Bulletin UASVM, Veterinary Medicine 67(1)/2010
ISSN 1843-5270; Electronic ISSN 1843-5378

The Identification and Characterization of f-SWCNT from Tissue Samples by Confocal Laser Scanning Microscopy

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Abstract. Carbon nanotubes are new material with biomedical applications which present also novel challenges for toxicity testing. The first challenge is the difficulty of their identification once they are systematically administrated. This problem is due to their size, containing only carbon atoms in their structure, which makes these nanostructures very hard to detect in biological tissues. This study aims to identify using the Confocal Laser Scanning Microscopy technique, the presence of SWCNT in the tissues and the preferential organs in which these nanoelements are stocked. The identification of the accumulation patterns related to the time passed until CNT are accumulated in organs, will allow the establishing of a preliminary conclusion regarding the body distribution of these nanostructures. In this respect we used some fluorescent labeled SWCNT that were injected intraperitonealy in a single dose at laboratory rats. The animals were sacrificed in dynamics at certain time intervals. Using the confocal technique, high accumulations of SWCNT were found in the mesentery, grite omentum and abdomial parietal lymph nodes and a discrete presence in splenic, lung, renal and liver tissues. In the pancreas and encephalon the nanotubes were not found.

Keywords: carbon nanotubes, fluorescent, biodistribution, pharmakinetics.

INTRODUCTION

The carbon nanotubes (CNT) are one-dimensional (1-D) nanomaterials from the class of fullerenes, being composed by rolled up graphene sheets. According to the rolling's number of the graphene sheet, the CNT are classified as single-walled carbon nanotubes (SWNT) or multi-walled carbon nanotubes (MWNTs) (2).

Because of their unique combination in size, physico-chemical and mechanical properties, the carbon nanotubes (CNT) are investigated for biomedical application, one of the main research directions being the implementation of new strategies for cancer therapies. The CNT are used as carrier systems for pharmacologic agents, nucleic acids and proteins in order to detect or treat cancer cells (1).

The biomedical application of carbon nanotubes is conditioned by the behavior of these structures once they are administrated systemically. (3) Until now the fate of the carbon nanotubes inside the living organism is not so well known. The main problem is due to the difficulties in tracking these structures in biological environments because of their multiple variables which can strongly influence the distribution and toxicity of these particles. The characteristics such as: dimension, purity, fictionalization and characteristics of adherent

particle, route of administration, and last but not least the detection system used for identification of carbon nanotubes, can strongly influence the results of these studies. These are some of the potential causes of the inconstant conclusion on the biodistribution and toxicity of carbon nanotubes. The fluorescence labeling of carbon nanotubes makes the trafficking of these structures possible, in the ideal scenario the fluorescent molecule giving the exact information on the CNT location in tissues. The difficulties in applying this technique arise from the autofluorescnece that some biomolecules have doubled by the autofluorescence which appear after histological preparation of slides microscopy. The auto fluorescence appearance can act out as tracked fluorocrome and by this, giving a false positive location of the carbon nanotubes. The clear advantage of the confocal microscopy in comparison with the classical epifluorescence microscopy is a higher specificity in fluorochrome detection, a lower background autofluorescence and, indirectly, a much better detection of the nanotubes.

MATERIALS AND METHODS

Obtaining the functionalized SWCNT suspension with single stranded DNA and analyzing the SWCNT suspension:

In order to form a homogenous suspension, a non covalent functioning of the nanotubes was made after the purification step, by connecting the single salmon strand DNA, sequence (5'-3') TGGACAAGTGGTATG-Cy3. The Cy3 fluorocrome was selected to label the DNA molecule. The SWCNT suspension was obtained by non covalent functionalizing, starting connections between the spiral DNA molecule fluorescent marked and the nanotube's wall, consecutively making ultrasonications in baths of ice, followed by repeated series of centrifugations and supernatant isolation.

This step was made using the confocal laser technique and also the direct one through optical microscope. In parallel with the image acquisition we also performed the measuring of the carbon nanotube's length in the fluorescent suspension, as well as the reconstruction of the three-dimensional structure of the tubes found isolated in the solution.

The SWCNT administration was done intraperitoneally, using a quantity of 390 mg/L. The volume of saline solution with functionalized SWCNT with single stranded DNA was of 1,5 ml, at the reference lot being intraperitoneally administrated only the saline solution.

After the administration the animals were sacrificed at 3, 6, 24 and 48 hours from the start of the nanotubes solution administration. The detailed necropsy examination of the sacrificed animals was followed by the gathering and conservation of the tissue samples represented by: mezentery, omentum, liver, spleen, pancreas, lung, kidneys, encephalon, long bone, ileac lymph nodes and skeletal muscle. .

The Confocal Microscopy

The images were acquired using a Zeiss LSM 710 confocal laser scanning unit equipped with argon and HeNe lasers mounted on a Axio Observer Z1 inverted microscope. Because of the poorer backround fluorescence we used the cryosection tehnique. The slides were obtained by criosection with Leica BX 1850 criothome. The fluorescent staining of the tissues was made with the help of the Blue Pseudocolor (DRAQ 5, Cell Signalling) flurocrome for the nucleous, and for fluorescent marking of the carbon nanotubes the cyanide fluorocrome Cy3 was used.

RESULTS AND DISCUSSIONS

The confocal laser analysis of the functionalized nanotube suspension with Cy3 marked DNA revealed o good dispersion of the fluorescent marked elements in the solution, elements marked in a form of a dot or slightly linear, and also a quantity of unfunctionalised nanotubes found in the analyzed suspension under the aspect of blackish bulks with small stainless sizes.

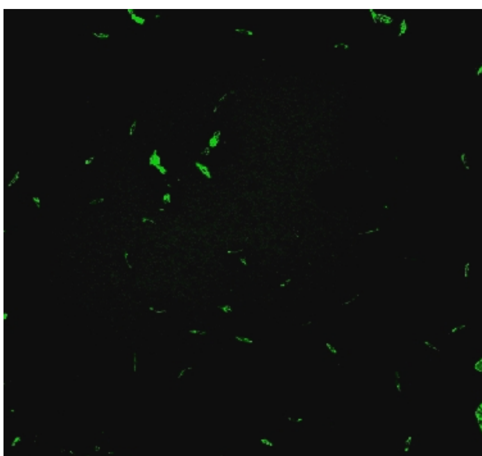


Fig.1 Confocal image of ss-DNA-cy3 SWCNT solution, linear green fluorescence with dark center corresponding to nanotubes , ob. Apoplan x63

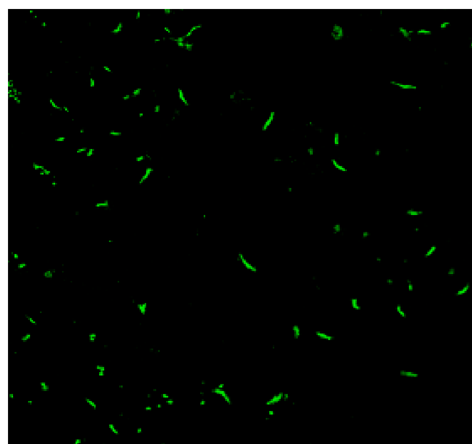


Fig.2 Confocal image of ss-DNA-cy3 SWCNT solution, linear green fluorescence specific to Cy3 can be observed, ob. Apoplan 63

The lengths measured had values ranging between 1,5 and 8,75 micrometers, with an average of 2,28 microns. The majority of the nanotube's lengths measured were falling in between 2-3 microns.

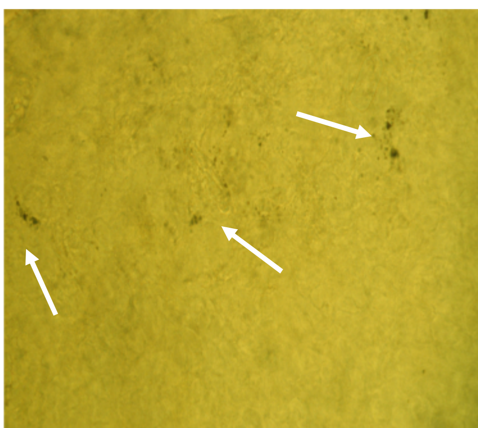


Fig. 3 Light microscopy image of an lymph node, black accumulations corresponding to intracellular CNT bulks (white arows) can be saw in the contrast with the unstained tissue, ob. Apoplan x63

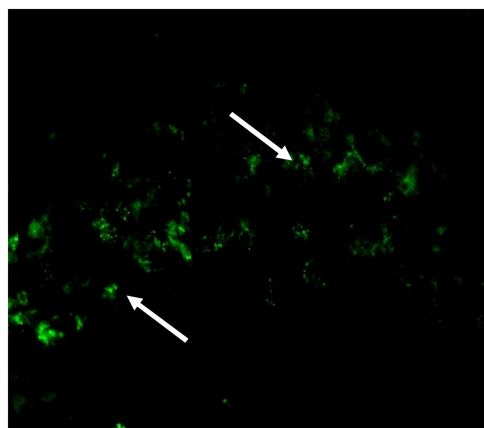
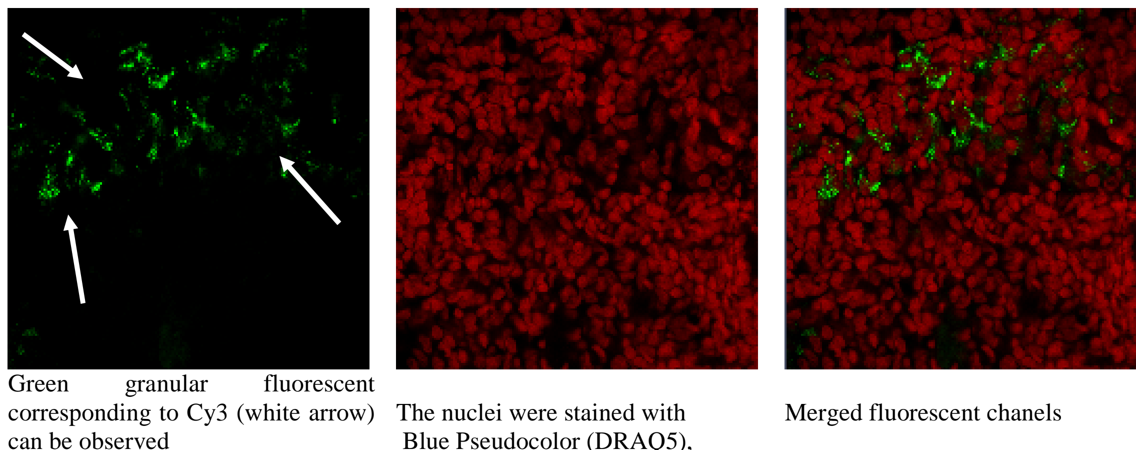


Fig. 4 Confocal fluorescent visualization of the prior image. The black accumulations are confirmed to be CNT by the specific green fluorescent of the Cy3, ob. Apoplan x63

At three hours from the administration we found at the laser confocal microscopy as well as at the optical microscopy but in a more discrete way, the presence of intracellular bulks of nanotubes, in high levels in the omentum, hepatic tissue, pulmonary tissue as well as

spleen tissue. There was also observed a rapid transport of the nanotube injected intraperitoneally, in the entire organism.



Green granular fluorescent corresponding to Cy3 (white arrow) can be observed | The nuclei were stained with Blue Pseudocolor (DRAQ5), | Merged fluorescent chanels

Fig.5. Lymph node, confocal image, ob.Apoplane x63

The rapid diffusion of the functionalized nanotubes with DNA in the organism consecutive to their administration intraperitoneally is in concordance with the ones observed by the Borowiak-Palen et al., the majority of the researchers finding a rapid adsorption and distribution of the nanotubes after their intravenous or intraperitoneal administration.

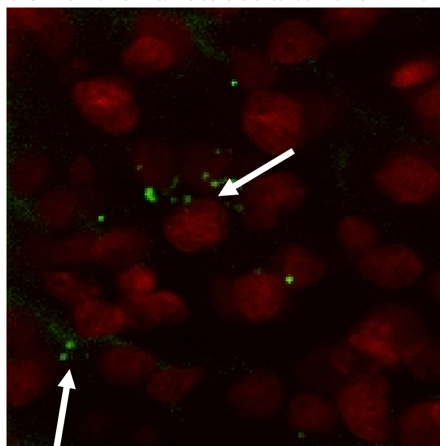


Fig. 6 Confocal image of liver 6 hours after the administration of the nanotubes suspension. The discrete, granular aspect of the nanotubes can be observed (white arow). The hepatocites nuclei were stained with DRAQ5; Digitaly amplified images, ob Apoplane x63

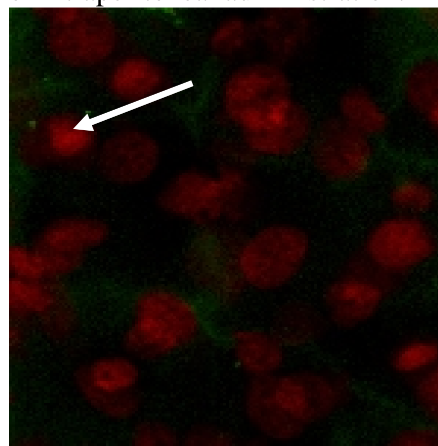


Fig.7 Confocal image of liver 6 hours after the administration of the nanotubes suspension. The discrete, granular aspect of the nanotubes can be observed (white arow). The hepatocites nuclei were stained with DRAQ5; Digitaly amplified images, ob Apoplane x63

The possible lymphatic transport is suggested by the high quantity of nanotubes internalized in the reticular cells from the parietal abdominal lymph nodes, a transport path suggested also by Alberto Bianco et al. The absorbtion and lymphatic transport, since we are talking about molecules with lengths reaching 7 micrometers, is due probably to the high permeability of stomata from the intercelular space of the mesothelial layer. These gaps are situated between the lateral borders of the mesothelial cells that are covering the roof of the

lymphatic lacunae of the diafragm (Yoffey J.M. et al. 2006). These stomata can permit the passage of objects as large as 23 mm in diameter (Ramesh Khanna Robert 2006),

The large quantities of nanotubes identified in the omentum is due to their passing in the peritoneal layer and their cell internalization , a high amount of the peritoneal adsorption being dependent to the intestinal lymphatic circulation. (Becker L et al. 2001), the entrance of the nanotubes in the intracellular space conducting to their persistence at this level throughout the entire period since the first scarification (at 3h) until the final one (at 48 hours). Nevertheless, in our case the lymphatic transport could be due to the fact that the adsorption and particle mobility at the peritoneal level is made mainly though the lymphatic way (Baddour C. E. et al., 2005).

CONCLUSIONS

The complete adsorption of the functionalized nanotube with DNA suspension administrated intraperitoneally and the lack of peritoneal reaction noticeable histological.

The nanotube transport in the organism has a major lymphatic component, a thing underlined by the high amounts of nanotubes in the parietal abdominal lymph nodes and the moderate quantities in the other lymph nodes found in the organism.

The persistence of the nanotubes in the liver, spleen, kidney tissues was noticed also at medullar level at 48 hours from the administration.

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