UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

AVALIAÇÃO DA TOXICIDADE DE QUANTUM DOTS DE TAMANHOS MÁGICOS DE CdSe/CdS DO TIPO CORE SHELL NO MODELO ANIMAL *C. elegans*

Aluno (a): Victor Alexandre Félix Bastos

Orientador (a): Prof. Dr. Luiz Ricardo Goulart Filho

Co-orientador (a): Prof.ª Dr.ª Anielle Christine

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_____

(Nome do Orientador)

Faça boa arte!

(Neil Gaiman)

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Lista de Abreviaturas

%	Porcentagem
º C	Graus Celcius
rpm	Rotações por minuto
λ_{exc}	Comprimento de onda de excitação
λ_{em}	Comprimento de onda de emissão
mL	Mililitro
mg	Miligrama
μg	Micrograma
ηmol	Nanomol
mmol	Micromol
M	Mol
eV	Eletron volt
QD	Quantum dot
MSQD	Magic-sized quantum dot
USQD	Ultramall quantum dot
CSQD	Core-shell quantum dot
CdSe	Seleneto de cádmio
$CdCl_2$	Cloreto de cádmio
Cd^{2+}	Ion de cádmio
FRET	fluorescence resonance energy transfer
GFP	Proteína verde fluorescente
RFP	Proteína vermelha fluorescente
FudR	5-Fluoro-2'-deoxyuridine
NGM	Nelmint growth médium
cDNA	DNA complementar
PCR	reação em cadeia da polimerase
qRT-PCR	PCR quantitativa

Apresentação

Os quantum dots (QD) são nanocristais inorgânicos e fluorescentes, com propriedades óticas singulares. Foram inicialmente desenvolvidos em 1982 por Rossetti & Brus e aplicados no campo biológico a partir de 1998. Em comparação a fluoróforos orgânicos, os QDs apresentam vantagens como: maior foto estabilidade, amplo espectro de absorção, alta luminescência, baixa taxa de degradação, e espectro emissão controlável. Desde o desenvolvimento inicial dos QDs, inúmeros esforços são feitos com o intuito de produzirem QDs com melhores propriedades óticas, mais estáveis e mais seguros para utilização em sistemas biológicos. Tais esforços resultaram na criação de QDs de tamanhos mágicos (MSQD) e ultrapequenos (USQD). OS MSQD e USQD são mais adequados para utilização em ensaios biológicos, pois são muito pequenos (2 nm), possuem alta eficiência quântica, alta estabilidade, baixa toxicidade, são capazes de se difundir passivamente por membranas biológicas, além de manter sua florescência estável por mais de 36 horas. Estas características diferem os MSQD e USQD dos QDs convencionais, e os tornam excelentes ferramentas teranósticas. Apesar de inúmeras vantagens, os QDs despertam preocupações em relação a sua toxicidade, principalmente QDs que possuem Cd em sua estrutura. Além disso, por se tratarem de nanocompostos, suas propriedades físico-químicas são diferentes dos materiais macromoleculares de origem. Por este motivo testes de toxicológicos são extremamente importantes e necessários, para cada tipo de QD ou nanocompostos.

Ensaios toxicológicos com organismos modelo são preferíveis do que ensaios *in vitro,* pois demonstram de maneira mais fidedigna complicações fisiológicas que podem acontecer. Entretanto a escolha de um organismo modelo deve levar em consideração fatores como custo, quantidade de animais, praticidade e homologia com outros organismos. O organismo modelo *Caenorhabditis elegans (C. elegans*), é um nematóide bacterívoro de vida livre, encontrado em todo o mundo na fase intersticial líquida do solo, e é um dos organismos modelo mais utilizados para avaliação de efeitos tóxicos e impactos ambientais causados por compostos

químicos. Além de características comuns a outros organismos modelo, o *C. elegans* se destaca pela similaridade de seus processos fisiológicos com outros organismos mais evoluídos e por sua homologia genética com genes humanos, cerca de 60% dos genes humanos e 40% de genes associados com doenças humanas, são encontrados como ortólogos no genoma de *C. elegans.*

Outro importante organismo modelo é o peixe *Danio rerio (D. rerio)*, conhecido como zebrafish. O zebrafish vem sendo utilizado na pesquisa científica desde 1930, e foi inicialmente empregado como modelo animal para estudos de desenvolvimento embrionário e formação neuronal, entretanto, características particulares como fertilização e desenvolvimento embrionário externos, ovos e embriões transparentes, aumentaram o interesse na utilização desse organismo modelo para os mais variados fins, incluindo ensaios toxicológicos.

O presente trabalho avalia a potencial toxicidade de MSQDs e USQDs de CdSe/CdS, produzidos por nosso grupo, nos organismos modelo *C. elegans* e *D. rerio.* A dissertação está dividida em três capítulos. O Capítulo I apresenta uma breve introdução sobre o tema abordado. O capítulo II apresenta a avaliação da toxicidade de MSQDs no organismo modelo *C. elegans.* O Capítulo três apresenta a avaliação da toxicidade comparativa de MSQDs e USQDs no desenvolvimento embrionário do organismo modelo *D. rerio.*

Capítulo I

FUNDAMENTAÇÃO TEÓRICA

1. Quantum Dots

Quantum dots (QD) são nanocristais fluoróforos inorgânicos, compostos de elementos semicondutores e que possuem tamanhos que variam de 1 a 10 nm (Azzazy et al. 2007; Jamieson et al. 2007; Algar et al. 2010). Foram inicialmente desenvolvidos em 1982 por Rossetti & Brus, para avaliar processos referentes a cinética de oxirredução superficial em colóides de semicondutores. Entretanto, verificou-se que no caso de nanocristais de CdS, o rendimento quântico foi suficiente para produzir luminescência detectável. Além disso, a luminescência observada podia ser controlada de acordo com a concentração de elementos redutores na superfície dos nanocristais (Rossetti and Brus 1982; Azzazy et al. 2007).

De maneira geral, os QDs são formados por um núcleo composto da combinação de elementos dos grupos II e IV ou III e V, e uma casca constituída por uma liga de elementos semicondutores que apresentem um espectro de banda proibida mais amplo do que os elementos do núcleo (Alivisatos et al. 2005; Azzazy et al. 2007). A presença da casca, torna os QDs mais estáveis, diminui a liberação de íons provenientes da degradação do núcleo, e potencializa o rendimento quântico (Jaiswal and Simon 2004; Ozkan 2004; Azzazy et al. 2007).

A fluorescência característica dos QDs, se deve ao efeito de confinamento quântico, este efeito é observado em semicondutores menores do que 20 nm (Reed et al. 1988; West and Halas 2003; Bruchez 2005; Azzazy et al. 2007), e ocorre quando o tamanho do QD é menor do que o raio de excitação de Bohr. Nessa condição, quando o QD é atingido por luz, um fóton com mais energia do que o *bandgap* do elemento semicondutor é absorvido e o QD entra em um estado de alta excitação. Nesse estado, a absorção de energia é favorecida. Quando o estado de excitação retorna a níveis inferiores, ocorre a emissão de fluorescência, geralmente em um espectro curto e simétrico (Michalet et al. 2005; Azzazy et al. 2007).

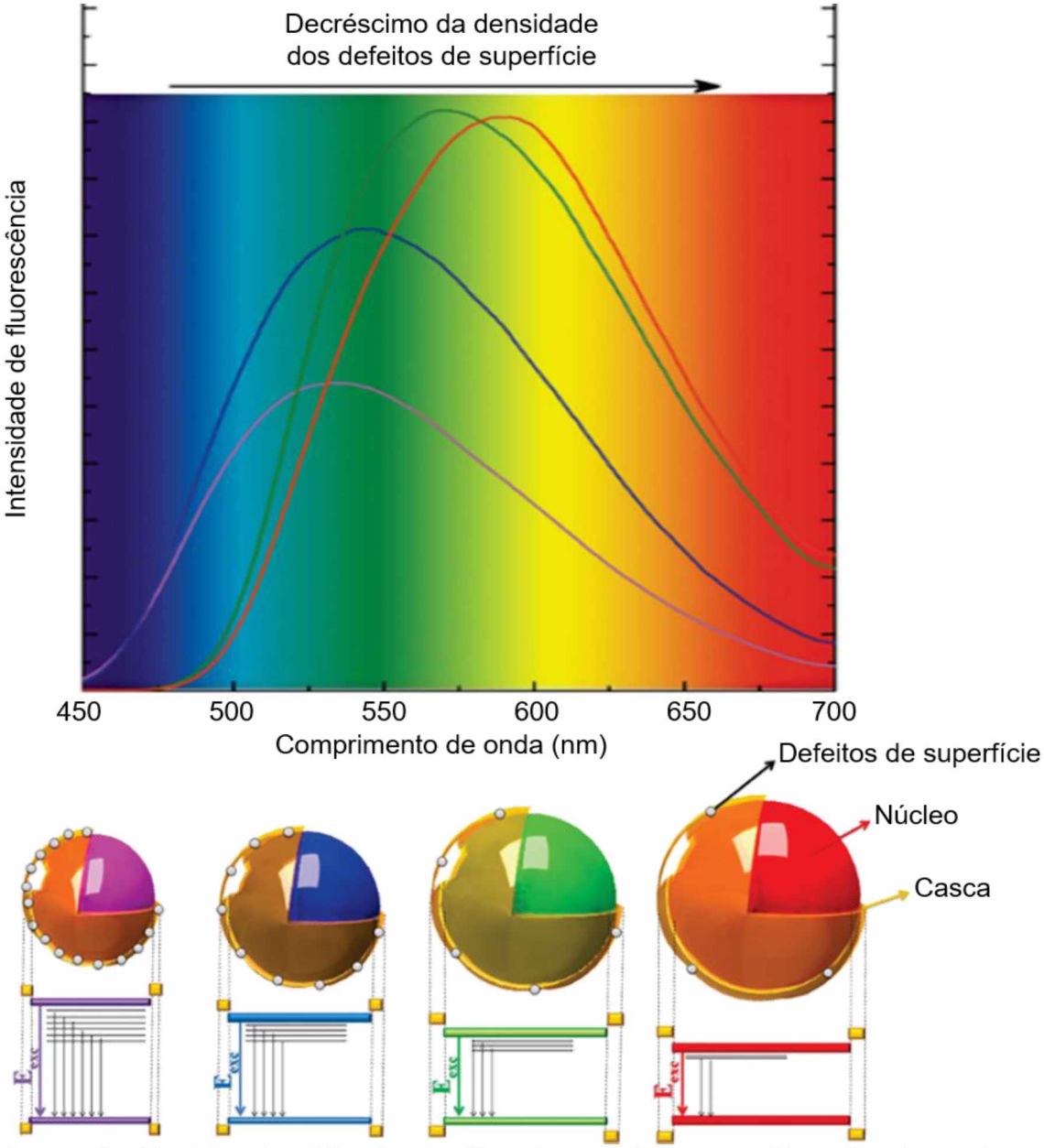

Figura 1: Modelo simplificado de Quantum dots, seus diferentes tamanhos e correspondentes alterações em seus espectros de fluorescência. (Adaptado de Almeida Silva et al. 2014).

1.1. Importância e aplicações

Em comparação a fluoróforos orgânicos, os QDs apresentam inúmeras vantagens, incluindo maior foto estabilidade, amplo espectro de absorção, alta luminescência, baixa taxa de degradação, e espectro de emissão controlável (Michalet et al. 2005; He and Ma 2014). Apesar dos QDs possuírem um espectro de absorção muito amplo, podendo ser excitados por vários comprimentos de onda

(Azzazy et al. 2007; Jamieson et al. 2007), eles podem possuir espectros de fluorescência estreitos ou amplos, ajustáveis de acordo com o tamanho e a composição dos Qds, variando geralmente entre 450 nm e 850 nm. Tais características permitem que múltiplos QDs sejam utilizados em conjunto, sendo excitados por uma mesma fonte de luz e emitindo diferentes fluorescências (Yezhelyev et al. 2006; Azzazy et al. 2007; Jamieson et al. 2007).

Desde sua primeira utilização no campo biológico em 1998 por Alivisatos et al., os QDs demonstraram um potencial incrível e muita versatilidade, podendo ser utilizados para diagnóstico, como biomarcadores fluorescentes em ensaios de imunomarcação, ensaios celulares de acompanhamento, transferência ressonante de energia por fluorescência (FRET), detecção de patógenos e proteínas, monitoramento relacionado a entrega de fármacos, imageamento in vivo e demarcação de estruturas em procedimentos cirúrgicos (Parak et al. 2003; Alivisatos 2004; Azzazy et al. 2007).

Tabela 1: Exemplos da utilização clínica de QDs.

Aplicação	Descrição	Referência
Detecção de patógenos e proteínas	QDs conjugados com anticorpos para detecção de receptores sinápticos.	(De Koninck et al. 2007)
	Detecção de bactérias utilizando QDs funcionalizados com phagos.	(Edgar et al. 2006)
Imageamento *In vivo*	Detecção do biomarcador para câncer de próstata, PSA, por QDs conjugados com anticorpos.	(Gao et al. 2004)
	Marcação de linfonodos para cirurgia de câncer esofágico.	(Parungo et al. 2005)
Diagnóstico	Marcação de anticorpos circulantes para detecção de esclerose sistêmica.	(Sukhanova et al. 2007)
	Detecção de biomarcadores para câncer de ovário.	(Wang et al. 2004)

1.2. Quantum dots de tamanhos mágicos e ultra pequenos

QDs de tamanhos mágicos (MSQDs) e QDs ultrapequenos (USQDs) possuem propriedades óticas e eletrônicas diferentes dos QDs convencionais. O processo de síntese de MSQD e USQD é similar ao de QDs convencionais, entretanto, pequenas alterações na composição, estrutura superficial, porcentagem da liga e espessura da casca podem modificar e aprimorar de maneira significativa as propriedades físicas e fotônicas dos MSQD e USQD, qualificando-os como uma classe particular de QDs (Li et al. 2008; Li et al. 2009; Riehle et al. 2009; Dukes et al. 2010).

MSQD e USQD são mais adequados para aplicação em sistemas biológicos, pois são muito pequenos (2 nm), possuem alta eficiência quântica, alta estabilidade, baixa toxicidade, são capazes de se difundir passivamente por membranas biológicas, além de manter sua florescência estável por mais de 36 horas (Nguyen et al. 2010; Silva et al. 2014). Estas características diferenciam os MSQD e USQD de QD convencionais, tornando-os potenciais ferramentas teranósticas.

1.2.1. Toxicidade de MS e US CdSe/CdS CS-QD

De uma maneira geral, a toxicidade de nanocompostos levanta várias questões de segurança tanto para utilização clínica quanto a respeito de possíveis impactos ambientais. Como os nanocompostos possuem propriedades físico-químicas que diferem daquelas de seus respectivos materiais macromoleculares, testes completos de toxicidade devem ser realizados para cada tipo de composto.

A toxicidade de nanopartículas que possuem Cd em sua composição, é principalmente relacionada com a liberação de íons Cd^{2+} e sua interferência em vários processos biológicos (Martelli et al. 2006; Oh et al. 2016; Singh 2016). Entretanto, os mecanismos envolvidos não são bem determinados, visto que a toxicidade de nanopartículas está intimamente relacionada com o processo de síntese das nanopartículas, proporção de Cd presente, presença de casca protetora, e condições de utilização (Jamieson et al. 2007; Yong and Swihart 2012; Singh 2016).

Nosso grupo desenvolveu MS e US Core-Shell CdSe/CdS QDs pelo método de solução aquosa (Silva et al. 2013), deste modo, a estrutura cristalina da casca formada diminui a degradação do núcleo, fazendo que que os QDs sejam menos tóxicos, mais estáveis e com melhores propriedades óticas (Linkov et al. 2016). Os MSQDs apresentam um amplo espectro de luminescência (520 – 680 nm), que é explicado pela quantidade de íons Cd^{2+} em sua superfície, enquanto que os USQDs apresentam um espectro de luminescência mais estreito (500 – 580 nm) e uma baixa densidade de íons Cd^{2+} em sua superfície (Almeida Silva et al. 2014; Silva et al. 2014; Silva et al. 2016).

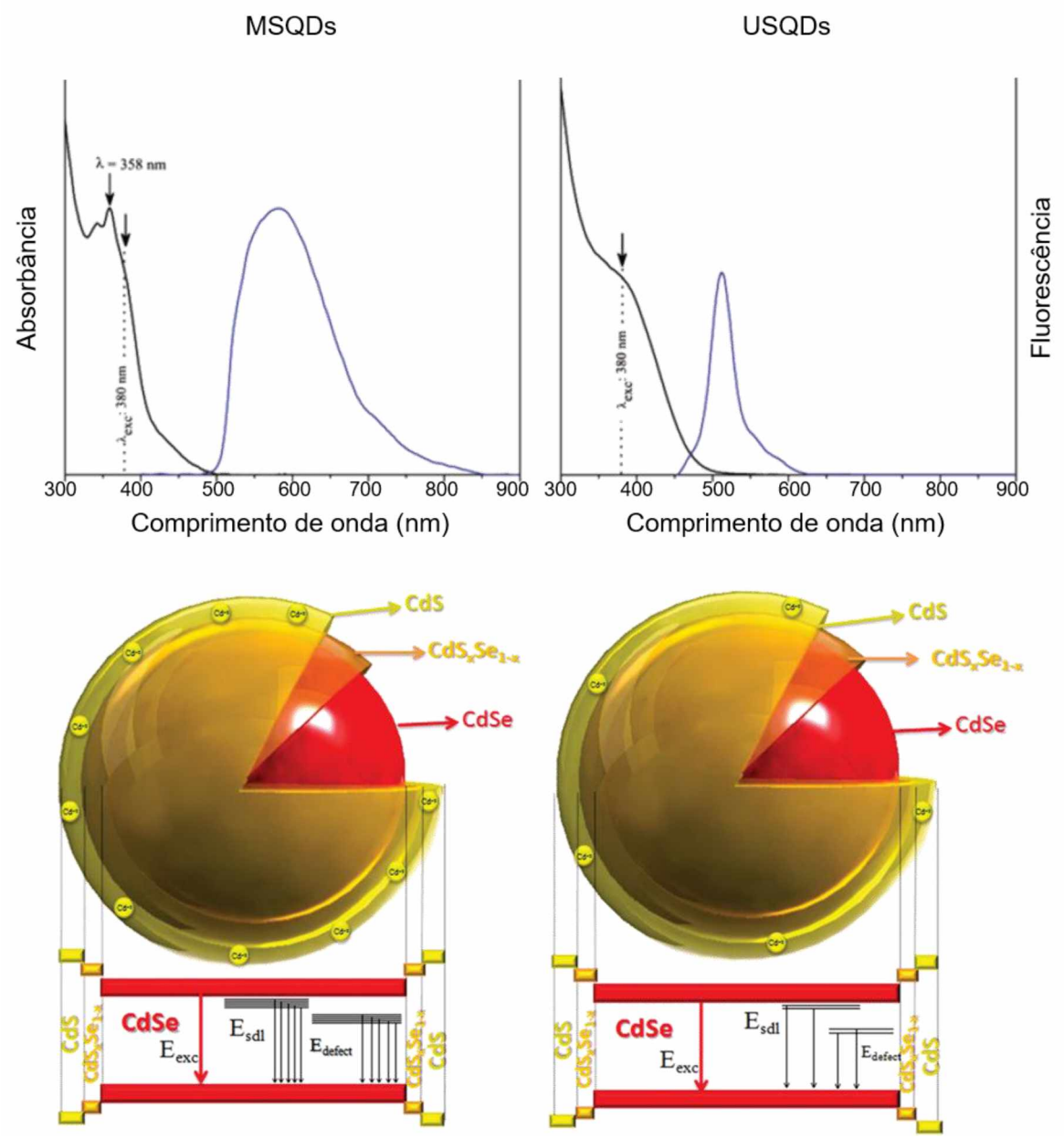

Figura 2: Modelo representativo da estrutura dos MSQDS e USQDs, com seus respectivos espectros de fluorescência.

Mesmo com o desenvolvimento de novos e melhores QDs, testes de toxicidade devem ser realizados de maneira específica para cada tipo de QD, tanto para elucidar mecanismos gerais sobre a toxicidade de nanocompostos quanto para determinar doses seguras e contraindicações a respeito da utilização de nanocompostos específicos (Li et al. 2008; Yong and Swihart 2012).

2. Organismos modelo

Apesar de representarem apenas uma pequena parte da grande biodiversidade existente na Terra, os organismos modelo auxiliam na compreensão de processos fisiológicos, de hereditariedade, de desenvolvimento e de causa e efeito (Hedges 2002). Os organismos modelo fazem parte do cenário cientifico há mais de 100 anos. Acredita-se que Mendel tenha sido o primeiro a realmente caracterizar e utilizar um organismo como modelo (Müller and Grossniklaus 2010).

A utilização de organismos modelo se baseia na similaridade e conservação do mecanismo de ação de processos fisiológicos básicos entre espécies. Entretanto, se faz necessária a utilização de vários modelos, desde vírus e procariontes até vertebrados para que se atinja uma compreensão mais ampla dos processos biológicos envolvidos (Griffiths et al. 2012).

De maneira geral, as principais características para um organismo modelo são: curto tempo de vida, geração de prole com muitos indivíduos, tamanho pequeno, fácil manutenção e manipulação. Além disso, a quantidade de conhecimento acumulado sobre tal organismo é fundamental para sua escolha e implementação como organismo modelo (Müller and Grossniklaus 2010; Griffiths et al. 2012).

2.1. *Caenorhadbitis elegans (C. elegans)*

Caenorhabditis elegans (C. elegans), é um nematóide bacterívoro de vida livre, encontrado em todo o mundo na fase intersticial líquida do solo. É um dos organismos modelo mais utilizados para avaliação de efeitos tóxicos e impactos ambientais causados por compostos químicos (Leung et al. 2008; Kumar et al. 2015).

Este nematóide, foi originalmente proposto como organismo modelo por Sydney Brenner em 1963 (Wood 1988), graças a sua simplicidade frente a outros organismos multicelulares (Müller and Grossniklaus 2010). Desde então, sua utilização no campo científico só aumentou, devido a características como fácil manutenção, genoma completamente sequenciado, linhagem celular completamente descrita, ciclo de vida curto e alto número de progênie (Tejeda-Benitez and Olivero-Verbel 2016).

Além de características comuns a outros organismos modelo, o *C. elegans* se destaca pela similaridade de seus processos fisiológicos com outros organismos mais evoluídos (Corsi et al. 2015). Cerca de 60% dos genes humanos e 40% de genes associados com doenças humanas, são encontrados como ortólogos no genoma de *C. elegans* (Culetto and Sattelle 2000; Kaletta and Hengartner 2006; Leung et al. 2008; Rodriguez et al. 2013), fato que qualifica esse nematóide como um excelente modelo para estudo e compreensão da fisiologia humana em vários casos.

2.1.1. Ciclo de vida

C. elegans possuem um ciclo de vida curto, evoluindo de ovos até adultos férteis em 3 dias. Cada adulto pode gerar de 300 a 1000 novos indivíduos em seu período de vida (Corsi 2006; Corsi et al. 2015).

A embriogênese em *C. elegans* leva em média 16 horas, nesse período, o ovo é formado após a fecundação, ele possui uma casca praticamente impermeável, fazendo com que o embrião se desenvolva completamente isolado de seu progenitor (Corsi et al. 2015). Os ovos levam cerca de 9 horas para eclodirem em larvas (L1), caso não exista alimento no meio, as larvas podem se manter nesse estágio larval por até dois dias. O estágio L1 dura por cerca de 12 horas, e cada estágio subsequente (L2, L3 e L4) dura cerca de 8 horas. Após cada estágio, as larvas passam por uma muda, trata-se de um período de letargia, onde uma nova cutícula é formada, permitindo o crescimento das larvas. Cerca de 8 horas após a muda do estágio larval L4, os animais hermafroditas começam a produzir ovos por cerca de 2 a 3 dias (Raizen et al. 2008; Corsi et al. 2015).

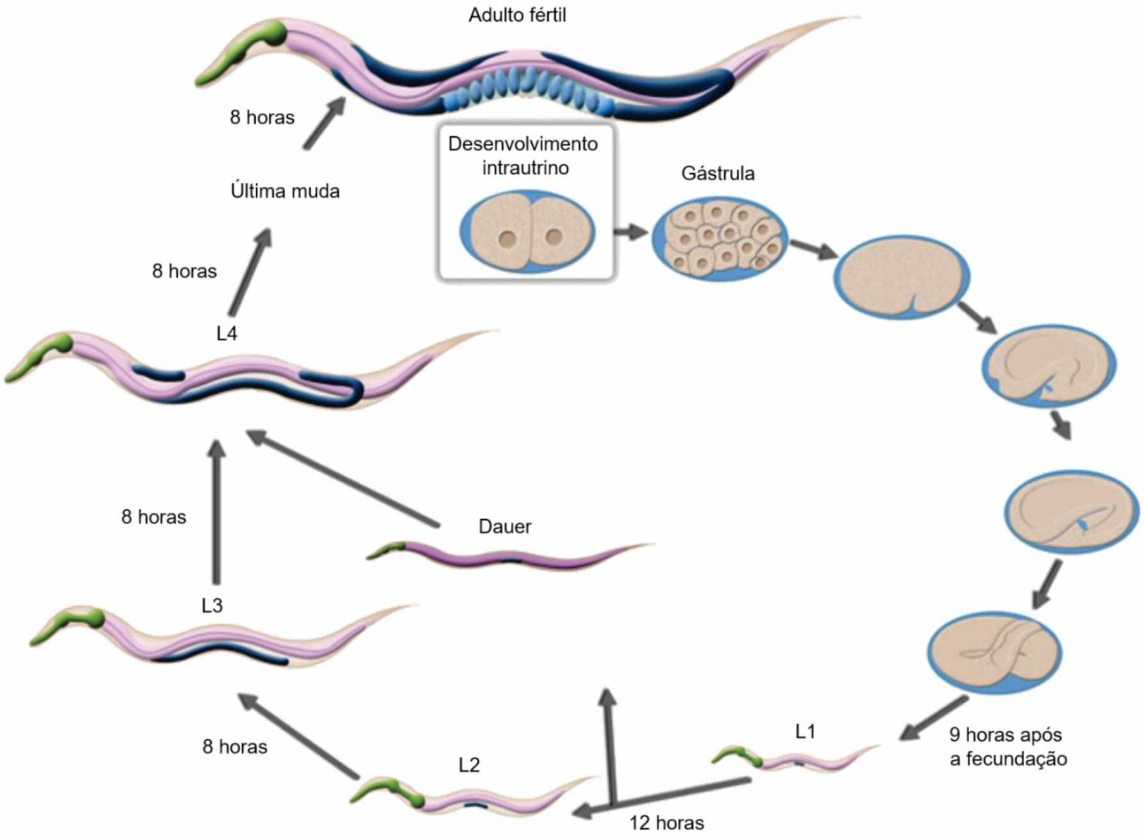

Figura 3: Ciclo de vida do nematóide *C. elegans.* (Adaptado de Altun and Hall 2009).

Além dos estágios larvais mencionados (L1-L4), as larvas L1/L2 podem assumir outra forma, chamada de "dauer" no caso de escassez alimentar (Golden and Riddle 1984; Hu 2007). Nesta forma mais resistente, a cutícula cobre toda a larva, inclusive a boca, impedindo que a larva se alimente, mas garantindo que sobreviva por até 4 meses. Quando houver alimento disponível novamente, a cutícula retorna ao normal e a larva pode se alimentar e se desenvolver normalmente para a fase L4 (Corsi et al. 2015).

2.1.2. Ensaios toxicológicos

Dentre os parâmetros mais utilizados para analisar a toxicidade de compostos utilizando *C. elegans* estão: letalidade, crescimento, reprodução, fertilidade, longevidade, locomoção, desenvolvimento, expressão de gênica, estresse oxidativo, apoptose, danos ao DNA, dentre outros. Sendo que tais parâmetros podem ser divididos entre feitos biológicos e marcadores moleculares, para facilitar

a escolha e compreensão dos testes aplicados (Tejeda-Benitez and Olivero-Verbel 2016).

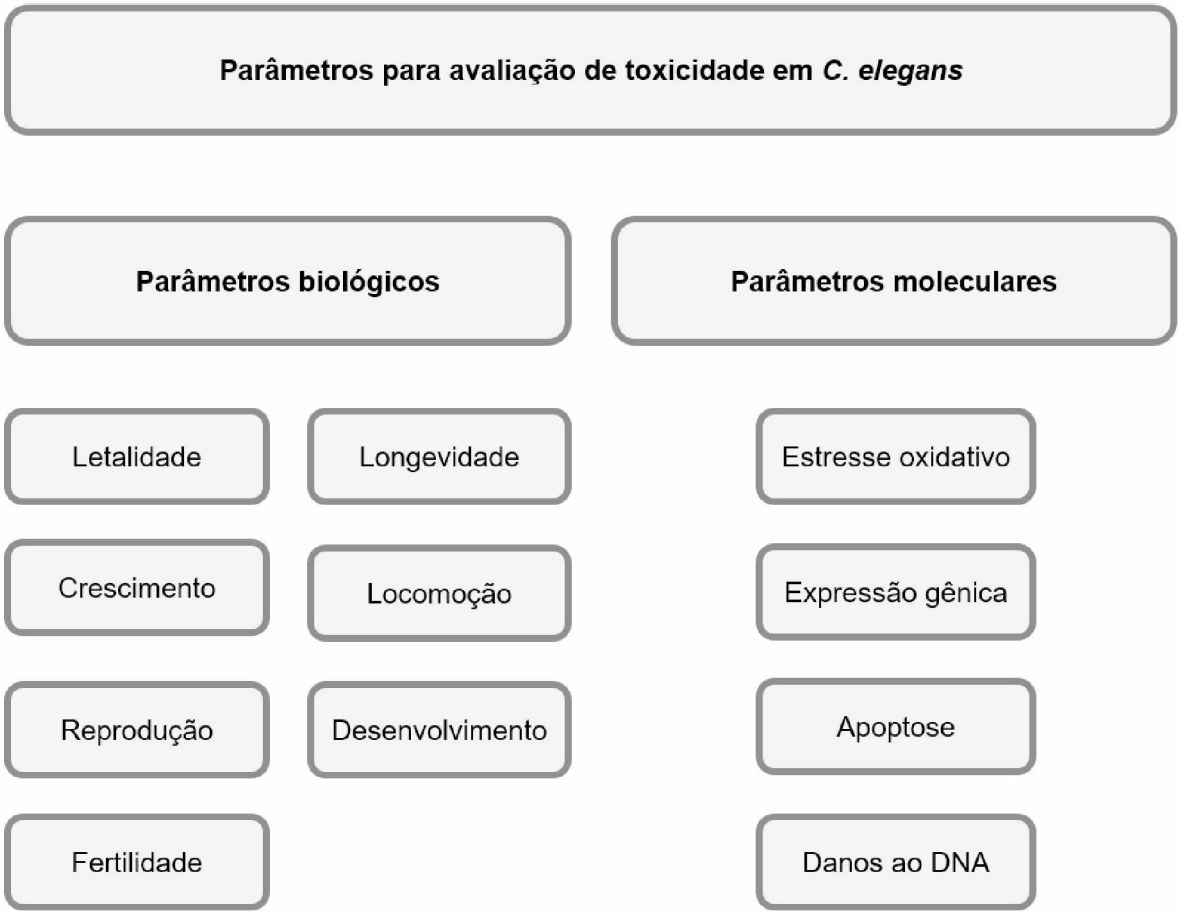

Figure 4: Parâmetros para avaliação de toxicidade em *C. elegans.*

Graças a versatilidade de parâmetros toxicológicos, o modelo, *C. elegans,* vem sendo empregado na avaliação de toxicidade dos mais variados compostos, tais como, amostras de solo e água (Menzel et al. 2009; Turner et al. 2013), pesticidas (Anbalagan et al. 2013; Leelaja and Rajini 2013), metais pesados (Roh et al. 2009; Shen et al. 2009; Yu et al. 2013), drogas (Boyd et al. 2010; Taki et al. 2014) e nanocompostos (Wu et al. 2012; Chen et al. 2013; Zhao et al. 2015).

As características particulares apresentadas pelo nematóide *C. elegans,* o qualificam como uma poderosa ferramenta para estudos toxicológicos, auxiliando na predição de efeitos em outros organismos. Características chave, como, baixo custo, a vasta quantidade de animais, corpo transparente, genoma sequenciado,

facilidade de manipulação e de criação de mutantes, além da possibilidade de análise de múltiplos parâmetros simultâneos, fazem com que ensaios realizados com *C. elegans* sejam altamente significativos e complementares a estudos com culturas celulares e modelos vertebrados.

2.2. *Danio rerio (D. rerio)*

Danio rerio (D. rerio), conhecido como zebrafish, peixe-zebra ou paulistinha, é um pequeno peixe tropical originário do norte da Índia, pertencente ao gênero *Danio* (Parng et al. 2002; Westerfield 2007).

O zebrafish vem sendo utilizado na pesquisa científica desde 1930, e foi inicialmente empregado como modelo animal para estudos de desenvolvimento embrionário e formação neuronal (Streisinger et al. 1981; Schulte-Merker 2003). O interesse na utilização de zebrafish como um organismo modelo é fundamentado em algumas características do animal: trata-se de um animal pequeno, com baixo custo de manutenção, progênie numerosa (100 a 200 ovos), ciclo de vida curto (2 a 3 meses), sua fertilização e desenvolvimento embrionário são externos, e seus ovos e embriões são transparentes, o que facilita a observação e o acompanhamento de seu desenvolvimento (Laale 1977; Parng et al. 2002; Lieschke and Currie 2007; Jang et al. 2014).

Atualmente, o zebrafish é utilizado para vários fins, pois trata-se de um vertebrado, com genoma completamente sequenciado e com alta homologia, mais de 80% de seus genes possuem um correspondente em humanos (Barbazuk et al. 2000; Dooley 2000; Schulte-Merker 2003; Howe et al. 2013). Além disso, o desenvolvimento de técnicas de clonagem, mutagênese e transgenia aplicadas ao zebrafish, permitiu a criação de diversos modelos de doenças humanas (Grunwald and Streisinger 1992; Geisler 2002; Lieschke and Currie 2007).

2.2.1. Ciclo de vida

Além de produzir um grande número de embriões por acasalamento, o Zebrafish apresenta um desenvolvimento embrionário muito rápido, completando os estágios iniciais de desenvolvimento em cerca de 24 horas após a fertilização. Após 5 dias,

os alevinos estão completamente formados e abandonam a alimentação de sua reserva de vitelo. Com 90 dias os adultos já estão aptos a reproduzir novamente (Schulte-Merker 2003; Giannaccini et al. 2014).

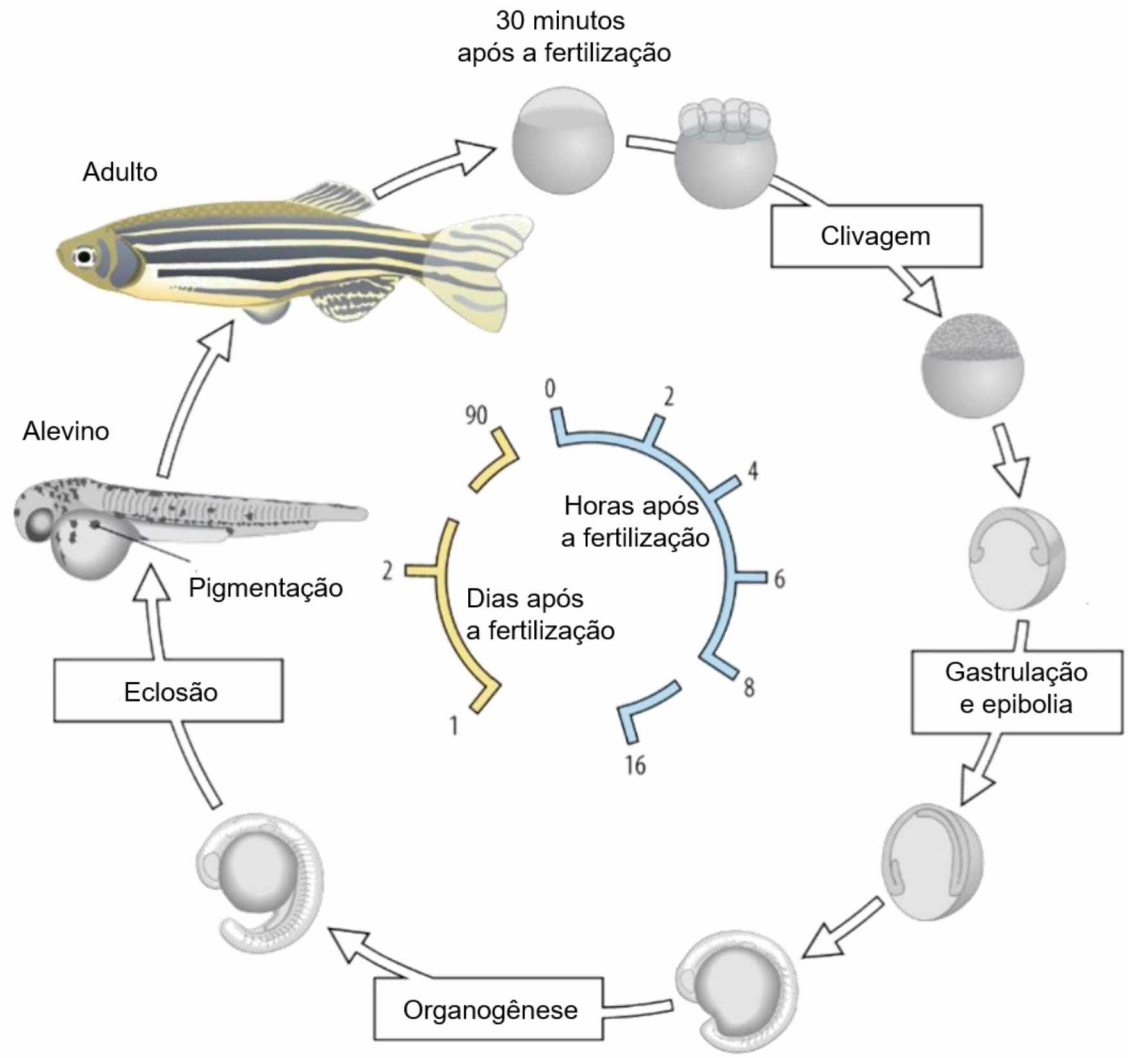

Figura 5: Ciclo de vida de Zebrafish (*Danio rerio*) (Adaptado de Wolpert and Tickle 2011).

2.2.2. Ensaios toxicológicos

Por possuir um ciclo de vida curto e um rápido desenvolvimento, diferentes testes podem ser aplicados, utilizando como parâmetros de toxicidade alterações em cada uma das fases do desenvolvimento do Zebrafish (Parng et al. 2002; Lieschke and Currie 2007; Giannaccini et al. 2014).

No que diz respeito a toxicidade de nanocompostos e seu impacto ambiental, o Zebrafish é um modelo extremamente útil, e vem sendo utilizado com sucesso,

tanto em ensaios com embriões como com animais adultos (Powers et al. 2011; Zhang et al. 2012; Duan et al. 2013; Jang et al. 2014). Além disso, testes com embriões de Zebrafish são particularmente interessantes, visto que os embriões se mantêm transparentes por até 72 horas após a fertilização, onde o tecido começa a ficar denso e pigmentado, possibilitando a observação direta de alterações morfológicas, principalmente no cérebro, coração e notocorda (Hill et al. 2005).

Outros ensaios, que avaliam a viabilidade, crescimento, morfologia, função cardíaca e locomoção em Zebrafish são muito utilizados, e graças ao baixo custo de manutenção, facilidade de manejo e alta homologia com o genoma humano, o zebrafish vem tomando o lugar de organismos modelos mais complexos e dispendiosos, como o *Mus musculus* (Hill et al. 2005; River 2014).

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Capítulo II

ASSESSMENT OF MAGIC SIZED CORE/SHELL CDSE/CDS QUANTUM DOTS TOXICITY ON CAENORHABDITIS ELEGANS

*Instructions according to Archives of Toxicology

Resumo: Quantum dots de tamanhos mágicos (MSQD) são nanocristais estáveis e fluorescentes, com tamanhos menores que 2 nm, e incríveis propriedades óticas e eletrônicas. Visto que a toxicidade de quantum dots que utilizam cádmio em sua composição é um tema controverso, o nosso objetivo foi avaliar os efeitos de CdSe/CdS CS-MSQD no modelo animal *Caenorhabditis elegans* (*C. elegans*), um importante modelo para testes de toxicidade de nanocompostos *in vivo*. Nós expomos os nematoides a várias concentrações de MSQDs e avaliamos os seguintes parâmetros: toxicidade aguda, tempo de vida, crescimento, expressão de genes relacionados a toxicidade e bioacumulação. Os MSQDs apresentaram pouca ou nenhuma toxicidade, o LC50 após 24 horas de exposição foi calculado em 1815.039 μg/mL, enquanto que o LC50 do $CdCl_2$ foi 825.254 μg/mL. Os nematoides expostos não apresentaram diferenças significativas no tempo de vida ou no crescimento. Além disso, a análise da expressão genica demonstrou comportamentos diferentes para nematoides expostos aos MSQDs e para $CdCl_2$. Conseguimos ainda detectar altos níveis de fluorescência derivada dos MSQDs internalizados pelos nematoides.

Palavras-chave: *C.elegans*, quantum dots, toxicidade, cádmio

Abstract: Magic-sized quantum dots (MSQD) are highly stable fluorescent nanocrystals with sizes < 2 nm, and remarkable optical and electronic properties. Since toxicity of cadmium composed quantum dots is highly controversial, our aim was to assess the effects of CdSe/CdS CS-MSQD on the animal model *Caenorhabditis elegans* (*C. elegans*), an important *in vivo* model for analysis of nanocompounds toxicity. We have exposed the nematodes to several concentrations of the MSQD, and evaluated the following endpoints: acute toxicity, life spam, growth, expression of stress related genes and bioaccumulation. The MSQD presented little to no toxicity, with a 24 h LC50 of 1815.039 μg/mL while $CdCl_2$ LC50 was 825.254 μg/mL. MSQD exposed nematodes showed no significant difference in life spam or growth analysis. Furthermore, gene expression analysis of MSQD exposed nematodes demonstrated a diverse behavior from nematodes exposed to $CdCl_2$. In addition, we could detect high stable fluorescence derived from MSQD within the exposed nematodes.

Keywords: *C.elegans*, quantum dots, toxicity, cadmium

1. Introduction

Quantum dots (QDs) are semiconductors nanocrystals with remarkable electronic and optical capabilities. They have been widely used for industrial and research purposes (Galian and Guardia 2009; Bera et al. 2010; Vasudevan et al. 2015). Characteristics such as high quantum efficiency, adjustable bandgap, stable fluorescence, low toxicity and immunogenicity make QDs exceptional tools for development of probes, drug delivery systems or biological labeling (Algar et al. 2010; Byers and Hitchman 2011; Valizadeh et al. 2012; He and Ma 2014).

QDs are normally composed by 2B and 6A family elements, which can rise safety concerns about their toxicity. A range of commercial QDs present cadmium (Cd) in their composition. However, Cd is known to be highly toxicity and harmful to the nervous system, rendering those QDs not suitable or recommended for biological assays (Jamieson et al. 2007; Su et al. 2009; Ji et al. 2014). However, scientific advances in the field of nanotechnology and in toxicological research opened new doors for the usage, development and improvement of novel QDs, even in the presence of Cd^{2+} ions (Li et al. 2008; Yong and Swihart 2012).

Our group has developed Ultrasmall (US) and Magic-sized (MS) Core-Shell (CS) CdSe/CdS QDs by aqueous solution method (Silva et al. 2013), in which their crystal structure limit the core degradation, rendering the CS-QDs less toxic, highly stable, and with improved optical properties (Linkov et al. 2016). The USQD presents narrow luminescence spectrum (500 – 580 nm) and low Cd^{2+} ions density on the surface, while the MSQD presents a very wide luminescence spectrum (520 – 680 nm), explained by a large number of Cd^{2+} ions on its surface. However, is important to emphasize that even with greater density of Cd^{2+} ions on the surface, the MSQD proved to maintain high fluorescence for extended periods with little toxicity (Almeida Silva et al. 2014; Silva et al. 2014; Silva et al. 2016).

It is well established that toxicity of cadmium-based nanoparticles is mainly related to the release and interference of Cd^{2+} ions in many biological pathways (Martelli et al. 2006; Oh et al. 2016; Singh 2016). However the mechanisms involved are yet to be determined, as it is intimately related to the nanoparticle's synthesis process, Cd proportion, presence of a protective shell, and conditions of applications (Jamieson et al. 2007; Yong and Swihart 2012; Singh 2016). Thus, toxicological studies are still required to better understand general and specific nanoparticles toxicity mechanisms.

The nematode *Caenorhabditis elegans (C. elegans*) is one of the most used animal models to evaluate toxic effects and environmental impacts of chemical compounds. This free-living soil nematode has short life cycle, is easily maintained in laboratorial culture either in solid or liquid medium, and has his genome complete sequenced (Brenner 1974; Leung et al. 2008). Among the most common features analyzed in toxicological assays with *C. elegans,* are physiological endpoints such as acute toxicity response, growth, lifespan, reproduction, production of reactive oxygen species, development, and motility, which respond well to small alterations in culture conditions. For these reasons *C. elegans* has become a widely used model to assess nanomaterials ecotoxicology and environmental toxicity (Dengg and Van Meel 2004; Leung et al. 2008; Ma et al. 2009).

In this work, we analyzed the potential toxicity of the CdSe/CdS CS-MSQD, synthesized by our group, in the animal model *C. elegans*, assessing lethal concentration, effects on lifespan, nematode growth and toxicity related gene expression, as well as determining the most efficient concentrations for exposure, biodistribution and fluorescence detection.

2. Materials and Methods

2.1. Materials

Wild type *C. elegans* strain N2 (Bristol) and *E. coli* strain OP50 were kindly provided by Dr. Carlos U. Vieira (Laboratory of Genetics, Institute of Genetics and Biochemistry, Federal University of Uberlândia, Brazil). CS-CdSe/CdS MSQD were synthesized and characterized as previous described (Silva et al. 2013), and provide by Dr. Noelio O. Dantas (Laboratory of New Insulating Materials and Semiconductors - LNMIS, Physics Institute, Federal University of Uberlândia, Brazil). 5-Fluoro-2'-deoxyuridine (FUdR) and cadmium chloride ($CdCl_2$) were purchased from Sigma, St. Louis, MO. All other materials were obtained from specialized commercial sources and used without further purification steps.

2.2. Synthesis and characterization of CS-CdSe/CdS MSQD

The physical-chemical characterization and synthesis of CS-CdSe/CdS MSQD were conducted in aqueous solutions at room temperature as described elsewhere (Silva et al. 2014). Briefly, 2 mmol of $Cd(ClO_4)_2.6H_2O$ and 5 mmol of 1-thioglycerol were mixed in ultra-pure water and the pH was adjusted to 6 with 0.1 M NaOH at room temperature. The resulting suspensions were precipitated with ethanol and centrifuged four times at 6,000 rpm

for 10 minutes. The resulting nanopowders were dried in a vacuum mechanical pump at room temperature and dispersed in ultra-pure water.

2.3. *C. elegans* culture

Nematodes were cultured at 20 ºC on solid nematode growth medium (NGM) petri dishes, seeded with *E. coli* OP50 as food source (Brenner 1974). To achieve synchronized age at L1 stage culture, NGM plates with gravid adult nematodes were washed with 10 mL M9 buffer (3g KH_2PO_4, 6g $Na_2HPO_4 \cdot 7H_2O$, 5g NaCl, 0.25g $MgSO_4 \cdot 7H_2O$, H_2O to 1 liter. Sterilize by autoclaving) and collected in 15 mL conical tubes. The tubes were centrifuged and the supernatant was discarded. The worms pellet was resuspended and treated with 10 mL of 20% alkaline hypochlorite solution for 10 minutes. Once most of the worms were dissolved, the tubes were centrifuged and the pellet was rinsed with M9 buffer three times, then resuspended and maintained in light agitation until the eggs hatched into L1 stage worms.

2.4. Acute toxicity and LC_{50} estimation

L4 stage worms were exposed to six concentrations of CS-CdSe/CdS MSQD, ranging from 1000 to 31.25 μg/mL. The test was conducted on 24 well tissue culture plates, at 20°C for 24h without food sources. $CdCl_2$ was used in the same concentrations as reference toxicant. The assay was conduct in triplicate with 30 (± 3) worms per well (~90 worms for each concentration). Death was assumed when no detectable movement was observed after stimuli with platinum wire. LC_{50} were estimated by Probit analysis (Gaddum 1948), with 95% CI.

2.5. Lifespan analysis

Tissue culture plates with 24 wells were prepared with 1 mL agar-NGM medium with 2 mM FUdR to prevent worms' reproduction. *E. coli* OP50 lawn was used as food source. CS-CdSe/CdS MSQD or $CdCl_2$ solution were added to wells at test concentrations. The plates were incubated at 37 °C for 1h before adding the worms and then at 20 °C after worms' addition. For each concentration, 30 (± 3) L1 stage worms per well (~90 worms) were analyzed daily and live worms were recorded. Each treatment was realized in triplicate, survival was plotted using Kaplan-Meier survival curves, and analyzed by log rank test using Graph Pad Prism 5.0.

2.6. Growth assessment

For growth analysis, L1 stage worms were cultured for 4 days in the same conditions as the life span assay. Every day a subset of 15 (± 4) worms were pipetted out in glass slides, heated to 50 °C, to straighten out and photographed in an inverted microscope (EVOS FL Cell Imaging System®, Thermo Fisher Scientific). Body length was measured using NIH Image J software.

2.7. RNA extraction

Age synchronized L4 worms were exposed to treatments (CS-CdSe/CdS MSQD or $CdCl_2$) for 4h or 24h. After exposure, worms were washed with M9 buffer and collected by centrifugation. Total RNA was extracted using TRIzol reagent (Invitrogen), according to manufacturer's protocol. Total RNA quantification was performed with Nanodrop ND-1000 spectrophotometer (Nano- Drop Technologies). Samples quality was determined by 260/280 and 260/230 absorbance ratios. All treatments were performed in triplicates.

2.8. cDNA synthesis and qRT-PCR

Complementary DNA (cDNA) was synthesized by reverse transcription of total nematode RNA (1 μg) using the M-MLV Reverse Transcriptase (Thermo Fisher Scientific). Real time PCR was performed using power SYBR Green (Applied Biosystems). Amplification was conducted on a 7300 Real-Time PCR System (Applied Biosystems) in the following conditions: 95ºC for 10 min for polymerase activation, followed by 40 cycles of: denaturation at 95ºC for 15 sec and primer annealing and elongation at 61ºC for 60 sec. Primers sequences used for qRT-PCR are described on Table 1. Relative quantification was determined by ΔΔCt method (Schmittgen and Livak 2008) after Ct normalization against the reference gene t*ba-1*. Samples were run in triplicates.

Table 2: Primer information of selected genes.

Gene	Locus tag	Related function	Forward primer	Reverse primer
cdr-1	F35E8.11	Cadmium stress	TCTTCTCTCAATTGGCAACTG	TTTGGGTAAACTTCATGACGA
hsp-70	C12C8.1	Stress, heat shock	TGAAATTGAAGCAAAGGACAA	TGTGGATAATTGCTGGAATGG
dhs-26	ZK816.5	Cell metabolism related dehydrogenase	ATCGCAAATATGCGTAGGAAGA	AGCTGACATCCAGAGGGTCT
tba-1	F26E4.8	Tubulin alpha	TCAACACTGCCATCGCCGCC	TCCAAGCGAGACCAGGCTTCAG

2.9. Bioaccumulation and fluorescence

To visualize internalized QDs, L1 stage synchronized worms were treated with 500 μg/mL CS-CdSe/CdS MSQD for six days. Images were obtained every two days using an inverted fluorescence microscope (EVOS FL Cell Imaging System®, Thermo Fisher Scientific) equipped with GFP (λ_{ex}: 470/22 λ_{em}: 525/50) and RFP (λ_{ex}: 531/40 λ_{em}: 593/40) Light Cubes to detect fluorescence.

2.10. Statistical analysis

All experiments were realized in triplicates. $CdCl_2$ was used as reference toxicant and non-treated animals as control. Data were analyzed with SPSS 22 statistical software and Graph Pad Prism 5.0 with appropriated tests for each assay.

3. Results

3.1. Comparative acute toxicity and LC50 determination for $CdCl_2$ and CS-CdSe/CdS MSQD

Observed mortality increased within higher concentrations of both treatments. The 24h LC50s were estimated to be 828.254 and 1815.039 μg/ml for $CdCl_2$ and CS-CdSe/CdS MSQD, respectively (Table 2). In control groups less than 10% of worms responded in all cases. The toxicity of CdSe/CdS MSQD was significantly lower than $CdCl_2$ alone.

Table 3: LC50 estimation in 24h treated *C. elegans*.

Treatments	24 h LC_{50} (μg/mL)	Confidence interval (95%)
$CdCl_2$	828.254	673.668 < LC 50 < 1082.686
CS-CdSe/CdS MSQD	1815.039	1271.318 < LC 50 < 3275.690

3.2. Lifespan analysis

C. elegans is widely used for toxicological studies. Previous studies demonstrated their use to assess nanoparticles toxicity by prolonged exposure of L1 larvae to adult nematodes (Wang et al. 2009; Cha et al. 2012; Wu et al. 2013). To assess the toxicity of the CS-CdSe/CdS MSQD, nematodes were cultivated from L1 larval stage until death in the presence of CS-CdSe/CdS MSQD in three test concentrations, 1000, 500 and 250 μg/mL. $CdCl_2$ was used as reference in the same concentration. Control groups were composed of

non-treated worms. $CdCl_2$ showed higher toxicity. At day six, all subjects treated with the highest concentration of $CdCl_2$ (1000 µg/mL) were dead, and at day 21, all subjects in any concentration were dead as well. For the CS-CdSe/CdS MSQD treatments, nematodes exposed to the higher concentration (1000 µg/mL) were still alive until 19 days post-exposure. For the lowest concentration (250 µg/mL) worms were alive for 21 days. Non-treated control groups survived for 24 days.

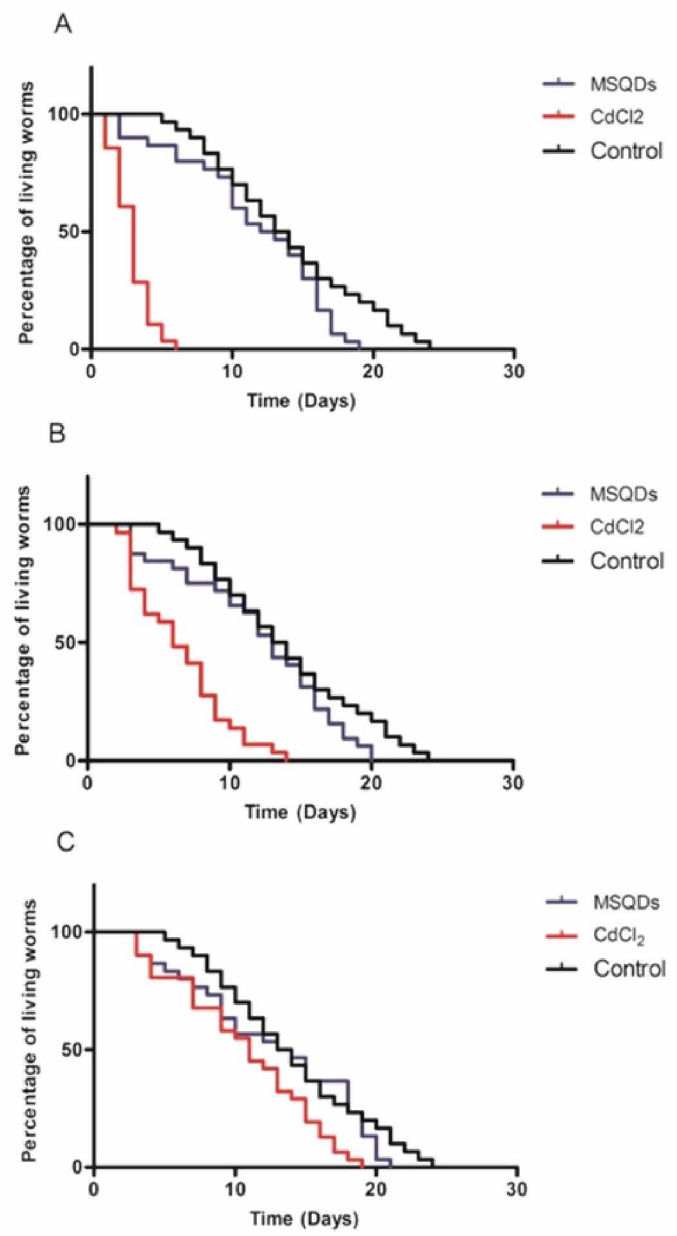

Fig. 6 Lifespan analysis of *C. elegans* exposed to different concentrations of CS-CdSe/CdS MSQD and $CdCl_2$ from L1 larvae stage to L4-adult nematodes. Exposure concentrations (A) 1000 µg/mL, (B) 500 µg/mL (C) 250 µg/mL.

3.3. *C. elegans* growth analysis

Growth was evaluated by daily body length measures from L1 larvae stage up to 4-day old nematodes. For each treatment, 15 (± 4) worms were analyzed in an inverted microscope, and the worms were measured in nm using the NIH Image J Software (Schneider et al. 2012). Mean ± standard deviation (SD) for all observed days are described in Table 2.

Table 4: Daily growth measuraments of *C. elegans* exposed to different concentrations of CS-CdSe/CdS MSQD and $CdCl_2$.

Treatments	Body length (nm)			
CS-CdSe/CdS MSQD	**Day 1**	**Day 2**	**Day 3**	**Day 4**
1000 μg/mL	222.75 ± 23.85	224.24 ± 13.62	228.40 ± 17.16	315.52 ± 55.87
500 μg/mL	228.85 ± 22.61	225.80 ± 19.84	240.97 ± 31.71	368.21 ± 91.89
250 μg/mL	230.64 ± 20.14	237.54 ± 21.69	300.52 ± 49.34	478.48 ± 169.21
$CdCl_2$				
1000 μg/mL	199.71 ± 26.01	248.92 ± 23.04	347.66 ± 25.91	216.41 ± 20.49
500 μg/mL	201.65 ± 10.66	267.13 ± 30.47	372.63 ± 66.89	268.75 ± 59.35
250 μg/mL	208.95 ± 15.51	286.63 ± 26.53	426.52 ± 95.06	304.98 ± 76.25
Control	236.83 ± 21.95	332.77 ± 42.58	539.09 ± 78.89	564.58 ± 230.2

Significant differences were observed within two days. Effects on the nematodes growth proved to be dose dependent for both treatments. CS-CdSe/CdS MSQD presented less effect than $CdCl_2$ in all test concentrations. After four days, only the concentration of 1000 μg/mL of CS-CdSe/CdS MSQD presented statistical significance in nematode growth, while in all $CdCl_2$ concentrations tested nematodes were 40% smaller than controls.

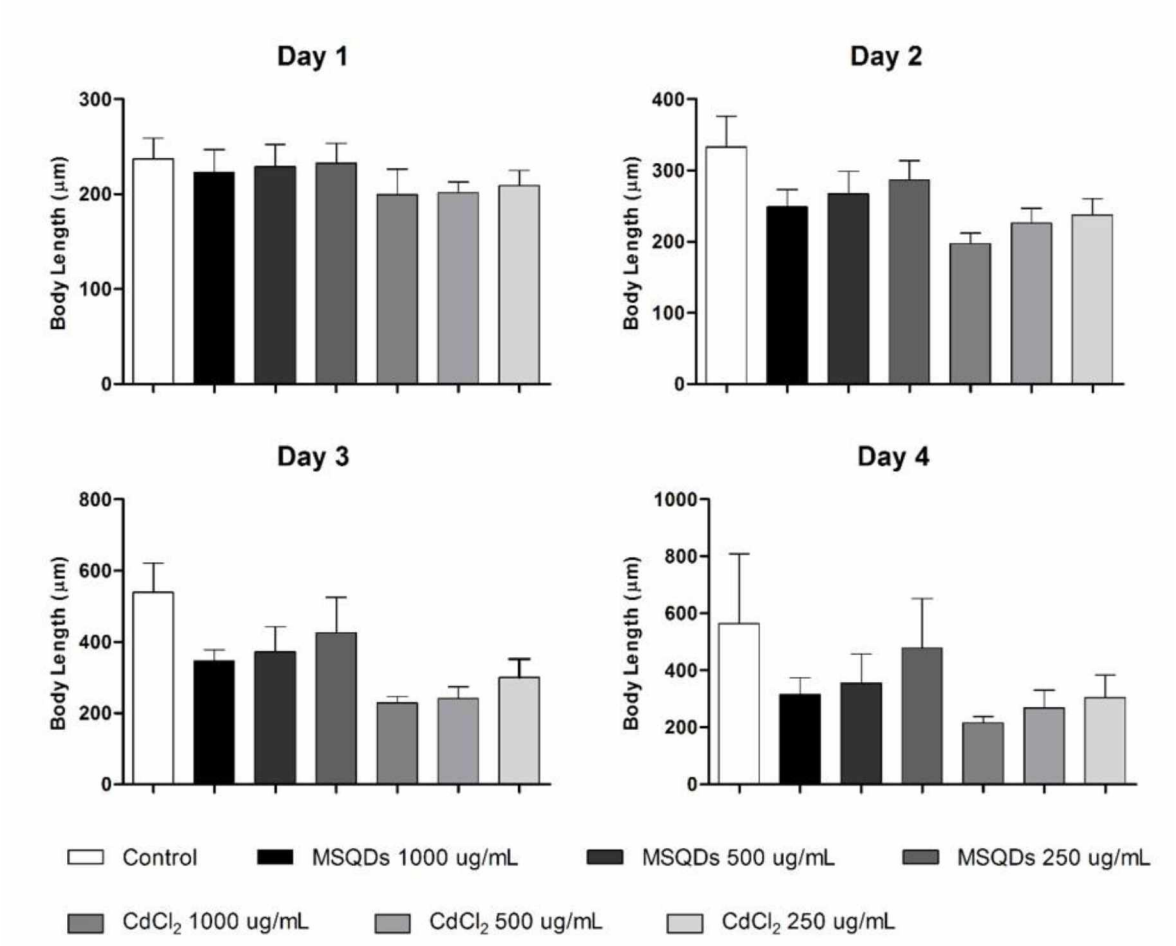

Fig. 7 Effects of different concentrations of MSQD and CdCl2 on nematodes growth. The growth was assessed by body length. Exposure was performed from the L1 larvae stage, and the endpoint was examined daily for four days.

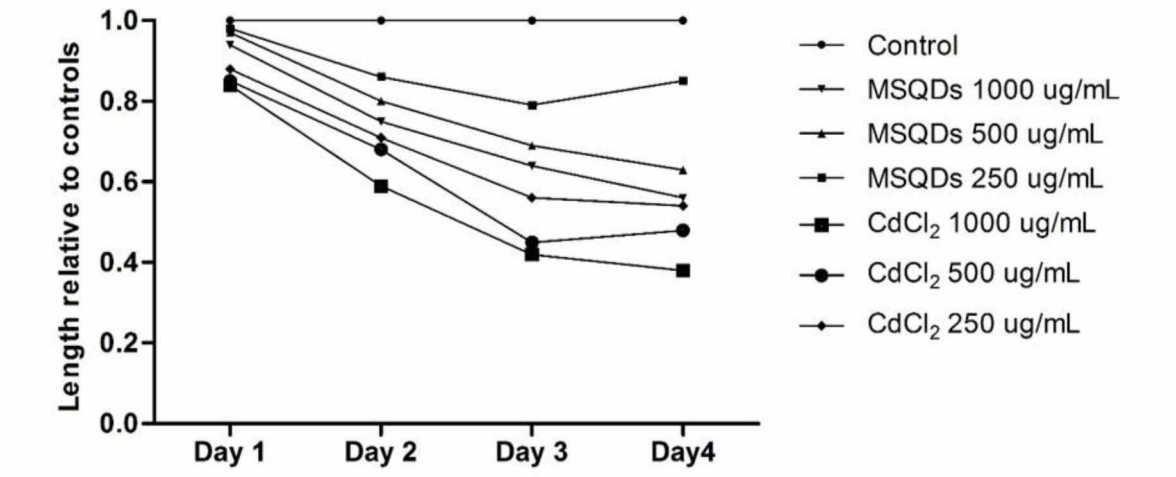

Fig. 8 Relative representation of nematodes growth under MSQD and CdCl2 stimuli.

3.4. Bioaccumulation and fluorescence

To observe internalization, biodistribution and bioaccumulation of the CS-CdSe/CdS MSQD on nematodes, L1 larvae were cultivated on agar plates containing previous exposed *E. coli* as food source. Bacteria were incubated for 1 hour with 500 μg/mL of CS-CdSe/CdS MSQD. This concentration is below the assessed LC50 for CS-CdSe/CdS MSQD (1815.039 μg/mL) which maintained stable fluorescence and allowed visualization after internalization into nematodes. Observations were done every two days, in an inverted fluorescence microscopy and the increasing fluorescence intensity can be observed on Figure 4. Since our CS-CdSe/CdS MSQD can emit detectable fluorescence in both GFP and RFP filters, we compared both emissions to assess fluorescence intensity, and confirmed CS-CdSe/CdS MSQD bioaccumulation.

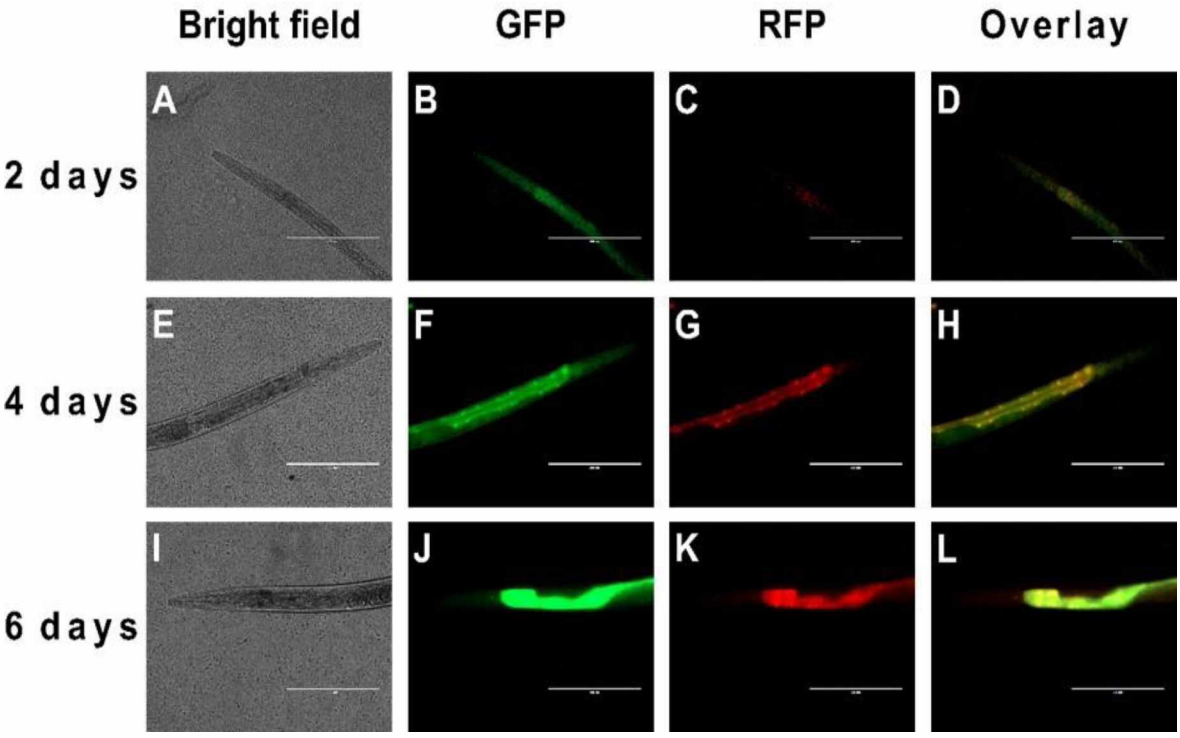

Fig. 9 The pictures show the MSQD accumulation in cultured C. elegans. Nematodes were exposed to 500 μg/mL of CS-CdSe/CdS MSQD and observed every 2 days. After 2 days of exposure (A) Bright field, (B) GFP filter, (C) RFP filter, (D) Overlay; after 4 days exposure (E) Bright field, (F) GFP filter, (G) RFP filter, (H) Overlay; and after 6 days exposure (I) Bright field, (J) GFP filter, (K) RFP filter, (L) Overlay.

3.5. Gene expression of cadmium-responsive genes by qRT-PCR

The expression of four genes (*tba-1, cdr-1, hsp-70* and *dhs-26*) was evaluated by ΔΔCt method after 4h and 24h after exposure at three different concentration of MSQD or $CdCl_2$. *Tba-1* was evaluated as reference gene. *Cdr-1* expression is directly related to Cd toxicity (Cui et al. 2007), functioning by counteracting the Cd toxic mechanisms. We observed increased expression of *cdr-1* after 4h exposure of MSQD in a dose dependent manner. On the other hand, the $CdCl_2$ exposure at concentrations higher than 250 μg/mL presented decreased *cdr-1* expression, probably due to the greater amount of death in larger concentrations. After 24 h exposure, $CdCl_2$ exposed nematodes presented a dose dependent increase in *cdr-1* expression, while for MSQD expose nematodes, *cdr-1* relative expression reached levels as low as 2-fold. *Hsp-70* is a heat sock protein related to inducible stress caused by toxic substances, including metals (Kumar et al. 2015). After 4 h exposure to MSQD, the relative expression of *hsp-70* increased with the concentration of MSQD. However, after 4 h exposure to $CdCl_2$, expression levels of *hsp-70* was six-fold below in each concentration, again due to greater death rates observed. After 24 h exposure to MSQD, relative expression of *hsp-70* at all concentrations were below three-fold. While for $CdCl_2$ treated samples, relative expression of *hsp-70* increased from 11-fold at 250 μg/mL to 48-fold at 500 μg/mL, and 31-fold at 1000 μg/mL. Dhs-26 is responsible for coding a short-chain dehydrogenase, related to organism growth and development, which have been found to be down-regulated upon cadmium exposure (Cui et al. 2007). After 4 h exposure, the relative expression of *dhs-26* decreased in a dose dependent manner for both treatments. In $CdCl_2$ exposed nematodes, the higher relative expression was 21-fold at the 250 μg/mL concentration. After 24 h exposure, the relative expression reached maximum values of 4.8-fold and 2-fold for MSQD and $CdCl_2$ exposed nematodes, respectively, maintaining a similar behavior as the 4h exposures.

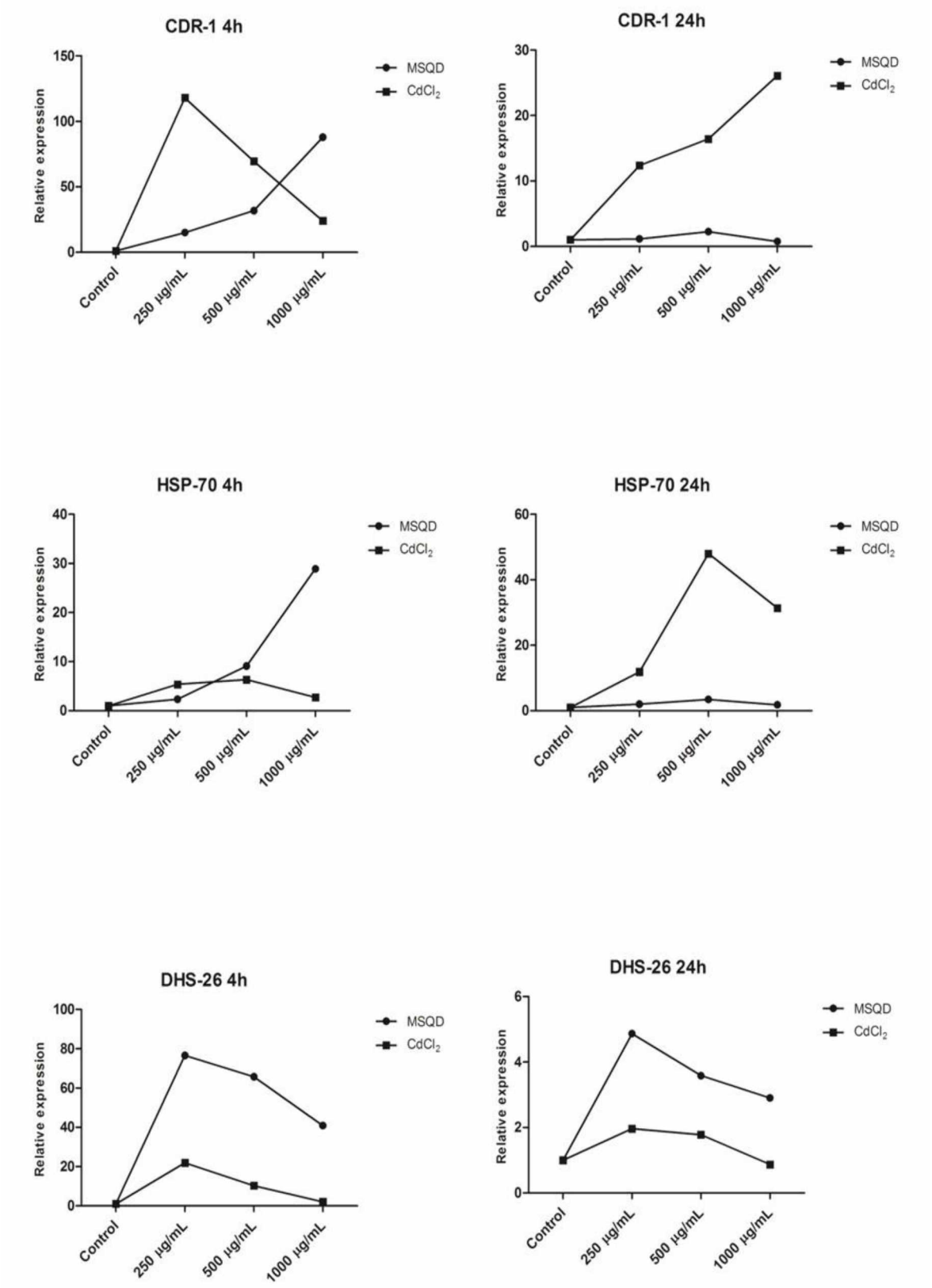

Fig. 10 Expression of C. elegans toxicity and stress related genes after 4h and 24h exposure to MSQD and CdCl2 in different concentrations.

4. Discussion

Cadmium composed nanoparticles toxicity is still controversial, and the toxic effects related to Cd^{2+} ions release is not well explained either as nanoparticles alone or as a synergic effect of both (Li et al. 2009; Yong and Swihart 2012). Some studies have demonstrated that the toxicity is mainly related to Cd^{2+} ions release as a causative interference in biological pathways (Su et al. 2009). However, the QDs toxicity is still on debate and no conclusions have been reached yet (Hardman 2006). The general consensus about nanoparticles toxicity is that every nanoparticle need to be assessed specifically and that is not safe or wise to generalize.

We have developed a magic-sized (MS) core-shell (CS) CdSe/CdS QD by aqueous solution method (Silva et al. 2013), in which their crystal structure limits the core degradation, rendering the CS-QDs less toxic, highly stable, and with improved optical properties (Silva et al. 2014). This new MSQD presents potential application as fluorescent probes or markers with a very broad luminescence spectrum (520 – 680 nm), explained by a large number of Cd^{2+} ions on its surface, which is also able to maintain high fluorescence for extended periods with little toxicity (Almeida Silva et al. 2014; Silva et al. 2014; Silva et al. 2016). Although the MSQD has not caused *in vitro* toxicity (Almeida Silva et al. 2014; Silva et al. 2014; Silva et al. 2016), *in vivo* toxicity analyses have not been performed, and we investigated whether the greater density of Cd^{2+} ions on the surface might have an effect on the MSQD bioaccumulation and biodistribution in animal models. In this study, we evaluated the optical properties and potential applications of CdSe/CdS CS-MSQD, as well as their toxicity in the animal model, *C. elegans*.

The animal model, *C. elegans*, has been extensively used as a toxicological model for various nanoparticles (Hsu et al. 2012; Zhang et al. 2012; Ahn et al. 2014; Gonzalez-Moragas et al. 2015). Among the endpoints used to toxicity assessment in *C. elegans* are lifespan, growth and gene expression of stress related genes (Power and De Pomerai 1999; Wah Chu and Chow 2002).

Firstly, we determined the LC50 for both MSQD and $CdCl_2$. Our findings regarding the $CdCl_2$ are similar to those presented elsewhere (Roh et al. 2006), with an LC50 of 828.254 μg/mL in the nematodes after 24-h treatment. However, the MSQD showed to be far less toxic, with a LC50 value of 1815.039 μg/mL.

In the lifespan assay, we found that $CdCl_2$ had greater negative effect on nematodes' survival, while the MSQD exposure seemed to have little or no effect. $CdCl_2$ mortality was dose-dependent and was more prevalent at the highest concentration (1000 μg/mL). All three concentrations of MSQD presented similar period of nematodes survival with approximately 20-day lifespan.

C. elegans presents developmental regulatory mechanisms that are similar to its arthropod and vertebrate relatives, which provides complementary insights on how growth and patterning events are integrated during development. Besides, its development has close association with food abundance and abnormalities in ideal culture conditions (Lambie 2002). To assess the effects on the nematodes' development, daily observations were conducted, and showed that both MSQD and $CdCl_2$ treatments hampered the development of the nematodes in a dose-dependent manner.

C. elegans DNA microarrays have been used to monitor global changes in the nematode transcription profile following cadmium exposure, and 290 genes were differentially expressed following a 4-h or 24-h exposure to cadmium. Among them, several genes were involved in metal detoxification, including *mtl-1*, *mtl-2*, *cdr-1* and *ttm-1*. Interestingly, the expression of cadmium-responsive genes was maximally induced after only 4 h; whereas, the general stress-responsive genes reached their highest expression levels after 24 h (Cui et al. 2007). To confirm the effects of MSQD exposure, we evaluated the relative expression of three specific genes, *cdr-1* (related to cadmium exposure), *hsp-70* (heat sock and stress) and *dhs-26* (cellular metabolism), and compared them under $CdCl_2$ exposure.

Cdr-1 relative expression after 4-h treatment with MSQD increased in a dose-dependent manner; however, after 24h exposure there were no significant changes in *cdr-1* expression. Contrarily, after 4-h $CdCl_2$ exposure, a significant increase in *cdr-1* expression was observed at the concentration of 250 μg/mL, but followed by decreased expressions at 500 and 1000 μg/mL, probably due to acute toxicity and high mortality rate of nematodes at those concentrations, which explains the reduced expression of the metal detoxification gene early in the development. This is further corroborated by the absence of *cdr-1* expression after 24-h exposure.

Hsp70 proteins are central components of the cellular network of molecular chaperones and folding catalysts. *Hsp70* interacts with key regulators of many signal transduction

pathways controlling cell homeostasis, proliferation, differentiation and cell death. *Hsp70* disturbances of the cellular system induced by environmental, developmental or pathological processes act on these signal transduction pathways (Mayer and Bukau 2005; Cui et al. 2007). The *hsp-70* expression showed similar behavior as *cdr-1* after 4 h and 24 h exposure, first increasing the expression accordingly to the concentration of MSQD, and without significant expression after 24 h. For $CdCl_2$ treated nematodes, the initial response (4 h) was relatively low, and even though the expression of *hsp-70* decreased in concentrations higher than 250 μg/mL, the difference was not significant. After long exposure (24 h), *hsp-70* expression assumed other behavior, increasing substantially in 250 μg/mL and 500 μg/mL $CdCl_2$ treatments, and decreased again in samples treated with 1000 μg/mL. We hypothesize that the initial response of *hsp-70* is due to physical stress caused by the MSQD size (~2 nm) and the lather response is associated with cadmium toxicity. Even if the high concentration of $CdCl_2$ (1000 μg/mL) and the longer exposure period (24 h) promoted high expression of *hsp-70*, it affected the nematodes in an unexpected way, probably due to interference of Cd^{2+} ions in various metabolic pathways.

Dhs-26 is related to cell metabolism, and have been shown to be under-expressed upon cadmium exposure (Cui et al. 2007). The *dhs-26* expression times presented similar behavior in both exposure periods for both MSQD and $CdCl_2$, although the MSQD induced a significant intensification in the nematodes' metabolism. Such MSQD effect is probably due to the increased cell uptake of MSQD aggregates. It is possible that heterogeneity in the surface modified by *E. coli*, the food source, may have led to hydrophobic patches on the MSQD surface, resulting in aggregation, which may have led to increased uptake stimulating specific cell metabolism, such as phagocytosis.

Besides the presence of Cd, results proved that the MSQD presents low toxicity in *C. elegans*. This fact may be due to the presence of the CdS alloy shell, which limits the release of Cd^{2+}. This is further corroborated by *in vivo* observations of *C. elegans* fed with *E. coli* OP50 submitted to 500 μg/mL of MSQD for six days. The accumulation of MSQD in nematodes observed in every two days increased with the exposure time, but both MSQD concentration and exposure time proved to be safe for nematodes.

5. Conclusion

In conclusion, the CdSe/CdS CS-MSQD proved to have little or no toxicity effect in the animal model *C. elegans*. Furthermore, the MSQD showed high and stable fluorescence in the nematodes. Our results encourage the use of MSQD in many biological applications, such as probes, drug delivery systems or biological labeling, and also demonstrates that the presence of Cd^{2+} ions in the surface of nanocrystals are safe to use. But, it remains to be demonstrated whether such effect were due to synthesis procedure, the core-shell structure, the small number of cadmium ions in the surface, or yet because of the greater stability of this novel quantum-dot. However, more studies are still needed, aiming the functionalization of MSQD for specific targets, as well as to evaluate their potential toxicity after functionalization, which may change the particles properties and probably inhibit even more the release of Cd^{2+} ions and their toxic effects.

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Capítulo III

TOXICITY EFFECTS OF ULTRA-SMALL AND MAGIC-SIZED CORE/SHELL CDSE/CDS QUANTUM DOTS ON DANIO RERIO EMBRYONIC DEVELOPMENT

*Instructions according to Archives of Toxicology

Resumo: Quantum dots de tamanhos mágicos (MSQDs) e ultrapequenos (USQDs) são nanocristais altamente fluorescentes com tamanhos menores do que 2 nm, mas diferindo em suas propriedades óticas e eletrônicas únicas. Considerando a semelhança no tamanho das nanopartículas, mas suas diferentes propriedades; nosso objetivo foi comparar os efeitos no desenvolvimento embrionário do modelo animal, *Danio rerio* (zebrafish), um importante modelo para análises de toxicidade. Ambos quantum dots foram avaliados por absorção ótica e espectroscopia de fluorescência, demonstrando que os MSQDs apresentam um espectro de fluorescência mais amplo do que os USQDs. Ovos fertilizados de zebrafish foram expostos a diversas concentrações de QDs, e a toxicidade foi avaliada durante o desenvolvimento embrionário, observando-se alterações morfológicas e anatômicas. Os MSQDs apresentaram maior toxicidade do que os USQDs na concentração de 10 μg/mL, visto que as larvas de zebrafish apresentaram um desenvolvimento anormal. Os embriões não eclodiram quando expostos a cloreto de cadmio na mesma concentração (controle). A toxicidade dos MSQDs pode ser explicada pela concentração de íons Cd^{+2} na superfície dos QDs. O modelo para os testes *in vivo* se demonstrou uma importante ferramenta para testes de toxicidade de nanocompostos, principalmente no caso de materiais com pouca alteração em sua composição ou estrutura.

Palavras-chave: Quantum dots de tamanhos mágicos, quantum dots ultrapequenos, CdSe/CdS core shell, zebrafish.

Abstract: Magic-sized (MSQDs) and ultra-small quantum dots (USQDs) are highly stable fluorescent nanocrystals with sizes < 2 nm, but differing on their unique optical and electronic properties. Because of their similar size, but different properties, our aim was to compare their effects on the embryonic development of *Danio rerio* (zebrafish), an important *in vivo* model for toxicity analyses. Both QDs were evaluated by optical absorption and fluorescence spectroscopy (FL), demonstrating that the MSQDs presented broader fluorescence spectrum than USQDs. We have exposed fertilized eggs of zebrafish to several concentrations of both QDs, and evaluated toxicity during embryonic development by observing anatomical and morphological changes. The CdSe/CdS CS-MSQDs presented higher toxicity than CdSe/CdS CS-USQDs at 10 μg/mL since larvae displayed abnormal development. The embryos did not hatch when cadmium chloride was applied in the same concentration (control). The CdSe/CdS CS-MSQD toxicity may be explained by the increasing concentration of Cd^{+2} ions on the surface, causing larger surface defects. The *in vivo* zebrafish model demonstrated to be an important tool for toxicity tests mainly caused by small variations on QDs nanostructures.

Keywords: magic-sized quantum dots, ultra-small quantum dots, CdSe/CdS core shell, zebrafish.

1. Introduction

Magic-sized quantum dots (MSQDs) and ultra-small quantum dots (USQDs) are nanostructures that present unique optical and electronic properties, which render them numerous applications in biological processes, differentially from conventional quantum dots (QDs) (Chen et al. 2005; Xia and Zhu 2008; Riehle et al. 2009; Dukes et al. 2010; Nguyen et al. 2010).

Both QD classes are preferable for biological purposes, due to their very small size, higher quantum efficiency, greater stability, little cytotoxicity and capability of passively diffuse into cells, but while MSQDs present broader luminescence spectrum, USQDs present narrow luminescence spectrum. The long-term fluorescence of MSQDs that can be maintained for over 36 hours coupled to the facile transport through cell membranes of both MSQDs and USQDs, due to their ultra-small size, represent key characteristics that cannot be achieved by conventional QDs and dyes, qualifying them as potential theranostic tools (Silva et al. 2014b).

However, the possible toxic effects of such ultra-small QDs on living organisms and in the environment have not been investigated. Although nanotoxicology is an expanding research field with applications of several models and assays to elucidate such effects (Li et al. 2009), there is no established sensitive model to test small variations between very similar nanomaterials.

The syntheses of ultra-small and magic-sized quantum dots are quite similar. But, minimal changes in the synthesis parameters can generate significant alterations on photonic and physical properties, such as the percentage of alloy and shell thickness, which have led to the concepts USQDs and MSQDs (Li et al. 2008; Li et al. 2009; Riehle et al. 2009; Dukes et al. 2010). The CdS_xSe_{1-x} alloy in the core/shell synthesis decreases the concentration of Cd^{+2} ions on the surface, diminishing the possible exposure of Cd^{+2} ions and release by both in CdSe/CdS CS-MSQDs (Li et al. 2009; Pilla et al. 2013; Silva et al. 2013; Almeida Silva et al. 2014; Silva et al. 2014a; Silva et al. 2014b), and CdSe/CdS CS-USQDs (Pan et al. 2005; Zhang et al. 2010; Ma et al. 2011; Ahamefula et al. 2012). However, although smaller concentrations of Cd^{+2} ions are tolerable by cells, certain modifications may still confer higher toxicity to QDs than the Cd^{+2} ions alone (Guo et al. 2007; Su et al. 2010; Bradburne et al. 2013; Silva et al. 2014a). For this reason, all QDs must be subjected to extensive *in* vitro *and in vivo* toxicity tests, but unfortunately, an efficient assay, able to detect small

effects has not been established yet. The use of *in vivo* animal models has been extremely important and fundamental to understand the behavior of novel nanomaterials in the environment and in living organisms. The vertebrate model *Danio rerio* (zebrafish) has been attracted the attention and has been successfully used in such assays (Parng et al. 2002; Lieschke and Currie 2007) due to their small size (3-4 cm), fast reproductive rate, known genomic sequence, transparent embryos that facilitate observation, low maintenance cost, and for their significant similarities to mammalians (Giannaccini et al. 2014). For those reasons, they constitute an excellent experimental model for behavioral, genetic and toxicological studies, and have been widely used in toxicity tests of nanoparticles (Powers et al. 2011; Zhang et al. 2012; Duan et al. 2013a; Zhang et al. 2013; Duan et al. 2013b).

We have synthesized core/shell MSQDs and USQD with CdS_xSe_{1-x} alloy and a CdS shell, termed CdSe/CdS CS-MSQDs and CdSe/CdS CS-USQDs, respectively, by an aqueous solution method at room temperature (Silva et al. 2013; Almeida Silva et al. 2014; Silva et al. 2014a). Modifications of their physical properties were generated only by modifying the pH of the reaction. We hypothesized that small changes in the surface of these ultra-small nanocrystals with similar sizes would lead to variable toxicity. This study demonstrated that different physical properties of both CdSe/CdS CS-MSQDs and CdSe/CdS CS-USQDs, assessed by optical absorption (OA) and fluorescence spectroscopy (FL), have exerted differential toxicity effects on developmental stages of zebrafish embryos.

2. Methods

2.1. Materials

Selenium powder (Se - 99.999%), sodium borohydride (NaBH4-98%), cadmium perchlorate hexahydrate (Cd(ClO4)2.6H2O - 99.999%), sodium hydroxide (NaOH) and 1-thioglycerol (>97%) were all purchased from Sigma-Aldrich (Brazil) and used without further purification. Ultra-pure water used in the preparation of aqueous solutions was obtained from the QUIMIS system.

2.2. Syntheses of CdSe/CdS CS-MSQDs and CdSe/CdS CS-USQDs

Both QDs were grown in aqueous solutions at room temperature based on the methodology described elsewhere (Ahamefula et al. 2012). Briefly, 2 mmol of Cd(ClO4)2.6H2O and 5mmol of 1-thioglycerol (xT) were mixed in ultra-pure water and the pH was adjusted to 7 for MSQDs (Silva et al. 2013; Silva et al. 2014b) and to 11 for USQDs (Silva et al. 2014a) by adding 0.1 M NaOH at room temperature. The resulting suspensions

were precipitated with ethanol and centrifuged four times at 6,000 rpm for 10 min. The resulting nanopowders were dried in a vacuum mechanical pump at room temperature and further dispersed in ultra-pure water.

2.3. Zebrafish maintenance

The wild type zebrafish were obtained from a local pet store and kept in an aquarium with water circulation system in a controlled environment room (26°C). The photoperiod was set to a cycle of 14h light/10h dark. Adult fish were fed daily with *Artemia sp* to favor oviposition and dry food. All the assays were approved by the Ethics Committee for Animal Experimentation of the Federal University of Uberlândia, under the project number 122/2015.

2.4. Zebrafish embryos and larvae exposure to quantum dots

Fertilized eggs were collected and selected under a stereomicroscope (ZEISS STEMI SV6) with 4 hours post-fertilization (hpf). All embryos were obtained from the same spawn eggs for statistical comparisons between control and treated groups. Healthy embryos were placed in a 24-well plate (10 embryos in 1-mL solution/well). The experiments were performed in triplicates for each group. Embryos were treated with CdSe/CdS CS-MSQDs and CdSe/CdS CS-USQDs at concentrations of 0.1, 1, 10, 100 and 1000 μg/mL from 4 to 96 hpf (4, 24, 48, 72 and 96). The embryos and larvae were observed with an inverted microscope (EVOS® FL) to assess the viability and possible malformations.

2.5. Statistical analyses

Normality was tested using the Kolmogorov-Smirnov test. Two-way ANOVA were used to test differences among treatments. Mean comparisons were performed by Bonferroni post-tests, and significance was considered when $p < 0.05$. Values were expressed as mean ± standard error of mean (SEM) in all experiments16. All statistical analyses were carried out using GraphPad Prism 5.00.

3. Results

The optical absorption spectrum (OA) of MSQDs (Figure 1A) presents a 358-nm wavelength band at 3.5 eV, which refers to the excitonic transition of CdSe QDs. This band has higher energy compared to bulk CdSe (1.74 eV), indicating that the CdSe/CdS CS-MSQDs exhibits a quantum confinement effect (Silva et al. 2013; Almeida Silva et al. 2014; Silva et al. 2014a; Silva et al. 2014b). Furthermore, we have also observed an apparent absorption band at longer wavelengths, centered at 358 nm, evidencing the formation of a second group of low density QDs. The presence of a narrowband and discontinuous growth

are strong indications of MSQD. The normalized luminescence spectrum was relatively broad due to its internal atomic defects present in the MSQD structure, with extra or missing atoms that can create a non-radiative trap state, another characteristic feature of MSQDs (Zhang et al. 2010). The formation of CdS shell was performed based on the methodology (Pan et al. 2005; Riehle et al. 2009; Dukes et al. 2010; Ahamefula et al. 2012). The OA of CdSe/CdS CS-USQDs (Figure 1B) showed the lowest energy excitonic band (OAexc) at 373 nm (3.3 eV), with higher energy when compared to the bulk CdSe (1.74 eV), also confirming the presence of a quantum confinement effect (Silva et al. 2013). In addition, the observed excitonic energy levels were in the expected range for USQDs, and the normalized luminescence spectrum was relatively narrower, differing from the MSQDs (Zhang et al. 2010; Silva et al. 2014a). The formation of CdS shell was performed based on the methodology (Almeida Silva et al. 2014).

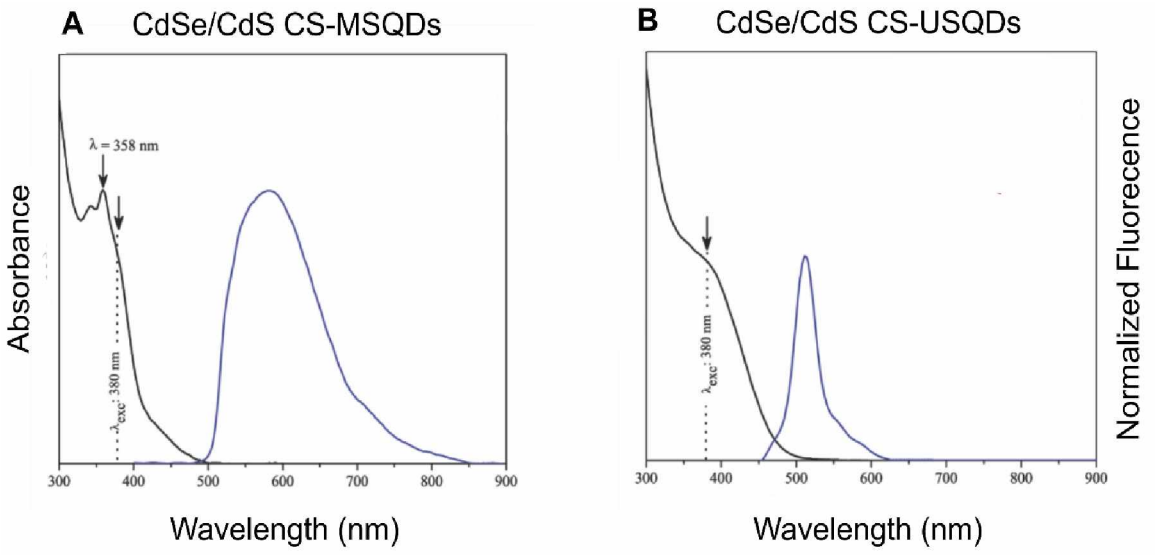


Fig. 11 Optical absorption (OA) and normalized fluorescence spectra of colloidal solutions containing CdSe/CdS CS-MSQDs (A) and CdSe/CdS CS-USQDs (B).

In order to demonstrate whether differences in fluorescence behavior would affect toxicity, we tested different concentrations of ours QDs on embryos hatching of zebrafish (Figure 2), in which variations in embryo development were recorded at 72- and 96-h post exposure. Short term exposure (< 72h) did not present significant differences and showed no effect on viability. We have observed that delayed hatching rates caused by QDs on zebrafish embryos were dose-dependent; concentrations higher than 1 μg/mL for CdSe/CdS CS-MSQDs (A) and higher than 10 μg/mL for CdSe/CdS CS-USQDs (B) showed significant decrease in embryos hatching rates ($p < 0.01$). At the QDs concentration limits, besides the delayed embryonic development, the embryos also presented some important anatomical

alterations, such as malformation of the yolk sack and changes in the axial curvature, whereas at higher concentrations, when toxicity levels were reached, the embryos did not hatch (Figure S1).

Comparisons of effects at 96 h (Figure 2C) demonstrated that CdSe/CdS CS-MSQDs displayed higher inhibitory effect with concentrations 10 times lower in relation to the CdSe/CdS CS-USQDs. This effect may be explained by surface defects in the CS-MSQDs, with higher Cd^{+2} ions at the surface (Silva et al. 2014a), and possibly by interactions they may present with the embryo, since both QDs used are smaller than 2 nm, which may enable passive diffusion through pores of the embryos' chorion, with a diameter of approximately 0.5-0.7 μm (Almeida Silva et al. 2014).

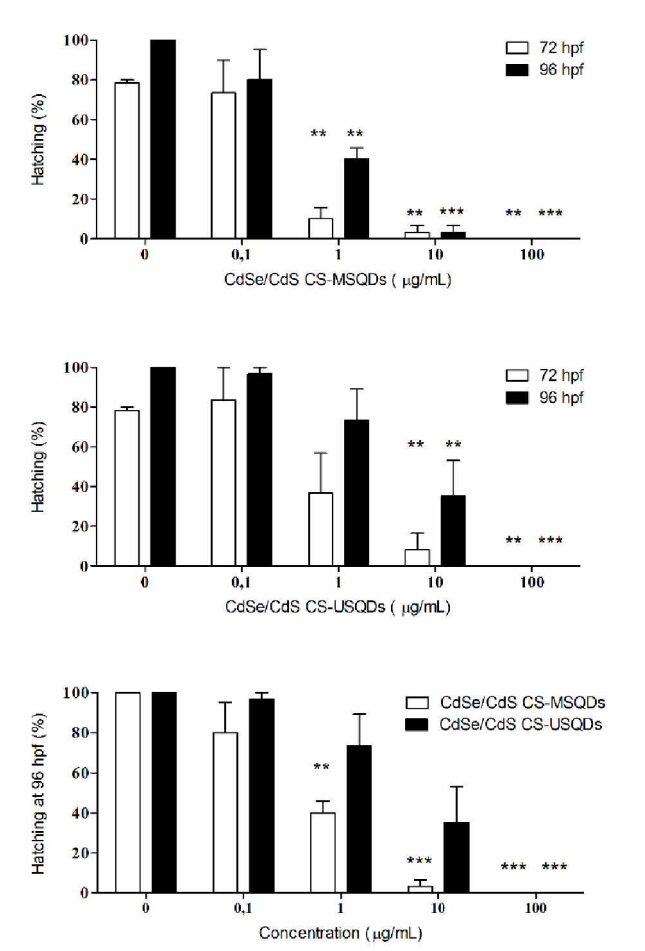


Fig. 12 Egg hatching rates of zebrafish embryos upon (A) CdSe/CdS CS-MSQDs and (B) CdSe/CdS CS-USQDs exposure for 72 and 96 h with different concentrations (0, 0.1, 1, 10,

100 μg/mL), and (C) comparative hatching ratios of CS-MSQDs and CS-USQDs at 96 h of exposure.

In Figure 3, we have observed that both QDs internalized the embryos' chorion upon 72-h exposure, and seem to accumulate at the surface of the chorion. At 72-h exposure, only the CdSe/CdS CS-MSQDs at the concentration of 10 μg/mL was lethal (C), although all treatments presented delayed embryonic development when compared to the control (A). At 96 h, the concentration of 10 μg/mL of CdSe/CdS CS-MSQDs was lethal to all embryos, whereas the concentrations of 1 μg/mL for both QDs and 10 μg/mL for CdSe/CdS CS-USQDs caused a delay in the embryos development. In all cases, the QDs seems to cross the chorion, and spread evenly inside the embryos.

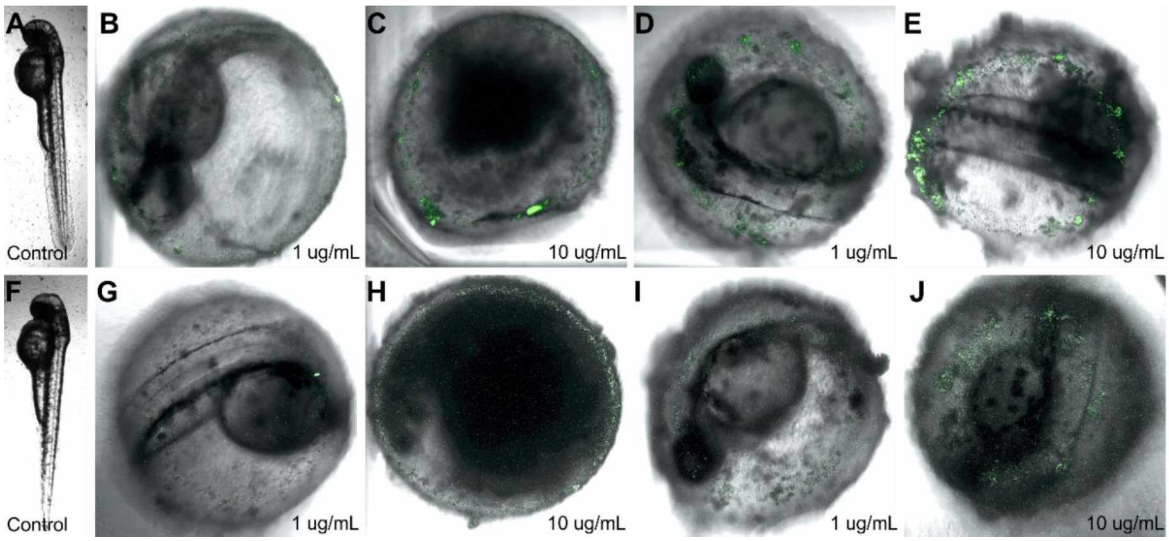


Fig. 13 Confocal images of embryos treated with CdSe/CdS CS-MSQDs and CdSe/CdS CS-USQDs. (A, F) Control group, (B) embryo treated for 72 h with 1 μg/mL of CdSe/CdS CS-MSQDs, (C) embryo treated for 72 h with 10 μg/mL of CdSe/CdS CS-MSQDs, (D) embryo treated for 72 h with 1 μg/mL of CdSe/CdS CS-USQDs, (E) embryo treated for 72 h with 10 μg/mL of CdSe/CdS CS-USQDs, (G) embryo treated for 96 h with 1 μg/mL of CdSe/CdS CS-MSQDs, (H) embryo treated for 96 h with 10 μg/mL of CdSe/CdS CS-MSQDs, (I) embryo treated for 96 h with 1 μg/mL of CdSe/CdS CS-USQDs, (J) embryo treated for 96 h with 10 μg/mL of CdSe/CdS CS-USQDs.

4. Discussion

In the last years, the utilization of quantum dots have grown exponentially due to the variety of applications in biological, medical and chemical fields. Notwithstanding, the knowledge about their toxic effects is still insufficient for making available their wide utilization *in vivo* (Chen et al. 2012). We have chosen the zebrafish model to show *in vivo*

toxicity and the negative effects of the exposure to CdSe/CdS CS-MSQDs and CdSe/CdS CS-USQDs because cadmium (Cd) is known to cause damage during embryonic development, leading to different types of deformities, including hypopigmentation, cardiac edema, changes in the yolk sac, altered axial curvature and tail malformations. The frequency of such defects increases in a dose-dependent manner (Powers et al. 2011; Chen et al. 2012; Jemec et al. 2012; Zhang et al. 2013; Jang et al. 2014). It has also been demonstrated that some QDs can cause a delay in the embryonic development besides causing morphological changes in zebrafish larvae. Such changes have also been reported for other types of nanoparticles (Powers et al. 2011; Duan et al. 2013a; Duan et al. 2013b; Jang et al. 2014) . Therefore, considering the high susceptibility of the zebrafish model to Cd^{+2} ions, we hypothesized that small defects on the surface of QDs could be detected, which was confirmed by our evidences.

In order to understand the relative higher toxicity of CdSe/CdS CS-MSQD when compared to CdSe/CdS CS-USQDs, we propose a representative model based on the FL spectra (Figure 4).

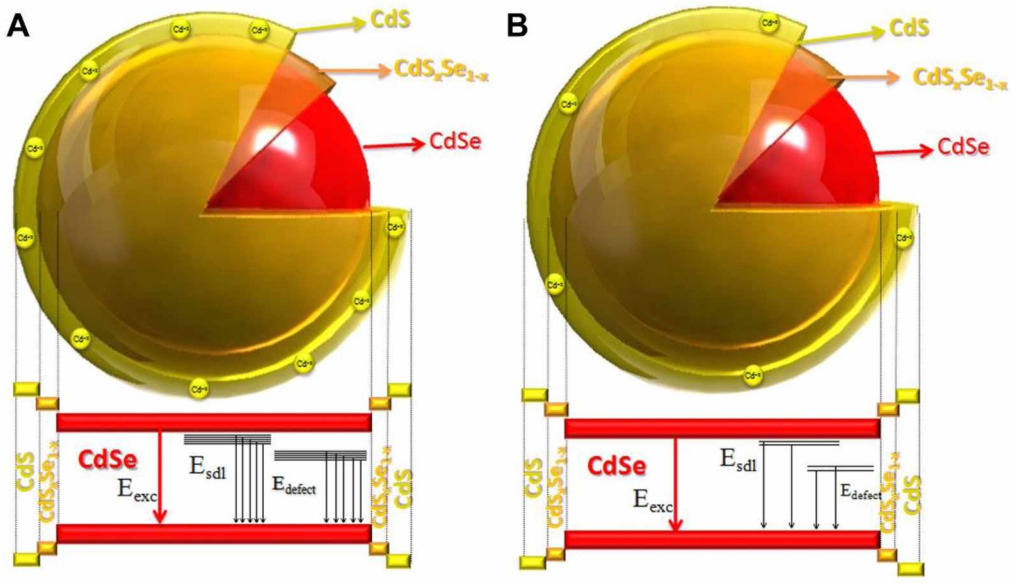


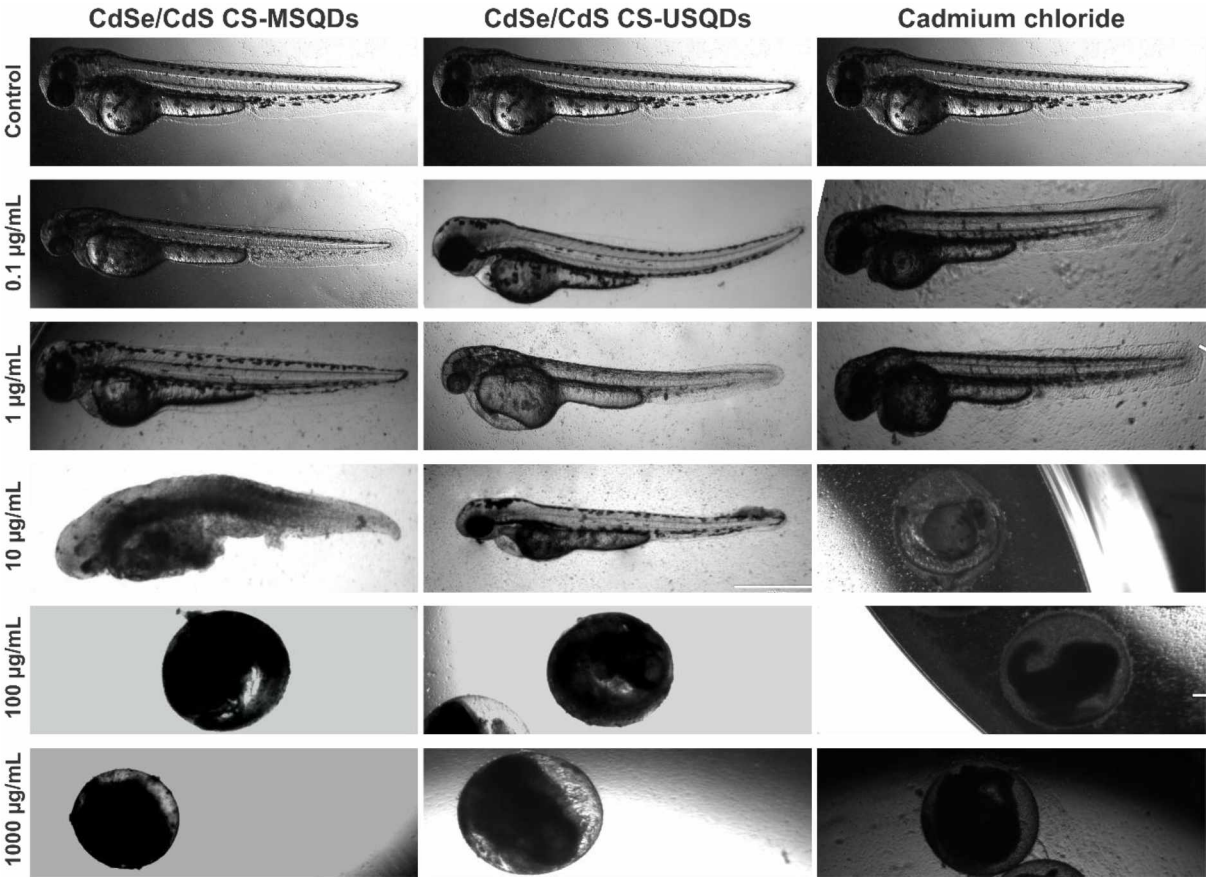
Fig. 14 Representative model for the structure of the CdSe/CdS CS-MSQDs (A) and CdSe/CdS CS-USQDs (B) and their excitonic energy levels based on their concentration of Cd+2 and defects at the surface.

By this model, we can confirm the higher density levels of surface defects (SDL) and the presence of internal atomic defects (E_{defect}) in CdSe/CdS CS-MSQDs. Even if the formation of CdS_xSe_{1-x} alloy and the CdS shell decreases the density of Cd^{+2} ion on the

CdSe/CdS CS-MSQDs and CdSe/CdS CS-USQDs surface, a small quantity of those ions still remains distributed in both QDs surfaces (Ahamefula et al. 2012). Furthermore, previous work, demonstrated that high pH facilitate the annexation of stabilizing molecules on the surfaces of QDs, decreasing considerably the density of Cd^{+2} on the surface, which favors the formation of more crystalline QDs by decreasing the density of internal atomic defects (Bradburne et al. 2013).

In particular, we conclude that the quantum dots tested presented toxic effects in a dose-dependent manner and caused a significant delay in embryos development. Nevertheless, the CdSe/CdS CS-USQDs exhibits a lower toxic potential when compared to CdSe/CdS CS-MSQDs due to the surface cadmium density on the surface. Analyzing the embryo hatching, we observed a significant difference ($p<0.001$) by showing slow hatching phase at concentrations $\geq$ 1 µg/mL for CdSe/CdS CS-MSQDs and $\geq$ 10 µg/mL for CdSe/CdS CS-USQDs. This fact shows that the CdSe/CdS CS-MSQDs presents an inhibitory concentration 10 times lower than the CdSe/CdS CS-USQDs, consequently presenting higher toxicity. QDs distribution in the embryos of zebrafish early post exposure seemed to cluster at the surface of the chorion, but at 96-h post exposure, the QDs seemed able to cross the chorion membrane and spread evenly inside the embryos. Since the Cd^{+2} dispersed on the surface of the QDs are directly associated with their potential toxicity, our findings confirm that CdSe/CdS CS-USQDs are less toxic than the CdSe/CdS CS-MSQDs, since they have a lower Cd^{+2} surface density, rending them more stable and less prone to lose cadmium ions when in solution.

Supplementary material



Supplementary figure 1: Anatomical and morphological changes of embryos and larvae of Danio rerio after exposure to CdSe/CdS CS-MSQDs and CdSe/CdS CS-USQDs, and cadmium chloride. Anatomical and morphological deformities were observed in various degrees in all treatments. Cadmium chloride was used as a positive control in the same concentrations as the CdSe/CdS CS-MSQDs and CdSe/CdS CS-USQDs, which generated the greatest deformities and eggs did not hatch in concentrations greater than 10 µg /mL.

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