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# Nanotubos de carbono de parede simples funcionalizados com polietilenoglicol: avaliação de parâmetros *in vivo* e *in vitro*

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“Se eu vi mais longe, foi por estar de pé sobre ombros de gigantes.”

**Isaac Newton**

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**Sumário**

## Resumo

WEBER, Gisele Eva Bruch. **Nanotubos de carbono de parede simples funcionalizados com polietilenoglicol: avaliação de parâmetros *in vivo* e *in vitro***. 2013. 60p. Dissertação de Mestrado. Programa de Pós-Graduação em Ciências Fisiológicas: Fisiologia Animal Comparada. Universidade Federal do Rio Grande, Rio Grande.

Os nanomateriais apresentam uma escala na qual ao menos uma das dimensões varia entre 1 e 100 nm e possuem propriedades químicas, físicas ou biológicas dependentes da nanoestrutura e que lhes confere características funcionais de interesse para fins comerciais ou aplicações na área médica. Dentre os nanomateriais mais estudados e utilizados, destacam-se os de carbono, que incluem os fulerenos e os nanotubos de carbono (NT). Uma potencial utilização dos nanomateriais de carbono é na área biomédica, já que estes podem interagir com os sistemas biológicos em nível molecular e supramolecular com alto grau de especificidade. Em contrapartida, é importante considerar que os nanotubos de carbono podem exercer efeitos tóxicos, tendo como possível mecanismo o estresse oxidativo. Sendo assim, o objetivo desse trabalho foi investigar a ação dos nanotubos de carbono de parede única funcionalizados com polietilenoglicol (SWNT-PEG) em *Danio rerio* "zebrafish" (Teleostei, Cyprinidae). Avaliaram-se parâmetros bioquímicos, histológicos, comportamentais e de biodistribuição para entender como esse material se comporta *in vitro* e *in vivo.* Foi observado que o tipo de funcionalização é determinante para a ação desse material em meio biológico. No experimento *in vitro* o SWNT-PEG não mostrou efeito pró-oxidante nas avaliações de peroxidação lipídica, capacidade antioxidante total, conteúdo de GSH e atividade de GCL. Na exposição intraperitoneal em *zebrafish* constatou-se a agregação e geração de processo inflamatório, o que sugere que a cadeia de PEG utilizada para a funcionalização dos NT possui um tamanho inadequado e/ou uma funcionalização ineficiente para manter a estabilidade do material em meio biológico e evitar uma resposta inflamatória por parte do organismo exposto. Possivelmente devido a esta característica do nanomaterial, nas análises de biodistribuição, através de espectroscopia Raman, não se observou distribuição de SWNT-PEG no sistema nervoso central de *zebrafish*. No entanto, através da análise histológica foi observado processo inflamatório no tecido nervoso central, bem como alterações comportamentais avaliadas na tarefa de campo aberto.



Palavras-chave: SWNT-PEG; biodistribuição; toxicidade; zebrafish.

## Abstract

WEBER, Gisele Eva Bruch. **Nanotubos de carbono de parede simples funcionalizados com polietilenoglicol: avaliação de parâmetros *in vivo* e *in vitro***. 2013. 60p. Dissertação de Mestrado. Programa de Pós-Graduação em Ciências Fisiológicas: Fisiologia Animal Comparada. Universidade Federal do Rio Grande, Rio Grande.

Nanomaterials have a scale on which at least one dimension ranging between 1 and 100 nm and have a chemical, physical or biological nanostructure dependent giving them functional characteristics of interest for commercial purposes or applications in the medical field. Among the most studied and used nanomaterials, we highlight the carbon one, including fullerenes and carbon nanotubes (NT). One potential use of the carbon nanomaterial is in the biomedical field, because they can interact with biological systems at the molecular and supramolecular levels with high specificity, moreover, it is important that the carbon nanotubes may have toxic effects, and as possible oxidative stress mechanism. Therefore, the aim of this work was to investigate the action of carbon nanotubes single-walled, functionalized with polyethylene glycol (PEG-SWNT) in *Danio rerio* "zebrafish" (Teleostei, Cyprinidae). We evaluated biochemical, histological, behavioral and biodistribution approaches in an attempt to understand how this material behaves *in vitro* and *in vivo*. It was observed that the functionalization type is crucial for the action of this material in a biological environment. The SWNT-PEG showed no pro oxidant effect on assessments of lipid peroxidation, total antioxidant capacity, GSH levels and GCL activity. In the zebrafish intraperitoneally exposure we found that the functionalization with PEG chain has an inadequate size for maintaining product stability in a biological environment, occurring aggregation and an inflammatory process. Hence, the biodistribution analysis by Raman spectroscopy was not observed distribution of SWNT-PEG to the central nervous system of *zebrafish.* However, also an inflammatory process was observed in central nervous system as well as behavioral changes assessed in the open field task.



Keywords: SWNT-PEG; biodistribution; toxicity; zebrafish.

## Lista de abreviaturas

NT - nanotubos de carbono

SWNT-PEG - nanotubos de carbono de parede única funcionalizados com polietileno glicol

PEG - polietilenoglicol

SNC - sistema nervoso central

ROS - espécies reativas de oxigênio

CNS - central nervous system

TGA - thermogravimetric analysis

TEM - transmission electron microscopy

AFM - atomic force microscopy

HRTEM - high resolution transmission electron microscopy

EDS - Energy-dispersive X-ray spectroscopy

## Lista de figuras

## 1. Introdução

A nanotecnologia compreende o desenvolvimento e a utilização de materiais na escala nanométrica (1 nanômetro corresponde à bilionésima parte do metro) sendo considerados nanomateriais aqueles que apresentam ao menos uma das dimensões menor que 100 nm (Silva, 2006; Medina *et al*., 2007)(figura a). Sabe-se que os efeitos quânticos se sobressaem as leis da física clássica quando o tamanho das partículas está abaixo de 50nm, portanto, esses materiais possuem propriedades químicas, físicas ou biológicas dependentes da nanoestrutura e que lhes confere características funcionais de interesse para aplicações na área médica (Oberdörster *et al*., 2005; Zhang *et al*., 2005, Tang *et al*.,*2012*).

A manipulação da matéria em nível atômico permite obter materiais e dispositivos com propriedades especiais, o que vem impulsionando a produção de nanomateriais em larga escala (Service, 2004). Essas características especiais dos nanomateriais são diretamente afetadas devido a sua grande área de superfície por unidade de massa, sendo que esse acréscimo na área de superfície também aumenta a reatividade, uma vez que o número de grupos potencialmente reativos presentes na superfície das partículas será maior (Buzea *et al*., 2007).

Os nanomateriais abrangem estruturas de diferentes composições e arranjos atômicos, dentre os quais os mais comuns são os compostos puramente de carbono, os metais e óxidos metálicos, as nanopartículas poliméricas e os lipossomos (Donaldson *et al.*, 2004; McNeil, 2005). Entre os nanomateriais mais estudados e extensamente utilizados, destacam-se os nanomateriais de carbono, que incluem os fulerenos e os nanotubos de carbono (NT) (Medina *et al*., 2007).

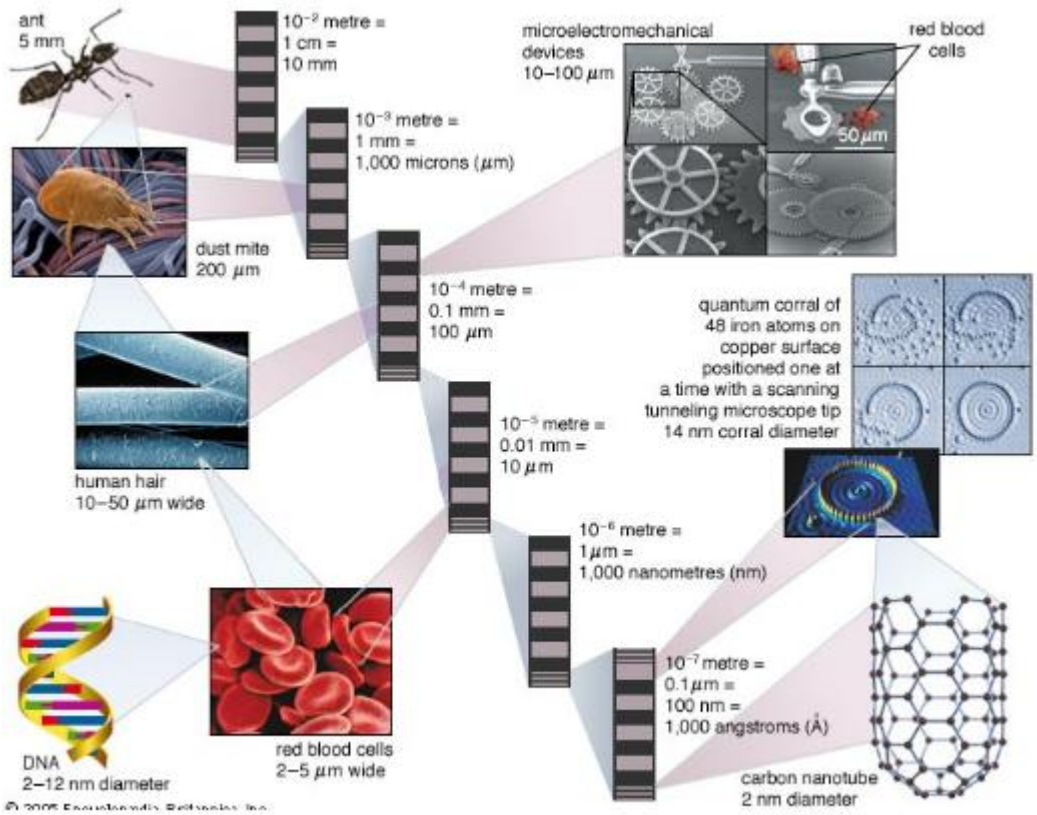


Figura a. Exemplo de escala de dimensões micrométricas a manométricas.
Fonte: Encyclopædia Britannica, 2010.

## 1.1 Nanotubos de Carbono

### 1.1.1. Estrutura e características

Os nanotubos de carbono (NT) foram descobertos em 1991 por Ijima (Ijima, 1991, Ijima e Ichihaschi, 1993), os quais, como outros nanomateriais possuem estrutura singular e propriedades físicas diferenciadas (Ajayan, 1999; Baughman *et al*., 2002). Os NT são alótropos do carbono e podem ser descritos como estruturas cilíndricas formadas por folhas de grafeno enroladas em tubos de diâmetro nanométrico e comprimento na ordem de mícrons (Donaldson *et al*., 2006, Wu *et al*., 2010). Estes nanomateriais podem ser agrupados em duas categorias: nanotubos de parede única ou simples (SWNT, do inglês *single-wall carbon nanotubes*), constituídos por apenas uma camada cilíndrica do grafite, e os nanotubos de paredes múltiplas (MWNT, do inglês multi-wall carbon nanotubes),

formados por vários cilíndricos concêntricos de grafite (Ajayan, 1999) (Figura b). As dimensões, estrutura e topologia dos NT conferem a estes nanomateriais propriedades mecânicas, elétricas e óticas especiais (Ajayan, 1999), que despertam o interesse de pesquisadores e indústrias para as mais diversas aplicações, como em dispositivos nanoeletrônicos e de armazenamento de energia, fontes de emissão de campo elétrico, sensores, sondas e componentes de compósitos poliméricos (Baughman *et al*., 2002).

Os NT possuem uma relação comprimento diâmetro de cerca de 1000, podendo ser considerado uma estrutura unidimensional. Além disso, os NT podem ser sintetizados por diferentes métodos: descarga por arco, ablação por laser e deposição química a vapor. Via de regra, são uma mistura de tubos com diâmetro, comprimento e quiralidade diferentes, contendo, na sua maioria, impurezas, nanopartículas de carbono amorfo e resíduos catalíticos.

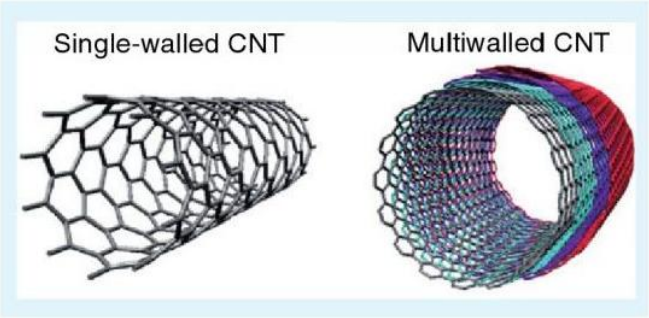


Figura b. Estrutura de nanotubo de carbono de parede única (SWNT) e de parede múltipla (MWNT). Fonte: Zhang, Bai, Yang, 2010.

Nos últimos anos, foram conduzidas inúmeras pesquisas avaliando possíveis aplicações de NT em materiais e dispositivos para fins de diagnóstico, transporte e liberação controlada de fármacos e cultura celular (Kam *et al*., 2005; Medina *et al*., 2007; Foldvari e Bagonluri, 2008; Liang e Chen, 2010).

Para a utilização dos NT na área biomédica é necessário utilizar estratégias para sua purificação e dispersão em meio fisiológico (Ajayan, 1999), pois se sabe que estes

nanomateriais são altamente hidrofóbicos e formam agregados em meio aquoso, de modo que precisam ser modificados ou funcionalizados para que ocorra sua dispersão em soluções compatíveis com os sistemas biológicas (Liu *et al*., 2009).

### 1.1.2. Importância da funcionalização dos nanotubos de carbono

A incorporação de polímeros hidrofílicos à superfície das nanopartículas, como o polietileno glicol (PEG) (Figura c), é uma estratégia bastante utilizada para aumentar a biocompatibilidade, prolongar a permanência na circulação sanguínea e diminuir a absorção hepática das nanopartículas (Liu *et al*., 2007; Yang *et al*., 2008a,b; Ilbasmiş-Tamer *et al*., 2010). Sendo assim, é importânte ressaltar que se o nanomaterial de interesse não estiver bem envolto por uma substância que evite o seu reconhecimento pelo sistema imune ele pode gerar um processo inflamatório grave, estresse oxidativo e acúmulo de nanopartículas em órgãos do sistema retículo endotelial (RES - baço, fígado, rim, entre outros).

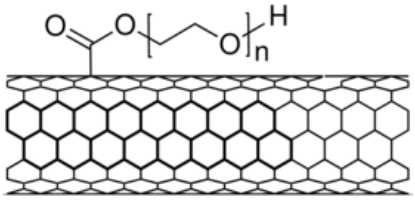


Figura c. Nanotubo de carbono funcionalizado com polietilenoglicol.
Fonte: http://www.sigmaaldrich.com

Para evitar o ataque das opsoninas uma das estratégias utilizadas é modificar a superfície do nanomaterial com a ligação de polímeros hidrofílicos e agentes tensoativos não iônicos. Um dos mais eficazes é o polietilenoglicol (PEG), pois é altamente hidrofílico e flexível (Gref *et al.*,2012).

Uma das principais barreiras que os nanotubos precisam atravessar é o reconhecimento por parte de componentes do sistema retículo endotelial (RES). Os

macrófagos do RES podem remover em pouco tempo partículas da corrente sanguínea através do reconhecimento de opsoninas (proteínas do sistema do complemento) que se ligam a superfície dessas partículas. Inicialmente as opsoninas entram em contato com partículas estranhas por movimento browniano aleatório e, em seguida, se ligam à superfície das partículas dirigidas por forças atrativas específicas (tais como hidrofóbicas, eletrostáticas, de Van der Waals, entre outros). Depois de ter ocorrido a opsonização, as partículas são facilmente reconhecidas e internalizadaspelos macrófagos do RES. Seguindo a fagocitose, as partículas são transportadas em vesículas específicas (lisossomos) e atacadas por enzimas digestivas (Gref *et al.*,2012).

Algumas teorias tentam explicar de que forma ocorre à repulsão de proteínas a superfícies hidrofóbicas planas. Uma dessas teorias tem como base a ligação das cadeias de PEG a uma extremidade da cadeia hidrofóbica em uma configuração de "escova". Foi elaborado um modelo matemático levando em consideração quatro tipos de interações entre uma proteína e um substrato hidrofóbico. As melhores condições para repulsão da proteína foram encontradas para um comprimento de cadeia longa de PEG e com uma densidade superficial elevada desse polímero (Jeon *et al*., 1991; Gref *et al.*,2012). Conforme o modelo matemático proposto na teoria, no caso de proteínas pequenas (aproximada como esferas com um raio de 2 nm), a distância entre a ancoragem ao substrato de duas cadeiass de PEG ligadas deve ser cerca 1 nm, enquanto que para proteínas maiores (6-8 nm), D deve ser cerca 1,5 nm (Gref *et al.*,2012) (Figura d).

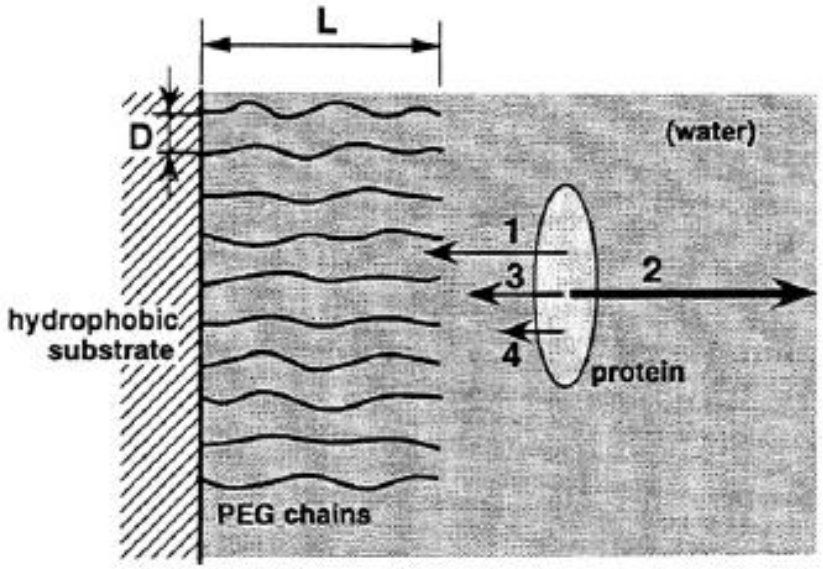


Figura d. Interação entre proteína e substrato hidrofóbico com cadeias de PEG ligadas. 1. atração hidrofóbica entre a proteína e o substrato. 2. repulsão estérica resultante da constrição das cadeias de PEG. 3. atração de forças de van der Walls entre a proteína e o substrato. 4. atração de forças de van der Walls entre a proteína e as cadeias de PEG.
Fonte: Gref *et al.*, 2012.

Estudos *in vitro* foram elaborados para esclarecer aspectos da farmacocinética e da biodistribuição de nanomateriais funcionalizados com PEG, os quais demonstraram que SWNT-PEG podem ser incorporados pelas células por mecanismos distintos, fagocitose ou transporte passivo, e se acumular em diferentes organelas, como lisossomos, mitocôndrias (Zhou *et al*., 2010) e núcleo celular (Cheng, *et al*., 2008).

### 1.1.3. Aplicações biológicas dos SWNT-PEG

As nanopartículas em geral vêm sendo estudadas como potenciais carreadores de fármacos para o sistema nervoso central (SNC), pois constituem veículos com potencial capacidade de ultrapassar a barreira hematoencefálica e liberar substâncias ou agir diretamente sobre alvos intracelulares (Silva, 2008; Bhaskar *et al*., 2010; Yang *et al*., 2010). Para esse tipo de aplicação é necessário desenvolver um material que possa ser bem absorvido desde seu local de aplicação e ao ser transportado pela corrente sanguínea,

atravesse as barreiras endoteliais e celulares e possua afinidades pelo sítio de ação desejado. Também é importante que após liberar o fármaco e atingir seu objetivo, este material seja excretado sem maiores efeitos tóxicos para o organismo (Lacerda *et al*, 2006).

Uma potencial aplicação para os SWNT funcionalizados com PEG foi proposta por Roman e colaboradores (2011) que demonstraram que a administração local deste nanomaterial após a lesão na medula espinal promoveu reparo axonal e recuperação funcional em ratos, reportando pela primeira vez o efeito neuroreparador deste nanomaterial *in vivo*. A partir disso, postula-se que NT funcionalizados podem atuar como ferramentas diagnósticas e terapêuticas para intervenções dirigidas a células e compartimentos subcelulares específicos.

Dessa maneira, percebe-se que a neurociência constitui um campo de estudo bastante promissor para as aplicações da nanotecnologia, especialmente para os dispositivos e materiais a base de NT (Silva, 2006; Malarkey & Parpura, 2007). Existem estudos que demonstram a biocompatibilidade de substratos contendo NT, sobre os quais as células nervosas são capazes de crescer e se diferenciar (Mattson *et al*., 2000; Liopo *et al*., 2006; Galvan-Garcia *et al*., 2007). Além disso, modificações na estrutura dos NT podem modular o desenvolvimento dos neurônios em cultura (Bekyarova *et al*., 2005; Sucapane *et al*., 2009). A potencialidade dos NT em reestabelecer conexões sinápticas entre neurônios vêm sendo demonstrada em estudos *in vitro* que relataram o aumento da atividade elétrica (Lovat *et al*., 2005; Galvan-Garcia *et al*., 2007; Mazzatenta *et al*., 2007) e aumento da força sináptica em circuitos neuronais (Cellot *et al*., 2009). Recentemente um estudo que utilizou NT impregnados com células progenitoras neurais em ratos submetidos à isquemia cerebral focal demonstrou que a administração deste nanomaterial diretamente no local da lesão

contribuiu para a diferenciação celular necessária para a cicatrização do tecido lesionado (Moon *et al*., 2012).

Por outro lado, a internalização das nanopartículas pelas células pode desencadear reações que levam à morte celular (Nel *et al*., 2006; Porter *et al*., 2007), de modo que a investigação de potenciais efeitos tóxicos decorrentes das interações entre as NT e os constituintes celulares é de fundamental importância. Para que a investigação toxicológica seja mais abrangente possível, devem ser considerados também os aspectos acerca da biodistribuição e acumulação dos diferentes nanomateriais nos tecidos e órgãos dos organismos expostos. De acordo com a modificação da superfície e as características físico-químicas, os NT podem apresentar padrões distintos de biodistribuição, acumulação e toxicidade nos potenciais órgãos-alvo dos organismos expostos (Cheng *et al*., 2009; Kang *et al*., 2009; Johnston *et al*., 2010; Jain *et al*., 2011). Diferentes ferramentas têm sido empregadas para analisar a biodistribuição dos NT em tecidos, dentre as quais a marcação dos NT com isótopo radioativo (Deng *et al*., 2007; Guo *et al*., 2007), a fluorescência intrínseca do nanomaterial (Cherukuri *et al*., 2006) e a ligação dos NT à uma sonda fluorescente (Kang *et al*., 2009). Nos últimos anos, a espectroscopia Raman, uma técnica de espalhamento inelástico da luz que fornece informações sobre a estrutura atômica e eletrônica e presença de defeitos na estrutura dos NT (Jorio *et al*., 2004), tem se mostrado uma ferramenta bastante apropriada para a investigação da distribuição e meia-vida circulatória dos NT nos tecidos após a administração intravenosa (Liu *et al*., 2008; Schipper *et al*., 2008; Kang *et al*., 2009) ou subcutânea (Zavaleta *et al*., 2008) destes nanomateriais em roedores.

Além dos estudos de biodistribuição e acumulação dos nanomateriais nos diferentes órgãos, muito se têm pesquisado acerca da capacidade destes em atingir o sistema nervoso central (SNC) de animais expostos por diferentes vias de administração, como revisto por

Hu e Gao (2010). As características físico-químicas, principalmente relacionadas ao tamanho, lipofilicidade e presença de grupos funcionais das nanopartículas podem ser determinantes para a habilidade dos diferentes nanomateriais em ultrapassar a barreira hematoencefálica (Oberdöester *et al*., 2009; Sharma *et al*., 2009), sendo que alguns NT conseguem penetrar no SNC (Guo *et al*., 2007; Ren *et al*., 2012).

O SNC é particularmente vulnerável ao estresse oxidativo devido seu alto conteúdo de substratos oxidáveis, como ácidos graxos poliinsaturados e catecolaminas, elevado metabolismo aeróbico e capacidade antioxidante relativamente reduzida (Halliwell, 1992; Sayre et al, 2008). O estresse oxidativo é descrito com o possível mecanismo de ação tóxica destes nanomateriais, o qual pode ocorrer quando o aumento na quantidade de agentes oxidantes, como as espécies reativas de oxigênio (ROS do inglês *reactive oxygen species*), excede a capacidade das defesas antioxidantes celulares ou perturbam a regulação da sinalização redox celular (Jones, 2006). Esta condição pode ocorrer nos sistemas biológicos quando há uma deficiência na ação dos sistemas antioxidantes seguida de uma superprodução de espécies reativas, que pode causar a oxidação de lipídeos, proteínas e DNA (Valko *et al*., 2007).

Em condições severas, o dano oxidativo decorrente da superprodução de ROS pode culminar na apoptose, evento celular que envolve a ativação de proteases específicas denominadas caspases e a autofagia regulada da célula (Orrenius *et al*., 2007). Tendo em vista que os NT podem atingir o SNC e promover estresse oxidativo (Nel *et al*., 2006; Oberdörster *et al*., 2009), é possível que NT se acumulem nesse tecido e causem injúria neuronal (Zhang *et al*., 2010), o que implicaria em prejuízos funcionais para os organismos expostos. A neurotoxicologia comportamental constitui um instrumento importante na investigação das consequências funcionais de potenciais agentes neurotóxicos, cujas ações podem ocorrer em nível molecular, celular e genético, provocando perdas funcionais cujas

respostas podem ser acessadas em termos de alterações no comportamento de animais expostos (Wallace, 2005; Levin *et al*., 2009).

Sendo assim, é considerável a hipótese que a exposição aos nanomateriais, como os NT funcionalizados, pode ocasionar um quadro de estresse oxidativo, alterando as concentrações de ROS no SNC dos animais expostos, e causar alterações comportamentais como alteração da atividade locomotora.

### 1.1.4. *Zebrafish* como modelo animal para avaliação biológica de NT

A utilização de animais não mamíferos em estudos neurotoxicológicos tem oferecido uma alternativa na elucidação de mecanismos de toxicidade de substâncias químicas (Kokel and Peterson, 2008), dentre os quais o peixe teleósteo popularmente conhecido como *zebrafish* (*Danio rerio*). Seu pequeno tamanho e fácil reprodução proporciona menores custos na produção e manutenção desses animais em laboratório (Pyati *et al*, 2007). Este pequeno modelo vertebrado tem contribuído com pesquisas nas áreas da genética, farmacologia e neurociência (Darland & Dowling, 2001; Guo, 2001; Gerlai *et al*., 2000). O sequenciamento do genoma do *zebrafish* possibilitou avanços importantes na área da pesquisa e na implementação de modelos não mamíferos e estudos demonstram forte homologia entre mamíferos e peixes teleósteos (detalhes ver em http://zfin.org). Além disso, *zebrafish* e mamíferos apresentaram respostas fisiológicas similares a diversos estímulos estressores (Fako & Furgeson, 2009).

Tendo em vista a similaridade entre muitos sistemas neuroquímicos e estruturas neurais do *zebrafish* e outros vertebrados, este pode contribuir para o entendimento do funcionamento do SNC (Best & Alderton, 2008) e constitui um importante modelo biológico nos estudos de neurotoxicologia (Linney *et al*., 2004, Barros *et al*., 2008; de

Castro *et al*., 2009), inclusive para avaliação dos efeitos bioquímicos e comportamentais de nanomateriais (Fako & Furgeson, 2009).

## 2. Objetivos

### Objetivo Geral

Investigar os possíveis efeitos *in vitro* e *in vivo* dos nanotubos de carbono de parede única funcionalizados com polietileno glicol (SWNT-PEG) em *Danio rerio* "zebrafish" (Teleostei, Cyprinidae).

### Objetivos específicos

- Avaliar a indução do sistema de defesa antioxidante através da determinação da capacidade antioxidante total e medida da atividade das enzimas glutamato-cisteína ligase (GCL) e conteúdo de glutationa reduzida (GSH) em homogeneizados de tecido nervoso de zebrafish expostos ao SWNT-PEG;

- Realizar a quantificação de dano oxidativo em nível lipídico (TBARS) em homogeneizados de tecido nervoso de zebrafish expostos ao SWNT-PEG;

- Verificar as possíveis alterações comportamentais em zebrafish (*Danio rerio)* tratados com dispersão de SWNT-PEG através da atividade locomotora na tarefa de campo aberto;

- Avaliar a biodistribuição da dispersão aquosa de SWNT-PEG em zebrafish;

- Avaliar os parâmetros inflamatórios através de analises histológicas de órgãos da cavidade abdominal e cérebro.

**Artigo**

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# Biodistribution and toxicology study of PEGylated single wall carbon nanotubes in zebrafish nervous system (*Danio rerio*)

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**Abstract**

Nanotechnology has been proved as increasingly compatible with pharmacological and biomedical applications. Therefore, we have studied carbon nanotubes functionalized with polyethylene glycol (SWNT- PEG) on its biological interactions. For this purpose, we analyzed biochemical, histological, behavioral and biodistribution parameters in attempt to understand how this material behaves *in vivo* and *in vitro*, using the fish *Danio rerio* (zebrafish) as biological model. I*n vitro* results for fish brain homogenates indicated that the SWNT-PEG had no effect upon lipid peroxidation, total antioxidant capacity, GSH levels and GCL activity assessments. However, in the intraperitoneally exposure, SWNT-PEG proved to be lower biocompatible and able to form aggregates. Possibly this characteristic is implicated in the very lower, or practically absent, biodistribution of SWNT-PEG in zebrafish tissues as verified by Raman spectroscopy. Hence, there is an accumulation of material in the abdominal cavity that generate an inflammatory process and behavioral disturbance, as evaluated by histology analysis and the open field test, respectively. These results provide some evidence about how effective is the surface modification of SWNT with short chain PEG in improve the biocompatibility of this nanomaterial and can guide further studies on biomedical applications of functionalized carbon nanotubes.

**Introduction**

Nanomaterials, including single wall carbon nanotubes, have chemical, physical and/or biological properties dependent on the nanostructure and that gives them functional characteristics of interest for applications in the biomedical field[1, 2]. It is known that these nanomaterials can interact with biological systems at the molecular and supramolecular levels with high specificity[3, 4, 5, 6]. In recent years, numerous studies have been conducted to evaluate potential applications of NT devices for diagnostic purposes, transport and controlled release of drugs and cell culture[7, 8, 9, 10, 11, 12].

Purification and strategies for dispersion in physiological medium are required to optimize nanotubes for biomedical applications[13]. Its high hydrophobic proprieties and the tendency to form aggregates in aqueous media, are a challenge to overcome. The main strategies to solve this issue are to modify or to functionalize to disperse it in compatible solutions with biological systems[14]. In this way, the incorporation of hydrophilic polymers to the surface of nanoparticles, such as polyethylene glycol (PEG), is a widely-used strategy to increase biocompatibility, increasing blood circulation and decreasing liver uptake of nanoparticles[14, 15, 16, 17].

Carbon nanotubes functionalized with polyethylene glycol (SWNT-PEG) have various applications in the biomedical field, however, this work focus on its applications in nervous tissue. The SWNT-PEG had its first neuroprotective effect *in vivo* reported by Roman and coworkers (2011) [18] where axonal repair and functional recovery was demonstrated in rats after spinal cord injury. In this manner, it is clear that neuroscience is a very promising field of study for nanotechnology applications, especially for devices and materials based on NT[3]. Studies have demonstrated the biocompatibility of NT containing substrates upon which nerve cells are able to grow and differentiate[19, 20, 21]. Moreover, changes into the structure of the NT may modulate the development of neurons in culture[22,

[23]. The internalization of nanoparticles by cells can trigger reactions that lead to cell death[24, 25], so that investigation of potential toxic effects arising from interactions between the NT and cellular constituents is of fundamental importance. For a comprehensive toxicological research to be possible, aspects about the biodistribution and accumulation of nanomaterials in different organs and tissues of exposed organisms should also be considered. According to surface modification and physicochemical characteristics, the NT may exhibit distinct patterns of biodistribution, toxicity and accumulation in target organs potentials of exposed organisms[26, 27, 28, 29]. Concerning Raman spectroscopy, a technique of inelastic scattering of light provides information about the atomic and electronic structure and the presence of defects in the structure of the NT[30], it has shown a very appropriate tool to search the distribution and half-life of NT circulatory tissues after intravenous[31, 32. 27] or subcutaneous[33] administration of these nanomaterials in rodents.

Besides the biodistribution studies and accumulation of nanomaterials in different organs, much has been studied on the ability of these to reach the central nervous system (CNS) of animals exposed by different routes of administration, as reviewed by Hu & Gao (2010)[34]. The physicochemical characteristics, mainly related to size, lipophilicity and the presence of functional groups of the nanoparticles can be crucial to the ability of different nanomaterials to overcome the blood-brain barrier[35, 36], and some NT penetrate the CNS[37, 38]. It is known that NT can reach the CNS and promote oxidative stress[24, 35], it is possible that the NT accumulate in the tissue and cause neuronal injury[39], which implies functional damage to exposed organisms.

Therefore, the aim of this work was to investigate the possible *in vitro* and *in vivo* effects of single wall carbon nanotubes functionalized with polyethylene glycol (SWNT-PEG) in *Danio rerio* "zebrafish" (Teleostei, Cyprinidae). Since the zebrafish has similarity of their neurochemical systems and neural structures with other vertebrates, it is an

important biological model in studies of neurotoxicology[40, 41, 42], including assessment of behavioral and biochemical effects of nanomaterials[43].

## 1. Experimental Section

### 1.1. Preparation of SWNT-PEG dispersion and sample characterization

The material studied in this work is a commercial sample (Sigma-Aldrich-652474-Lot MKBC 9435) single wall carbon nanotube (SWNT), synthesized via the electric arc discharge method and functionalized with polyethylene glycol (PEG, Mw = 600Da). The as-received SWNT-PEG material was found to contain ~ 25 wt % of grafted PEG, ~ 60 wt% of SWNT and ~ 20 wt % of 20-40 nm diameter carbon-coated catalyst particles (Ni – Y) as determined by thermogravimetric (TGA) (TA Instruments, SDT 2960, in dry air at a scanning rate of 5 $^{o}$C min$^{-1}$) and transmission electron microscopy (TEM) (FEI, Tecnai G2-20 SuperTwin, 200 kV) analyses.

For the production of stable dispersions in water, several steps of mechanical disintegration and centrifugation were necessary. The adopted procedure was adapted from the work of Kalinina *et a1* [44].

Initially, SWNT-PEG material was dispersed in water at a concentration of 3 mg/mL by ultrasonication for 48h using a bath sonicator (ColeParmer, model 08895-50, ~40 kHz). The mixture was subsequently placed in a high-shear rotor-stator mixer (IKA Labortechnik, Ultra-Turrax T8) for 1h30 min and centrifuged (Eppendorf, 5417C) for 30 min at 5,000 g to remove large particles and agglomerates. The supernatant was then carefully removed, diluted four times and placed in an ultracentrifuge (Sorvall, Ultra Pro 80) for 1 h at 170,000 g to remove the excess of unbound PEG, non-tubular carbon species and other impurities. In the next step, the resulting pellet was resuspended in water to give

a dispersion concentration of 2,1 mg/mL and subjected again to bath sonication for 4 h, shear mixing for 30 min, and centrifugation for 30 min at 5,000 g.

The morphology of PEG-functionalized SWNTs remained in the dispersion were evaluated by performing transmission electron microscopy (TEM), atomic force microscopy (AFM), zeta potential measurements and Raman spectroscopy. TEM and high resolution TEM (HRTEM) was performed on a FEI Tecnai G2-Spirit 120 kV and a FEI Tecnai G2-20 SuperTwin 200 kV microscopes, respectively. For TEM analyses, two or three drops of the SWNT-PEG dispersion was deposited on holey carbon-coated Cu-grids (300 mesh). The digitalized TEM images taken at different locations on several grids were analyzed using Image J Software. AFM observations were carried out with a NTegra Aura (NT-MDT Co.) microscope, and the samples were prepared by depositing a drop of the dispersion on $Si/SiO_2$ substrates. The silicon substrates were previously cleaned with a piranha solution, rinsed with isopropanol and deionized water and drying with nitrogen flow. Zeta potential was determined using the electrophoretic light scattering technique on a Zetasizer Nano- ZS ZEN3600 system (Malvern Instruments), after diluting the original dispersion 10 times and adjusting the ionic strength to 0.001 mol / L using NaCl. Raman spectra were obtained at 514 and 785 nm excitation wavelengths, using a modular Raman system (Horiba Jobin Yvon, RMS-550). For Raman measurements a drop of the dispersion was deposited onto a silicon substrate and subsequently dried. The final concentration of SWNTs in the dispersion was spectrophotometrically evaluated by performing optical absorption measurements using a Shimadzu UV-vis-NIR spectrophotometer UV-3600 over the wavelength range of 190 to 1100 nm. A standard curve of the absorbance *vs* SWNT concentration (based on Lambert-Beer law) was generated by diluting the original dispersion in water and measuring the optical absorbance at 700 nm.

### 1.2. *In vitro* experiment

#### 1.2.1. Animals and housing

Five hundred adult "wild type" (short fin) zebrafish (*Danio rerio*) were obtained through breeding and cultivation in aquariums performed at the Institute of Biological Sciences - ICB – FURG or from a commercial supplier (Red Fish, Porto Alegre, Brazil). All fish were acclimated for at least two weeks in the experimental room and housed in groups of 15 fish in 15 L, kept in a recirculating water system equipped with biological filter and disinfection using UV light, with controlled temperature (28 ± 2 °C), pH (7) and a 14–10 h day/night photoperiod and feeding was performed with balanced artificial diet (Tetra Color Tropical Granules) offered *ad libitum* twice daily. All protocols were approved by the Institutional Animal Care Committee (029/2011, CEUA-FURG) and followed Brazilian legislation, the guidelines of the Brazilian Collegium of Animal Experimentation (COBEA), and the Canadian Council for Animal Care (CCAC) guide on the care and use of fish in research, teaching, and testing.

#### 1.2.2. Preparation of brain homogenates

For organ dissection, fish were anesthetized and brains extracted. Organs were homogenized (1:3) in Tris-HCl buffer (100 mM, pH 7.75) plus EDTA (2 mM) and $Mg^{2+}$ (5 mM) and centrifuged at 10,000 *g* for 20 min at 4°C, the supernatant was exposed to SWNT-PEG[45]. This supernatant was used for the measuring of lipid peroxidation, GSH content, GCL activity and total antioxidant capacity. Before the biochemical analysis, total protein content was determined through the Biuret method (550 nm), in triplicate, using a microplate reader (BioTek LX 800).

### 1.2.3. Exposure to SWNT-PEG

Brain extract was exposed *in vitro* for 1, 2, and 4 h to 1, 10 and 100 mg SWNT-PEG/L. These concentrations were selected based on previous *in vitro* experiments performed with PC12 cells[46]. Control group was also run in parallel, without adding SWNT-PEG suspension. After each exposure time, aliquots were stored, and then lipid peroxidation was measured. The remaining material was used for further biochemical analysis.

### 1.2.4. Measurement of Biochemical analyses

First performed analysis was lipid peroxides content that was estimated by measuring thiobarbituric acid-reactive substances (TBARS)[47]. The concentration of TBARS (nmols/g wet tissue) was calculated by employing a standard curve of tetramethoxypropane (TMP; Acros Organics).

In order to determine the activity of the GCL and GSH content White et al., 2003 protocols were used[48].

Finally, total antioxidant competence against peroxyl radicals was analyzed through ROS determination in organ samples treated or not with a peroxyl radical generator[49].

## 1.3. *In vivo* experiment

### 1.3.1. Biodistribution of SWNT-PEG

Adult male zebrafish (*Danio rerio*, Teleostei, Cyprinidae) were used in the experiment (animal and housing as in item 2.2.1.). Animals were injected intraperitoneally for fivefold on alternate days with 10μL of SWNT-PEG at different concentrations (0.01 mg/mL, 0.1 mg/mL and 1.0 mg/mL). The control group received saline. 48 h after the last injection all the animals were anesthetized and killed for the subsequent tissue removal.

For the biodistribution assays only brain and heart were used, once the other organs were bathed by SWNT-PEG during the intraperitoneal injection. After the removal of the tissues they were homogenized in lyses buffer (1% SDS, 1% Triton X-100, 40 mM Tris acetate, 10 mM EDTA, 10 mM DTT) using a homogenizer and sonication (1 min for each sample)[31]. Shortly before the analysis in the equipment (Horiba T 64000 Raman spectrometer - laser excitation wavelength - 785 nm) tissues were heated at 70°C for two hours to obtain a clear lysate for Raman spectroscopy readings.

### 1.3.2. Open Field Test

Behavioral task was performed using an open field test. The apparatus consisted in a large rectangular glass opaque tank (12.3cm height×38.7cm width×47.3cm length) filled with aquarium water to the level of 12 cm[50]. In the test session 42 adult males of *zebrafish* (animal and housing as in item 2.2) were used. The animals were divided in groups as follows: saline group (n=9), SWNT-PEG 0.01 mg/mL (n=11), SWNT-PEG 0.1 mg/mL (n=11) SWNT-PEG 1mg/mL (n=11). They received intraperitoneal injections of 10μL of SWNT-PEG 24 hours before the test, and the tests were run in the morning.

Fish were placed in the tank and were videotaped for 5 minutes, divided into five separate trials. The trials were recorded via camera (Sony Handycam DCR-SR47, New York, NY) and analyzed off-line using Ethovision XT7 (Noldus IT, Wageningen, Netherlands). Later we proceeded to analyze the mean velocity (cm/s) and distance traveled (cm).

### 1.3.3. Histology examination

Animals were injected by the same protocol for biodistribution. Forty eight hours after the last injection, the animals were anesthetized and killed for the subsequent tissue

removal (organs of the abdominal cavity and brain). The organs were then taken and fixed in 4% neutral buffered formalin, processed routinely into paraffin, sectioned at 7 µm, stained with hematoxylin and eosin (H&E) and examined by a digital microscope Olympus BX41- DP72.

### 1.4. Statistical analysis

In the *in vitro* assay all variables were analyzed by means of ANOVA[51]. The assumptions of normality and homogeneity of variances were verified, and when at least one of them was violated, mathematical transformations were applied. Comparisons between means were then performed through the Newmann–Keuls *post-hoc* test. Orthogonal contrasts were applied in the TBARS assay (see Figure 4). In all cases, type I error probability was fixed at 0.05 ($\alpha = 0.05$).

In the *in vivo* experiment of open field test (OFT) variables were firstly analyzed by repeated measures ANOVA Bonferroni *post hoc* test. Then the endpoint values were calculated by taking the sum of the activity that occurred during 1–5 min for each individual fish, them these data were analyzed by one way ANOVA test of variance followed by Newman-Keuls *post hoc* Test. Data is presented as mean± standard error of the mean (S.E.M.); significance was set at $p<0.05$.

## 2. Results and discussion

In order to understand the actions of carbon nanotubes in biological systems, both *in vitro* and *in vivo*, it is essential to carried out a detailed characterization of the sample used in the experiments[52, 53]. In this work, many techniques were combined to characterized the as received powder material as well the dispersions obtained after each step of mechanical disintegration.

Low-resolution TEM analyses revealed that the commercial PEG-functionalized SWNT material forms large aggregates in water even after prolonged bath sonication (Figures 1A and 1B). The sample still contains a high amount of metallic catalyst as well as some graphitic impurities. The combination of bath sonication and high shear mixing followed by centrifugation steps was able to break the polymer mass and separated the bundles (Figure 1c). However, all attempts to break the bundles in isolated nanotubes and remove the catalyst nanoparticles were unsuccessful. A carefully HRTEM characterization of the final dispersion suggested that the original SWNT material was functionalized in the form of bundles rather than as individual tubes. A representative HRTEM image is shown in Fig. 1D. Besides SWNT bundles, the dispersion contains catalyst residues in the form of metallic nanoparticles surrounded by multiple graphitic layers (nano-onions) and many empty nano-onions. These undesirable materials are also PEG-functionalized and covalently linked to SWNT bundles, preventing their removal by using conventional centrifugation techniques. The metallic impurities were determined by EDS to be mainly composed of nickel and yttrium, two catalysts commonly used in the electric-arc synthesis of SWNTs[54].

Measuring around 100 objects from various areas of several TEM grids, the majority of SWNT-PEG bundles (~ 70%) were estimated to be between 5 and 15 nm, which is equivalent to 3-9 nanotubes (Figure 2a). The bundles have very heterogeneous lengths mostly between 600 nm and 800 nm (Figure 2b).

The concentration of the final dispersion was estimated by measuring the intensity of light absorption at 700 nm. It was possible to achieve a concentration of 2.1 ± 0.2 mg/mL after the entire process.

To verify how the nanotubes are functionalized we used Raman spectroscopy, which is a molecular vibrational spectroscopy widely used to characterize carbon

nanomaterials[55]. This technique provides important information about the structure and purity of the nanotubes. D and G bands are characteristic Raman features of graphitic carbons. The D band is associated with defects and it is present in the Raman spectra of all disordered $sp^2$ carbons in the region 1250-1400 $cm^{-1}$. The G band appears around 1580 $cm^{-1}$ and originates from the in-plane stretching of the C-C bond. The cylindrical geometry and nanoscaled diameter of an SWNT split the G band in two components (longitudinal and transversal) and introduce a special feature in the range 100-500 $cm^{-1}$, the radial breathing mode (RBM), which corresponds to the in phase displacements of carbon atoms in the radial direction[55]. Chemical functionalization of SWNTs is expected to increase the intensity and width of the D band and broaden the RBM and G band [56]. The Raman spectra of SWNT-PEG dispersion taken at 514 and 785 nm showed well-defined and intense G band and RBM features, which was not expected to functionalized samples. This observation confirms that not all tubes were functionalized, i.e. the functionalization occurs mainly on the SWNT bundle surfaces rather than on individual nanotubes (Figure 3).

The zeta potencial is an indicator of the stability of colloidal systems. Because of the electrostatic characteristics, colloidal particles with zeta potential greater than 15 mV tend to be stable. Already stabilized polymer particles and zeta potential of less than 15 mV can be stable, but this is due to steric effects generated by coating the particle with polymer[57]. Measurements of the zeta potential have already been used to discuss the density of acidic sites on the surface of carbon nanotubes and the stability of colloidal carbon nanotube dispersions. The increase in the zeta potential, improves electrostatic repulsion which results in a better dispersion stability [58,59]. Nance and cols[60] demonstrated that nanoparticles with a zeta potential less negative than -4 mV were able to diffusion throughout the nervous tissue of rats. However particles with zeta potential lower than -6 mV were not able to diffusion in this tissue.

The SWNTs in the aqueous dispersion was found to have a zeta potential of around -60 mV. This result may be an indication that the PEG-functionalized nanotubes have still many unreacted carboxylic acid groups (-COOH). Indeed, the zeta potential of SWNT-PEG decreased to around -30 mV with increasing NaCl concentration to 0.06 mol / L. Whereas in physiological media are a series of salts, proteins and other macromolecules which can interact with the nanoparticles, and taking into consideration a relatively negative zeta potential, is considered the possibility of aggregation of SWNT-PEG in biological media. Taking into account all these complex interactions with the nanoparticle constituents of biological media is essential to make analyzes *in vitro* and *in vivo* to evaluate the results of these interactions.

Once the characterization of the nanomaterial was performed, we started the evaluation of their intrinsic behavior when in presence of biological environment *in vitro*. For this we employed the homogenized brain tissue of *zebrafish* exposed to different concentrations of SWNT-PEG and evaluated representative oxidative stress parameters to verify the interactions of the nanomaterial with biomolecules. Regarding the toxicological evaluation of nanomaterials, there is a number of different results that depend on physicochemical characteristics and the type of synthesis of this material[59].

Furthermore, properties such as surface coating and solubility may possibly decrease or amplify the size effect, and as it is known, the size, shape and aggregation are nanomaterial characteristics that can culminate in reactive oxygen species (ROS) generation[61]. Oxidative stress is described with the possible mechanism of toxic action of the nanomaterials, which can occur in biological systems when the increase in the amount of oxidizing agents such as ROS exceeds the capacity of cellular antioxidant defenses or disturb the regulation of cellular redox signaling[62]. Overproduction of reactive species can result in oxidative damage to lipids, proteins and DNA[63].

In this regard, lipid peroxidation is the oxidative degradation of cell membranes initiated by the presence of ROS, which is most commonly measured by assaying the presence of malondialdehyde (MDA) or other thiobarbituric acid reactive substances (TBARS)[63]. In this study we found a significant decrease ($p < 0.05$) on lipid peroxidation in homogenized exposure to SWNT-PEG 1mg / L for 4h when compared with the control group (Figure 4). Wang and cols[65], demonstrated that there was a significant increase in the levels of MDA in PC12 cells treated with different concentrations of SWNT, which occurred in a time and dose-dependent manner. This difference may be due to the fact that SWNT used in this work is functionalized with PEG, similarly then used in another study with PC12 cells where the authors showed that functionalization with PEG significantly reduces the side effects of SWNTs, as well as the disturbance of oxidative stress-related gene expression[46].

Among the oxidative stress parameters studied on tissue homogenates, we chose the exposure of 4 hours, because this was the only time in which a significant modulation in lipid peroxidation was observed. The next parameter analyzed was the total antioxidant capacity, where there has been no significant change ($p > 0.05$) in treatments assessed (Figure 5).

We also analyzed glutathione (GSH) content once it is the major intracellular low molecular-weight thiol compound that plays a critical role in the cellular defense against oxidative stress through scavenger ROS directly or indirectly in cells[66]. Our results show a significant decrease in the content of GSH in the extract exposed to lower concentrations of SWNT-PEG (Figure 6) that corroborated with the results of [46, 64]. Thus, it can be inferred that depletion of GSH content induced by SWNT-PEG may be due to increased reactive oxygen species (ROS). However, other results of our study show that this increase

in ROS was not enough to cause damage in lipids of the brain extract and does not increase the activity of GCL (Figure 6).

Besides the *in vitro* analysis, an *in vivo* study was conducted. First we evaluated the biodistribution of the nanomaterial by Raman spectroscopy. However, if SWNT-PEG was indeed distributed through zebrafish organs, it reached tissues at concentrations below $10^{-5}$ mg/mL (Figure 7) since the Raman spectra of SWNT-PEG G band was not observed for the brain tisuues. The SWNT-PEG G band could be observed for control samples with SWNT-PEG concentration as low as $10^{-5}$ mg/mL. In another study, performed with mouse and SWNT noncovalently functionalized with different PEG chains, also did not observe distribution of these nanomaterials to the brain. This study also assessed blood tissue, liver, spleen, among others, and in these tissues it was possible to verify the presence of PEGylated SWNT[31].

To access indirect toxic whole effects caused by the nanotubes, we conducted the open field test on animals 24h after exposure to different concentrations of SWNT-PEG. In this task were evaluated locomotor and exploratory activity. Only the animals exposed to the higher concentration of SWNT-PEG (1mg/mL) had a significant increase in overall distance traveled (Figure 8). Troughand cols[67], also observed abnormalities in locomotor activity in animals exposed to gold nanoparticles, showing the validity of this test to check changes in central nervous system.

Histological changes of animals that received intraperitoneal injections of PEG-SWNT-PEG were also analyzed (figure 9). In this experiment we observed increased tissue injury and inflammation in a dose-dependent manner. In the abdominal cavity organs we observe injuries caused directly by interaction of tissues and the nanomaterial. Although nervous tissue damage occurred in an indirect way, the animals presented an inflammatory response demonstrated by histological assay. In a study using mouse, it was shown that

intraperitoneal injection of magnetic $Fe_3O_4$-nanoparticle induces hepatic and renal tissue injury and the tissue injury is also dose dependent[68].

## 4. Conclusions

The results found in this study demonstrated that the type of functionalization of the carbon nanotube is extremely decisive for their biological interaction. We have been looking for a nanomaterial with high biocompatibility and potential biomedical applications mainly in the field of neuroscience. Although *in vitro* experiment showed that the SWNT-PEG produced subtle changes in the oxidative stress parameters analyzed, with a discrete antioxidant effect on lipid peroxidation and an opposite pro-oxidant effect by the depletion of GSH. These nanomaterial has some limitations, how demonstrated by *in vivo* study, in which the SWNT functionalized with PEG 600 is not able to biodistribution, probably due to its instability in saline medium. In biological medium, it was observed aggregation of the nanotubes which resulted in damage to the blood vessels that lead to thrombus formation and resulted in tissue necrosis. Thus, there was an extensive inflammatory process once the nanomaterial was trapped in the abdominal cavity where it was introduced. Also in the central nervous system several histological modifications were observed in addition to the behavior changes observed in open field test. However, since nanomaterials are promising in the biomedical area, further studies are necessary, especially with SWNT functionalized with larger PEG chain.

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**Figures and captions**

**Figure 1.** TEM images of SWNT-PEG dispersion before (A, B) and after (C) purifying and breaking the polymeric mass. (D) HRTEM image of the final dispersion.

**Figure 2.** Histograms of bundle size (A) and length (B) distributions of SWNTs-PEG presented in the final dispersion.

**Figure 3.** Raman spectrum of SWNT-PEG dispersion at laser excitation of 514 nm.

**Figure 4.** Concentration of thiobarbituric acid reactive substances (TBARS) in brain extracts after 1, 2, or 4 h exposure to 1, 10 and 100 mg/L of SWNT-PE. Data are expressed as means±1 standard error. * significant statistical difference ($p$<0.05) by means of orthogonal contrast.

**Figure 5.** Total antioxidant capacity against peroxyl radicals in brain extracts after 4 h of exposure to 1, 10 and 100 mg/L SWNT-PEG. Data are expressed as means±1.0 standard error. Higher bars mean low total antioxidant capacity against peroxyl radicals.

**Figure 6.** A) Reduced glutathione (GSH) content in brain extracts after 4 h of exposure to 1, 10 and 100 mg/L SWNT-PEG. Data are expressed as means±1.0 standard error. * significant statistical differences ($p$>0.05). B) Glutathione-cysteine ligase (GCL) specific activity in brain extracts after 4 h of exposure to 1, 10 and 100 mg/L SWNT-PEG. Data are expressed as means±1.0 standard error.

**Figure 7.** Raman spectra of organs of *Danio rerio* after the injections of SWNT-PEG dispersions at different concentrations. The first line is a positive control of SWNT-PEG.

**Figura 8.** Total distanced traveled (A and C) and velocity (B and D) were recorded for 5 min in animals exposed to 3 concentrations of SWNT-PEG (0,01, 0,1 and 1,0 mg/mL).

**Figure 9.** The pictures A, B, C and D are photos of the peritoneal cavity of zebrafish 24h after intraperitoneal injection of different concetrações of SWNT-PEG. The other images

are slides of zebrafish tissues stained with hematoxylin and eosin. Slide E show exocrine pancreas with acini normal aspect en the peritoneal fat (20X), slide F has midgut with incipient inflammatory infiltrates in the submucosa and dense mononuclear infiltrates in peritoneum (10X), slide G show intestine with dense inflammatory infiltrates in the mucosa and submucosa, with superficial mucosal necrosis (40X), slide H shows peritoneal tissue with dense inflammatory infiltrates mainly by macrophages. Melanin deposits is observed in macrophages (40X), slide I consists of brain cortex with slight edema observed and normal cells avenulares (40X), slide J e K are from cortex with glial proliferation marked and focal edema (40X) and slide L shows brain with glial proliferation, edema, signs of neuronal distress with nuclear pallor and satellitosis (40X).

**Figure 1**

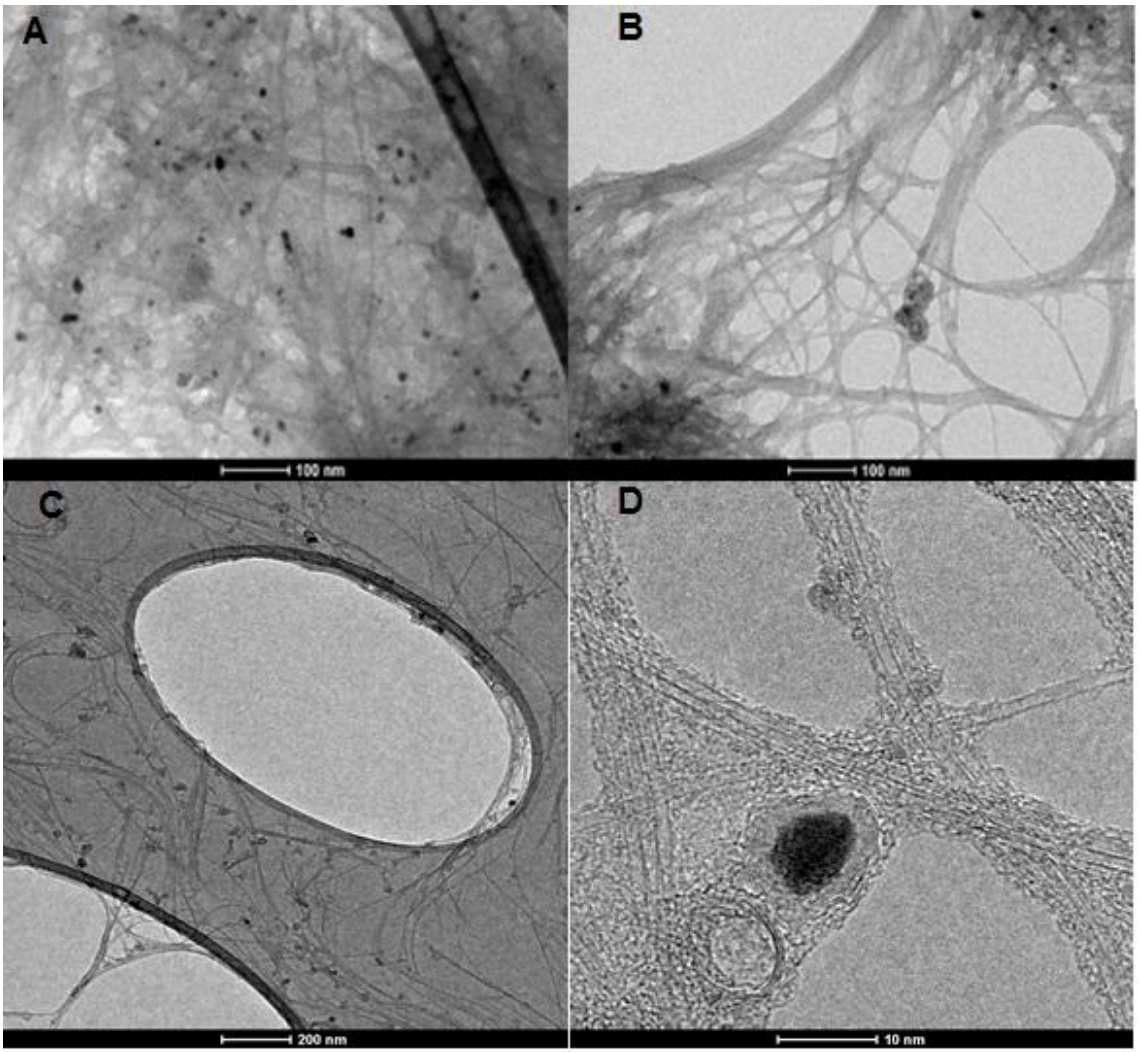


**Figure 2**

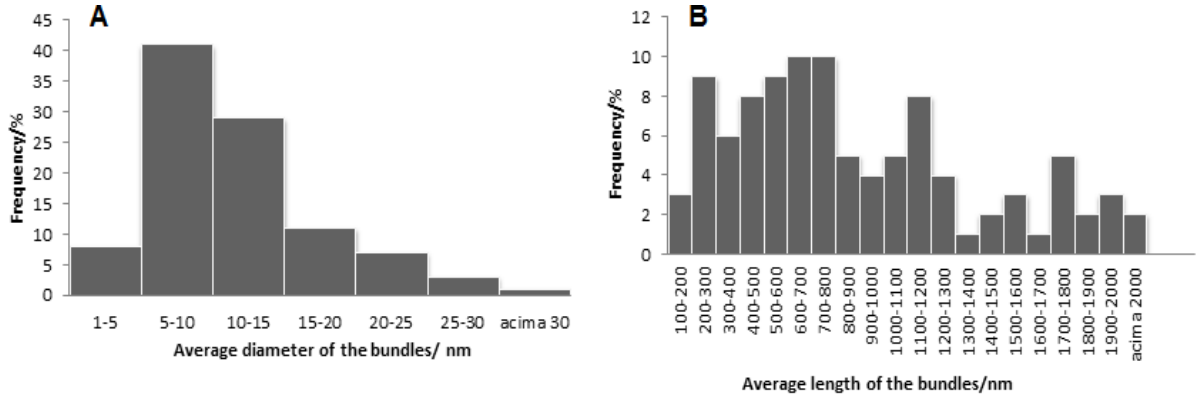

A
Frequency/%
45
40
35
30
25
20
15
10
5
0
1-5
5-10
10-15
15-20
20-25
25-30
acima 30
Average diameter of the bundles/ nm
B
Frequency/%
12
10
8
6
4
2
0
100-200
200-300
300-400
400-500
500-600
600-700
700-800
800-900
900-1000
1000-1100
1100-1200
1200-1300
1300-1400
1400-1500
1500-1600
1600-1700
1700-1800
1800-1900
1900-2000
acima 2000
Average length of the bundles/nm

**Figure 3**

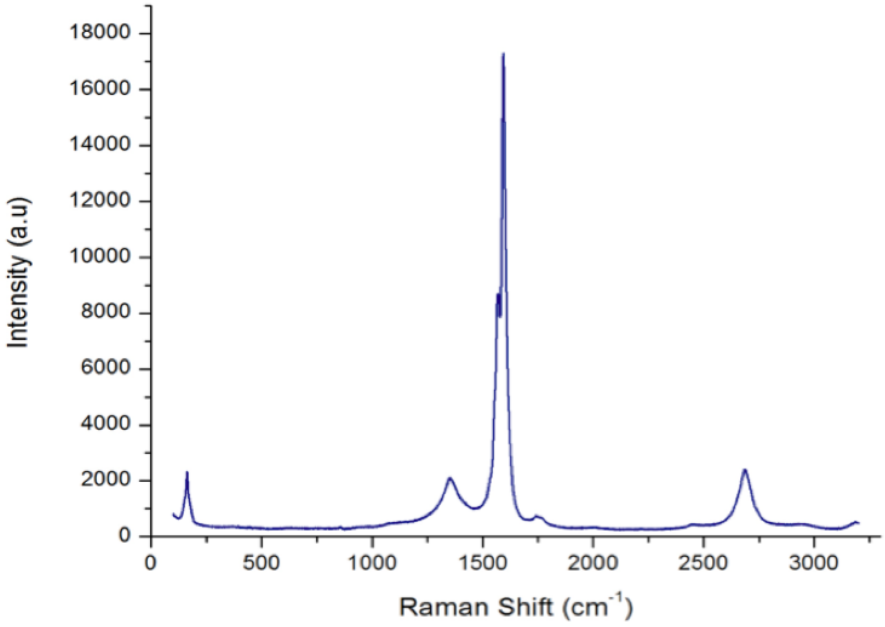

Intensity (a.u)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0
500
1000
1500
2000
2500
3000
Raman Shift (cm-1)

**Figure 4**

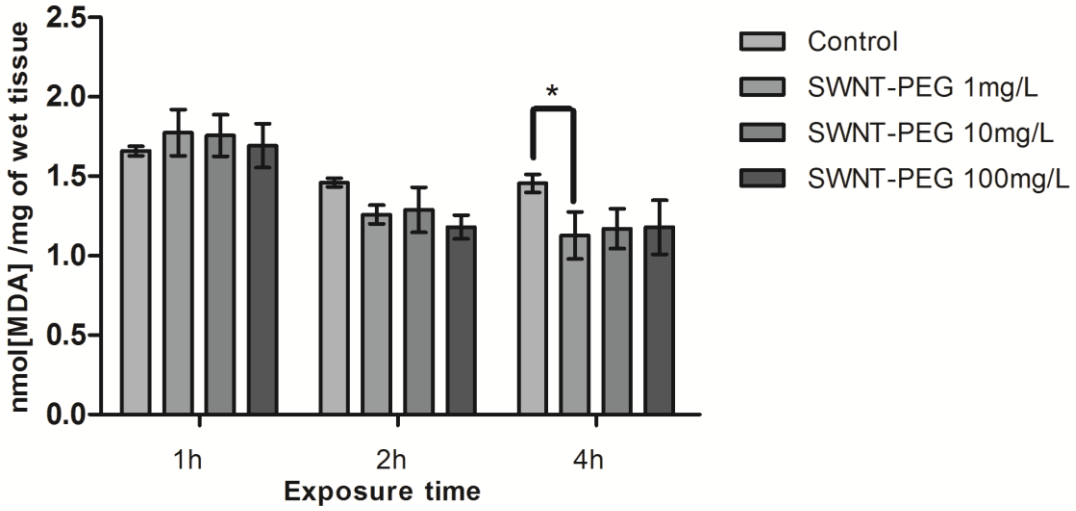

nmol[MDA] /mg of wet tissue
2.5
2.0
1.5
1.0
0.5
0.0
*
1h
2h
4h
Exposure time
Control
SWNT-PEG 1mg/L
SWNT-PEG 10mg/L
SWNT-PEG 100mg/L

**Figure 5**

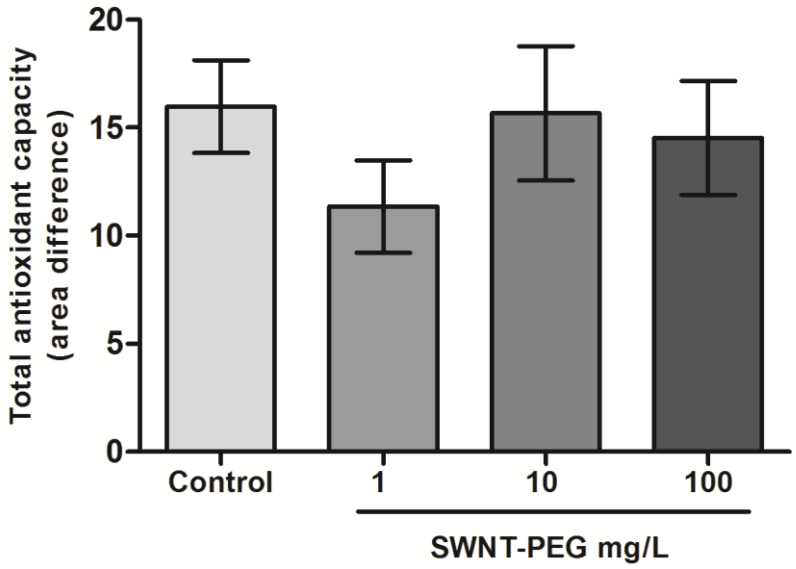

Total antioxidant capacity
(area difference)
20
15
10
5
0
Control
1
10
100
SWNT-PEG mg/L

**Figure 6**

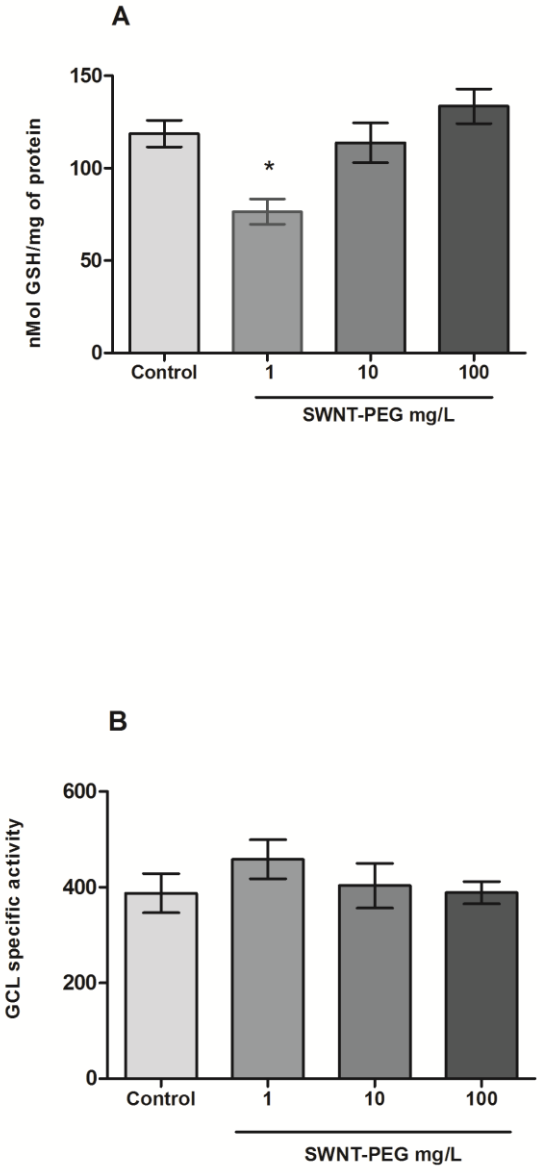

A
150
100
50
0
nMol GSH/mg of protein
*
Control
1
10
100
SWNT-PEG mg/L
B
600
400
200
0
GCL specific activity
Control
1
10
100
SWNT-PEG mg/L

**Figure 7**

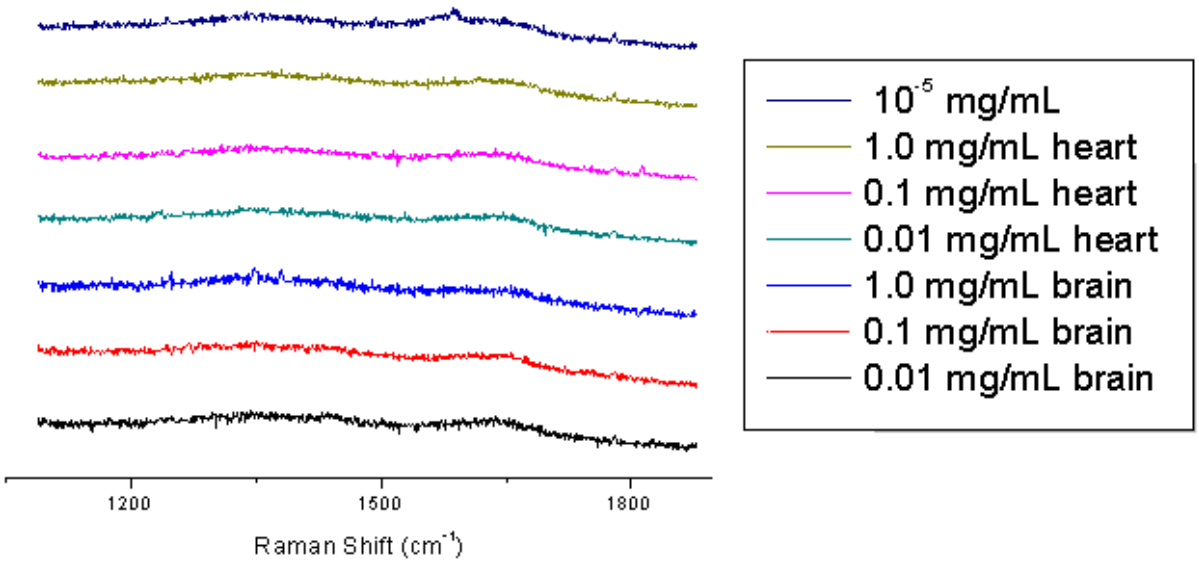

10-5 mg/mL
1.0 mg/mL heart
0.1 mg/mL heart
0.01 mg/mL heart
1.0 mg/mL brain
0.1 mg/mL brain
0.01 mg/mL brain
1200
1500
1800
Raman Shift (cm-1)

**Figure 8**

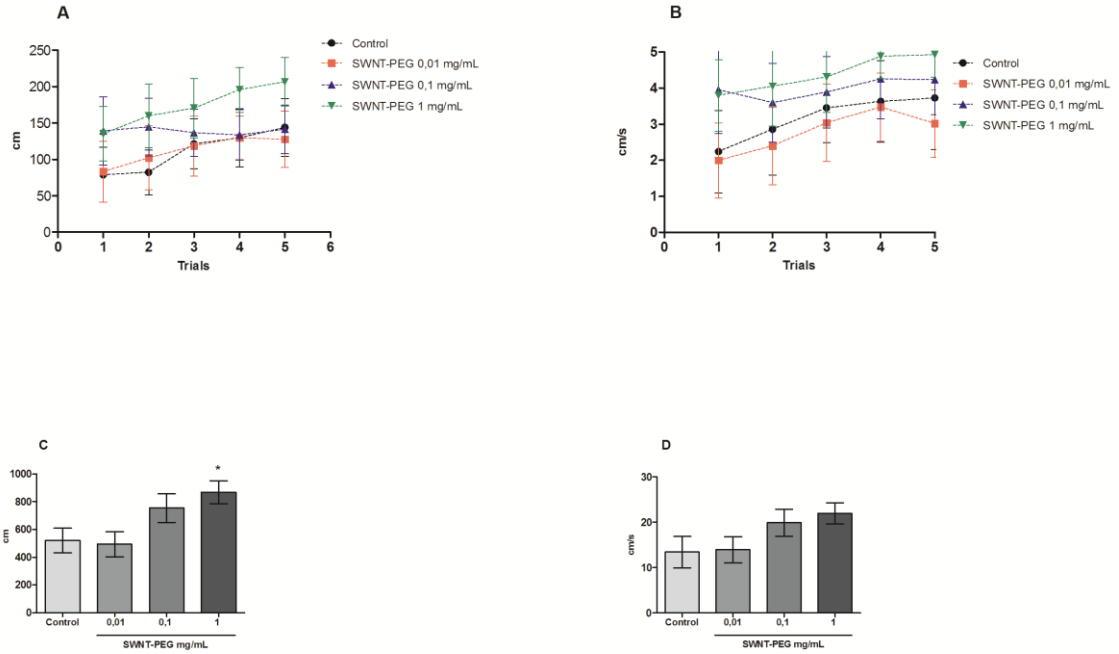
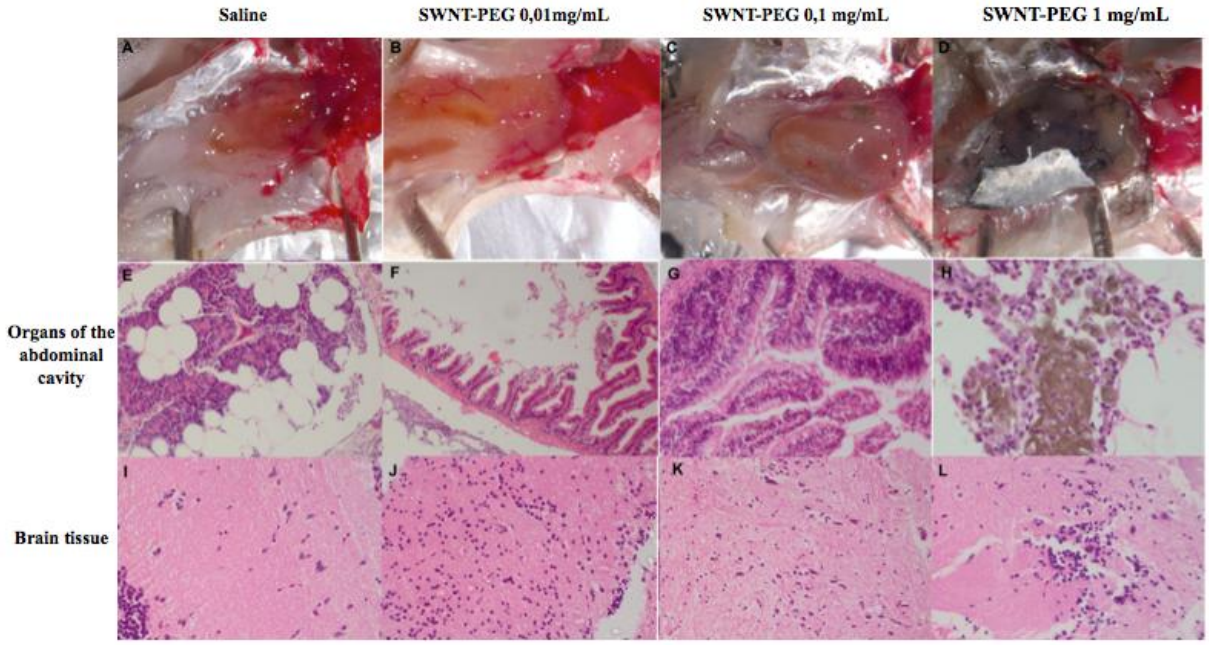

A
cm
Trials
Control
SWNT-PEG 0,01 mg/mL
SWNT-PEG 0,1 mg/mL
SWNT-PEG 1 mg/mL
B
cm/s
Trials
Control
SWNT-PEG 0,01 mg/mL
SWNT-PEG 0,1 mg/mL
SWNT-PEG 1 mg/mL
C
cm
*
Control
0,01
0,1
1
SWNT-PEG mg/mL
D
cm/s
Control
0,01
0,1
1
SWNT-PEG mg/mL

**Figure 9**


Saline
SWNT-PEG 0,01mg/mL
SWNT-PEG 0,1 mg/mL
SWNT-PEG 1 mg/mL
A
B
C
D
Organs of the abdominal cavity
E
F
G
H
Brain tissue
I
J
K
L

**Considerações finais**

Este estudo fornece informações importantes para a compreensão da interação entre um promissor nanomaterial, formado por nanotubos de carbono funcionalizados, e um modelo biológico amplamente estudado, o peixe *Danio rerio*. Tendo em vista a crescente produção de nanomateriais e a variedade de possibilidades que estes oferecem nas mais diversas áreas, a investigação dos seus efeitos nos organismos vivos se torna fundamental. Sendo assim, o estudo em um modelo biológico cuja genética e fisiologia são bem conhecidos pode permitir a elucidação dos mecanismos envolvidos nos efeitos biológicos dos nanomateriais.

Os resultados encontrados neste trabalho apontam para a necessidade de se investigar minuciosamente a influência do tipo de funcionalização dos nanotubos de carbono sobre seus efeitos biológicos. Como demonstrado neste trabalho, mesmo um grupo funcional altamente hidrofílico e biocompatível, como o PEG, pode ser ineficiente na dispersão e biodistribuição dos nanotubos em meio biológico. Características como o tamanho, a reatividade e a ramificação da cadeia de PEG devem ser consideradas, além da presença de impurezas, resíduos ácidos e grau de agregação do nanomaterial em meio aquoso.

Concluímos que os resultados de toxicidade encontrados neste trabalho são decorrentes da incapacidade do nanomaterial em se dispersar no meio biológico. O experimento *in vitro* corrobora com esta conclusão ao demonstrar que o SWNT-PEG não gerou um quadro de estresse oxidativo quando em contato com as biomoléculas, sendo que as respostas nocivas verificadas *in vivo* estão diretamente relacionadas à agregação do material e a extensa resposta inflamatória e dano tecidual verificada onde houve acumulação dos SWNT-PEG.

Este trabalho evidenciou a importância de se realizar experimentos *in vitro* e *in vivo* a fim de se conhecer tanto as interações de nanomateriais com os constituintes celulares

além das respostas orgânicas e comportamentais que podem ser decorrentes da exposição aos mesmos. Estes estudos são extremamente importantes para a seleção de nanomateriais com potenciais aplicações na área biomédica.

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