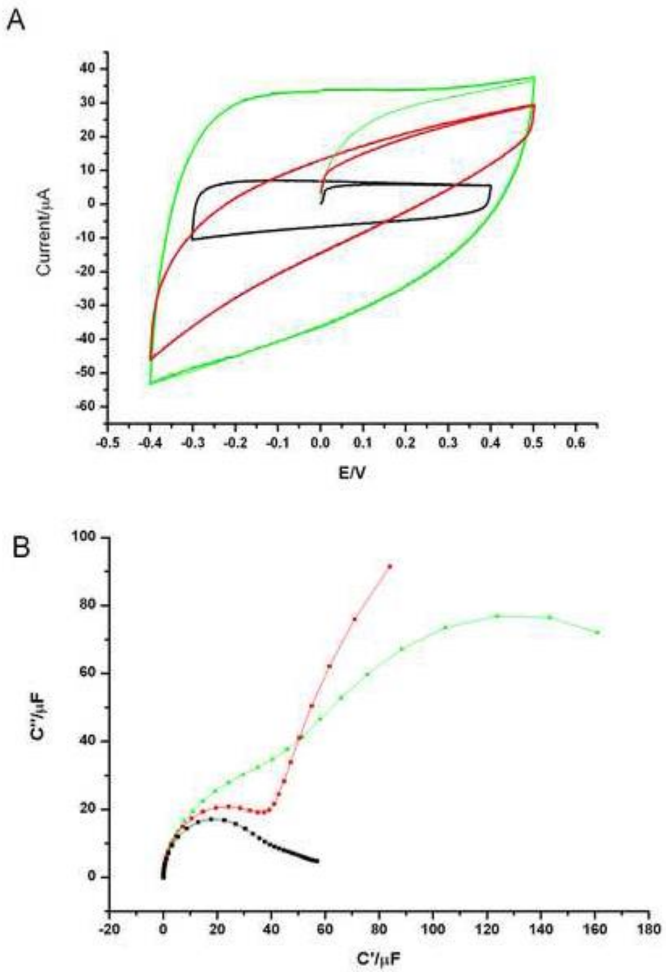
✓MWNTs (as prepared) contain cobalt, number of walls =/> 15

✓a-MWNTs (annealed at 2400°C) does not contain cobalt but a considerable amount of graphite (graphene sheets changed into graphite plaques and tubes became faceted); number of walls =/>15)

Energy Dispersive X-ray analysis

No	Sample	O	Si	Y	Ni	Co	Mg	Ca	Al	Ti	Fe	S	Cl
1	Synth. Graphite	4.73											
2	Carbon Black ENSACO porous	5.19											
3	Carbon Black ENSACO less porous	4.05											
4	SWNTs purified	7.94	0.75		0.18								
5	SWNTs heated at 800 °C in vacuum	13.20	1.03		0.19	0.71	0.7		0.09	1.15			
6	Carbon Black CNRS Toulouse	6.51											
7	Carbon Black CNRS Toulouse	4.06											
8	SWNTs MPI Stuttgart	7.24		0.06	0.70								
9	SWNTs T. Swan	4.90									0.41	0.09	0.08
10	Fe Catalyst T. Swan	38.72					31	0.24			0.71		
11	MWNTs heated at 600 °C, Univ. Orleans	8.09				0.45							
12	MWNTs heatd at 2400 °C, Univ. Orleans	8.33											
13	DWNTs Toulouse	8.44				0.27						0.16	
14	MWNTs Orleans	3.89										0.27	
15	MWNTs Orleans	6.10				1.31			1.30				

Cyclic Voltammetry (CV) and Electrochemical Impedance Spectroscopy (EIS) of MWCNTs



- **(A) Cyclic voltammetry curve of bare ITO (black line), as prepared MWCNTs on ITO (red line) and a- MWCNTs on ITO (green line) in PBS solution, scan rate 200 mV/s.**
- **(B) Electrochemical Impedance Spectrum (c-plot, complex capacitance) of bare ITO (black line), MWCNTs on ITO (red line) and a-MWCNTs on ITO (green line) in PBS solution.**
- **The current-potential curves recorded during the CV experiments showed the typical pseudo-capacitive potential of this material.**
- **The as-prepared MWCNTs film showed a diminished electrical performance with respect to a-MWCNTs: the latter film was in fact largely more conductive and with an increased electrical capacity**

Serafino A. et al, Nanomedicine, *2012, 8, 299*

Effects on biological parameters sensitive to electro-chemical stimuli

- ✓ **Intracellular Ca^{++} ion levels and cell membrane potential in electrically-sensitive nervous cells** (AtT20 cell line from rat anterior pituitary gland)

  by visualizing under fluorescent microscopy (Olympus IX51) AtT20 cells labelled with a calcium fluorescent probe (Oregon green) and then treated with both MWNTs (60μg/ml). CNTs samples were added to the cell cultures after 20 msec and Ionomycin (a Ca^{++} transporter that let Ca^{++} ions cross the cell membrane) after 70 msec from the beginning of the observation

- ✓ **Reorganization of the cytoskeleton network (Actin polymerisation) in Human Monocyte-Derived Macrophages (MDMs)** from peripheral blood by visualising, under CLSM, MDMs cells treated with MWNTs (60 μg/ml) after staining with TRITC-conjugated phalloidin.

  Polymerisation of G-actin is mediated by electro-chemical mechanisms

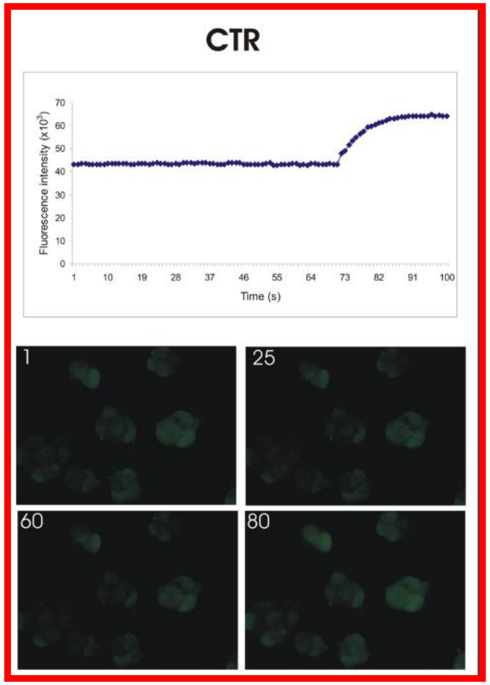
Measurement of cell membrane potential

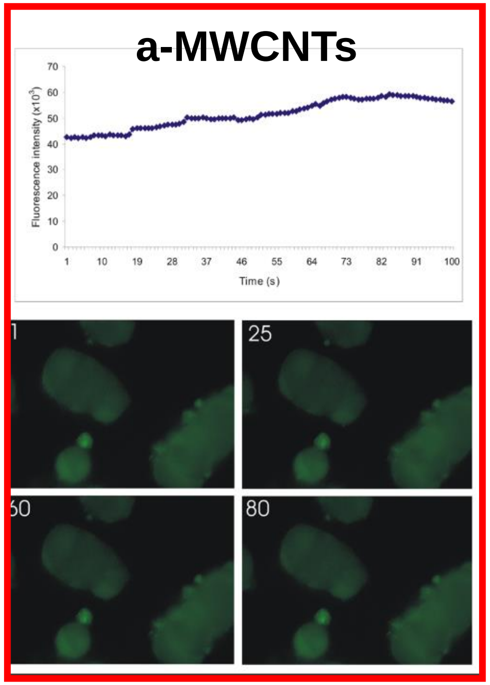
- Ionomycin, a Ca^{++} transporter that let Ca^{++} ions cross the cell membrane, is used to infer functional information on cell membrane voltage-sensitive ion channels

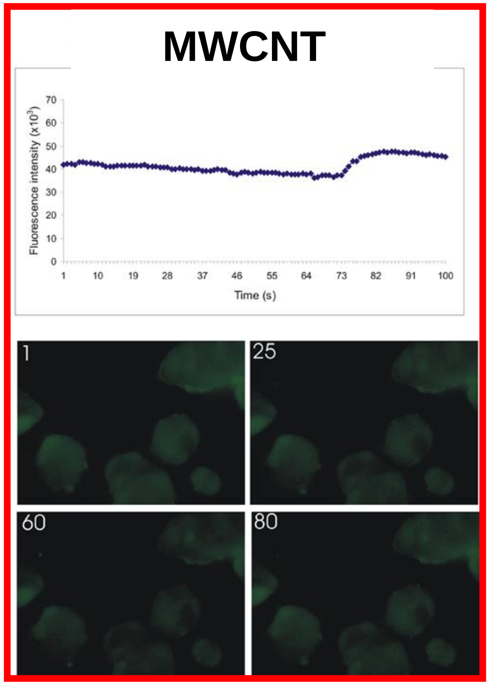
- Ionomycin is inhibited by the depolarization of the cell membrane

- **Thus, inhibition of ionomycin in presence of a-MWNTs is due to cell membrane depolarization induced by a-MWNTs**

Intracellular Ca^{++} Levels and Cell Membrane Depolarization



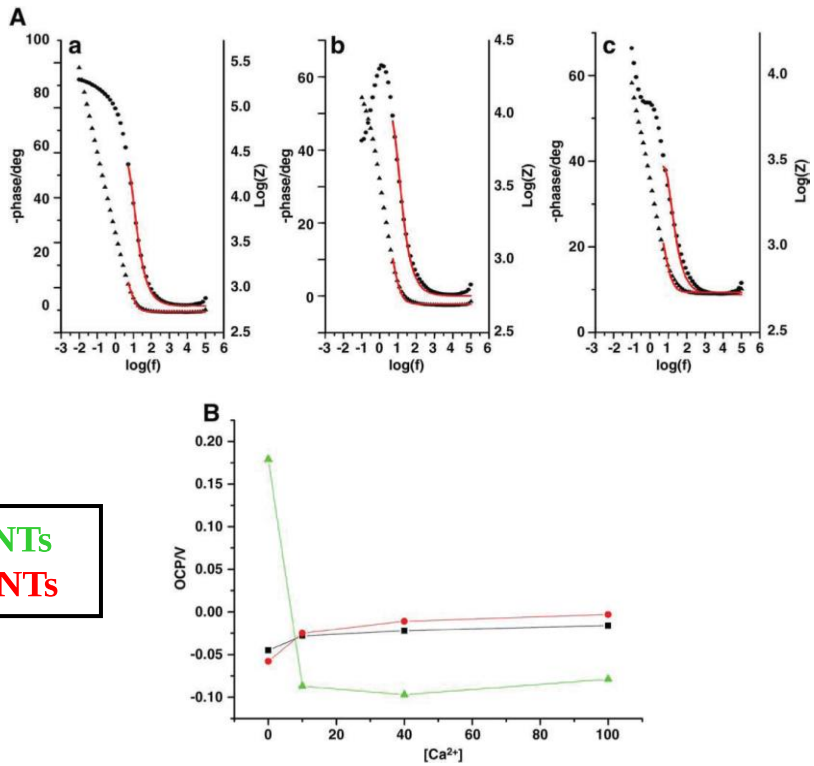




✓**marked increase in cytoplasmic calcium fluorescence intensity** in the pituitary corticotropin-derived cell line AtT20 versus controls that started to appear immediately after the addition of a-MWNTs (20 sec) to the cell culture

✓addition of ionomycin after 50 seconds did not result in any further appreciable increase of intracellular calcium

Electrochemical impedance spectrum (EIS) and Open Circuit Potential (OCP) measurements of a-MWNTs and as preparedMWNTs



a-MWNTs
MWNTs

- a-MWCNTs display enhanced electron-accepting ability and
- exhibit affinity for calcium ions with respect to MWCNTs

(A) Electrochemical impedance spectrum [bode plot, circle for phase angle and triangles for log(Z)] of bare ITO (graph a), MWCNTs on ITO (graph b), and a-MWCNTs on ITO (graph c) in PBS solution at OCP potentials and relative fitting curves (red lines) for high-frequency behavior. The fitting curves were obtained using an R(RC) equivalent circuit.

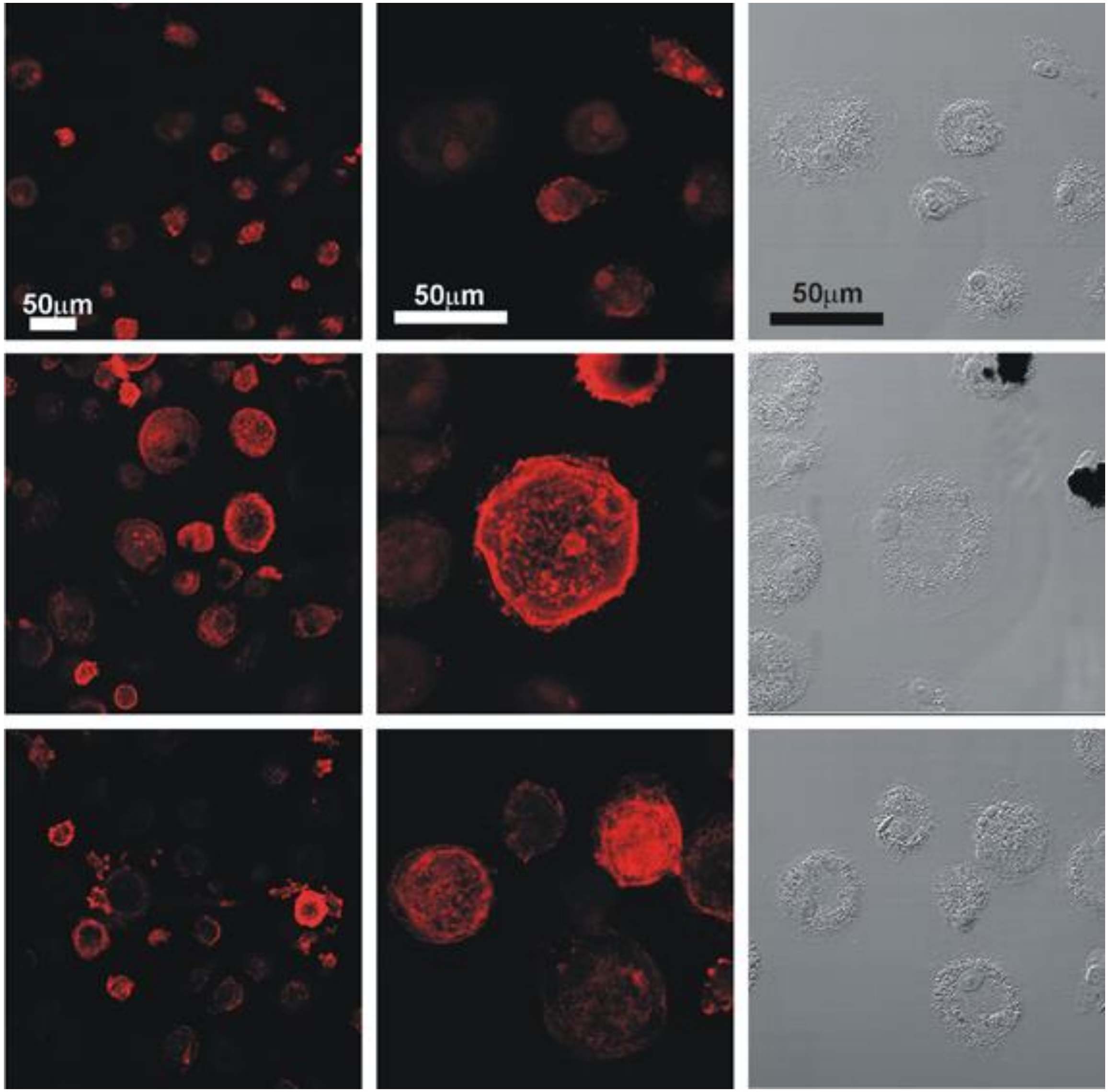
(B) Open circuit potential (OCP) values recorded for bare ITO (black line), MWCNTs on ITO (red line), and a-MWCNTs on ITO (green line) upon the addition of Ca2+ to the PBS solution. The starting OCP value of bare ITO in the PBS solution (−45 mV vs. Ag/AgCl) shifted in fact to 179 mV after the deposition of the a-MWCNTs film, thus indicating their ability to accept electrons from the ITO substrate. In the case of as-prepared MWCNTs almost the same OCP value was measured, indicative of no electron-accepting behavior.

Effect on the cytoskeleton network (actin polymerisation) in monocyte derived macrophage cells

control

a-MWNTs

MWNTs

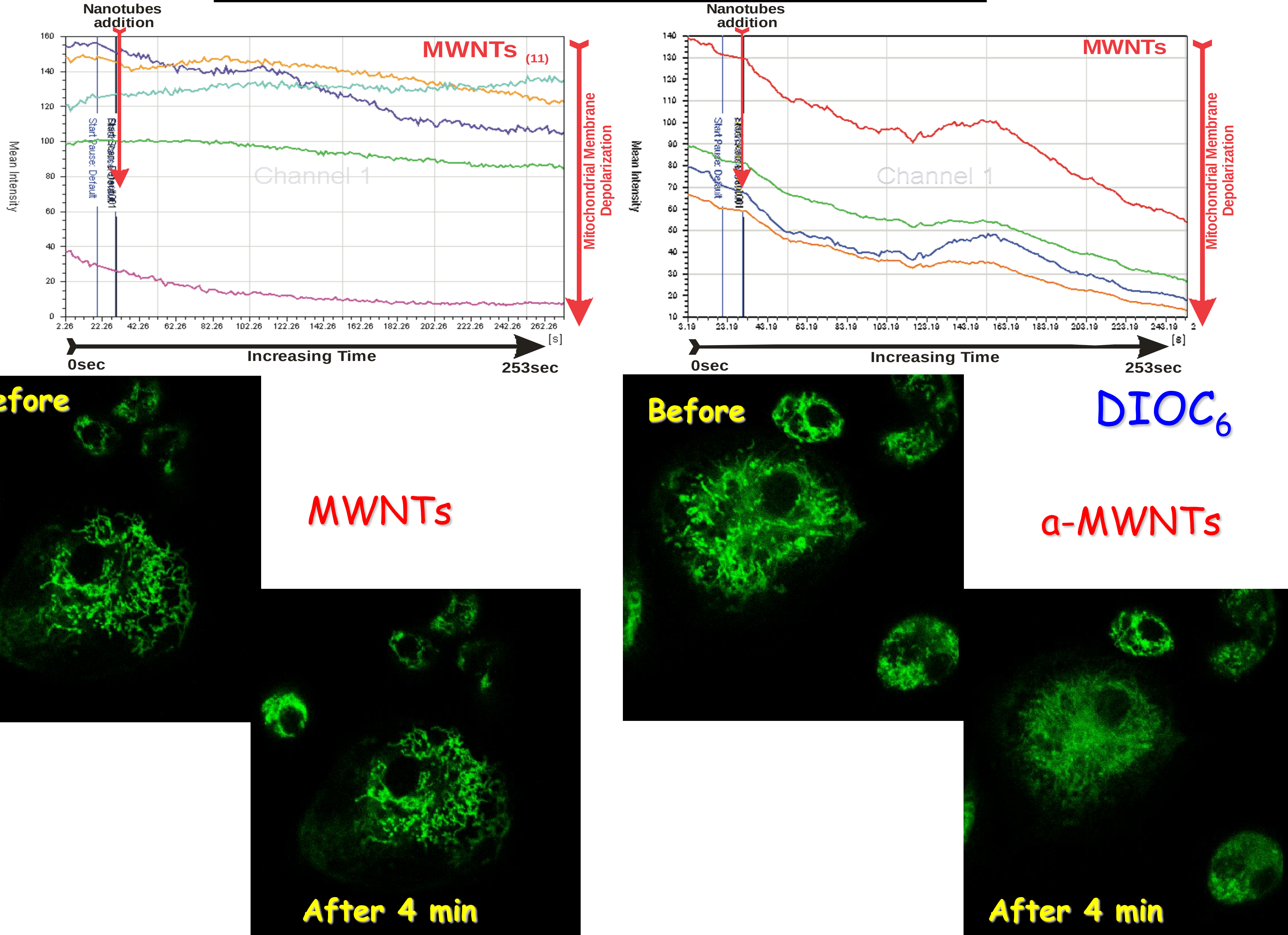


Evaluation of the mitochondrial membrane polarity*

- The inner mitochondrial membrane possesses a negative potential on the matrix side.
- A lipophilic molecule with a positive charge, such as DIOC6, accumulates in the matrix as a function of membrane potential
- If depolarization of the membrane occurs, the positive charged molecule cannot enter into the mitochondrion
- Depolarization of the mitochondrial membrane, from negative to positive is a critical event in the process leading to apoptosis (cell programmed death)

* by CLSM using the fluorescent dye DiOC*6*. **loss of membrane potential was detected as a reduced DiOC*6* fluorescent signal.** Changes in fluorescence intensity of DiOC*6* probe were measured by Live data Mode acquisition on living cells.

Mitochondrial Membrane Depolarization

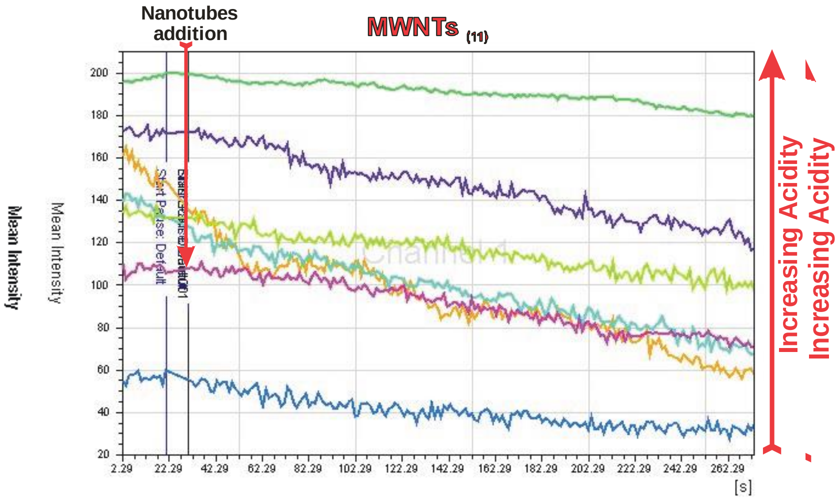


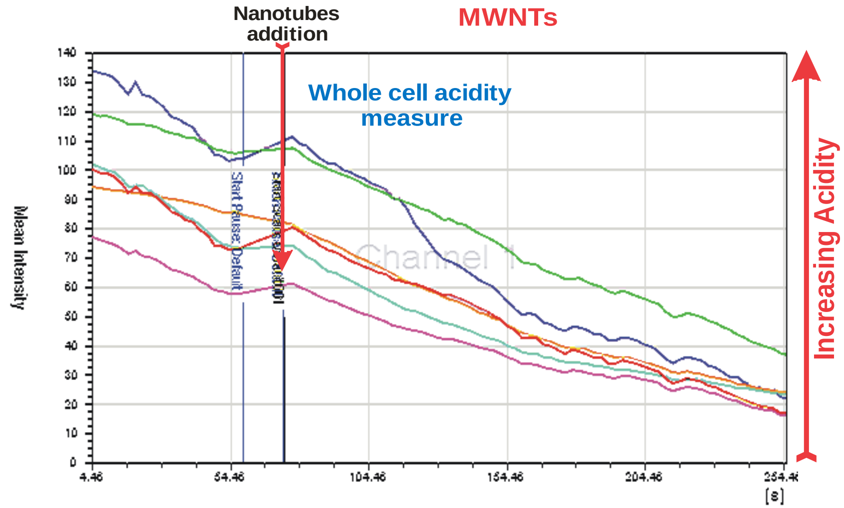
Effects on intracellular pH

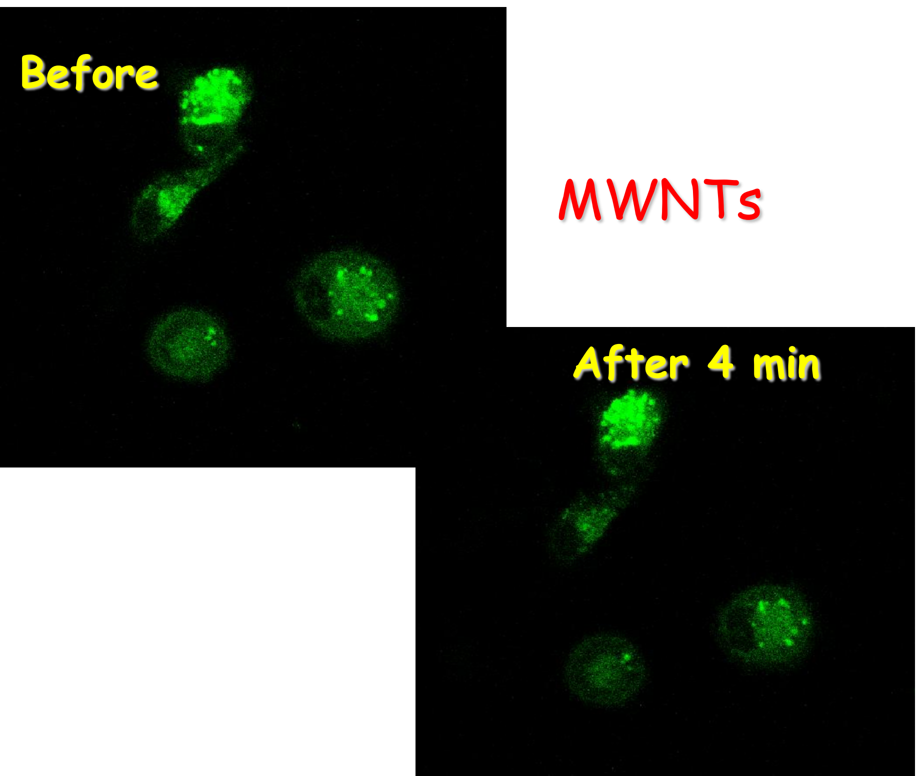
- **in Human Monocyte-Derived Macrophages (MDMs)** from peripheral blood

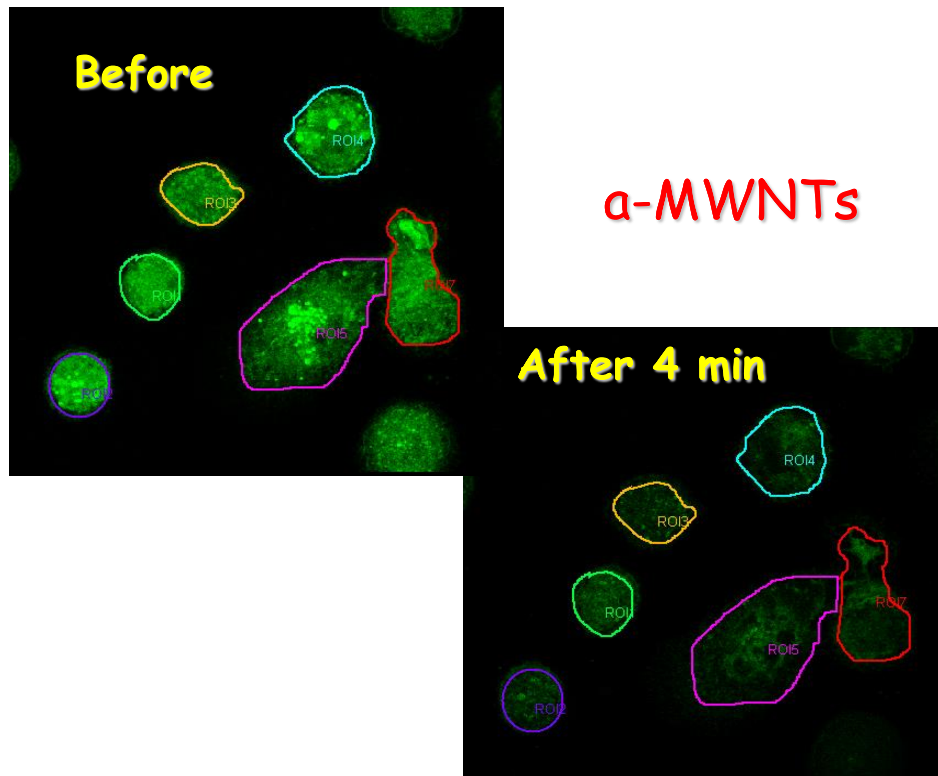
by CLSM using the LysoSensor Green DND-189 probe. The LysoSensor dye is an acidotropic probe that appear to accumulate in acidic organelles as the result of protonation (accumulation of hydrogen ions). **The Lysosensor exhibits a pH-dependent increase in fluorescence** intensity upon acidification. Changes in fluorescence intensity of lysosensor probe were measured by Live data Mode acquisition on living cells

Intracellular pH variations

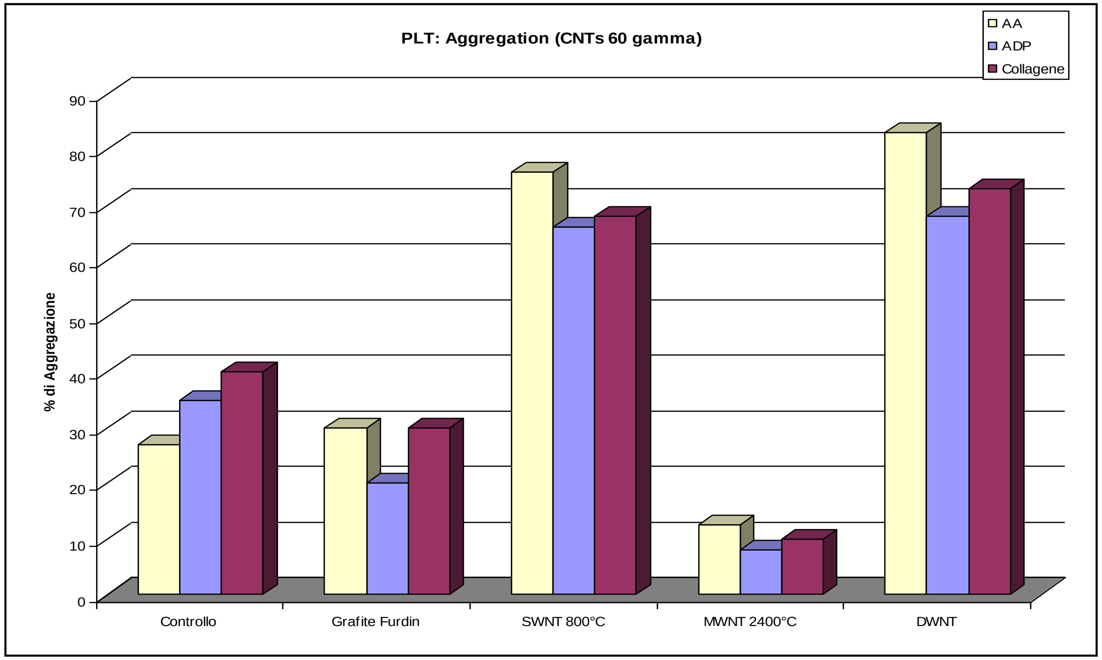




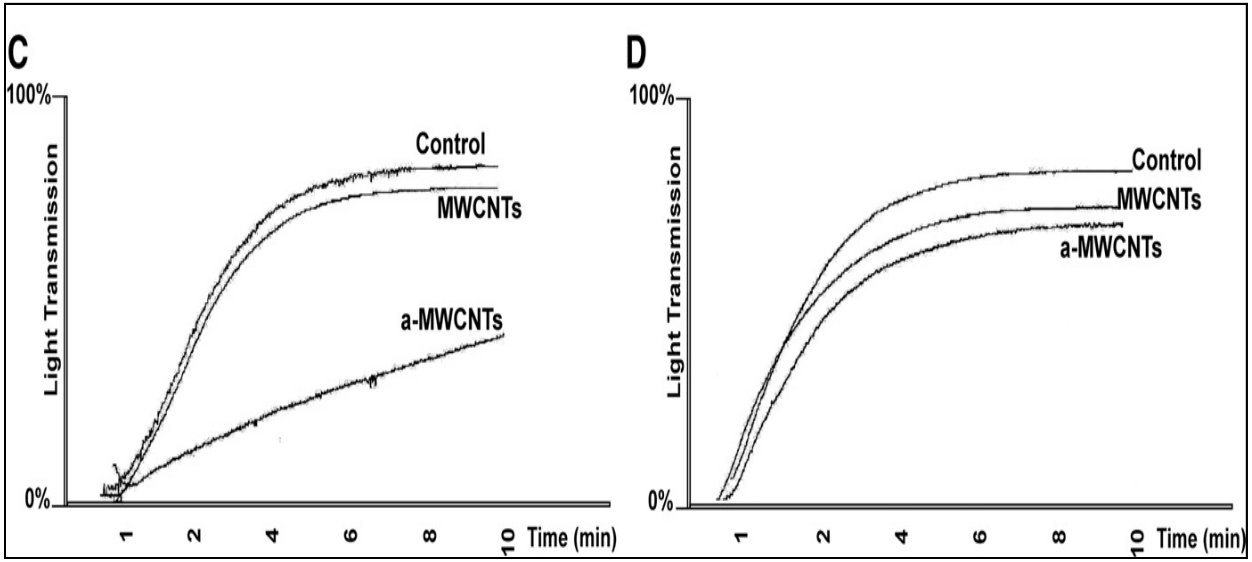




Inhibition of human platelet aggregation



Effect of both MWCNT samples on platelet-aggregating response induced in human platelet-rich plasma by arachidonic acid (AA), collagen (COLL), and ADP.



(C, D) Washed-platelet aggregation in Tyrode's buffer containing 0.09 mM Ca^{2+} (C) or 0.9 mM Ca^{2+} (D). The superimposed tracings were obtained by adding to washed-platelet suspension AA (150 µg/mL) 5 minutes after addition of a-MWCNTs or MWCNTs (60 µg/mL)

Effects of highly electro-conductive MWNTs on "charge-sensitive" cell parameters

- **In human macrophage cells**
  - **fast reorganization of the actin-cytoskeletal network**
  - **mitochondrial membrane depolarization**
  - **decrease of the intracellular pH (due to depletion of H+ in the intracellular compartments)**

  Fiorito S. et al, Carbon 2009, 47, 2789

- **in electrically sensitive neuronal-derived cells**
  - **depolarization of the cell membrane with a subsequent inhibition of voltage-sensitive Ca2+ channel functionality, with a consequent Ca2+ mobilization from the storage compartments**
  - **increase in intracytoplasmic calcium content, due to mobilization from intracellular storage compartments.**

  Serafino A., et al, Nanomedicine : NBM 2012, 8, 299

- **In platelet cells**
  - **Inhibition of platelet aggregation, dependent on the presence of cytoplasmic Ca2+ ions in the extracellular medium**

  Serafino A., et al, Nanomedicine : NBM 2012, 8, 299

Effects of highly electro-conductive MWNTs on “charge-sensitive” cell parameters

- **Due to the excess of negatively charged electrons on the aromatic units, an electrostatic attraction with positive charges could occur with the subsequent sorption of cationic ions, such as Ca^{2+} and H^{+}**

- **This is sustained by our data obtained by OCP measurements showing that the electroconductive a-MWCNTs exhibit a high Ca^{2+} affinity, not evidenced for the non-electroconductive MWCNTs.**

CARBON 78 (2014) 589–600

ELSEVIER

Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/carbon

Carbon

Redox active Double Wall Carbon Nanotubes show intrinsic anti-proliferative effects and modulate autophagy in cancer cells

CrossMark

Silvana Fiorito [a,d,*], Emmanuel Flahaut [b], Stefania Rapino [c], Francesco Paolucci [c], Federica Andreola [d], Noemi Moroni [d], Eugenia Pittaluga [d], Manuela Zonfrillo [d], Giovanni Valenti [c], Arianna Mastrofrancesco [e], Flavia Groppi [f], Enrico Sabbioni [g], Evangelos Bakalis [c], Francesco Zerbetto [c], Annalucia Serafino [d]

[a] Dipartimento di Medicina Clinica, Università Sapienza, Roma, Italy

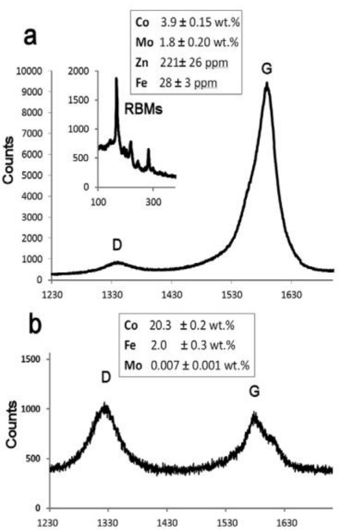
[b] Université Paul Sabatier-CNRS (CIRIMAT), Toulouse, France

[c] Dipartimento di Chimica "G. Ciamician", Università di Bologna, Bologna, Italy

[d] Institute of Translational Pharmacology, CNR, 00133 Roma, Italy

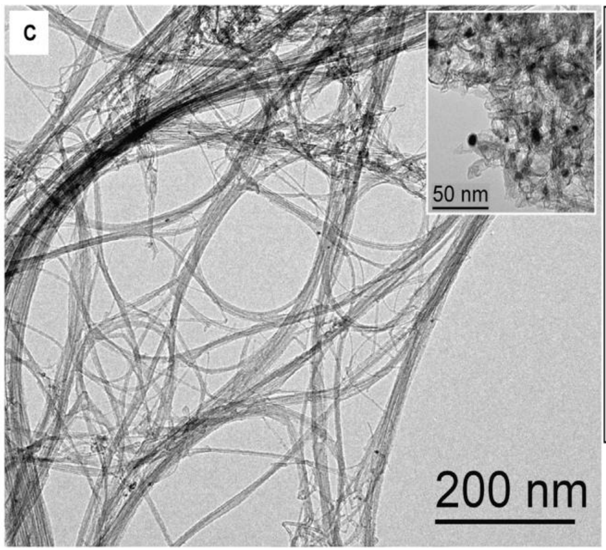
DWCNT effects on rat colon adenocarcinoma cell line DHD/K12/trb cells

Raman spectra

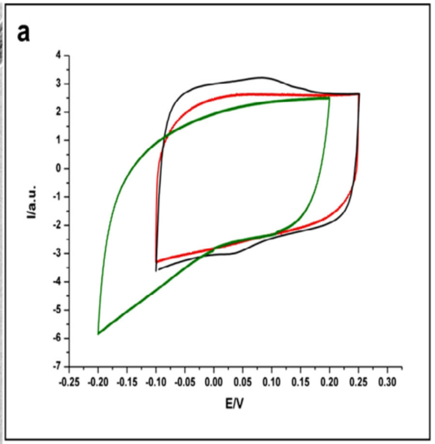


(a) DWCNTs
(b) Co@C

TEM

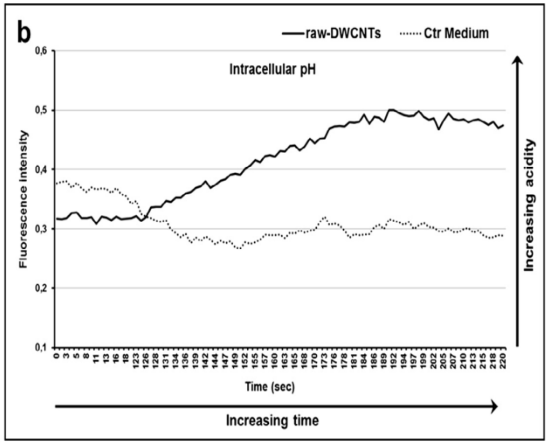


Cyclic voltammetry



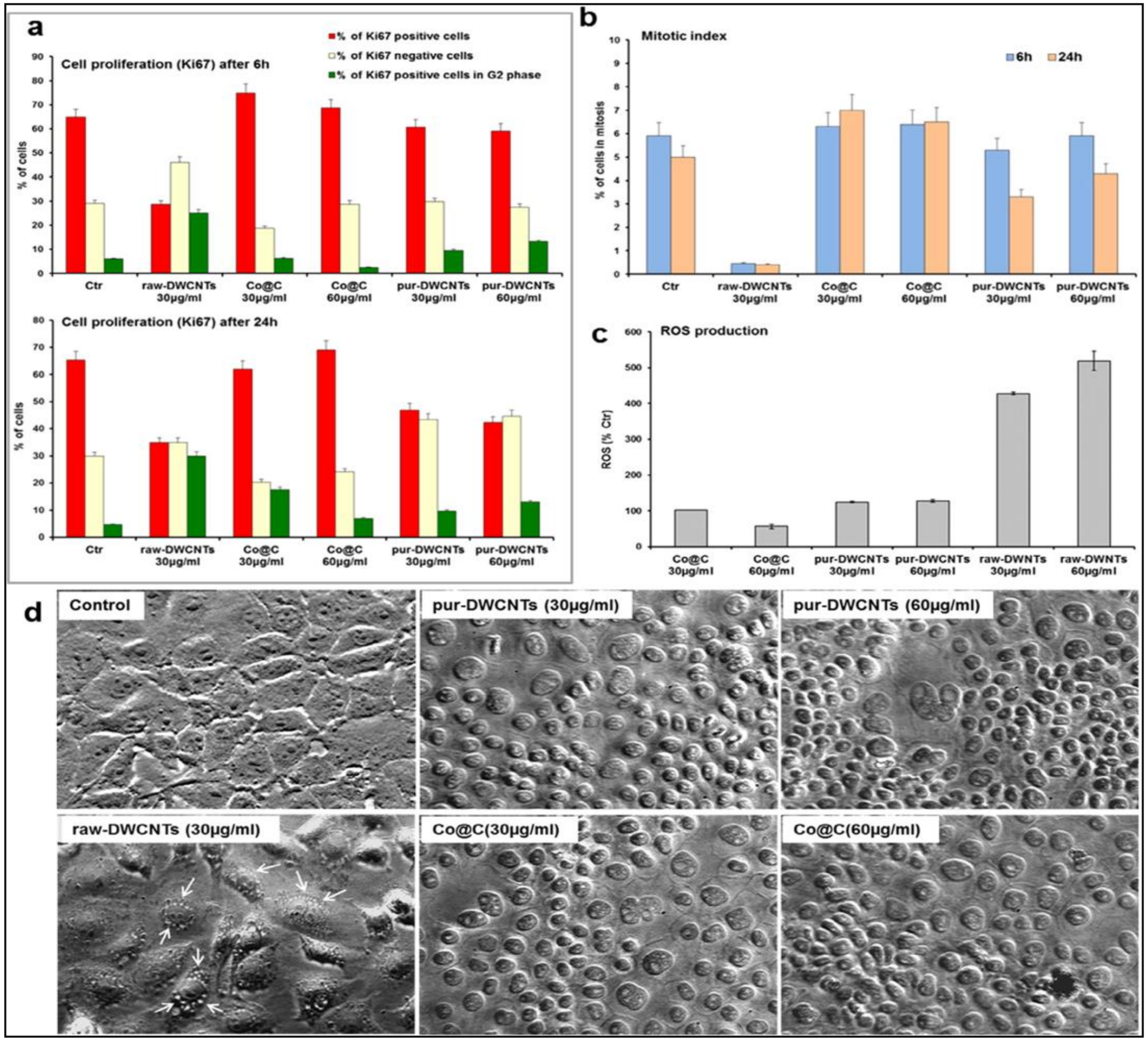
- Purified DWCNTs (red line),
- raw-DWCNTs (black line)
- Co@C nanoparticles (green line).

Changes of intracellular pH



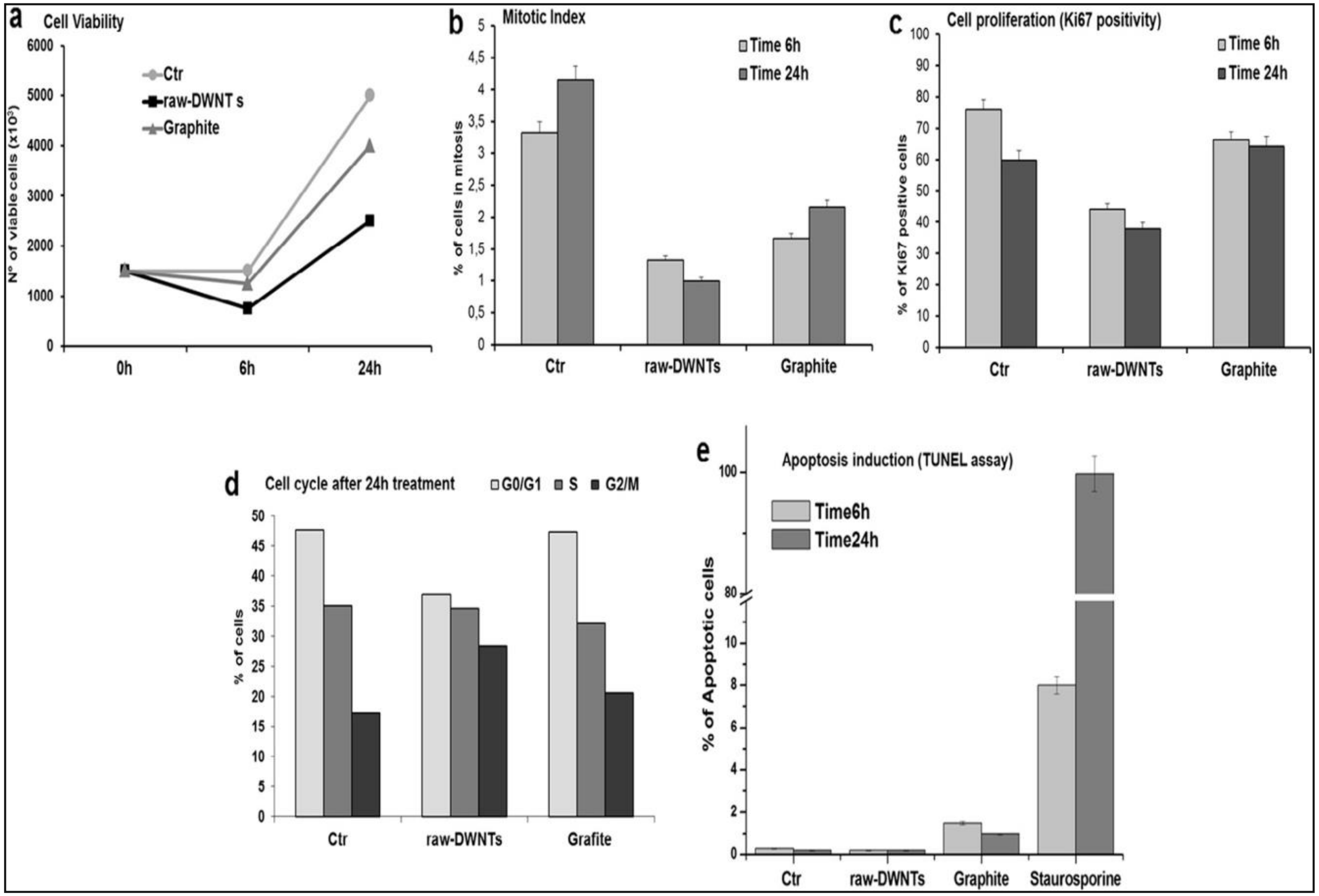
CV showed a redox peak, with E1/2 potential, at 70mV, attributable to metallic impurities left over from the catalyst used in the synthesis

Comparative analyses of the effects of raw-DWCNTs, Co@C particles and purified DWCNTs on DHD/K12/trb cells



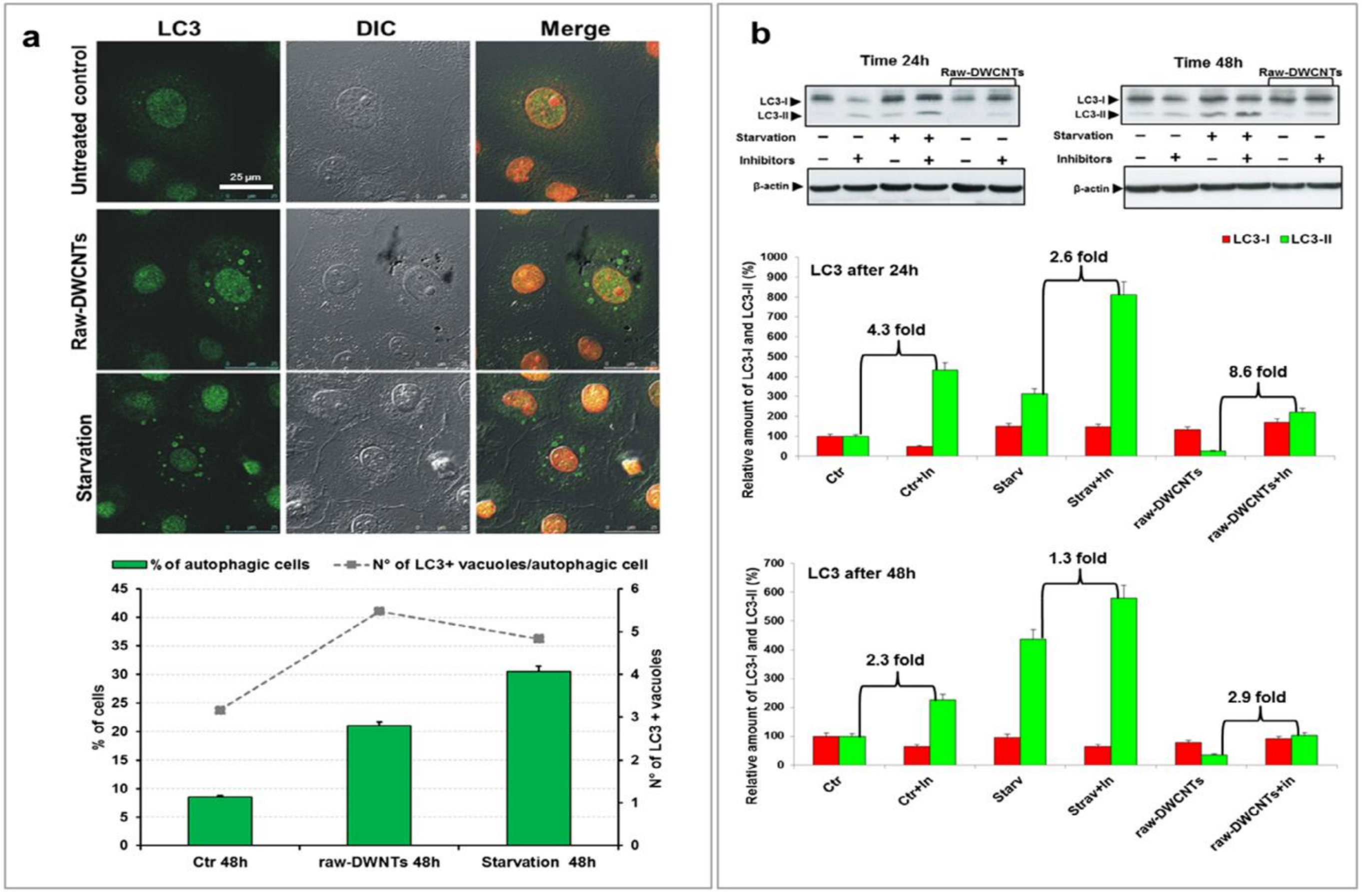
(a) Cell proliferation analyzed by immunofluorescent staining of Ki67;
(b) Mitotic index evaluation after 6 h and 24 h treatment.
(c) ROS production by cytofluorimetric analysis using CM-H2DCFDA as fluorescent probe
(d) Cell morphology of DHD/K12/trb cells analyzed by phase contrast microscopy after 48 h treatment

Effects of raw-DWCNTs on cell viability, proliferation, cycle and death in DHD/K12/trb cells



(a) Cell viability by Trypan blue exclusion method
(b) Mitotic index (MI) evaluation
(c) Cell proliferation evaluated by Ki67 immunofluorescent staining;
(d) Cell cycle analysis by flow cytometry.
(e) Apoptosis induction evaluated by TUNEL assay.

Induction of autophagy in DHD/K12/trb cells by raw-DWCNTs



(a) Autophagy analysis performed by evaluating the expression and redistribution at cytoplasmic vacuoles of the autophagy membrane marker LC3 after 48 h treatment with raw-DWCNTs.

(b) Western Blot analysis of the expression of LC3-I and LC3-II

Conclusions

- a-MWNTS (electro-conductive)

  - Induce changes in Ca^{++} balancing between intra and extracellular compartments
  - Induce changes of electrically sensitive cell parameters

  - Fast reorganization of the actin-cytoskeletal network
  - Fast mitocondrial and cell membrane depolarization
  - Fast changes (decreasing acidity) of the intracellular pH

- Redox active raw DWCNTs

  - **inhibit cancer cell proliferation**, likely through the induction of ROS generation and intracellular compartment acidification (increasing acidity)
  - **affect the autophagic machinery**, thus influencing a pathway critical for cancer cell survival.

Conclusions

- **There is evidence for electrochemical interactions between electro-conductive MWCNTs and redox-active DWCNTs and different cell types**
- **MWCNTs change cell membrane electrical properties, thus triggering chemical signalling pathways leading to cell activation**
- **DWCNTs inhibit cancer cell proliferation, thus possessing intrinsic properties of anticancer therapeutic tools**
- **Such specific behaviours may have an extremely high impact in a huge number of future applications in the biomedical field, not only concerning those cellular systems (neuronal and bone cells) sensitive to electrical stimuli, but also other cell systems (macrophages, tumor cells, platelets).**
- **Elucidating electro-chemical interactions between nanotubes and cells could provide new models of CNT-based biocompatible nanomaterials with the capacity to stimulate and/or inhibit different cell types**