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**Toxicidade de nanotubos de carbono de parede simples funcionalizados com polietileno glicol em embriões de *zebrafish* (*Danio rerio*)**

**Felipe Antonio Girardi**

Dissertação defendida no âmbito do programa de Pós-Graduação em Ciências Fisiológicas: Fisiologia Animal Comparada como parte dos requisitos para obtenção do título de MESTRE em Ciências Fisiológicas

Orientadora: Prof<sup>a</sup>. Dr<sup>a</sup>. Daniela Martí Barros

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## Resumo geral

GIRARDI, Felipe Antonio. **Toxicidade de nanotubos de carbono de parede simples funcionalizados com polietileno glicol em embriões de *zebrafish***. 2015. 90 p. Dissertação de Mestrado. Programa de Pós-Graduação em Ciências Fisiológicas: Fisiologia Animal Comparada. Universidade Federal do Rio Grande, Rio Grande.

A nanotecnologia tem possibilitado a geração de nanomateriais (NM) com características químicas e físicas singulares, despertando o interesse de diferentes áreas científicas e tecnológicas para suas potenciais aplicações. Os nanotubos de carbono de parede simples funcionalizados com polietileno glicol (*single walled carbon nanotubes*; SWCNTs-PEG) são materiais promissores para aplicações biomédicas, como dispositivos diagnósticos e sistemas de liberação controlada de fármacos. Porém, diversas questões sobre seu perfil toxicológico ainda não foram respondidas. Sendo assim, o objetivo desse trabalho é investigar a ação dos SWNT-PEG em *Danio rerio* “*zebrafish*” (Teleostei, Cyprinidae) nos níveis molecular, fisiológico, morfológico e comportamental. Embriões de *zebrafish* foram expostos individualmente a SWCNTs funcionalizados com duas cargas diferentes de peguilação: 600 Da (previamente caracterizados) e 2000 Da (caracterização inédita). Foram avaliados diversos parâmetros relacionados com toxicidade geral, desenvolvimento, dosagem de espécies reativas de oxigênio e ensaio cometa como avaliação de genotoxicidade. Os resultados mostram que os efeitos tóxicos, para ambos os nanotubos, ocorreram predominantemente na maior concentração testada (1 ppm), resultando em taxas de mortalidades significativamente elevadas, além de atraso no processo de eclosão. Foi observada também diminuição do comprimento total das larvas, para ambos os tratamentos, porém, sem alteração de parâmetros relacionados com desenvolvimento geral e do sistema nervoso central. A espectroscopia Raman não evidenciou a presença intracelular de nanotubos, mas o ensaio cometa mostrou efeito genotóxico significativamente elevado no tratamento com SWCNTs-PEG 600 Da (1 ppm). Mais estudos são necessários para entender quais mecanismos estão envolvidos, porém, acredita-se que o estresse oxidativo esteja entre os principais responsáveis pelos efeitos tóxicos observados, já que a quantificação de espécies reativas de oxigênio mostrou tendência à elevação nas maiores concentrações testadas. Os resultados deste estudo mostram que a presença de metais contaminantes pode ter importante papel nos efeitos observados e a funcionalização de nanotubos de carbono com diferentes pesos moleculares de PEG pode ser ineficiente na diminuição de sua toxicidade.

Palavras-chave: Nanotubos de carbono; toxicidade; *zebrafish*

## **1. Introdução geral**

### **1.1 Nanotecnologia**

A nanotecnologia tem possibilitado a geração de nanomateriais (NM) com características químicas e físicas singulares, despertando o interesse de diferentes áreas científicas e tecnológicas para suas potenciais aplicações. Nanomateriais compreendem estruturas com pelo menos uma de suas dimensões na escala nanométrica, diferentes composições e arranjos atômicos, dentre os quais se destacam os compostos puramente de carbono, os metais e óxidos metálicos, as nanopartículas poliméricas e os lipossomos (Mc Neil, 2005).

Com base no grande potencial de aplicabilidade de nanomateriais, espera-se que em um futuro próximo haja uma ampla produção com consequente exposições ocupacionais, bem como o desenvolvimento de medicamentos nanoencapsulados (Parpura *et al.*, 2013).

Dessa forma, uma análise criteriosa dos efeitos tóxicos destes materiais é de grande importância, já que estes podem interagir com os sistemas biológicos em nível molecular com alto grau de especificidade, podendo causar danos a tecidos e órgãos (Meng *et al.*, 2012).

A toxicologia tradicionalmente trata de questões envolvendo os efeitos adversos de substâncias químicas sobre os seres vivos. Parâmetros-chave na toxicologia clássica, como dose e tempo, são facilmente mensuráveis para substâncias químicas simples, além de grande parte dos ensaios já estarem plenamente definidos e padronizados. Porém, quando tratamos de nanotoxicologia, essas questões são agravadas pelas propriedades diferenciadas dos nanomateriais e falta de padronização dos ensaios toxicológicos. Isso se torna ainda mais complexo quando se extrapolam os resultados de sistemas *in vitro* para sistemas *in vivo* (Elsaesser e Howard, 2012).

Medicamentos baseados em nanoestruturas já são uma realidade, sendo usados experimentalmente para tratamento de diversas patologias, como inflamação e câncer e muitos outros usos estão em crescente investigação (Johnston *et al.*, 2010). Embora diversos estudos sobre a toxicidade de nanomateriais tenham sido realizados nos

últimos anos, um conhecimento abrangente de seus efeitos ainda está longe de ser obtido.

## 1.2 Nanotubos de carbono

Os nanotubos de carbono (*carbon nanotubes*, CNT), descritos inicialmente na década de 1990 (Iijima e Ichihashi, 1993), são alótropos do carbono formados por folhas de grafeno enroladas em forma de tubos de diâmetro nanométrico e comprimento na ordem de microns. Os nanotubos podem ser agrupados em dois principais grupos: parede simples (SWCNT, *single-walled carbon nanotubes*), constituídos por apenas uma camada cilíndrica de grafeno, e paredes múltiplas (MWNT, *multi-walled carbon nanotubes*), formados por vários cilíndricos concêntricos de grafeno (Fig.a).

A estrutura dos nanotubos confere a eles propriedades especiais, mecânicas e elétricas que despertam o interesse de pesquisadores e da indústria para diversas aplicações no campo da engenharia e física (Baughman *et al.*, 2002). A estrutura molecular destes compostos confere, por exemplo, elevada resistência mecânica, propriedades ópticas distintas além de e uma excelente condutividade elétrica (Bekyarova *et al.*, 2005).

Os CNTs podem ser sintetizados por diferentes métodos: descarga por arco elétrico, ablação por laser e deposição química a vapor. Quase sempre constituem uma mistura de estruturas com ampla variação de diâmetros e comprimentos, podendo apresentar impurezas como carbono amorfo e resíduos catalíticos como ferro e níquel (Firme e Bandaru, 2010).

Na última década, os CNTs foram intensamente explorados em sistemas de liberação de fármacos, devido a sua facilidade de penetração nas células sem causar citotoxicidade aparente. As características físico-químicas fazem com que nanotubos se comportem como "agulhas" (*needle-like*), facilitando a penetração nas células, podendo carregar consigo moléculas de interesse para a nanomedicina, por exemplo, drogas, genes e moléculas úteis para fins diagnósticos (Liang e Chen, 2010; Shao *et al.*, 2013).

A preocupação com o perfil toxicológico dos nanotubos cresce da mesma maneira que crescem suas possíveis aplicações. Alguns estudos na literatura sugerem que tal preocupação é infundada, e que os CNTs podem ser bem tolerados



biologicamente (Liu *et al.*, 2013). Inconsistências na literatura quanto à segurança de nanotubos se devem principalmente a diferenças no procedimento experimental das pesquisas e falta de caracterização físico-química adequada dos compostos estudados (Ong *et al.*, 2014). De maneira geral, os dados disponíveis na literatura indicam que estas estruturas são biocompatíveis e pouco tóxicas para finalidades biomédicas (Yang *et al.*, 2012).

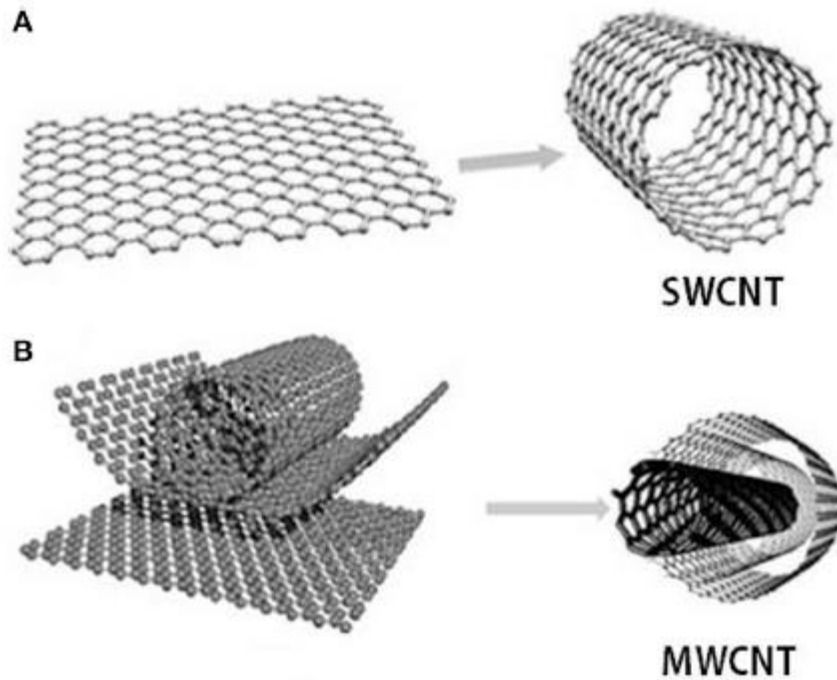


Figura a: Folhas de grafeno enroladas sobre si mesmas, formando nanotubos de carbono de paredes simples (A) e paredes múltiplas (B). Fonte: (Vidu *et al.*, 2014)

### 1.3 Funcionalização de nanotubos de carbono

Por sua natureza puramente composta de carbono, os nanotubos são intrinsecamente insolúveis em meio aquoso, gerando os fenômenos de aglomeração e agregação. Tais fenômenos envolvem a adesão das partículas, umas às outras, principalmente por forças de van der Waals, devido à elevada relação entre área de superfície e volume (Elsaesser e Howard, 2012). Ocorrendo a aglomeração, as propriedades físico-químicas se alteram, modificando seus efeitos biológicos (Navarro *et*

*al.*, 2008). Este se torna um dos principais fatores limitantes para o uso na área biomédica.

Uma das formas para contornar os problemas relacionados com a hidrofobicidade desses compostos é a biofuncionalização com moléculas orgânicas ou polímeros biocompatíveis. A funcionalização é o processo pelo qual os nanotubos de carbono, que normalmente formam densos agregados, são separados e revestidos com certas moléculas. Diversas abordagens químicas e físicas são possíveis para funcionalização de nanotubos, sendo genericamente categorizadas em modificações covalentes e não covalentes.

As modificações não covalentes se valem da natureza hidrofóbica dos CNTs para adsorção de moléculas anfifílicas, surfactantes de peso molecular pequeno, ou mesmo polímeros, que envolvem a superfície, sem modificar a estrutura molecular dos nanotubos, de maneira que a porção hidrofílica destas moléculas interage com solventes polares, e a porção hidrofóbica interage com a superfície dos nanotubos (Shao *et al.*, 2013). Por sua vez, a modificação covalente gera alterações de superfície causadas por reações químicas, seguidas de conjugação com moléculas orgânicas ou polímeros hidrofílicos, rendendo melhor solubilidade em água. Esse tipo de modificação, além do aumento de solubilidade, oferece também a possibilidade de incorporação de agentes terapêuticos (Georgakilas *et al.*, 2002).

Diversos estudos, *in vivo* e *in vitro*, evidenciam que a funcionalização com polietileno glicol (PEG), um tipo de modificação covalente, gera um perfil farmacocinético e toxicológico mais favorável aos CNTs (Bottini *et al.*, 2011). A incorporação de PEG é uma estratégia largamente utilizada para aumentar a capacidade de dispersão em água e também diminuir a toxicidade desses compostos (Ilbasmiş-Tamer *et al.*, 2010). Além disso, este material é liberado para uso interno em humanos pelo FDA (*Food and Drug Administration*), nos Estados Unidos e ANVISA (Agência Nacional de Vigilância Sanitária), no Brasil.

O PEG é um poliéter, cuja estrutura se baseia na repetição simples de grupos HO-(CH<sub>2</sub>-CH<sub>2</sub>-O)<sub>n</sub>-H (Fig.b), com massa molecular de 44 g/mol. Sendo assim, uma

molécula PEG de 1k Dalton (Da) consiste em aproximadamente  $n=23$  unidades repetidas (Wattendorf e Merkle, 2008).

Devido as suas características químicas relacionadas com a hidrofiliidade e neutralidade elétrica, polímeros como o PEG possuem capacidade de "repelir" proteínas. Acredita-se que superfícies peguiladas formem camadas flexíveis que se organizam espacialmente em forma de "escova" (*brush-like*), evitando assim a adsorção com proteínas e demais biomoléculas. A magnitude de tal efeito será dependente do comprimento e densidade das cadeias PEG, e de maneira geral quanto maior e mais densa a cadeia menor a interação com proteínas e tendência a agregação (Peracchia *et al.*, 1997). Este aspecto é particularmente importante quando pensamos em meios biológicos, tendo em vista a elevada quantidade de proteínas na superfície celular e que as interações se tornam mais fortes e potencialmente prejudiciais quanto maior for o grau de adsorção dos nanotubos.

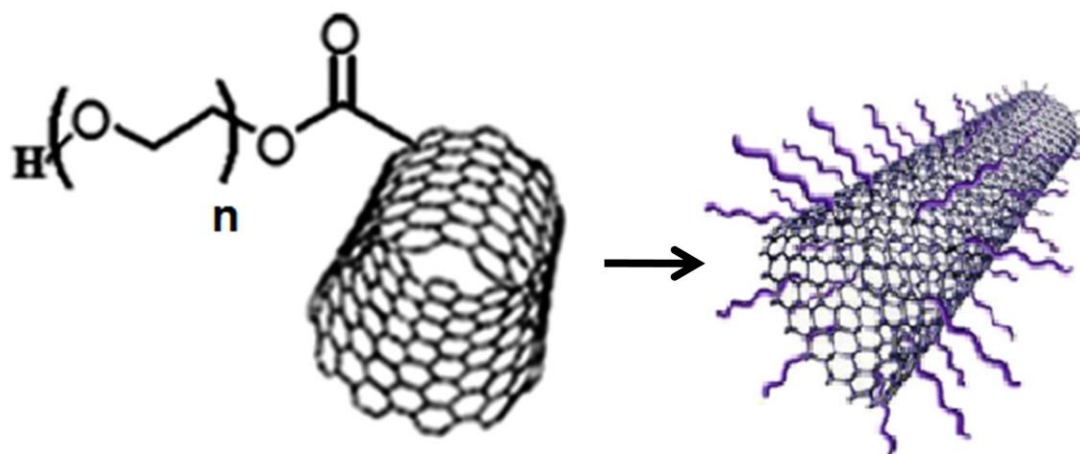


Figura b: SWCNTs-PEG. Adaptado de (Huang *et al.*, 2014; Kishore *et al.*, 2014)

Comparando diversos graus de peguilação, Andersen e colaboradores (2013) mostraram que a resposta imunológica do sistema complemento diminui conforme aumenta o grau de peguilação de CNT, apesar de não a suprimir completamente esta ativação. O estudo de Weber e colaboradores (2014) mostrou que a incorporação de PEG (600 Da) a SWCNTs pode ser ineficiente na dispersão e biodistribuição dos nanotubos em meio biológico, causando prejuízos inflamatórios e comportamentais em

*zebrafish*, e sugere que cadeias de peso molecular mais elevado poderiam atenuar os prejuízos. Neste sentido, outros estudos da literatura sugerem que o uso de cadeias mais densas tende a diminuir a toxicidade dos nanotubos, principalmente evitando sua adsorção e melhorando a estabilidade das suspensões em meios fisiológicos.

Apesar de outros métodos de funcionalização estarem disponíveis, como a carboxilação ou hidroxilação, a peguilação ainda se mostra como a forma mais eficiente de melhorar o comportamento farmacocinético de nanotubos de carbono. O desenvolvimento de técnicas fáceis, rápidas e reprodutíveis de peguilação se torna crucial para as futuras aplicações biomédicas destas partículas (Yang *et al.*, 2012).

Considerando o quão crítico a peguilação é para o comportamento de nanopartículas, estudos comparativos de diferentes cargas de densidade PEG são um pré-requisito para o desenvolvimento e produção de novas formulações (Rabanel *et al.*, 2014) bem como a definição das características de cadeias PEG mais adequadas ao uso biomédico.

#### **1.4 *Zebrafish* como modelo animal na investigação toxicológica de nanomateriais**

Apesar da crescente popularidade dos nanomateriais, ainda faltam dados toxicológicos realizados *in vivo*, principalmente em relação aos efeitos tóxicos sobre um organismo em desenvolvimento (Pietrojusti *et al.*, 2011). Nos últimos anos tem crescido o interesse da comunidade científica nos efeitos de nanomateriais em modelos não-mamíferos de embriologia e toxicologia, visando uma compreensão mais ampla dos mecanismos de toxicidade e de como uma dada nanopartícula é capaz de afetar o curso normal do desenvolvimento embrionário de certa espécie. Além disso, a investigação de potencial teratogênico de substâncias farmacêuticas é um aspecto fundamental na pesquisa de novos produtos.

Vários animais têm sido testados com essa finalidade no estudo de nanomateriais de carbono, incluindo camundongos (Pietrojusti *et al.*, 2011), o nematódeo *C. elegans* (Chen *et al.*, 2013), galinhas (Roman *et al.*, 2013) e a mosca *Drosophila* (Liu *et al.*, 2014). (Chesneau *et al.*, 2008)(Chesneau *et al.*, 2008)(Chesneau *et al.*, 2008)(Chesneau *et al.*, 2008)(Chesneau *et al.*, 2008) Por sua vez, o *zebrafish*, está provando ser um

modelo rápido e prático para avaliar de forma eficaz a toxicidade e avaliação de risco teratogênico dos nanocompostos (Duan *et al.*, 2013).

O peixe *Danio rerio* (Teleostei, Cyprinidae), conhecido popularmente por *zebrafish*, é um organismo que compartilha muitas características celulares, anatômicas e fisiológicas com outros vertebrados, e tem recebido destaque em pesquisas nas áreas da genética, farmacologia e neurociência (Guo, 2004). O embrião de *zebrafish* é um modelo de pesquisa útil pelo seu tamanho pequeno, transparência, fácil manutenção, rápida embriogênese e reprodução contínua (Fig. c, d). Uma vez que os embriões permanecem transparentes ao longo da maior parte do desenvolvimento, a formação dos principais órgãos pode também ser observada em tempo real (Kimmel *et al.*, 1995). O processo de desenvolvimento embriológico dos peixes teleósteos apresenta alta homologia com demais espécies de vertebrados, tornando o desenvolvimento desta espécie largamente comparável com o de mamíferos. Um número crescente de estudos tem demonstrado que embriões e larvas de *zebrafish* podem servir como uma plataforma conveniente para avaliação farmacológica e triagem de possíveis efeitos adversos de medicamentos. Além disso, o genoma desta espécie foi inteiramente sequenciado, o que coloca este peixe de água doce em uma posição privilegiada para estudos toxicológicos.

Diversas evidências sugerem que o *zebrafish* apresenta importante relevância pré-clínica, sendo um modelo de destaque para avaliação de substâncias químicas e atraindo cada vez mais atenção da indústria farmacêutica. Estágios embrionários do *zebrafish* são cada vez mais utilizados como modelo na investigação biomédica e (eco) toxicológica.

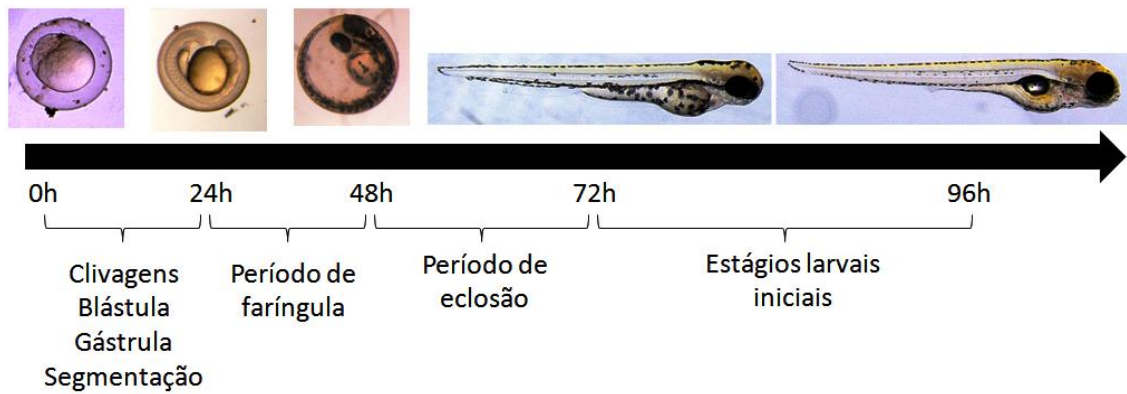


Figura c: Representação esquemática dos primeiros estágios de vida do *zebrafish* em 96 horas após fertilização (hpf). Entre 10 e 24 hpf ocorre a organogênese primária, desenvolvimento da cauda e são observados os primeiros movimentos espontâneos. Entre 24 e 48 hpf, podem ser observadas a circulação sanguínea, pigmentação corporal e os primeiros batimentos cardíacos. Entre 48 e 72hpf o embrião pode eclodir e a formação primária dos órgãos e sistemas está completa. A partir das 72 hpf a bexiga natatória se infla, a larva passa a apresentar comportamentos complexos como procura ativa por comida, comportamento exploratório e reflexo de fuga.

A vantagem de avaliar as consequências da exposição de nanomateriais durante o desenvolvimento embriológico é que pequenas perturbações do programa de desenvolvimento podem levar a respostas facilmente detectáveis (Usenko *et al.*, 2007). Os primeiros estágios de desenvolvimento oferecem uma “janela temporal” única na qual se pode avaliar uma ampla gama de vias de sinalização ativas, biomoléculas essenciais e suas interações com qualquer nanomaterial, interações estas que apresentarão suas consequências na vida adulta (Zon e Peterson, 2005). Durante a embriogênese, diversos mecanismos celulares e moleculares estão ativos em uma complexa rede que leva a formação dos tecidos e órgãos. Tais mecanismos podem ser influenciados de maneira mais ou menos específica por um grande número de agentes, com possível impacto na organização e diferenciação celular, inclusive em nível macroscópico. Diversos estudos com diferentes nanopartículas evidenciaram efeitos tóxicos e ocorrência de malformações (Cheng *et al.*, 2011; Cheng e Cheng, 2012; Duan *et al.*, 2013; Ong *et al.*, 2013), mostrando que o desenvolvimento embriológico desta espécie pode ser fortemente prejudicado por nanopartículas.



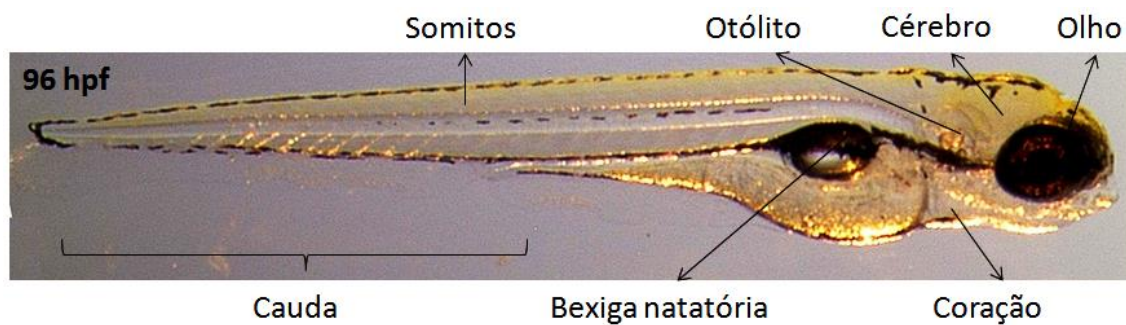


Figura d: Detalhe das principais partes anatômicas da larva do *zebrafish* em 96 hpf.

Este modelo toxicológico, além de flexível e versátil para a nanotoxicologia (Kim, 2013), tem ainda a vantagem de avaliar os efeitos da substância em estudo diretamente sob o processo de desenvolvimento, sem a complexa influência do metabolismo materno, que poderia gerar falsas interpretações, além de fornecer informações em diferentes níveis de organização: fisiológico, morfológico e comportamental (Frayse *et al.*, 2006; Hermesen *et al.*, 2011). Qualquer nanomaterial ou molécula pequena pode ser testada com o embrião do *zebrafish*, os embriões podem ser expostos continuamente por todo o período embrionário e larval por simples adição dos compostos à água onde se encontram os mesmos (Fako e Furgeson, 2009; Kim e Tanguay, 2014).

Este modelo animal também representa um ótimo sistema para o estudo do desenvolvimento do sistema nervoso central, tendo em vista que larvas com poucos dias de fertilização apresentam ampla gama de comportamentos complexos (Ahmad e Richardson, 2013). Exemplos de ensaios comportamentais customizados para larvas de *zebrafish* incluem, por exemplo, aprendizado não associativo (Best *et al.*, 2008) e suscetibilidade a convulsão (Berghmans *et al.*, 2007).

O cérebro em desenvolvimento é mais sensível do que o cérebro adulto, e a exposição a químicos durante a embriogênese tem sido implicada em doenças neurológicas e retardo mental (Andersen *et al.*, 2000). Em comparação com marcadores morfológicos e bioquímicos mais tradicionais, respostas comportamentais são indicadores bastante sensíveis da exposição a agentes tóxicos (Little *et al.*, 1990), e poucos estudos têm tentado vincular simultaneamente mudanças comportamentais com

marcadores e mecanismos fisiológicos (Sloman e Mcneil, 2012). A atividade locomotora do *zebrafish*, avaliada no teste de campo aberto (*open field*) se mostra um bom parâmetro na avaliação de prejuízos neurológicos durante o desenvolvimento (Tierney, 2011).

Muitos estudos da literatura evidenciam a capacidade de penetração celular de CNTs, o que nos leva a considerar a hipótese de que SWCNTs funcionalizados com PEG também podem penetrar nas células de embriões de *zebrafish*, causando ampla gama de danos, que podem ser mensurados por parâmetros simples, como mortalidade e taxa de eclosão, além da ocorrência de malformações embrionárias e alteração do comportamento.

### 1.5 Mecanismos gerais de toxicidade de nanotubos de carbono

Devido ao tamanho reduzido dos NM, a probabilidade de internalização e interação com constituintes celulares, organelas e macromoléculas é alta. Estas interações podem danificar tais componentes por dano físico ou modulação de vias bioquímicas. A definição de mecanismos de toxicidade é um passo fundamental no estudo da nanotoxicologia. Em linhas bastante gerais, podemos dividir os mecanismos de toxicidade em dois grupos principais: aqueles relacionados com indução de espécies reativas de oxigênio (*reactive oxygen species, ROS*), e os mecanismos não oxidativos relacionados com a interferência física de nanotubos com estruturas celulares (Kumar e Dhawan, 2013) (Fig. e).

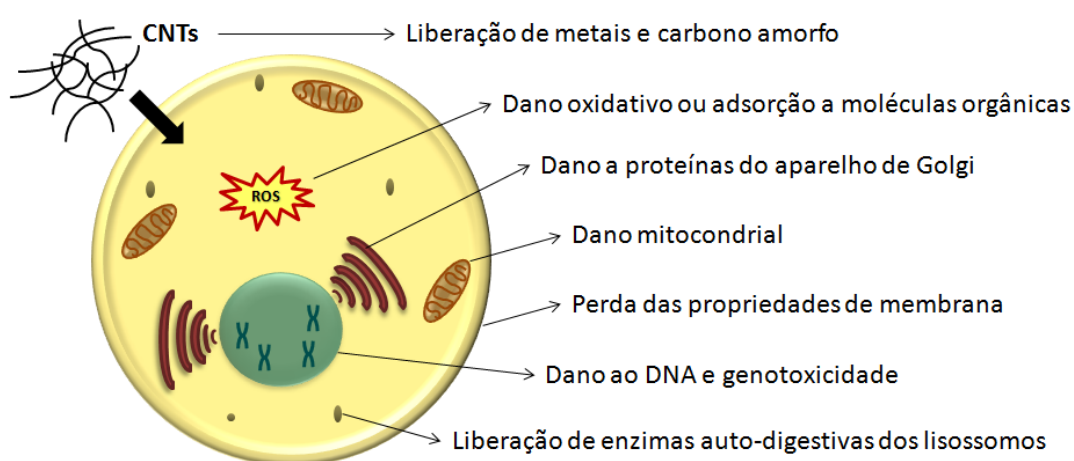




Figura e: Representação esquemática de algumas das possíveis interações de CNTs com componentes celulares.

### **1.5.1 Mecanismos relacionados ao estresse oxidativo:**

A regulação do processo embriológico é influenciada pela sinalização celular de espécies reativas de oxigênio, sendo que o dano oxidativo ao material genético e demais componentes do embrião tem função chave na etiologia da teratogênese (Wells *et al.*, 2005).

Para nanotubos de carbono, diversos trabalhos mostram a indução e produção de ROS (Manna *et al.*, 2005; Murray *et al.*, 2009). As nanopartículas, além de apresentarem capacidade intrínseca de geração de ROS, podem estimular a sua produção por vários componentes celulares em resposta a sua presença. Em condições severas, o dano oxidativo decorrente da superprodução de ROS pode levar a oxidação de lipídeos, proteínas e DNA perturbando de maneira decisiva a função destas moléculas e podendo levar a morte celular.

Dentre as maiores fontes de radicais livres, podemos citar a presença de metais de transição contaminantes, usados na produção de NP não-metálicas, e presença de defeitos na estrutura das mesmas, gerando elevada reatividade, além de grupos reativos oriundos do processo de funcionalização (Shvedova *et al.*, 2012). Cobalto, ferro e níquel são os materiais mais comuns utilizados na síntese de CNT, sendo utilizados como catalisadores para promover o processo de crescimento dos nanotubos de carbono durante sua síntese, e a ação destes metais sempre deve ser levada em consideração nos estudos nanotoxicológicos (Liu *et al.*, 2009a). Além disso, a presença de metais, ainda que em pequenas quantidades, é capaz de induzir diversos tipos de malformações embrionárias no *zebrafish* (King-Heiden *et al.*, 2009).

### **1.5.2 Mecanismos não oxidativos da toxicidade de NP:**

Diversos mecanismos de efeitos tóxicos não oxidativos são documentados para nanomateriais. Mecanismos potenciais de dano se dão pela interferência física de

nanotubos de carbono com constituintes celulares e extracelulares, alterando processos vitais da célula, podendo causar vários graus de dano e até mesmo morte.

Já foi demonstrado que nanopartículas de carbono de dimensões específicas podem causar bloqueio estérico de canais iônicos (Park *et al.*, 2003) e que SWCNTs podem se ligar especificamente a certos domínios proteicos, formando complexos estáveis, indicando um alto potencial de danos às proteínas (Shi *et al.*, 2013).

A similaridade de SWCNTs com microtúbulos do fuso mitótico foi a causa de citotoxicidade em cultura de células e reconstituição tridimensional mostrou interação direta entre nanotubos e o centrômero de cromossomos (Sargent *et al.*, 2012). Em outro estudo, a presença intracelular de SWCNTs causou agregação dos filamentos de actina com forte prejuízo para a atividade proliferativa (Holt *et al.*, 2010).

As propriedades funcionais da membrana celular, bem como sua integridade, também podem ser severamente afetadas pela presença de MWCNTs (Hirano *et al.*, 2008). Estudos demonstram que nanotubos de carbono podem interagir com modelos de bicamadas lipídicas alterando a organização estrutural das mesmas, reduzindo sua fluidez e flexibilidade (Wallace e Sansom, 2009). Estudos *in vitro* demonstraram que SWCNTs funcionalizados com PEG podem ser incorporados pelas células por mecanismos distintos como fagocitose e transporte passivo, e se acumular em diferentes organelas, como lisossomos, mitocôndrias e núcleo celular (Zhou *et al.*, 2010).

Os efeitos teratogênicos, capacidade de induzir malformações embrionárias (Fig. f), de nanopartículas podem estar diretamente relacionados com os mecanismos descritos acima, tanto oxidativos como não oxidativos. Genotoxicidade induzida por NPs pode ser atribuída a diversos fatores, como por exemplo, interação direta das partículas com DNA, dano indireto causado por ROS, liberação de íons tóxicos, material residual ou interação direta com constituintes celulares (Magdolenova *et al.*, 2012). Neste sentido, os efeitos genotóxicos das nanopartículas devem ser investigados com cuidado, tendo em vista que a instabilidade do material genético está diretamente associada com desenvolvimento de câncer (Karlsson, 2010).

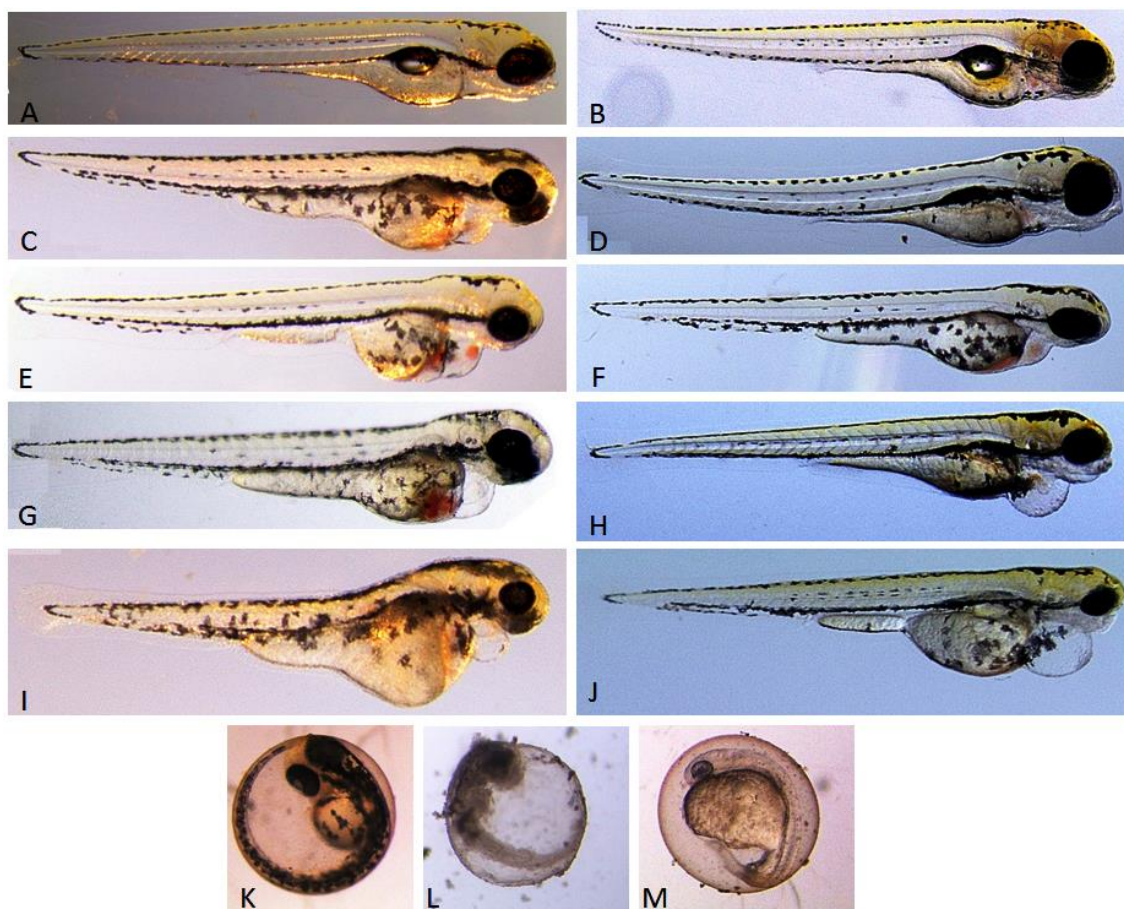


Figura f: Exemplos de malformações embrionárias. (A, B) Controles 96 hpf. (C, E) Malformação de vitelo e edema de pericárdio. (D) Curvatura anormal da coluna. (F, G) Edema de pericárdio com acúmulo anormal de eritrócitos. (H) Edema de pericárdio. (I, J) Edema de saco vitelínico, malformação de olhos e cabeça. (K) Embrião controle dentro do córion, em 48 hpf. (L, M) Morte embrionária e malformação evidente ainda dentro do córion, em 24 hpf.

O ensaio cometa é o principal teste de investigação de agentes genotóxicos e é aplicável para virtualmente qualquer tecido experimental (Singh *et al.*, 1988). O embrião do *zebrafish* é um modelo animal sensível a agentes genotóxicos e seu uso como fonte de células para o ensaio cometa é validado por diversas metodologias (Jarvis e Knowles, 2003; Kosmehl *et al.*, 2008). O ensaio cometa detecta danos primários ao DNA induzidos por uma série de agentes químicos, intercalantes, alquilantes e oxidantes. Os danos detectados são principalmente quebras simples e

duplas na fita de DNA, sítios alcalilábeis, danos oxidativos, ligações cruzadas entre DNA e outras moléculas. O ensaio cometa não é utilizado para detectar mutações, mas sim, lesões genômicas que, após serem processadas, podem resultar em mutação. Diferente das mutações, as lesões detectadas por esse teste ainda podem ser reparadas por sistema adequado (Burlinson *et al.*, 2007).

Um passo fundamental no ensaio do cometa é a análise de imagens obtidas por microscopia de fluorescência. Embora a análise manual ou visual seja extremamente validada (Aguilera e García-Muse, 2013), os *softwares* de análise de imagem têm sido utilizados exponencialmente em trabalhos (Fig. g). Devido à automação, *softwares* como o OpenComet são mais precisos, menos propensos a viés humano, e mais rápidos do que a análise manual (Gyori *et al.*, 2014).

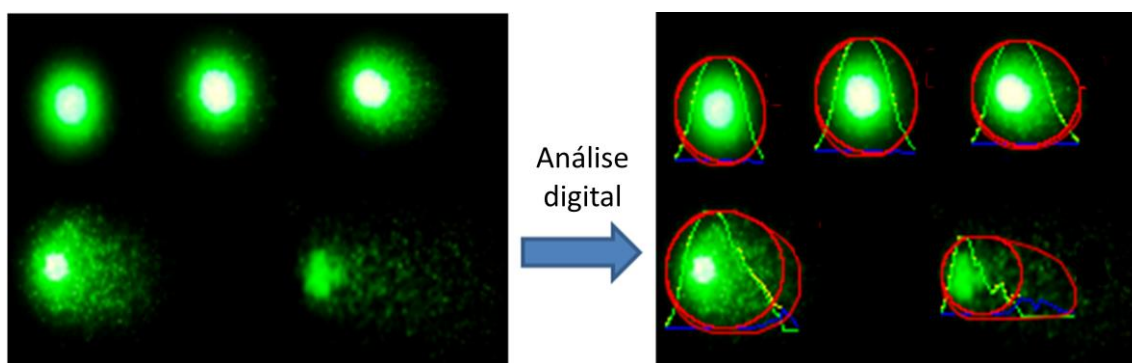


Figura g: Exemplo de diversos graus de dano ao material genético. Nucleoides obtidos por microscopia de fluorescência e analisados digitalmente pelo *software* livre OpenComet.

As características específicas de alguns NP podem resultar em interferência, direta ou indireta, com testes toxicológicos. De acordo com Karlsson e colaboradores (2014), não há nenhuma evidência, até o momento, que os nanotubos de carbono possam interferir significativamente com o ensaio cometa, seja durante a migração eletroforética do DNA ou mesmo na análise dos nucleoides obtidos.

Levando em consideração os possíveis mecanismos envolvidos na teratogênese e efeitos genotóxicos por NP, torna-se importante investigar tais efeitos após a exposição

de embriões de *zebrafish* a SWCNTs, e definir se o estresse oxidativo ou a presença intracelular de nanotubos possuem influência neste processo.

### **1.5.3 Influência da estrutura no perfil toxicológico**

Devido a sua natureza hidrofóbica, NP interagem com a cauda hidrofóbica dos ácidos graxos componentes das membranas celulares, causando alteração da tensão de membrana e consequente ruptura da mesma (Lai *et al.*, 2012). Agregados de NP perturbam membranas de maneira menos significativa em comparação a NP singulares de dimensões similares (Thomassen *et al.*, 2011). A hidrofobicidade da superfície das partículas também está intrinsecamente relacionada com o seu tamanho. Em geral, à medida que o tamanho da partícula diminui, o consequente aumento de curvatura de superfície torna mais difícil que moléculas de água formem uma camada de hidratação ao redor da NP (Chang *et al.*, 2008).

Devido à carga neutra e a formação de densa “capa de hidratação”, partículas de PEG são bem toleradas em sistemas biológicos (Cui *et al.*, 2015). Reduzindo a absorção de partículas por células fagocíticas, PEG eleva o tempo de circulação sanguínea de substâncias de interesse, "escondendo" as mesmas da fagocitose (Wattendorf e Merkle, 2008). Esta característica dos materiais peguilados é dependente da estrutura e densidade das cadeias PEG, sendo o planejamento e construção de cadeias PEG de alta relevância para os futuros sistemas de liberação controlada de fármacos (Yang *et al.*, 2014).

Estudos mostram que pesos moleculares mais elevados e cadeias PEG mais ramificadas conferem aos CNT um perfil farmacocinético favorável e menor toxicidade (Huang *et al.*, 2010). Diversos relatos na literatura mostraram que a funcionalização de nanotubos com PEG 2000 resultou em meia vida plasmática de 1.2h, enquanto que PEG 5000 aumentou em 5h este parâmetro toxicocinético (Liu *et al.*, 2008). O uso de cadeias ramificadas de PEG pode aumentar o tempo de circulação para até 15h (Yang *et al.*, 2012).

Para Elsaesser e Howord (2011) mais atenção deve ser dada ao tipo de funcionalização e química de superfície das NP do que o seu núcleo, visando desenhar partículas mais seguras para usos biomédicos.

Modificações da superfície dos CNT, como peguilação ou alteração de cargas elétricas, sabidamente modificam as interações com bicamadas lipídicas, modificando a tomada e retenção das partículas e consequente toxicidade (Ahamed *et al.*, 2008). O lado interno da membrana celular de mamíferos, e demais vertebrados, exibe discreta carga residual negativa, tornando possível a interação eletrostática com NP carregadas positivamente (Goodman *et al.*, 2004).

NP carregadas positivamente apresentam maior eficiência em cruzar membranas biológicas, enquanto que partículas sem cargas elétricas parecem ter pouca ou nenhuma interação com bicamadas lipídicas, porém a densidade e peso molecular da funcionalização ao redor também exercem papel fundamental na penetração em barreiras celulares (Beddoes *et al.*, 2015). Em estudo com embriões de *zebrafish* e nanopartículas de ouro, foi demonstrado que NP sem carga elétrica não apresentaram toxicidade, enquanto que partículas positivas causaram elevada mortalidade e partículas com cargas negativas causaram diversos tipos de malformações. Essa diferença dependente de cargas elétricas pode ser explicada por alterações no sistema redox celular (Harper *et al.*, 2011)

CNTs são cilindros com estrutura alongada e fina, possuindo uma elevada relação entre comprimento e diâmetro, podendo ser considerados uma estrutura unidimensional. Em termos de formato, nanopartículas com extremidades finas e curvadas parecem apresentar maior eficiência na translocação de membranas (Beddoes *et al.*, 2015). De um modo geral, nanopartículas semelhantes a fibras são mais tóxicas que aquelas que se assemelham com esferas. É sugerido que ao contrário das partículas esféricas, a elevada área de superfície dos nanotubos dificulta a sua tomada por células fagocíticas, gerando uma “fagocitose frustrada”, causando liberação de citocinas inflamatórias e estresse oxidativo (Madani *et al.*, 2014).

Focando na comparação de características físicas de nanotubos, um estudo mostrou que MWCNTs de menor comprimento (0,1  $\mu\text{m}$ ) causam severa toxicidade em



embriões de *zebrafish*, com alta taxa de mortalidade, falha na iniciação da gastrulação e diversas malformações estruturais, enquanto que equivalentes de maior comprimento (0,7  $\mu\text{m}$ ) não foram tão agressivos (Cheng e Cheng, 2012).

A organogênese de mamíferos também pode ser afetada pela exposição a nanotubos. A administração de SWCNTs (oxidados ou sem nenhum tipo de funcionalização) em camundongos durante o período de gestação foi capaz de induzir diversos tipos de malformações nos fetos, como atraso no desenvolvimento dos membros e torção do eixo corporal (Pietrojusti *et al.*, 2011). Outro estudo mostrou vários tipos de malformações esqueléticas após injeção intraperitoneal de MWCNTs em ratas prenhas (Fujitani *et al.*, 2012). Para Campagnolo e colaboradores (2013) o uso de sistemas de liberação de fármacos baseados em SWCNTs-PEG deve ser avaliado com muito cuidado durante a gravidez.

Muitos dos estudos citados avaliaram os efeitos citotóxicos, porém, tais efeitos tendem a ocorrer em doses mais elevadas enquanto que efeitos mais sutis podem ocorrer em doses menos agressivas, sem necessariamente causar morte celular. Diversos trabalhos em nanotoxicologia *in vitro* usam doses que excedem em muito a reprodutibilidade em sistemas *in vivo*, resultando em dados praticamente impossíveis de serem aplicados para a saúde humana. Estudos *in vivo* também estão focados em respostas que ocorrem após exposição a altíssimas doses (Oberdörster *et al.*, 2005). Tais pesquisas são válidas no sentido de definir mecanismos de toxicidade, mas são de difícil extrapolação para situações práticas (Maynard *et al.*, 2011).

Em nanotoxicologia é importante avaliar doses reprodutíveis a fim de obter conclusões que possam ser usadas na regulamentação do uso das NP visando a proteção da saúde pública. Neste contexto, as pesquisas devem fazer uso de doses mais baixas e períodos de exposição prolongados ou períodos críticos, como o desenvolvimento embrionário (Elsaesser e Howard, 2012). Além disso, as doses testadas devem ser apresentadas de maneira simples, como massa/volume (mg/mL ou ppm), afim de permitir fácil comparação com outros estudos e fornecer unidade aplicável para a indústria e para fins de regulação (Harper *et al.*, 2011).

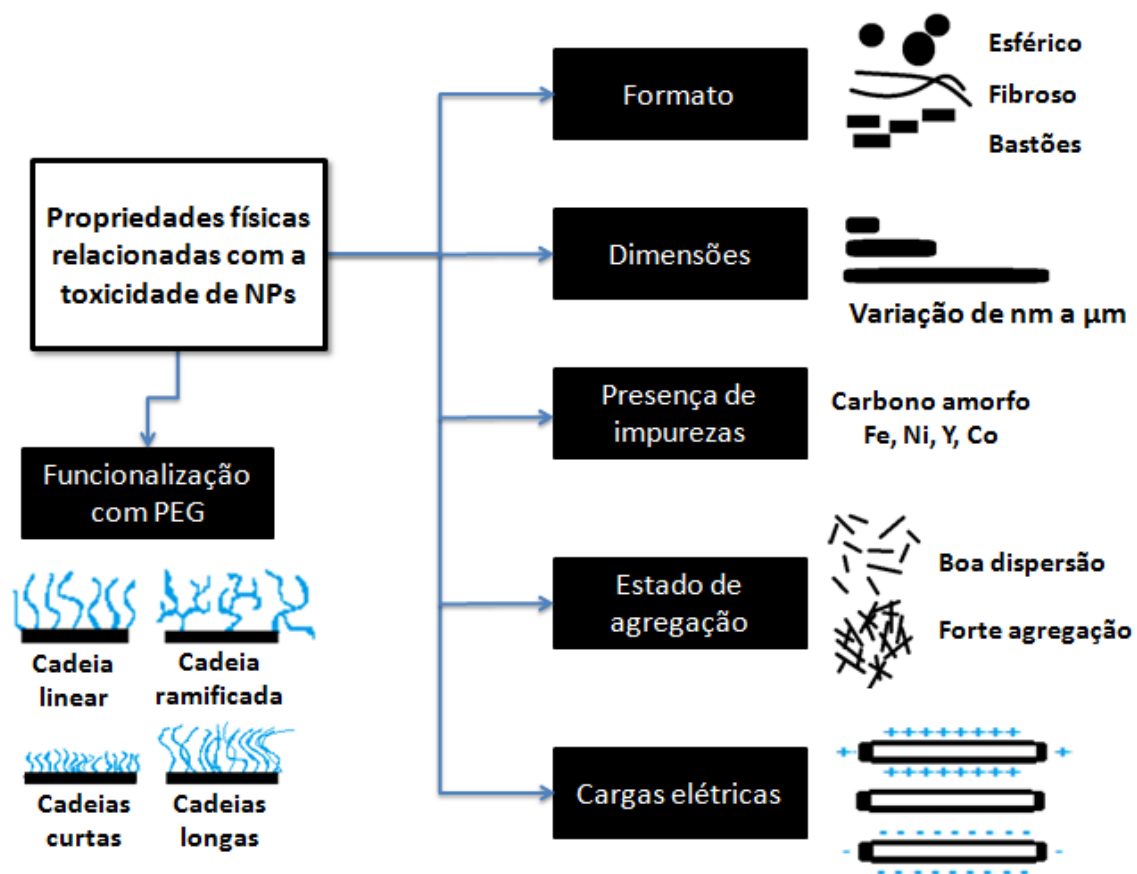


Figura h: Representação esquemática de algumas das possíveis variáveis relacionadas com o perfil toxicológico de nanopartículas



Na farmacologia, é definido que pequenas mudanças estruturais em moléculas de drogas influenciam decisivamente suas características, e em nanotoxicologia, cada vez mais esforços são somados em busca de uma compreensão global da relação entre estrutura e atividade biológica para diferentes NP. Os estudos que relacionam estrutura e efeitos são escassos em *zebrafish* (Lin *et al.*, 2013).

Tendo em vista os exemplos citados sobre as variações estruturais (Fig. h) e suas implicações, bem como a reconhecida sensibilidade do *zebrafish* a nanopartículas, é coerente hipotetizar que embriões desta espécie sejam afetados de maneira distinta por diferentes cargas de peguilação de SWCNTs. Assim, as diferenças físico-químicas resultantes dos graus de peguilação podem influenciar desigualmente o desenvolvimento embrionário do *zebrafish*. Os diversos estudos encontrados na literatura sugerem que quanto maior a carga de peguilação, menores são os efeitos deletérios produzidos, porém, as demais características dos materiais testados devem ser levadas em conta, principalmente a presença de contaminantes oriundos dos diferentes processos de fabricação. O conhecimento dos mecanismos subjacentes será útil no futuro *design* de CNTs menos tóxicos.

## **2. Objetivos**

### **2.1 Objetivos gerais**

Investigar os efeitos *in vivo* de nanotubos de carbono funcionalizados com dois tipos distintos de polietileno glicol (SWCNTs-PEG) no modelo animal *zebrafish* (*Danio rerio*).

### **2.2 Objetivos específicos**

- Expor embriões de *zebrafish* a concentrações diluídas de SWCNTs com diferentes cargas de pegulação e avaliar os perfis toxicológicos de cada;
- Avaliar as curvas de toxicidade geral, relacionadas com morte, taxa de eclosão dos embriões expostos;
- Realizar a quantificação de espécies reativas de oxigênio em homogeneizados de larvas de *zebrafish*;
- Investigar efeito genotóxico dos SWCNTs em larvas;
- Investigar alterações comportamentais em larvas expostas a SWCNTs-PEG;
- Investigar a passagem dos nanotubos pelas barreiras biológicas e sua presença intracelular em larvas de *zebrafish*;

**Artigo**

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**Toxicity of Single-wall carbon nanotubes functionalized with polyethylene glycol in  
*zebrafish* (*Danio rerio*) embryos**

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## Abstract

Nanotechnology has enabled the generation of nanomaterials (NM) with unique chemical and physical characteristics, attracting the interest of different scientific and technological fields with their potential applications. Single-wall carbon nanotubes functionalized with polyethylene glycol (SWCNTs-PEG) are promising materials for biomedical applications such as diagnostic devices and controlled drug release systems. However, several questions about their toxicological profile remain unanswered. Thus, the aim of this study was to investigate the action of SWNT-PEG in *Danio rerio* zebrafish embryos at the molecular, physiological, morphological and behavioral levels. Zebrafish embryos were individually exposed to functionalized SWCNTs with two different loads of PEGylation: 600 Da and 2000 Da. The toxic effects for both nanotubes occurred predominantly at the highest concentration tested (1 ppm), resulting in a high rate of mortality and a delayed hatching process. A decrease in the total length of larvae for both treatments was also observed, but this decrease was not accompanied by changing parameters related to the development of the central nervous system. FT-Raman spectroscopy revealed no intracellular presence of nanotubes, but the comet assay indicated significantly higher genotoxic effects in the treatment of SWCNTs-PEG-600 Da (1 ppm). More studies are needed to understand which mechanisms are involved; however, it is believed that oxidative stress is among the primary mechanisms responsible for the toxic effects observed, as the quantification of reactive oxygen species was more pronounced at the higher concentrations tested. The results of this study indicate that the functionalization of carbon nanotubes with different molecular weights of PEG may be ineffective in reducing toxicity.

Keywords: SWNT-PEG; embryotoxicity; zebrafish

## Introduction

Nanomaterials, including single-wall carbon nanotubes (SWCNTs), exhibit chemical, physical and/or biological properties that depend on the nanostructure and give nanomaterials functional characteristics of interest for applications in the biomedical field. Numerous studies have been conducted evaluating the possible applications of carbon nanotubes (CNT) for diagnostic purposes as well as the transport and controlled release of drugs (Kam et al., 2005; Medina et al., 2007).

With the development of nanomedicine, products based on nanomaterials can interact within organisms and can cause many toxic effects that are not yet completely understood. A careful analysis of the toxic effects of these materials is important, as these particles can interact with biological systems at different levels with high specificity and can damage tissues and organs.

By their nature composed of purely carbon, nanotubes are intrinsically insoluble in aqueous medium, generating agglomeration and aggregation phenomena. The incorporation of polyethylene glycol (PEG) is a widely used strategy to increase the dispersibility in water and to decrease the toxicity of these compounds (Ilbasmiş-Tamer et al., 2010).

Despite the growing popularity of SWCNTs in biological applications, there is a lack of *in vivo* toxicological data, especially regarding the toxic effects on a developing organism (Pietrojusti et al., 2011). In recent years, the zebrafish model has proven to be a quick and easy model to effectively evaluate the toxicity of NP (Duan et al., 2013). The embryological development process of teleost fish exhibits a high homology with other species of vertebrates, making the development of the zebrafish broadly comparable to that of mammals and humans (Giannaccini et al., 2013). The zebrafish embryo is a useful research model due to its small size, transparency, easy maintenance, rapid embryogenesis and continuous reproduction, and a growing number of studies have demonstrated that zebrafish embryo and larvae can serve as a convenient platform for pharmacological evaluation and screening of the possible adverse effects of drugs (Truong et al., 2012). The advantage of evaluating the consequences of exposure to nanomaterials during embryonic development is that small perturbations of the

developmental program can lead to easily detectable responses (Usenko et al., 2007).

Many published studies demonstrate the cell penetration capacity of CNTs, which leads us to consider whether SWCNTs-PEG can also penetrate the cells of zebrafish embryos to cause damage that can be measured by simple parameters such as mortality and hatching rate, in addition to the observation of embryonic malformations.

The interactions of nanotubes with different cellular components can damage structures by physical injury or the modulation of biochemical pathways, and the definition of toxicity mechanisms is a key step in the study of nanotoxicology. Furthermore, understanding how small changes in nanoparticle structure can affect their toxicological profile is an equally important challenge. It is well-known that the induction of reactive oxygen species (ROS) and non-oxidative mechanisms related to the physical interference of nanotubes with cellular structures are among the primary causes of cell damage (Kumar and Dhawan, 2013).

One of the primary types of NP-induced toxicity, with direct consequences to their possible uses in biomedical field, is genotoxic effects or DNA damage. Genotoxicity induced by NPs can be attributed to several factors, such as direct interaction of the particles with DNA, indirect damage caused by reactive oxygen species (ROS), release of toxic ions or direct interaction with cellular components (Magdolenova et al., 2012). Thus, the genotoxic effects of SWCNTs should be carefully investigated, given that the instability of the genetic material is directly associated with the development of cancer (Karlsson, 2010).

Due to their chemical characteristics related to the hydrophilicity and electrical neutrality, polymers such as PEG have the ability to "repel" proteins and other biomolecules by steric repulsion. The magnitude of this effect depends on the length and density of PEG chains; in general, larger and denser chains cause decreased interaction with proteins and aggregation tendency (Peracchia et al., 1997). Due to its importance, comparative studies of different PEG charges and densities are useful for the development and production of new formulations based on SWCNTs.

Considering the many examples in the literature regarding NP structural changes and their implications, as well as the recognized zebrafish sensitivity to nanoparticles,

we hypothesize that embryos of this species are differently affected by different PEGylation loads of SWCNTs. Thus, this study proposes to compare the possible toxic effects of SWCNTs functionalized with different polyethylene glycol loads (600 Da and 2000 Da) in zebrafish embryos to investigate whether these materials can penetrate cells and whether the induction of ROS is among the responsible mechanisms for the toxic effects. The knowledge of the underlying toxic mechanisms can be useful in the design of CNTs more appropriate to the biomedical proposes.

## **Material and methods**

### **Preparation of SWNT-PEG dispersion and sample characterization**

Briefly, we used a commercial sample of single-wall carbon nanotubes purchased from Sigma-Aldrich (652474- Lot MKBC 9435) synthesized via the electric arc discharge (AE) method and functionalized with PEG (MW = 600 Da). Details of the characterization and purification of this material are found in Weber et al., (2014).

The other SWCNTs sample used in this study was synthesized by high pressure carbon monoxide (HiPCo), and we followed the purification and PEGylation methods adapted from Kim et al., (2008) and Liu et al., (2009b). Briefly, the initial SWCNTs were placed in a high pH ethanol-water (80:20) solution and were sonicated for 4 hours in the ultrasound bath and subsequently allowed to stand for approximately 2 hours. The supernatant was extracted and discarded. The decanted (remaining solution) was filtered through a 0.1-mm polytetrafluoroethylene membrane and washed several times with an ethanol-water solution to remove excess NaOH and iron particles. Subsequently, the sample was washed with ethanol to remove excess water. The nanotubes were dried in a vacuum at room temperature.

After this purification process, we proceed with the PEGylation protocol with DSPE-PEG (MW= 2000 Da) as follows: The initial PEG-DSPE was added to water and sonicated for 30 minutes at Turrax, and the previously purified HiPCo-SWCNT was added in deionized water and sonicated for 30 minutes in an ultrasound bath. Both samples (nanotubes and DSPE-PEG) were mixed and sonicated for 1 hour at 22 ° C. The water bath was cooled on ice every 20 minutes to prevent heating. Subsequently,



this dispersion was centrifuged at 24000 g for 6 h at 22 ° C, and the supernatant was collected. The excess of non-bounded PEG was removed after three centrifugations at 4,000 g for 10 minutes each on an Amicon filter.

The morphological characteristics of the dispersion were analyzed by TEM and high-resolution transmission electron microscopy (HRTEM) performed on a FEI Tecnai G2-Spirit 120-kV and a FEI Tecnai G2-20 SuperTwin 200-kV microscope, respectively. We performed a thermogravimetric analysis (TGA) (TA Instruments, SDT 2960, in dry air at a scanning rate of 5 °C min<sup>-1</sup>) to determine the purity and the presence of amorphous carbon and residual metal in the sample. We also performed a Raman analysis (Horiba T 64000 Raman 226 spectrometer, laser excitation wavelength = 785 nm) to determine the nanotube boundedness and the functionalization. The final concentration of SWNTs-PEG 2000 Da in the aqueous 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, together with infrared spectroscopy. The final concentration obtained after the mentioned process was 0.005 mg/mL.

The initial SWCNTs- PEG 600 Da and 2000 Da suspensions were sonicated for 2 h and diluted with aquarium water, which was previously filtered with a 0.22 µm membrane to avoid contamination and the presence of organic matter, to final concentrations of 0.01 ppm, 0.1 ppm and 1 ppm, followed by another round of sonication (30 min) before exposure to the embryos.

### **Animal husbandry**

All protocols were approved by the Institutional Animal Care Committee (Pq004/2014, CEUA-FURG) and followed the guidelines of the Brazilian legislation respecting the CONCEA (*Conselho Nacional de Controle de Experimentação Animal*) determinations.

Adult wild-type zebrafish were obtained from a commercial supplier (Red Fish, Porto Alegre, Brazil) and cultivated in aquariums at the Institute of Biological Sciences - ICB – FURG. Fish were kept in groups of 15 fish in 15 L in a recirculating water

system equipped with a biological filter and disinfection using UV light, with controlled temperature ( $28 \pm 2$  °C), pH (7) and a 14–10 h day/night.

Embryos were obtained by photo-induced spawning over artificial green plants and were collected within 30 minutes after the lights were turn on. Subsequently, eggs were collected and rinsed several times with filtered (0.22  $\mu$ m) system water and were staged according Kimmel et al., (1995). The viable eggs that had reached the blastula stage were randomly transferred to 96-well plates, one embryo per well, in 200  $\mu$ L of prepared nanotube suspensions in a static exposure system. The 96-well plates were maintained in an incubator (Fluxo Tecnologia, FT1010) under the same conditions as the adult fish. In this study, “embryos” refer to zebrafish prior to hatching (0 – 72 h), whereas “larvae” refer to post-hatch animals (over 72 h).

### **Experimental set-up for embryotoxicity**

The embryos were distributed into 7 independent experimental groups: a control group (recirculating system water); groups treated with 0.01 ppm, 0.1 ppm and 1 ppm SWCNTs-PEG 600 Da; and groups treated with SWCNTS-PEG 2000 Da at the same concentrations, in quintuplicate (n=10). Each replica was considered valid if <10% of the control embryos exhibited mortality or malformations.

Embryonic development was monitored within 24 to 96 hours post-fertilization (hpf), observing different endpoints as described in the literature. Such endpoints are evaluated in a binary manner (normal or abnormal) to avoid subjective aspects of classification. Embryos were recorded as dead when no movement was detected or if the tissues changed from a transparent to an opaque appearance. Embryos were defined as normal when any of the endpoints mentioned in Table 1 were not visibly affected.

At the 24 and 48 hpf, we observed visually, with a stereomicroscope (Olympus, ZX-16), the embryos' spontaneous movement over 20 s and the heartbeat over 10 s. The hatching of the embryos was monitored between 24 and 96 hpf. At 96 hpf, images from embryos were captured (Olympus ZX16, equipped with Olympus SC30 camera) and performed morphometry (ImageJ - National Institutes of Health, NIH) of hatched

larvae. Head-to-tail length was measured as a metric of growth as described by Kimmel et al. (1995). At 96 hpf we observed the body position (noted as lateral or upside down positioning in water column) and tested the touch response. A dissecting needle was used to gently touch the caudal region of the larvae. Under normal conditions, this touch elicits a rapid and brief swim response, and the animals were considered responsive if they swam any distance in response to the stimuli. The clearly malformed larvae were not tested for these two last parameters because their responsiveness could be impaired. Throughout all treatment, the cumulative mortality was monitored, and the percentage of malformations was calculated as the ratio of malformed individuals over the number of embryos that were alive at 24 hpf, according Selderslaghs et al., (2009). Table 1 summarizes the observed parameters.

### **Preparation of histological sections and FT-Raman analysis**

Histological sections were prepared with 96 hpf larvae. Briefly, both treated and control animals were anesthetized with a tricaine methanesulfonate solution (MS-222, Sigma) at 0.3 g/L, and the larvae were fixed in 4% neutral buffered formalin, processed routinely into Paraplast Plus®, sectioned sagittally at 6  $\mu\text{m}$ , stained with hematoxylin and eosin (H&E) and examined by a digital microscope Olympus BX41-DP72.

To measure the presence of SWCNTs-PEG in zebrafish tissues, 5 slices of each treatment were used for the spectral analysis using Fourier Transform (FT) Raman spectroscopy. FT-Raman spectrometer (RFS 100/S – Bruker Inc., Karlsruhe, Germany) was used with an Nd:YAG laser at 1064 nm as an excitation source. The output power of the sample was maintained at 150 mW, and the data were recorded with 128 scans at an approximate resolution of 4  $\text{cm}^{-1}$ . Raman signals were collected using a liquid nitrogen cooled Ge detector. The tissue Raman spectra exhibited characteristic peaks of nanotubes at approximately 180, 1300 and 1600  $\text{cm}^{-1}$  that corresponded to the radial breathing mode (RBM) and the D and G bands, respectively (Liu et al., 2008). The histological slices were analyzed to cover the key internal structures, including the nervous, cardiac and muscular systems as well as the eyes and yolk sac.

### **Locomotor activity**

Locomotor activity of 96 hpf larvae was evaluated with an adapted protocol from Prieto, (2012). Briefly, larvae were placed individually in the center of a 6-well plate over a paper grid. The total field of each well (33.7 mm in diameter) was divided equally by four radial lines and also divided into a center zone and outer zone by a concentric circle (10.3 mm in diameter). Due to swim bladder inflation and the ability to swim along the water column, each well was filled with 2 mL embryo water (28° C) to allow only horizontal movement of larvae. After a 1-min acclimation period, the locomotor activity was measured as the number of times the animal crossed the gridlines in 1 min, and time spent in each zone was calculated as a percentage of total time as measure of thigmotaxis, according Schnörr et al., (2012). In this behavioral test, only larvae resulting from these treatments with no morphological changes were observed because obvious malformations or reduced body length would be expected to affect the locomotor efficiency of zebrafish larvae (Padilla et al., 2011).

The test was conducted in the morning period because time of day has an impact on the locomotor activity of the zebrafish larvae; larvae are more active in the morning than in the afternoon (MacPhail et al., 2009). All swimming patterns and other movements were digitally recorded with minimal manual intervention and were saved for later analyses.

### **ROS measurement**

Briefly, pools of 10 larvae were washed twice with cold PBS (pH = 7.4) and then homogenized in cold buffer (Tris-HCl 100 mM, EDTA 2 mM, MgCl<sub>2</sub>·6H<sub>2</sub>O 5 mM). The homogenate was centrifuged at 10,000 × g for 20 min at 4° C, and the supernatant was collected. Ten microliters of the homogenate was added to a 96-well plate and reaction buffer (HEPES 30 mM, KCl 200 mM, MgCl<sub>2</sub> 1 mM), and 10 µL of DCFH-DA stock solution (16 µM, in ethanol 100%) was added to each well. The fluorescence intensity was measured using a micro-plate reader (VICTOR 2D Fluorometer - PerkinElmer) with excitation and emission at 485 and 530 nm,

respectively, at 37 °C, over 120 min. The ROS concentration was expressed in arbitrary units (dichlorofluorescein mg/protein). Protein concentration was determined with a commercial kit (Microprote pirogalol - DOLES).

### **Alkaline comet assay**

Genotoxicity was evaluated using the method described by Jarvis and Knowles, (2003) with a few modifications. Briefly, a homogenate of cells of individual 96 hpf zebrafish larvae was prepared by transferring the larvae to a 1.5 mL Eppendorf tube and macerating the whole animal with a pipette tip in 20 µL of cold neutral PBS.

Following maceration, all of the 20 µL suspensions were combined with 30 µL of low melting point agarose at 34 °C. The total volume (50 µL) was then layered onto a frosted microscope slide coated with normal melting point agarose. A coverslip was applied, and the slide was placed on an ice-cold surface to facilitate solidification. The coverslips were removed from the slides, and the embedded cells were lysed at 4 °C for 90 min in lysing buffer (2.5 M NaCl, 100 mM NaEDTA, 10 mM Tris Base, NaOH to a pH of 10, 1% Na Sarconisate, 1% Triton X-100 and 10% DMSO). The slides were then incubated for 30 min in an alkaline electrophoresis solution (300 mM NaOH/1 mM EDTA, pH =14) to facilitate DNA denaturation. The slides were subjected to electrophoresis (0.7 V/cm and 300 mA, 15 min). After washing three times with neutralization buffer (0.4 M Tris–HCl, pH = 7.5), the slides were fixed for 5 min in 100% methanol.

The slides were stained in the dark with 10 µL of SYBR® Safe and analyzed with a fluorescence microscope at 510–560 nm. Individual images were captured with a camera. The DNA damage was assessed using ImageJ, with the plug-in OpenComet. Each group of exposed and control fish consisted of five larvae. Fifty comets were randomly assessed from each fish. A mean value of tail moment was obtained for each fish, and the average of these mean values for all fish in a group was analyzed.

## Statistical analysis

Data are expressed as the mean  $\pm$  SEM, and significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's test to compare the differences between groups. For the mortality and hatching rate, two-way ANOVA was used. Differences were considered significant at  $p < 0.05$ . All of the data analyses were performed using GraphPad Prism 6 software.

## Results and discussion

To understand the activity of carbon nanotubes in biological systems, the physicochemical features of the nanomaterials used in the experiments must be considered. As described by Weber et al., (2014), the SWCNTs-PEG 600 Da sample formed large aggregates in water, even after prolonged bath sonication, as shown by low-resolution TEM analyses (Figure 1A). The sample contained approximately 25 wt.% of grafted PEG, 60 wt.% of SWCNTs and approximately 20 wt.% of carbon-coated catalyst particles (Ni-Y), as determined by thermogravimetric analyses. In the aqueous dispersion, this material was found to exhibit a Zeta potential of approximately  $-60$  mV, indicating that this sample would be vulnerable to aggregation in biological systems.

The low-resolution TEM analyses of the SWCNTs-PEG 2000 Da showed the nanotubes are dispersed in the form of bundles. There is a great amount of residual PEG present in the form of a polymer mass, even after purification process, and this mass covers nanotubes forming agglomerated bundles (Figure 1B).

As we can see in Figure 2, the purification process was able to reduce the metal contamination from approximately 16 to 13 wt.%. There was a tightening in the derivative thermogravimetry curve (dTG), suggesting that the purified nanotubes exhibited a more homogeneous profile with respect to morphological characteristics compared with a commercial sample. The loss of weight observed at  $322$  °C is an indication that the purification process could impair some less sturdy nanotubes, forming amorphous carbon.

The peak approximately  $1338\text{ cm}^{-1}$ , the D band in the Raman spectroscopy, reflecting disordered graphitic structures, exhibited a relative intensity increase after the PEGylation process (Figure 3). This effect can be explained because PEG forms a polymeric mass that tends to interconnect nanotube bundles, resulting in a less organized structure.

The introduction of the PEG 2000 Da in the HiPco-SWCNTs sample clearly modified the infrared spectrum. The band at  $1652\text{ cm}^{-1}$  (Figure 4A) in the purified SWCNT sample spectrum is due to C=C stretching of carbon nanotubes, also observed in the SWCNT-PEG spectrum (Figure 4C). At  $2965\text{ cm}^{-1}$ , there is the C-H stretching band characteristic of these materials, but after interaction with the polymer, the band was not observed. Analyzing the spectrum of the DSPE-PEG 2000 Da (Figure 4B), we can highlight the C=O band for ester ( $1739\text{ cm}^{-1}$ ), the axial defect band C-H at  $2886\text{ cm}^{-1}$  and the C-O-C chain ( $1115\text{ cm}^{-1}$ ).

Once the characterization of the materials used is completed, it is important to define the rearing conditions of the tested animals. Previous studies have demonstrated that fish water can be used as a medium for embryotoxicity tests and that the embryos/larvae can survive for several days in a single well of a 96-well microplate using the yolk-sac nutrients (Hung et al., 2012).

Mortality, skeleton deformities and other teratogenic effects can result from water quality deterioration in wells, causing the accumulation of metabolites that may be harmful to the embryo, but for 96-well plates, this effect is only pronounced after 144 hours of treatment (Selderslaghs et al., 2009). It has previously been reported that appropriate rearing conditions and healthy genitors are suitable for a very low embryo mortality and low morphological heterogeneity (Kestemont et al., 2003). In this study, the frequency of mortality or abnormalities detected in the control groups was very low, less than 2%. In the various standards and guidelines, the validity threshold for the control group was 10% of undeveloped embryos at 24 hpf (Fraysse et al., 2006).

The survival or mortality rates are a direct parameter often used to indicate nonspecific toxicity. As shown in Figure 5, the cumulative mortality curves demonstrated an embryotoxic effect predominantly in the first 24 hours, which is the



most sensitive period of the early embryo development. For the lower concentrations tested (0.01 and 0.1 ppm), as the dose increased, the embryotoxic effect slightly increased relative to the control group. The most pronounced effects were observed for higher doses of both nanotubes, approximately 26 and 12%. Significant differences were observed throughout the treatment period for SWCNTs-PEG 600 Da at 1 ppm. For the same concentration of SWCNTs-PEG 2000 Da, we observed significant differences only after 72 hpf. In this case, the statistical analysis revealed that SWCNTs-PEG 600 Da exhibited a greater embryotoxic effect than did SWCNTs-PEG 2000 Da.

As shown in Figure 6, for both types of nanotubes at the lowest concentration (0.01 ppm) and controls, 100% of the survival embryos hatched within 96 hpf, and there was a time lag between the first and the last hatch of more than 24 h. There was a significant delay in the hatching process in animals treated with SWCNTs-PEG 600 Da (0.1 and 1 ppm) and SWCNTs-Peg 2000 Da (1 ppm). Previous studies have indicated that the hatching rate can be delayed by different nanoparticles, such as gold (Bar-Ilan et al., 2009) and silver (George et al., 2011). Delayed hatching may be a type of stress response in zebrafish embryos, and could leave an organism more susceptible to predators. If hatching is completely inhibited, it can lead to eventual death within the chorion, leading to important ecotoxicological implications (Ong et al., 2013).

Hatching is considered a key point in the life cycle of fish and represents a sporadic event that occurs over a period of several hours from 48 until 80 hpf. Thus, hatched embryos are not necessarily more developed than ones remains in the chorion (Kimmel, 1995). With respect to general development, this observation is consistent with the results for spontaneous movements (Figure 7A) and the heart rate (Figure 7B), demonstrating only a slight but insignificant decrease in these parameters at the higher concentrations tested (1 ppm) for both nanotubes. Alterations in the spontaneous movements and the heart rate of the zebrafish during early development have been widely documented, from a genetic and physiologic point of view and are good endpoints of developmental delay and toxicity (Hung et al., 2012). There are few studies that have evaluated these parameters for carbon nanoparticles. Tong et al., 2014) reported that exposure to MWCNTs can reduce the heart rate without affecting



spontaneous movements. The ability to swim in response to mechanical stimuli and to maintain the correct position in the water column (touch response and upside position) were also not affected by any treatment, indicating that the overall development was not affected.

Zebrafish locomotor activity and the open field test has proven to be viable endpoints for detecting neurological impairments during development (Tierney, 2011), and larvae are particularly well-suited for behavioral testing because of their relative maturity with respect to swimming capacity and the functionality of the motor, sensory, and stress-regulating systems (Schnörr et al., 2012). In the behavior test, we used only animals exposed to 0.1 and 0.01 ppm because the higher concentration tested impaired the total length of the larvae, and morphological changes would be expected to affect the locomotor efficiency of zebrafish larvae (Padilla et al., 2011). As shown in Figure 8A, no modification of the exploratory behavior was observed in the treated larvae compared with the control group. Novel and large environments, such as those experienced in the open field test, exhibit aversive properties that alter exploratory behavior and promote thigmotaxis in rodents and adult fish. Furthermore, zebrafish larvae exhibit characteristic patterns of exploratory behaviors in the standard open field, strongly avoiding the center of an arena and staying or moving in close proximity to the boundaries of a novel environment (Ahmad and Richardson, 2013). Again, no modification of this behavior endpoint was observed, as the time spent in center zone and outer zone was not modified, and the larvae clearly prefer the proximity to the walls of the arena (Figure 8B).

Taken together, the results of embryotoxicity at sublethal and behavioral levels indicate that the general development of the exposed embryos was not affected until 96 hpf relative to the control groups. However, we cannot exclude the possibility of alteration to the development of the central nervous system or late developmental stages for prolonged periods of treatment. For example, Powers et al., (2010) did not observe inhibition of spontaneous movement or response to touch with silver nanoparticles (100  $\mu\text{M}$ ), but when the same larvae were observed at 10 days post-fertilization, the distance the larvae swam was lower in exposed fish, demonstrating the validity of this test to

identify changes in the central nervous system. Furthermore, the study of Truong et al., (2012) also reported that early exposure to gold nanoparticles can lead to behavioral changes that persist into fish adulthood.

As we can see in Figure 9, the total length of the larvae was significantly reduced for both nanotubes at the concentration of 1 ppm. Similar results were reported in the study of Bai et al., (2010), in which ZnO nanoparticles induced mortality rates of approximately 65%, retarded embryo hatching, and reduced the body length of larvae without causing alteration of developmental endpoints. As mentioned by those authors, large ZnO aggregates might block the pore canals of the chorion, resulting in a shortage of the oxygen supply, essential to the fish growth.

The delay in the hatching process observed in this study and in various published papers, may be explained by the precipitation of NP and adhesion to the chorion, altering their mechanical properties and nutrient and oxygen exchange capacity (Jemec et al., 2012). The chorion is an acellular glycoproteic membrane protecting the embryo from external interference before hatching. Studies have demonstrate the passage of silver NP through chorion by Brownian motion (Nallathamby et al., 2008) and gold NP by passive diffusion (Browning et al., 2009). It is unclear whether nanoparticles have specific or non-specific interactions with the chorion (Duan et al., 2013), and contradictory results are found in the literature concerning the passage of NP through this membrane (Giannaccini et al., 2013).

Determining the biodistribution and accumulation of nanomaterials in the different organs and tissues of exposed organisms is also necessary for a comprehensive toxicological study (Weber et al., 2014). For approximately 72 hpf, the embryos are protected by the chorion, but after hatching, the larvae are fully exposed to suspensions present in the water. The FT-Raman spectroscopy of the histological slices (Figure 10) revealed that the tested nanotubes were unable to reach the internal tissues of the animals. The use of histology coupled with Raman analysis is used in some studies, like (Liu et al., 2008). Thus, we are led to believe that the observed toxicological findings are not directly related to the intracellular presence of the nanotubes, but indirect toxicity should be considered.

In the study of Cheng et al., (2007), scanning electron microscopic inspection revealed that the size of the pores on the embryo chorion was nanoscaled and that the size of SWCNT agglomerates was microscaled, indicating that the chorion was an effective barrier to raw SWCNT agglomerates. In this case, the hatching delay likely was induced by the residual Co and Ni (7.5 and 2.5%, respectively) used in the production of SWCNTs, and it is not clear whether delayed hatching can be attributed to the NPs or to the release of free metal from the NPs. In our study, the thermogravimetric analysis revealed relevant metal contamination in both samples tested. The values for SWCNT-PEG 600 Da and SWCNT-2000 Da were approximately 20% and 13%, respectively.

The presence of metals and amorphous carbon in the samples is an important consideration when we try to understand the toxicology of CNTs. Free metals, even at very low concentrations, affect many aquatic organisms at early stages of development because the chorion does not fully protect the embryo against metal penetration. Thus, metals accumulate in the egg and may adversely affect various metabolic processes in developing embryos, resulting in developmental retardation, morphological and functional anomalies, or death of the most sensitive individuals (Jeziarska et al., 2009). As we can see in Figure 11, the percentage of total malformations was significantly increased for only the SWCNTs-PEG 600Da (1 ppm) treatment, reaching approximately 18%. Embryos failed to develop the normal morphology, inducing typically pericardial edema, yolk sac edema, and tail and head malformations.

One of the primary mechanisms of toxicity and of the induction of embryonic malformations by nanomaterials is the production of reactive oxygen species (ROS). Among the major sources of ROS, we can highlight the presence of contaminants, the presence of defects in the structure of NPs, generating high reactivity groups derived from the functionalization process (Shvedova et al., 2012). For carbon nanotubes, several studies have reported the induction and production of ROS (Cheng et al., 2007; Manna et al., 2005; Zhu et al., 2007; Usenko et al., 2007; Murray et al., 2009). Under severe conditions, oxidative damage resulting from the overproduction of ROS can lead

to the oxidation of lipids, proteins and DNA, decisively disrupting the function of these molecules and potentially lead to cell death.

In this study, the ROS production was quantified by the reaction of DCFH-DA. The experiment was performed in homogenates tissues, composed by pools of 10 larvae, according Zhao et al.,(2013), in quadruplicate. As shown in Figure 12, we can observe graphically an increase in the ROS generation at the higher concentrations tested for both nanotubes; however, the statistical analysis indicates no significant difference for any treatments.

Many studies of nanotoxicology have focused on cytotoxicity. However, such effects often occur first at rather high concentrations, and the subtler effects that arise at lower concentrations, without necessarily causing cell death, also must be taken in account. In this sense, the genotoxic effects of nanoparticles should be considered because oxidative damage to genetic material plays a key role in the etiology of embryonic malformations and because the instability of the DNA is directly associated with the development of cancer (Wells et al., 2005; Karlsson, 2010). The comet assay is today the most common method for detecting DNA damage caused by NP. The alkaline version of the test has been shown to be even more sensitive and is the method of choice for low damage levels (Kumar and Dhawan, 2013; Karlsson et al., 2014).

Consistent result trends mentioned thus far, as shown in Figure 13, the highest concentrations tested for both nanotubes caused a clear increase in DNA damage. The genotoxic effect was only significant for the SWCNTs-PEG 600 Da at 1ppm. In our study, we did not observe the intracellular presence of the nanotubes in embryonic tissues. Therefore, we are led to exclude DNA damage induced by mechanical interaction with the genetic material. Although direct interaction has already been demonstrated (Holt et al., 2010; Sargent et al., 2012), ROS is believed to play a key role in DNA damage. However, we cannot say that a “oxidative stress” situation is happening, because we don't evaluate the antioxidant defenses of the larvae.

Several experiments found in the literature have described the genotoxic activity of SWCNTs. However, thus far, no carcinogenicity has been reported for these complex

materials. Nonetheless, carbon nanotubes should be treated with more care than simple carbon particles when used for industrial and commercial purposes (Toyokuni, 2013).

Due to the low solubility and high affinity for organic matter, carbon nanotubes are often deposited on the surface of cells, which is an inevitable factor in the nanotoxicological studies (Liu et al., 2013). NPs in aqueous suspension are dispersed due to the electrostatic and steric repulsion of the surface charge (positive/negative) present on them. The rate at which particles will aggregate or agglomerate depends on the concentration; higher concentrations confer a greater tendency to form aggregates (Maynard et al., 2011). In this sense, we use concentrations as low as 1 ppm also trying to avoid the aggregation and facilitate the diffusion and penetration of nanotubes in the zebrafish tissues.

PEGylated surfaces are believed to form flexible layers that organize themselves spatially in a "brush-like" structure, thereby preventing aggregation and adsorption to proteins and other biomolecules by steric repulsion. In general, larger and denser PEGylation decrease the chain interaction with proteins and the tendency to aggregate (Peracchia et al., 1997; Dos Santos et al., 2007). The study of Weber et al., (2014) demonstrated that the incorporation of PEG (600 Da) into SWCNTs can be inefficient in dispersing this material in a biological environment, suggesting that higher molecular weight chains could reduce the toxic effects observed.

In this regard, many published studies suggest that the use of more heavy chains tends to decrease the toxicity of nanotubes, especially avoiding their adsorption and improving the stability of suspensions in physiological media. However, despite the improvement of PEGylated material characteristics, it is not possible to achieve total protection against organic matter adsorption or aggregation by increasing the PEG molecular weight (Gref et al., 2000). For example, Andersen et al., (2013) reported that complement system activation by MWCNTs-PEG is independent of PEG molecular weight. These authors reported a decrease in complement activation as the degree of PEGylation increased, although complement activation was not completely suppressed.

Considering that we observe toxic effects at doses as low as 1 ppm, we must consider the ecotoxicity of this nanomaterial. According Chowdhury et al., (2012), the

synthesis methods of nanotubes (HiPCo and EA) resulted in distinctive toxicological profiles, which were directly correlated with metal content. These authors postulate that more toxic SWCNTs (greater metal content) will be transported in the environment, affecting several aquatic organisms. The primary mechanisms of the toxic action of metals, even at very low concentrations, are related to the osmotic disturbances and alterations of enzyme synthesis and activity (Jeziarska et al., 2009). Contaminants present in the SWNT-PEG sample, such as Yttrium (Y) and Nickel (Ni) are capable of inhibiting calcium channels, which could be the mechanism responsible for a variety of toxic effects related to these metals (Jakubek et al., 2009).

In this study, the toxic effects observed were more pronounced at higher concentrations (1 ppm) for both tested nanotubes; however, the SWCNTs-PEG 600 Da had a greater embryotoxic effect. We believe that metal contamination plays an important role in this effect. The smaller PEG chain (600 Da) tends to easily cause aggregation, leading to adsorption on the cell surface of the embryo. Although it is possible to quantify the damage, it is not possible to clearly identify which of these events was responsible for the induction of injury.

## **Conclusion**

The zebrafish embryo is very sensitive to environmental contamination and has been highlighted in the study of nanomaterials. However, despite the advantages of this model, fish development and embryo morphology differ from that of mammals, and findings for one class cannot be automatically translated to the other.

Many laboratories are involved in nanotoxicological studies *in vitro* and *in vivo*, but the results are often conflicting. Despite the efforts, it is difficult to draw any overall conclusion of toxicity (Darne et al., 2014). Inconsistencies observed in the literature are largely due to differences in experimental protocols and the lack of adequate characterization of the compounds tested (Ong et al., 2014).

Many studies use carbon nanomaterials without any functionalization, and often in high concentrations, intensifying problems with aggregation. Various studies *in vitro*

use doses that far exceed the reproducibility *in vivo*, resulting in data that is virtually impossible to apply to human health. In several cases, *in vivo* studies have focused on responses that occur after exposure to very high doses (Oberdörster, 2010). In this context, the concerns with public health are concentrated in lower doses and longer exposure times or critical periods (Elsaesser and Howard, 2012). The concentrations used in our study were chosen taking into account the literature data and using a mass/volume-based unit to enable easy comparison with other studies and to supply an applicable unit for industry and for regulation (Harper et al., 2011).

It is accepted in the literature that PEGylation enhances the toxicological profile of the nanotubes, but despite improvements, PEGylation cannot achieve total protection against aggregation. In this study, Raman spectroscopy showed that the two types of SWCNTs-PEG were not able to reach the intracellular environment of zebrafish embryos. Thus, we speculate that the corium acted as an effective barrier to the passage of the aggregates, as shown by several other studies.

ROS overproduction, oxidative damage to DNA, teratogenic (malformations) and embryotoxic effects are intrinsically related. If the damage is too severe for efficient repair, the cell will undergo apoptosis (Wells et al., 2005). Although we highlight malformations in the treatments, the toxicological data are nonspecific, and the tested samples represent a complex combination of materials. Therefore, we cannot classify SWCNTs-PEG as teratogenic. It is important to note in this study, and in several other experiments, that the observed effects were due CNTs mixed with catalyst metals and amorphous carbon, meaning that the results actually were a combination effect, making it hard to evaluate the true toxicity of the CNTs alone.

We conclude that the observed difference in the effect of nanotubes on hatching rate, mortality and larvae morphology might be due to the differences in the metal contamination, coupled with physicochemical characteristics. However, because these nanomaterials are promising in the biomedical area, further studies are necessary, especially with SWNTs functionalized with larger PEG chains and denser PEG coatings (Weber et al., 2014), and special attention should be given to purification protocols.

More studies of the underlying toxicity mechanisms may be useful in the future design of less toxic CNTs.

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### **Conflict of interest statement**

The authors declare that there are no conflicts of interest.



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## Figures and captions

**Table 1:**

Toxicological endpoints normal = score 0, abnormal = score 1	Developmental period			
	24 hpf	48 hpf	72 hpf	96 hpf
<b>Lethal</b>				
Coagulate/dead embryo	x	x	x	x
Tail detachment	x	x	x	x
Heartbeat	x	x	x	x
<b>Sublethal</b>				
Development of somites	x	x	x	x
Development of the eyes	x	x	x	x
Development of the otolith	x	x	x	x
Spontaneous movement	xx	x	x	x
Blood tail circulation			x	x
Heart rate		xx		
Hatching	xx	xx	xx	xx
<b>Teratogenic</b>				
Malformations		x	x	xx
Head-to-tail length				xx
Side-wise position				x

**Table 1:** Morphological characteristics evaluated as measures for the embryotoxic potency of SWCNT-PEG at the designated times points. Endpoints indicated as (x) are evaluated in a binary manner (normal = score 0, abnormal = score 1), and as (xx) are the quantified endpoints. An embryo was considered either normal (all scores = 0), or malformed (score = 1 for one or more morphological characteristics evaluated).

**Figure 1:** Comparative TEM images of SWNT-PEG 600 Da (A) and 2000 Da (B) (scale bar = 100 nm). Additional TEM images of SWCNT-PEG 2000 Da (C1, C2 and C3; scale bar = 50, 200 and 500 nm, respectively).

**Figure 2:** Thermogravimetry analysis of HiPco SWCNTs-PEG 2000 Da, before (A) and after (B) after the purification process.

**Figure 3:** Raman spectrum of purified SWCNTs before (A) and after (B) 2000 Da PEGylation process at a laser excitation of 514 nm.

**Figure 4:** FTIR spectrum of the samples: SWCNTs HiPco (A), DSPE-PEG (B), purified HiPco + DSPE-PEG (C).

**Figure 5:** Cumulative mortality curves. The percentage of mortality is increased only at 1 ppm treatments, for both nanotubes, compared with the controls. The data are expressed as the means  $\pm$  SEM from five independent experiments. Significant difference: \* $p < 0.05$  and \*\* $p < 0.01$ . In this case, SWCNTs-PEG 600 Da exhibited a greater embryotoxic effect relative to SWCNTs-PEG 2000 Da.

**Figure 6:** A delay in the hatching process was observed only at 72 hpf after embryos were exposed to both nanotubes. Significant differences relative to the controls were observed at 0.1 and 1 ppm of SWCNTs-PEG 600 and 1 ppm of SWCNTs-PEG 2000. The data are expressed as the means  $\pm$  SEM from five independent experiments (\* $p < 0.05$  and \*\* $p < 0.01$ )

**Figure 7:** Spontaneous movements at 24 hpf (A) and heart rate at 48 hpf (B) during the SWCNTs-PEG 600 Da and 2000 Da treatment. The data are expressed as the means  $\pm$  SEM from five independent experiments.

**Figure 8:** Behavior of 96 hpf larvae in the open field test, after continuous exposure SWCNTs-PEG 600 Da and 2000 Da. (A) Spontaneous swimming activity of zebrafish larvae (as number of lines crossed in 1 min) at 96 hpf,. (B) Pattern of zone preference in larval zebrafish: Thigmotaxis measured as the % time spent in outer zone. Data are expressed as the means  $\pm$  SEM (n=10).

**Figure 9:** Total length of 96 hpf larvae exposed to SWCNTs-PEG 600 Da and 2000 Da. The data are expressed as the means  $\pm$  SEM from three independent experiments (n=5) (\* $p < 0.05$ ).

**Figure 10:** Illustrative histology slice of a 96-hpf zebrafish larva stained with hematoxylin and eosin (H&E). Dashed lines represent the main areas covered by the FT-Raman signal.

**Figure 11:** Malformation of zebrafish embryos exposed to SWCNTs-PEG. The data are expressed as the means  $\pm$  SEM from five independent experiments (n=10) (\* $p < 0.05$ ).

**Figure 12:** Reactive oxygen species in 96 hpf zebrafish larvae exposed to SWCNT-PEG as measured by the DCFH-DA method. The experiment was performed in quadruplicate in pools of 10 homogenized larvae. Data are expressed in  $\pm$  SEM (\* $p < 0.05$ ).

**Figure 13:** Genotoxicity evaluated with the alkaline comet assay using tail moment (A) and tail length (B) as metrics of DNA damage. The data points represent the average of the means (from 50 comets) of five individual larvae from each exposure group. The increase in damage was found to be significant only for SWCNTs-PEG 600 Da at 1 ppm. Data are expressed as  $\pm$  SEM (\* $p < 0.05$ ).



**Figure 1:**

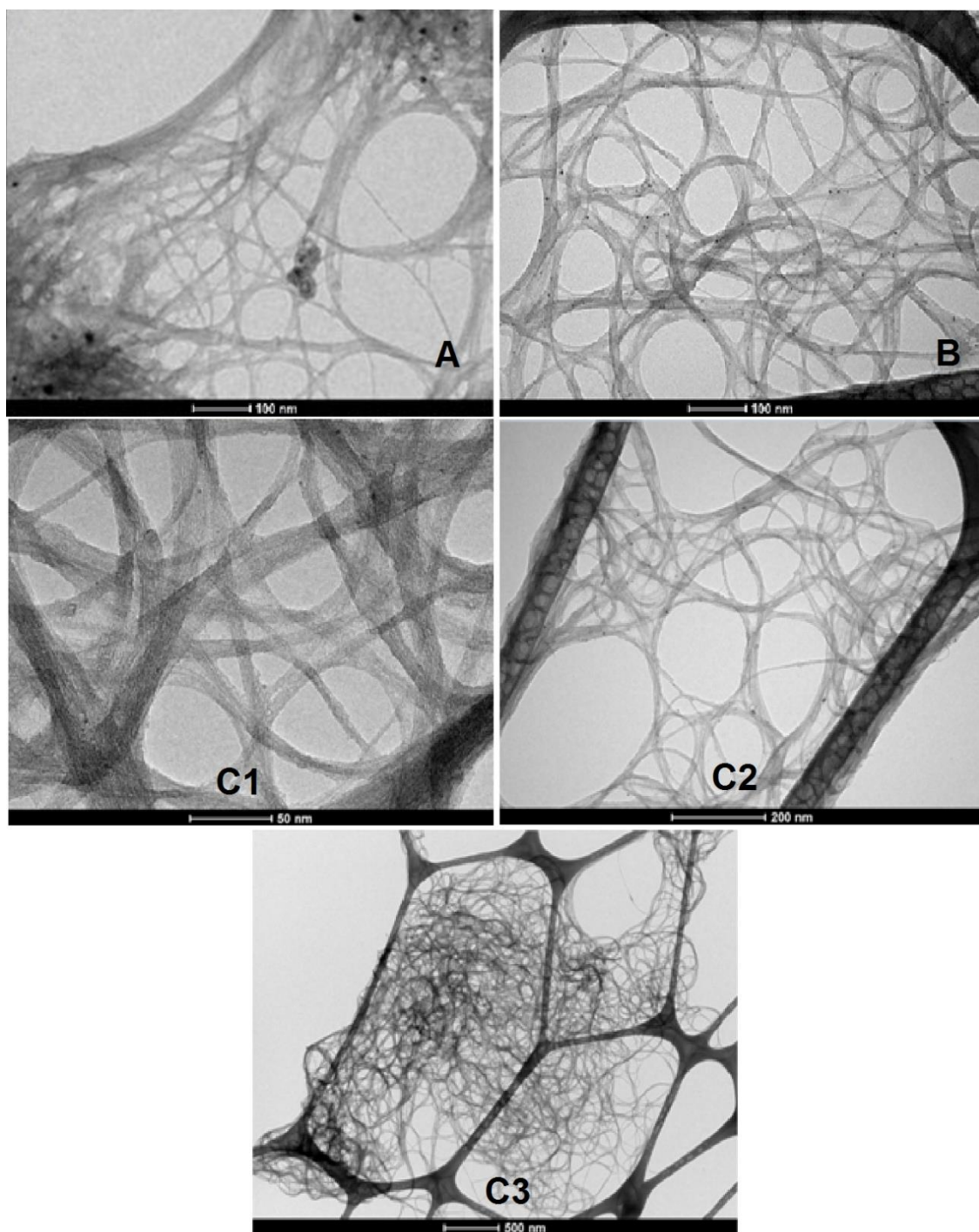




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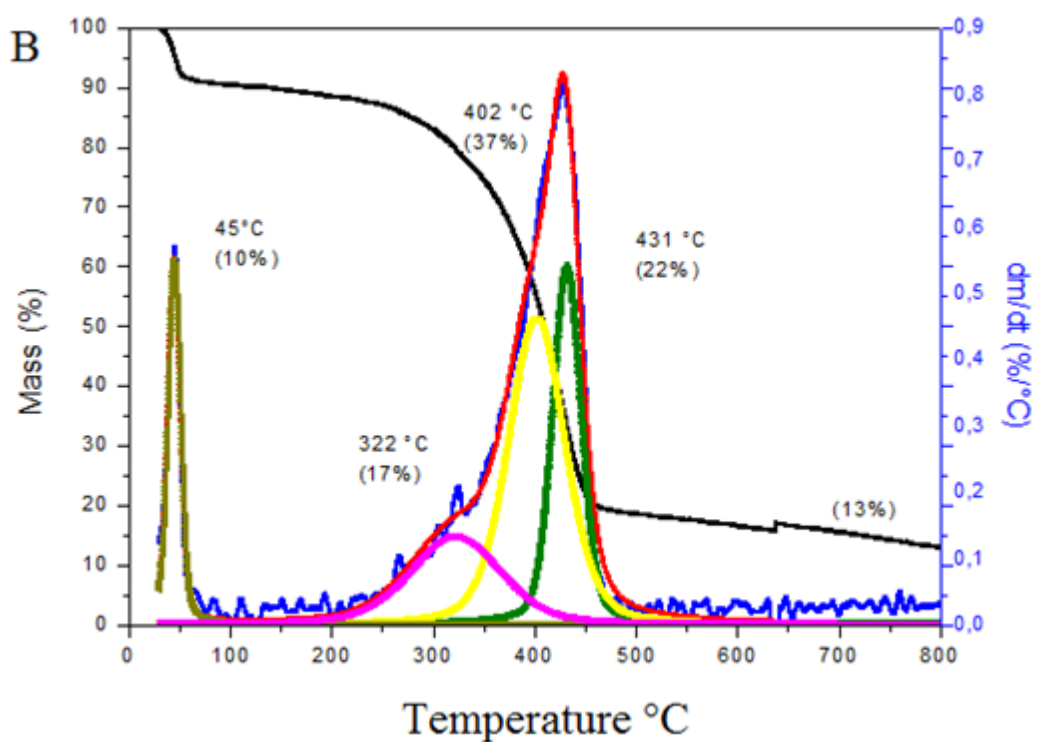
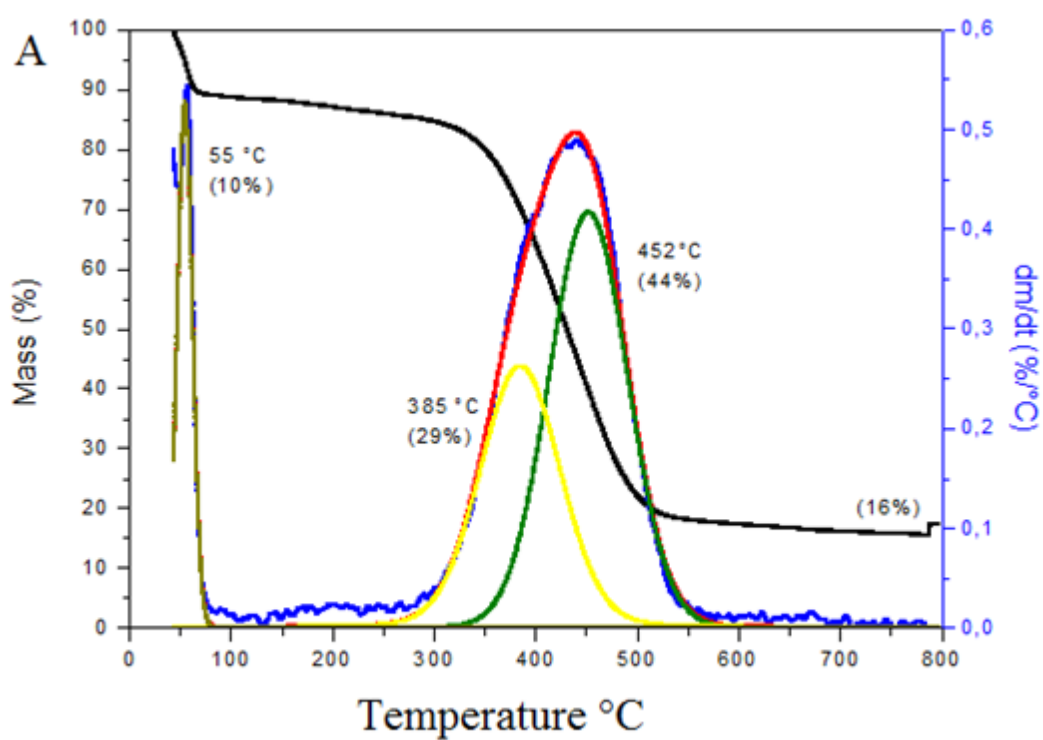


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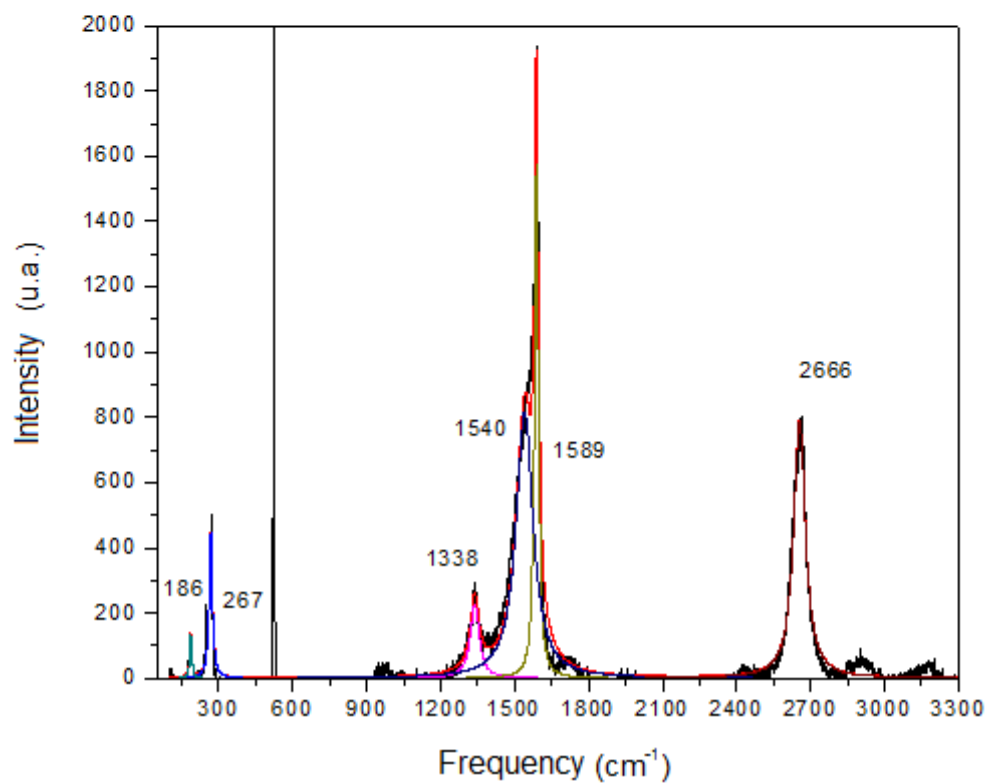
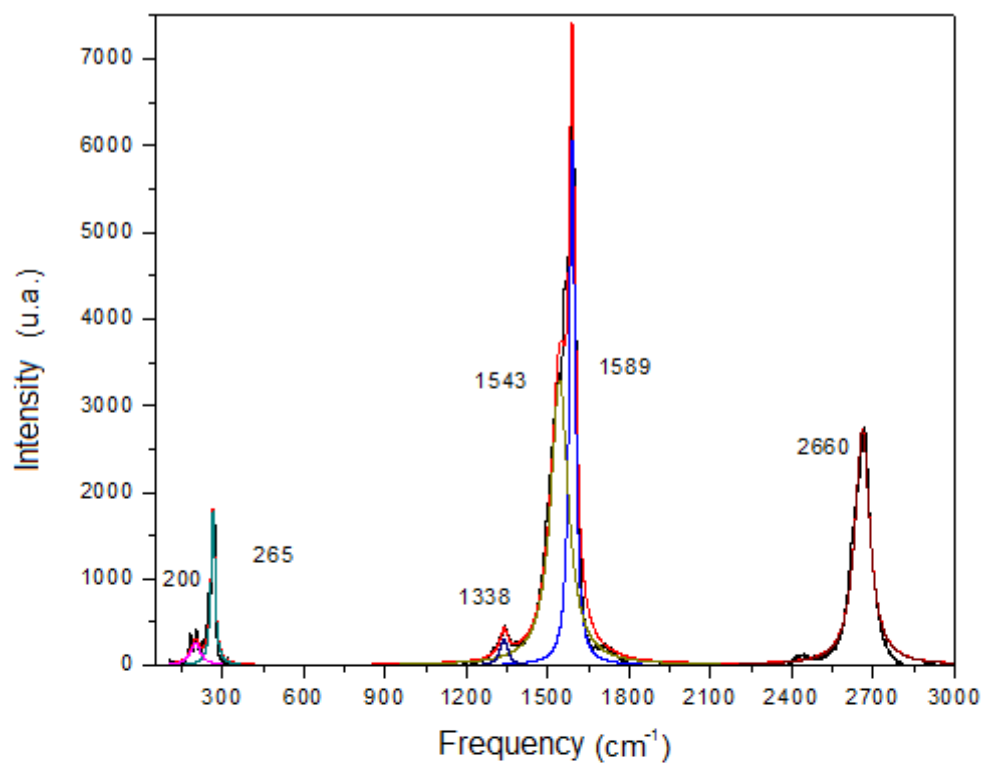


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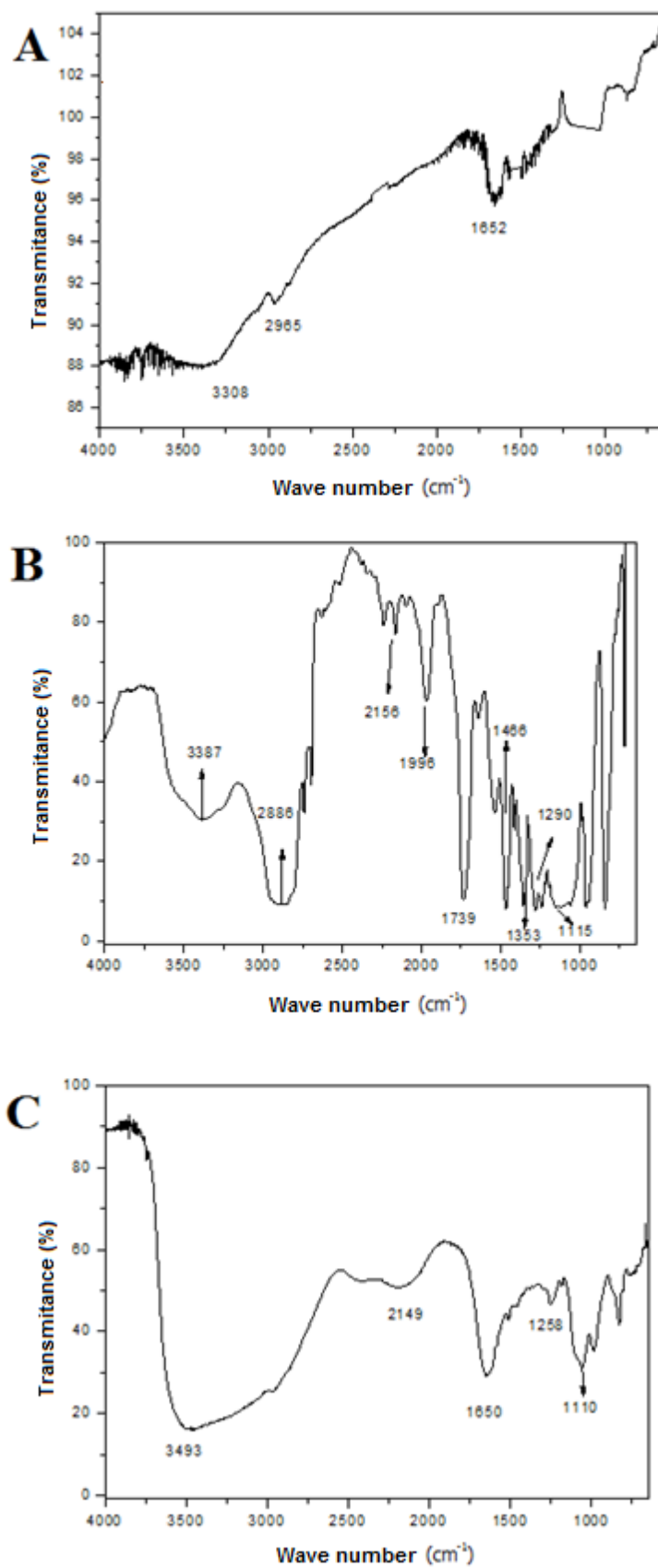


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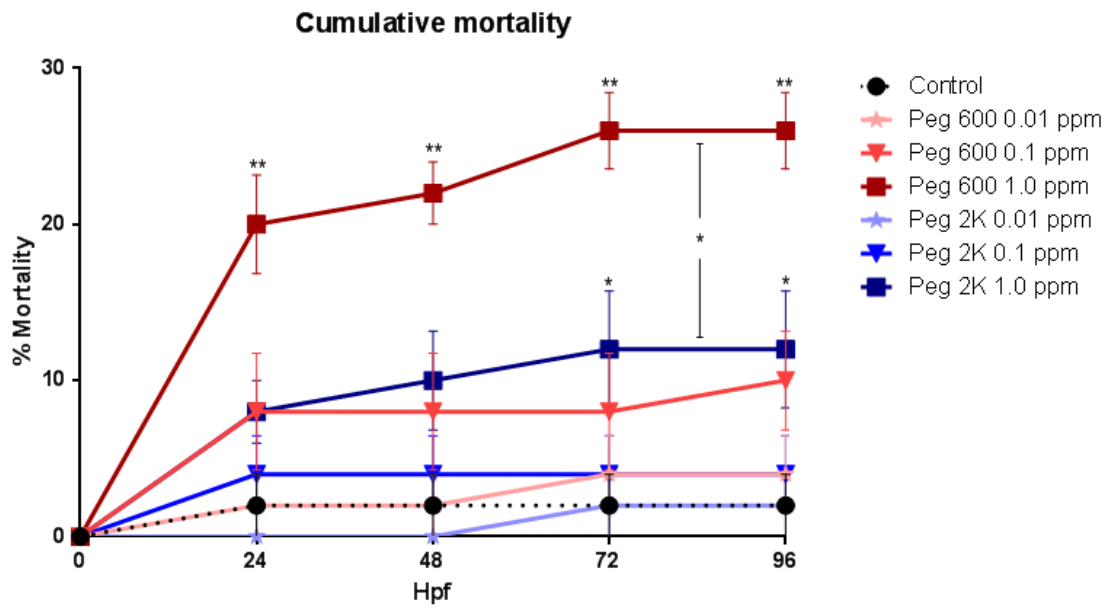


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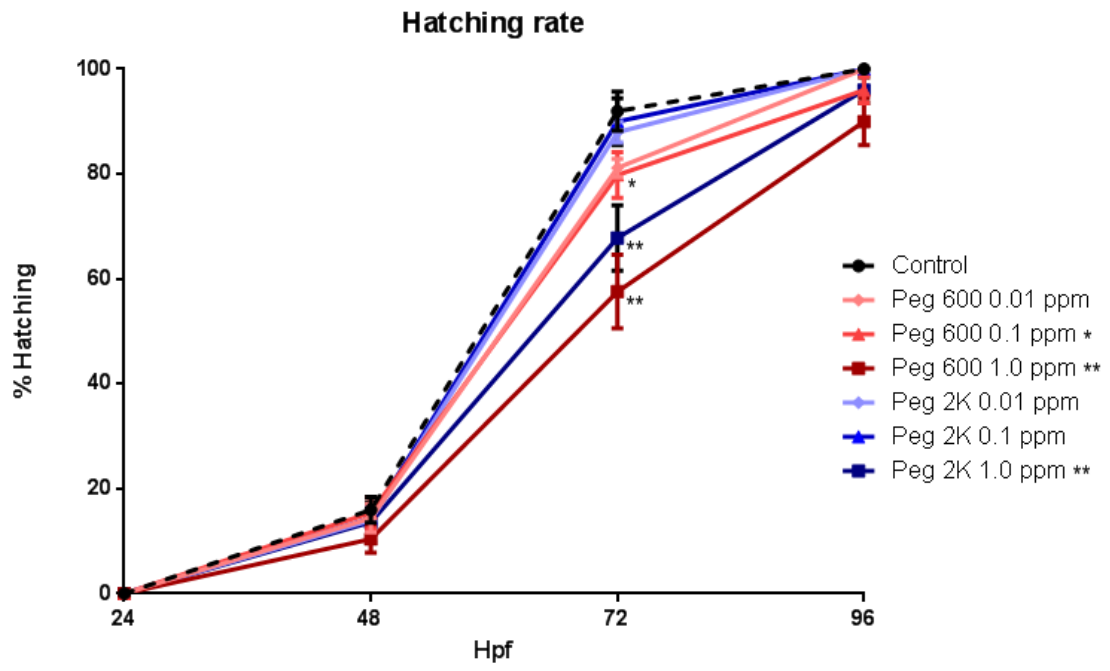


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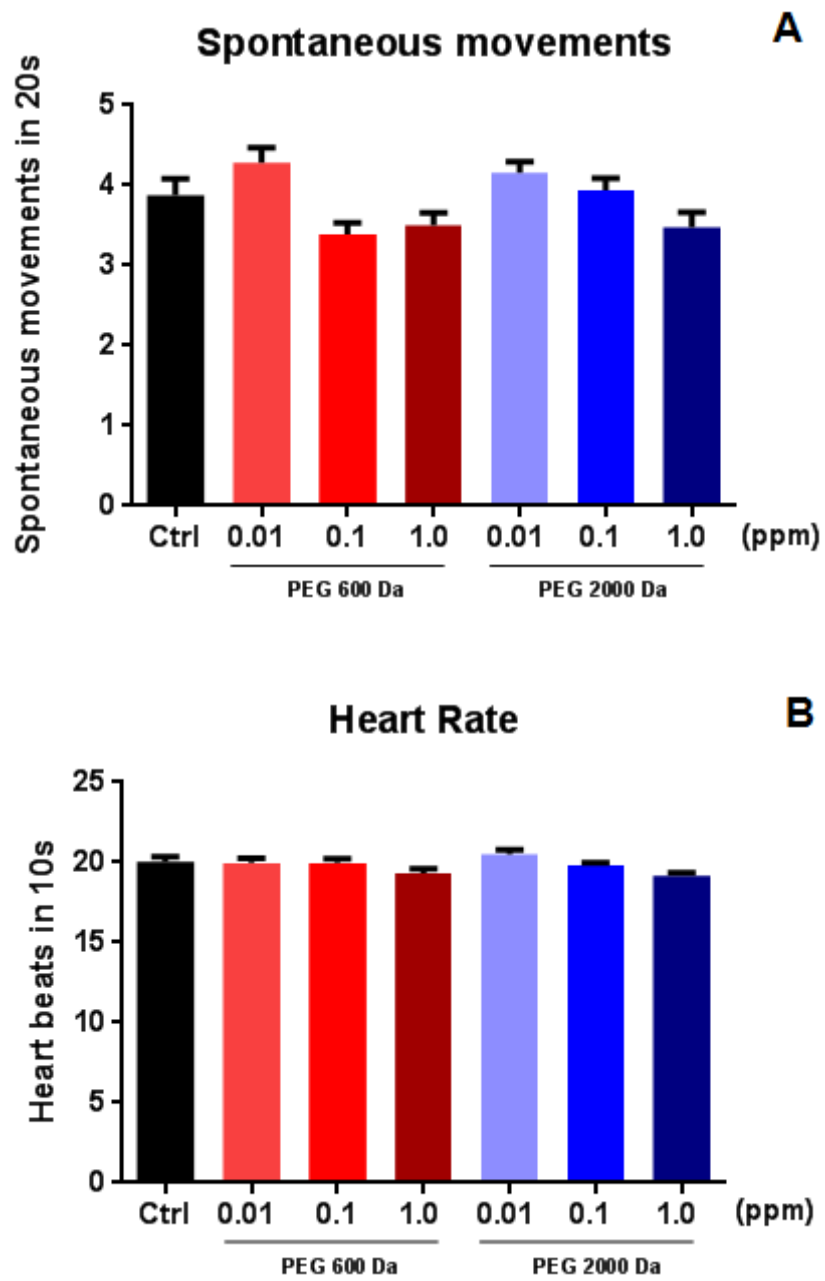


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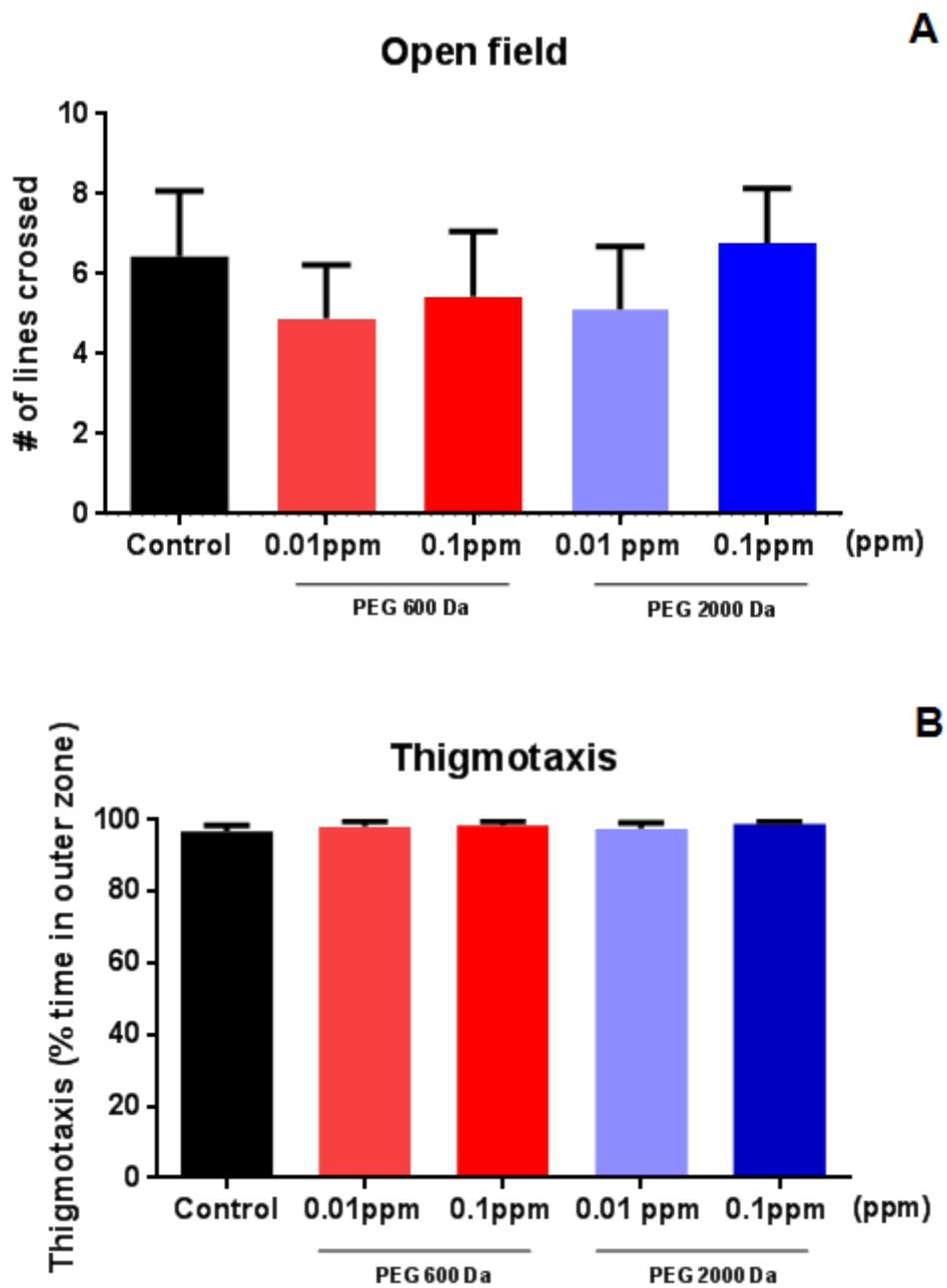
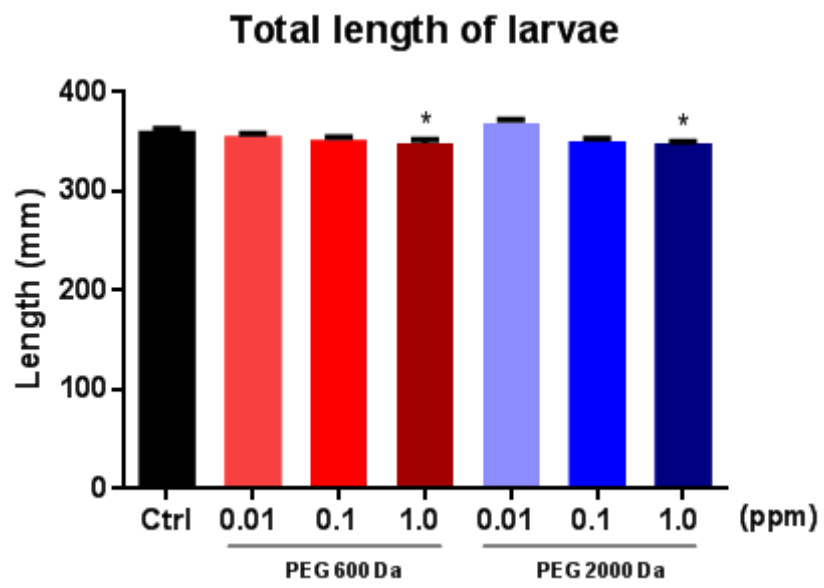


Figure 9:





**Figure 10:**



Figure 11:

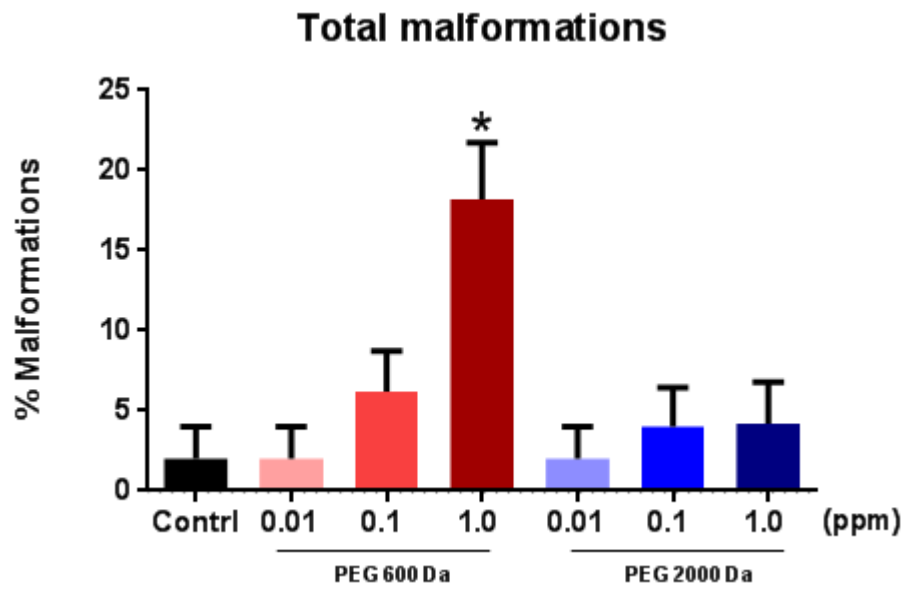


Figure 12:

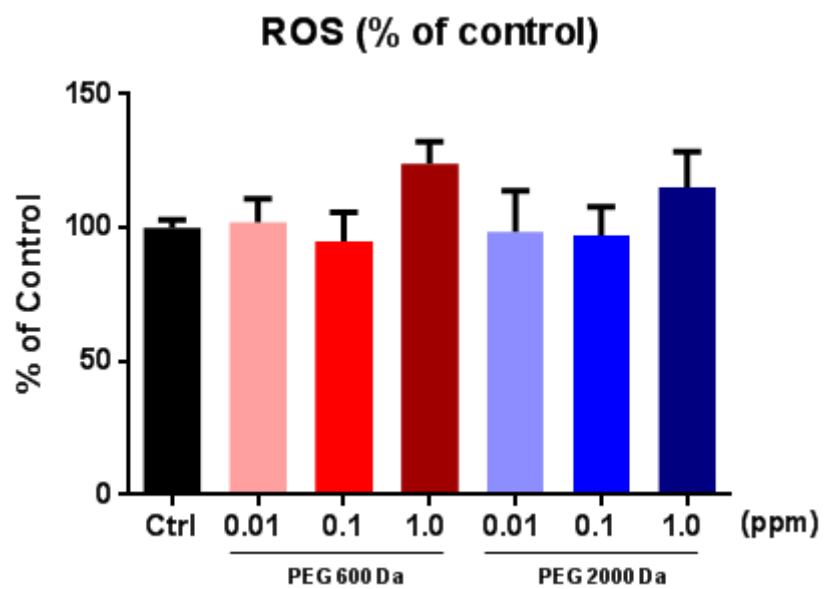
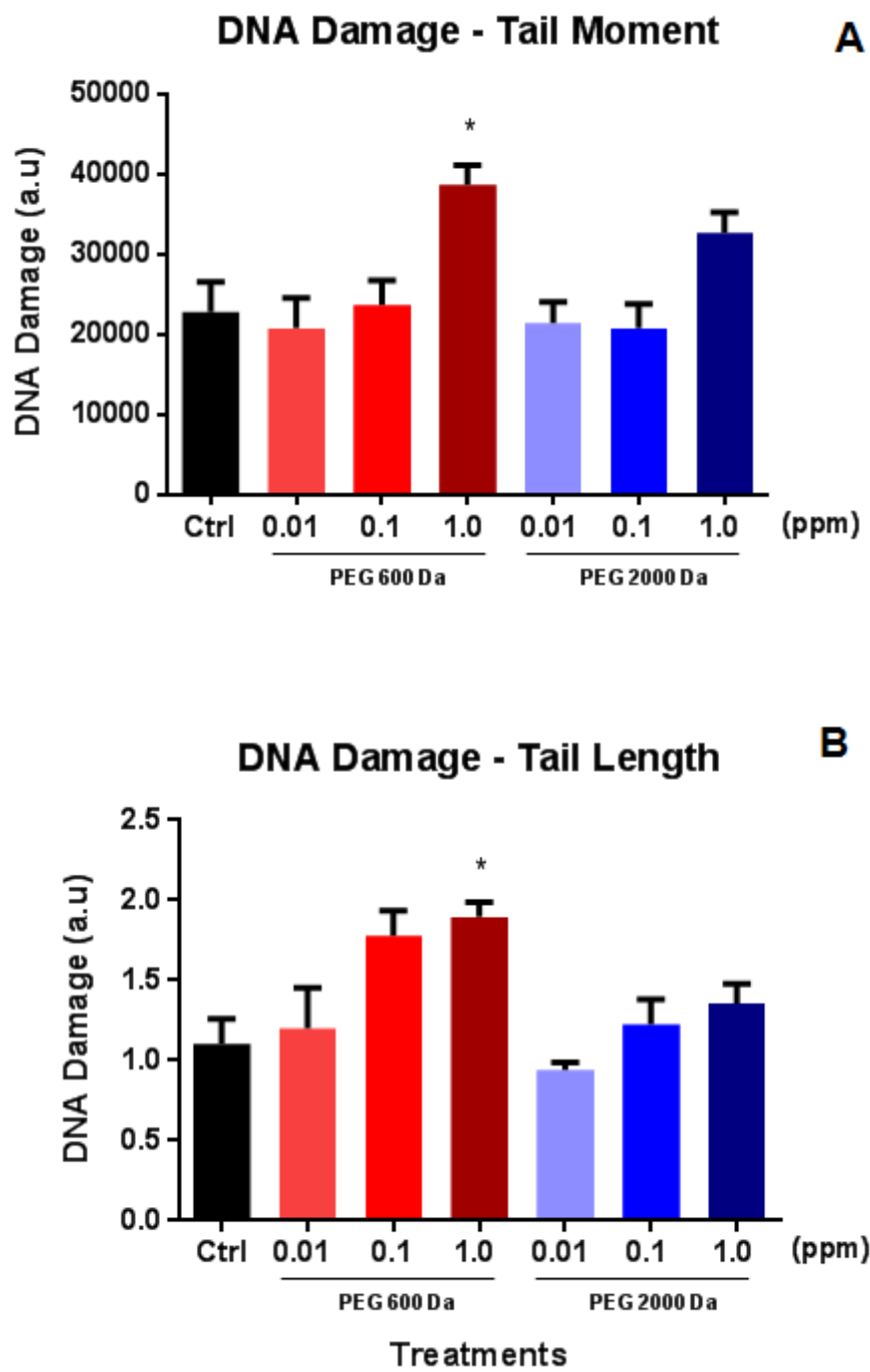


Figure 13:



#### 4 Discussão geral

O *zebrafish* é um excelente modelo para investigação toxicológica. A homologia genômica compartilhada entre humanos e teleósteos torna os estudos com esta espécie de grande relevância para a saúde humana (Giannaccini *et al.*, 2013), e sua sensibilidade a nanopartículas, especialmente na fase embrionária, é discutida em diversos estudos.

Assim como acontece com a maioria das pesquisas biomédicas, testes de toxicidade *in vitro* e *in vivo* direcionam o caminho para futuras aplicações de diversas substâncias. Este aspecto é particularmente importante em relação aos nanotubos de carbono, já que muitas questões sobre sua toxicidade ainda não foram completamente esclarecidas (Firme e Bandaru, 2010).

Avaliação criteriosa da toxicidade de nanomateriais irá fornecer dados críticos, melhorar a confiança do público no setor de nanotecnologia, ajudar órgãos reguladores para determinar os riscos ambientais e de saúde e proporcionar à indústria informações para direcionar o desenvolvimento de nanomateriais e produtos mais seguros (Lin *et al.*, 2013).

O presente trabalho evidenciou prejuízos causados por SWCNTs-PEG em diferentes níveis biológicos: morfológico, comportamental e molecular, contribuindo então com a construção do conhecimento sobre a complexa interação dos nanotubos de carbono com sistemas biológicos, principalmente no contexto das modificações estruturais e suas implicações toxicológicas.

Os resultados aqui descritos indicam que a peguilação, processo de funcionalização largamente estudado, pode ser ineficiente para garantir estabilidade de suspensões em meios biológicos, e neste caso, a toxicidade dos materiais testados é independente da funcionalização. Porém, especial atenção deve ser dada aos processos de purificação, destes materiais, buscando diminuir a presença de metais e outras partículas contaminantes.

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