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Evaluation of Biological Toxicity of CdTe Quantum Dots in *Trypanosoma cruzi*

Avaliação da toxicidade biológica de pontos quânticos de CdTe em *Trypanosoma cruzi*

Evaluación de la toxicidad biológica de los puntos cuánticos de CdTe en *Trypanosoma cruzi*

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Abstract

Luminescent semiconductor nanocrystals or quantum dots (QDs) emerge as important fluorescent probes for *in vitro* and *in vivo Trypanosoma cruzi* cells studies. However, to ensure applicability to living organisms, several tests still need to be done. Since several toxic events are caused by QDs, such as loss of mitochondrial membrane potential, ROS generation, DNA damage and cell death by autophagy. We performed a review of the literature on mechanisms of cellular uptake, internalization and citotoxicity of nanoparticles including our results about the evaluation of biological toxicity in *T. cruzi*. We evaluated the possible effects on parasite growth curves in a time - scale of control and incubated cells with different concentrations of CdTe – QDs (0.2; 2.0; 20 and 200μM) to determine the development cells changes. In addition, intracellular ROS were measured by Electron Paramagnetic Resonance Spectroscopy (EPR) technique. According our results, we can infer that the toxic effects of QDs in *T. cruzi* are dose-dependent and that high levels of ROS are involved in cellular toxicity promoted by higher concentrations of QDs. In summary, parasites labeled with low concentrations of nanoparticles are suitable and can be used as bioimaging tools for living parasites. However, more studies on QDs cytotoxicity need to be carried out.

Keywords: Protozoan parasites; Fluorescence nanoparticles; Bioimaging; Nanotoxicity.

Resumo

Nanocristais semicondutores luminescentes ou pontos quânticos (QDs) surgem como importantes sondas fluorescentes para estudos in vitro e in vivo de células de *Trypanosoma cruzi*. No entanto, para garantir a aplicabilidade aos organismos vivos, vários testes ainda precisam ser feitos. Uma vez que diversos eventos tóxicos são causados por QDs, como perda de potencial de membrana mitocondrial, geração de ROS, dano ao DNA e morte celular por autofagia. Realizamos uma revisão da literatura sobre os mecanismos de captação celular,

internalização e citotoxicidade de nanopartículas incluindo nossos resultados sobre a avaliação da toxicidade biológica em *T. cruzi*. Avaliamos os possíveis efeitos nas curvas de crescimento do parasita em uma escala de tempo de controle e células incubadas com diferentes concentrações de CdTe - QDs (0,2; 2,0; 20 e 200µM) para determinar as alterações nas células de desenvolvimento. Além disso, as ROS intracelulares foram medidas pela técnica de Espectroscopia de Ressonância Paramagnética Eletrônica (EPR). De acordo com nossos resultados, podemos inferir que os efeitos tóxicos dos QDs no *T. cruzi* são dose-dependentes e que altos níveis de ROS estão envolvidos na toxicidade celular promovida por maiores concentrações de QDs. Em resumo, parasitas marcados com baixas concentrações de nanopartículas são adequados e podem ser usados como ferramentas de bioimagem para parasitas vivos. No entanto, mais estudos sobre citotoxicidade de QDs precisam ser realizados.

Palavras-chave: Parasitas protozoários; Nanopartículas Fluorescentes; Bioimagem; Nanotoxicidade.

Resumen

Los nanocristales semiconductores luminiscentes o puntos cuánticos (QD) emergen como importantes sondas fluorescentes para estudios in vitro e in vivo con células de *Trypanosoma cruzi*. Sin embargo, para garantizar la aplicabilidad a los organismos vivos, aún deben realizarse varias pruebas. Dado que varios eventos tóxicos son causados por QD, como la pérdida del potencial de la membrana mitocondrial, la generación de ROS, el daño del ADN y la muerte celular por autofagia. Realizamos una revisión de la literatura sobre los mecanismos de captación celular, internalización y citotoxicidad de las nanopartículas, incluidos nuestros resultados sobre la evaluación de la toxicidad biológica en *T. cruzi*. Evaluamos los posibles efectos sobre las curvas de crecimiento del parásito en una escala de tiempo de control y las células incubadas con diferentes concentraciones de CdTe - QDs (0.2; 2.0; 20; 200µM) para determinar los cambios en las células de desarrollo. Además, las ROS intracelulares se midieron mediante la técnica de espectroscopia de resonancia paramagnética electrónica (EPR). Según nuestros resultados, podemos inferir que los efectos tóxicos de las QD en *T. cruzi* son dosis-dependientes y que los niveles altos de ROS están implicados en la toxicidad celular promovida por concentraciones más altas de QD. En resumen, los parásitos etiquetados con bajas concentraciones de nanopartículas son adecuados y pueden usarse como herramientas de bioimagen para parásitos vivos. Sin embargo, es necesario realizar más estudios sobre citotoxicidad QD**.**

Palabras clave: Parásitos protozoarios; Nanopartículas de fluorescencia; Bioimagen; Nanotoxicidad.

1. Introduction

Quantum dots (QDs) are nanoparticles of fluorescent semiconductor material with diameters that normally range from 2 to 10 nanometers (Nanda et al. 2017). They are composed by the elements from groups II - VI (CdSe, CdS, CdTe) as well as, although less common, from groups III - V (InP, InSb, InAs, GaAs) of the periodic table (Abbasi et al. 2016). Commercially, the application of QDs in the biomedical area has been explored to adjust its semiconductor properties to develop smaller and more complex devices with better performance (Field et al. 2020).

Due to the three-dimensional quantum confinement effects (the semiconductor material reduction to a few nanometers results in the reduction of energy levels to discrete values), these materials have unique optical and electronic properties (Vasudevan et al. 2015) such as:

1- **Size-tunable fluorescence:** as the size of the QDs can be regulated during the synthesis process, it is possible to produce QDs of the same material with emission of fluorescence in different spectrum regions, from the visible to the infrared (Banerjee et al. 2016; Nanda et al. 2017);

2- **Broad absorption spectrum: i**t allows the simultaneous excitation of different size QDs by a single wavelength (Probst et al. 2013);

3- **Narrow emission spectrum:** different material and size QDs can be used to generate multiplexed images without spectral interference (Probst et al. 2013);

4- **High quantum fluorescent performance**: PQs are efficient in terms of converting excitation light into emission (Banerjee et al. 2016);

5- **Photodegradation resistance:** this contributes to the long term QDs tracking in cells and biological systems (Banerjee et al.2016);

6- **Versatile surface for conjugation:** QDs can be conjugated to a wide variety of biological and organic molecules with specific recognition (Pereira et al.2019).

7- **Electrodense:** it allows identifying the ultrastructural location of QDs in samples by transmission electron microscopy (Szymanski et al. 2013).

All these properties make the QDs nanoparticles can be exploited for different applications in the biological and biomedical science areas.

2. Methodology

Quantum dots was prepared as described by Feder et al. (2009).

3. Composition of Quantum dots (QDs)

The QDs active center known as nucleus (Cadmium Selenide - CdSe), presents narrow band gap energy (Santos et al. 2012) (minimum energy necessary to excite an electron to an energy level above its ground state) (Santos et al. 2012). Due the fact that PQs are nanometric, a significant fraction of the atoms are located in the surface region and, therefore, do not have shared chemical bonds (Santos et al. 2012). Consequently, physical-chemical instability occurs, which can compromise the quantum fluorescence yield (ratio of photons absorbed to photons emitted by fluorescence) of the QDs nucleus (reviewed by Brkić 2017).

To stabilize the QD, a second semiconductor layer of distinct material and with larger band gap energy (eg ZnS) is added around the nucleus to interact with the surface atoms. To allow QD to stabilize, a second semiconductor layer of distinct material and with a greater gap energy (for example, ZnS) is added around the nucleus to interact with the surface atoms. This process is called passivation and results in a structure that receives the designation of QD core-shell (Singh et al. 2018). In terms of nomenclature a CdSe / ZnS QD for example corresponds to a zinc sulfide passivated cadmium selenide quantum dot.

Besides improving the nanoparticle stability and quantum fluorescence yield, the core - shell structure avoids the QDs aggregation (a phenomenon that naturally tends to occur in nanoparticulate systems), makes the surface atoms less sensitive to oxidation, photodegradation, pH and other chemical reactions with the surrounding medium (Singh et al. 2018).

The QDs are colloids (a mixture of at least two substances in different phases, in the case of QDs the dispersed phase is solid and the continuous phase is liquid) (Santos et al. 2012) being mostly synthesized in organic media, via organometallic precursors, which prevent its direct application in biological systems (Santos et al. 2012).

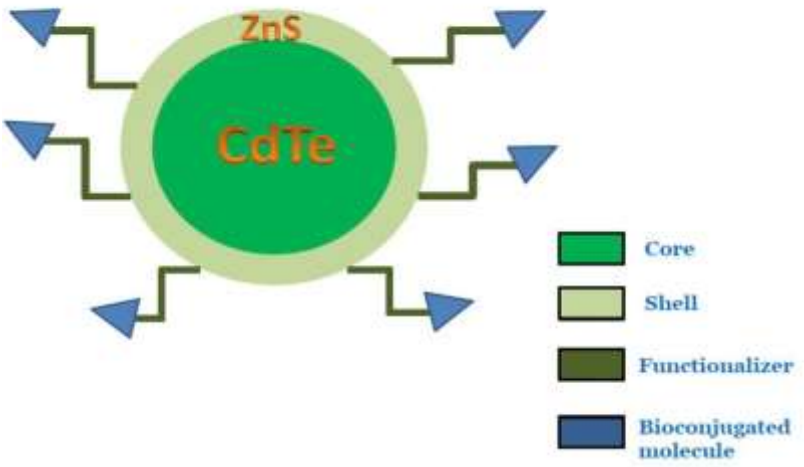
Several synthetic routes have been developed to obtain biocompatible QDs (Banerjee et al. 2016). One of the employed strategies is the synthesis directly in aqueous medium,

where organic molecules are used as functionalizing agents. Thiol-alkyl acids (consists of two terminal functional groups: thiol (-SH) and carboxylic (-COOH), including mercaptopropionic acid (3-MPA), mercaptoacetic acid (MAA), mercaptosuccinic acid (MSA) and dihydrolipoic acid (DHLA) are commonly used. The advantage of using these acids lies in the fact that mercapto-carbonic binders provide high water solubility to PQ, as well as high fluorescence efficiency (Banerjee et al. 2016, Pereira et.al.2019).

In QDs formed by a cadmium nucleus (Cd), these molecules can also, form passivation layers (i.e., act as stabilizing agents). The thiol group sulfur radical is adsorbed by the QD surface, forming a small layer of cadmium - CdS sulfide, resulting in physicochemical stability, thus increasing the quantum fluorescence yield (Santos et al. 2012). On the other hand, the carboxylic group present at the other molecule end provides solubility in water and can also be used as a bridge to conjugate QDs to specific recognition molecules (Santos et al. 2012, Banerjee et al. 2016; Pereira et al. 2019).

Quantum dots can be conjugated to a variety of biomolecules (lipids, proteins, oligopeptides, DNA) or synthetic molecules (drugs) which confers specificity and direction to nanoparticle (Santos et al. 2012; Banerjee et al. 2016; Pereira et al. 2019). Due to the large surface area and solubilization feasibility of these materials in aqueous medium, molecules and QDs can be linked forming fluorescent bioconjugates which can be used as tools that can help understand cellular mechanisms, diagnosis and treatment (Santos et al. 2012; Banerjee et al. 2016). In the Figure 1, we show a schematic of a functionalized QD for biological application.

Figure 1 - QD schematic illustration for biomedical and biology applications. The core consists of the semiconductor material responsible for the fluorescence properties. The shell is a material that stabilizes and protects the core, whereas the functionalizer provides anchoring sites to biological and organic ligands generating a target-specific QD.



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4. Quantum Dots Biological Applications

Quantum dots (QDs) are one of the main nanotechnologies integrated with life sciences (Abbasi et al. 2016). Due to the fact they present reduced photodegradation and exhibit a broad absorption spectrum (allowing multiple QDs to be excited by a single laser) these nanoparticles have been applied as fluorescent probes for labeling biological systems *in vitro* and *in vivo* (Chaves et al. 2008; Feder et al. 2009; Vieira et al. 2011; Chen et al. 2019; Koren et al, 2020). QDs have also been successfully introduced in bioimaging experiments for small animal (Zhao et al. 2017; Qiu et al. 2019), immunohistochemistry (Montón et al. 2012), nucleic acid detection (Chan et al. 2005), molecular biomarkers of cancer detection (Xiang et al. 2020), infectious diseases detection (Kim et al, 2018), guidance in surgical procedures (Li et al. 2012), drug screenings (Ku et al. 2011), drugs delivery (Mattea et al. 2017; Olerile et al.2017; Sangtani et al. 2018) and photodynamic therapy (He et al. 2016).

5. Mechanisms of Cellular Uptake of Nanoparticles

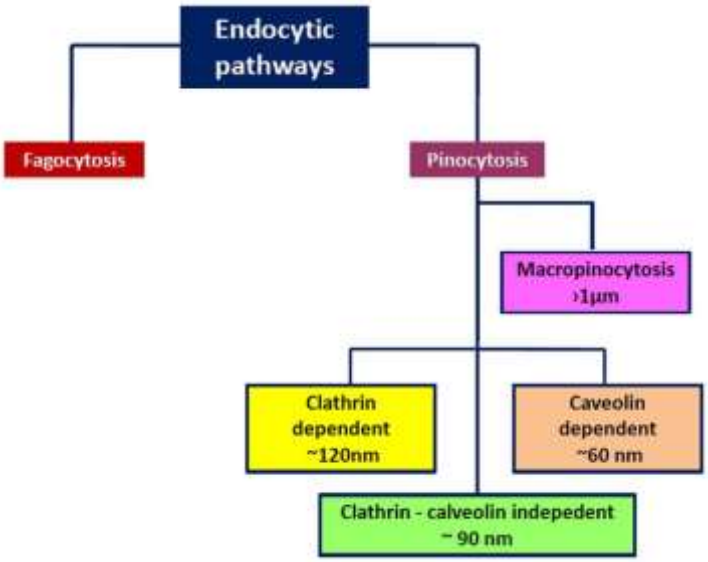
All cells are surrounded by a cellular membrane that acts as a permeable and selective barrier protecting them from the surrounding environment and helping to the intracellular homeostasis maintenance (Beddoes et al. 2015). In order to nanostructured systems have efficient biological application, these should be designed in such a way that they can overcome such barrier (Salatin and Yari Khosroushahi 2017).

Cellular uptake of the nanoparticles (NPs - as in the case of QDs) is determined by a series of events (cell, lineage, metabolic state, life cycle stage, healthy cell, cancer cell), and the type of interaction that the nanoparticle establishes with the surface of the plasma membrane and its physicochemical properties (chemical composition, surface coating, surface charge, size and synthesis medium) (Pelley et al. 2009; Santos et al. 2012).

The extracellular material uptake through the plasma membrane is well described and characterized by a variety of mechanisms ranging from passive diffusion (no energy expenditure) to active transport (with energy expenditure) (Panariti et al. 2012). Several studies indicate that the NPs cellular uptake occurs mainly through endocytosis (Feder et al. 2009, Zhang at al. 2019).

Endocytosis is an energy-dependent eukaryotic cell process that is involved in various biological events, such as the acquisition of extracellular nutrients, recycling of membrane lipids, surface receptor regulation, intracellular protein traffic, antigen presentation and also as a pathway entry of pathogens and toxins (Canton and Battaglia 2012).

Figure 2 - Portals of entry into the cells. The uptake of the NPs by cellular systems occurs with a process known as endocytosis. The endocytic pathways differ with a regard to the size of the endocytic vesicle (nanometer or micrometer) and the mechanism of vesicle formation. The nature of surface charge and size of NPs affects cell-membrane interactions.



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Cells conditions (lineage, metabolic status, life cycle stage, healthy cell, cancer cell) and physicochemical properties of nanoparticles (nucleus composition, size, nature of the surface coating, surface charge, means of synthesis, stability of PQs in aqueous media) play a significant role in the determinant uptake pathway (s) (Pelley et al. 2009; Canton and Battaglia 2012; Santos et al. 2012; Beddoes et al. 2015).

Regardless of the endocytic pathway used (see Figure 2), the material from the extracellular medium is confined to a vesicle (endosome or phagolysosome) consisting of plasma membrane lipids and proteins (Canton and Battaglia 2012). After the vesicle is released from the membrane, the endocytosed materials can be: (1) forwarded to other organelles; (2) redirected to the outside environment through recycling endosomes; (3) fusion with endosomes and lysosomes to be degraded or (4) exocytosed (Oh and Park 2014). QDs can be internalized by more than one endocytic pathway, which may result in distinct biological responses (Ferreira et al. 2014).

These observations demonstrate that understanding the dynamics of QD - cell interaction is fundamental to explore the potential of these nanoparticles in biological systems, and especially to understand the possible adverse and / or cytotoxic effects (Winnik and Maysinger, 2013).

6. The Cadmium Based - Quantum Dots Cytotoxicity

The nanotoxicology field has advanced considerably and numerous data have been reported aiming at understanding the mechanisms of cytotoxicity of QDs (Khalili Fard et al. 2015).

Although there are QDs with the metalloid core formed of Indium Phosphorus (InP), Zinc Selene (ZnSe) and Silicon (Si) that are considered less toxic, CdSe and CdTe based QDs present optical quality and greater fluorescence intensity, characteristics that are highly appreciated for biological applications (Santos et al. 2012).

However, as already commented, several reports demonstrate the success of QDs in the biological application evidencing little or no cellular toxicity and others report cytotoxic effects (Pelley et al. 2009). According Soenem et al. (2011) the inorganic nanoparticles (in the case of QDs) may not only exhibit different behavior when applied in biological systems but also induce cytotoxic effects.

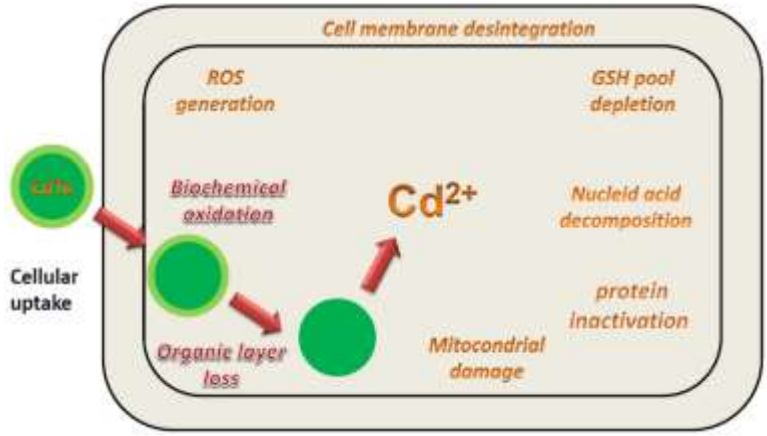
It is worth mentioning that the toxicity of QDs depends on many factors. Thus, it is essential to know the physical-chemical properties of these nanoparticles such as composition, size, surface coating, surface charge and means of synthesis, to better understand the cytotoxic effects. (Pelley et al. 2009; Caballero Diaz and Cases 2016).

Two primary sources of QDs toxicity commonly described in the literature involve: i) Cadmium ion leaching: after QDs internalization by the cell, they are exposed to various pH differences (7.4 in the extracellular environment, 5.5 in the late endosomes and 4.5 in the lysosomes) and several hydrolytic enzymes that can lead to degradation of the surface coating, resulting in the release of free cadmium ions (Cd2 +) into the intracellular environment (Wang et al. 2016). Cadmium (Cd) is a non-essential transition metal (it is not involved in enzymatic activities or other biological activities) (Caninno et al. 2009) and is known to cause acute and chronic toxicity in various organs and tissues (Joardar et al. 2019; Liu et al. 2019; Chandrasekaran et al. 2020). At the cellular level, Cadmium induces oxidative stress (He et al. 2019), damages DNA and inhibits repair mechanisms (Tan et al. 2019), alters protein expression (Meng et al. 2017), interferes with the signaling of intracellular calcium (Lin et al. 2015) and induces cell death (Yuan et al. 2018). Such effects occur depending on the dose, route of entry, cell type and frequency of exposure (Neminche and Guiraud, 2016).

ii) Electronic reactivity of the QD: the nuclei of the CdTe and CdSe QDs present a high level of electronic reactivity and are prone to photo-oxidation processes (Singh et al. 2018). Electrons from excited QDs can be transferred to the nearby oxygen molecule,

producing ROS in the cell (Wang et al. 2016). ROS (superoxide, hydrogen peroxide, radical hydroxyl, singlet oxygen) constitute a set of molecules with high reactivity and short half-life due to the tendency to supply or receive electrons for stability (Wang et al. 2016). In the intracellular environment, ROS are generated physiologically in various cell compartments including plasma membrane, cytosol, peroxisomes and endoplasmic reticulum and mitochondria membranes (reviewed in Di Meo et al. 2016), regulating various signaling pathways and important cellular processes such as proliferation (Lyublinskaya et al. 2015) and survival (Miller et al 2019). However, excessive ROS production in the intracellular environment can lead to oxidative stress causing damage to important macromolecules (Xiang et al. 2013)

Figure 3 - Simplified scheme of mechanisms of QD cytotoxicity - adapted from Geszke and Moritz 2013.



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As mentioned above, the literature also points out that other physical and chemical properties of QDs (size, charge, shape, concentration, surface coating (cappin material and functional groups), degree of aggregation) can dictate interactions and adverse biological responses. (Lai et al. 2016; Hu et al. 2019).

So far, several studies on the assessment of cadmium-based QDs cytotoxicity on prokaryotic organisms, eukaryotes and viruses have been carried out (see table 1).

Since QDs are not homogeneous materials, different combinations of physical chemical properties can result (or not) in varying degrees of toxicity (Lai et al. 2016; Hu et al. 2019). Moreover, different cells can interact and present different biological responses after exposure to the same type of QDs (Manshian et al. 2015; Hu et al. 2019), the time of exposure of the cell to nanocrystal being one of the critical parameters in the evaluation of cytotoxicity.

As we can see, a thorough analysis is needed on which properties (and in what proportion) contribute to the real toxicity of quantum dots.

The most toxicological tests with QDs are performed *in vitro*. However, the data obtained from *in vitro* experiments will not always reflect in the same way *in vivo* (Li et al. 2019). In fact, studies on the biological application of QDs have advanced our knowledge about cell uptake, biodistribution and mainly about the question of toxicity induced by QDs. The results obtained can assist in the elaboration of new designs of QDs in order to achieve the desired application proposal (image, diagnosis, therapy, delivery of medicines) (Zheng et al. 2013). For this to occur the chemical physical properties must be adjusted.

However, several strategies have been carried out to improve the biocompatibility of QDs. For example: QDs with a negative charge to decrease the intensity of interactions with the cell surface; surface coatings that reduce unwanted protein and/or antioxidant-based bonds to neutralize the formation of ROS by QDs in the cellular environment; reduction of the surface area of the QDs to decrease their dissolution, among others (Winnik and Maysinger, 2013; Buchman et al. 2019).

Table 1 - Cytotoxicity of cadmium - containing Quantum dots. Abbreviations: ND- Not determined; OH: hydroxyl radical.

Organism	QD type and mean size	Stabilizing/functionalizing agent	Exposure concentration	Toxicity	References
Bacteria (*E. coli)*	CdTe/ZnS (4.67 and 4,87nm)	3- mercaptopropionic acid (-) or Cysteamine (+)	0,1-1,6 µM	Cysteamine: more toxic Change in membrane fluidity	Lai et al. 2017
Fungus (*S.cerevisae*)	CdSe/ZnS (2.0-2.9nm)	Carboxylic acid	0.8-100µg/L	genes implicated in multiple cellular process were altered	Horstmann et al. 2019
Virus (Pseudorabies virus)	CdTe (3,2nm)	Glutathione	80nM		Du et al. 2015
Protozoan (*T. cruzi*)	CdTe (2,2nm)	Mercaptoacetic acid	2 and 200 µM	Changes in surface proteins Inhibition of replication	Marques et al. al.2014
Nematode (*C. elegans*)		3-mercatopropionoc acid	0,001-1µg/L	Dose and time dependent	

Source: Authors.

Table 1: Cytotoxicity of cadmium - containing Quantum dots. Abbreviations: ND- Not determined; OH: hydroxyl radical. (Source: Authors).

7. *Trypanosoma cruzi*

Trypanosoma cruzi the etiologic agent of Chagas disease (or American trypanosomiasis) is a flagellate protozoan of the order Kinetoplastida, family Trypanosomatidae (Chagas 1909; Cavalier-Smith 2010). According to the World Health Organization (WHO), there are around 6 - 7 million people infected with *T. cruzi* worldwide,

most of them located in Latin America, where it is considered endemic (WHO 2020). The transmission way for this trypanosomatid is mainly by means of insect vectors, but secondary pathways such as blood transfusion, organ transplantation, the congenital pathway and the oral one has been reported (Dias and Amato Neto 2011).

The *T. cruzi* vector is a hematophagous insect belonging to the order Hemiptera, family Reduviidae and subfamily Triatominae. The triatomine vectors belonging to the genera *Rhodnius*, *Triatoma* and *Panstrongylus* are considered the most epidemiologically relevant (Costa e Lourenço 2009).

During its life cycle, *T. cruzi* has three developmental stages: epimastigote, amastigote and trypomastigote. According De Souza (2002) the following aspects can differentiate these evolutionary stages: epimastigote can be found at the digestive tract of insect vector and axenic culture, is characterized by the elongated morphology, rodlike kinetoplast in position before the nucleus and perpendicularly oriented to the parasite's cellular body longitudinal axis. The flagellum emerges from the flagellar pocket located in the anterior region of the parasite, which has lateral opening, becoming free at the end of this region; amastigote presents a round morphology, with a cane-like kinetoplast located in the region anterior to the nucleus, with a short flagellum emerging from the flagellar pocket. This form can be found inside cells of infected vertebrate hosts, axenic culture, or infected animals' blood (De Souza 2009) and trypomastigote form presents an elongated and slightly flattened morphology with a rounded shape kinetoplast located in the region posterior to the nucleus and the flagellum adhering along the cell body, becoming free in the anterior region (De Souza 2002). This form is highly infective and can be found in the insect vector (posterior portion of the intestine and rectal ampulla) as metacyclic trypomastigote; in blood and intercellular space in vertebrate hosts, in cultures with infected cells and axenic culture, as blood trypomastigote **(**De Souza 2009).

8. Cell biology of Trypanosomatids

T. cruzi presents in its structural organization eukaryotic cell typical organelles and others that are specific to the trypanosomatidae family members. The exclusive organelles comprise the glycosomes, acidocalcysomes and reservosomes (De Souza 2002; De Souza 2009). The plasma membrane of *T. cruzi* consists of lipid bilayer composed of glycoprotein and glycolipids distributed on the outer face of the membrane forming the glycocalyx (De Souza 2009). It presents distinct regions: that of the membrane that lines the body of the

parasite which covers the flagellum and the region that covers the flagellar pocket. They are regions differentiated by the presence of sub-pelicular microtubules and the axonema (De Souza 2009). The subcellular microtubules are located underneath and distributed throughout the cell body (except in the region of attachment of the flagellum to the cell body and in the region of the flagellar pocket) and are connected to each other and to the plasma membrane, giving some mechanical rigidity to the cell (De Souza 2009). The axoneme microtubules of are associated only with the flagellum (De Souza 2009).

As for the single flagella, it is present in all the parasite developmental stages (De Souza, 2002). Surrounded by a flagellar membrane, the flagellum emerges from the basal corpuscle and is exteriorized by the region of the flagellar pocket that alternates its location (anterior or posterior) according to *T. cruzi* developmental stages (De Souza 2009). In the epimastigote and trypomastigote, next to the axonema is the paraflagellar structure that helps in flagellar beat (De Souza 2009). The flagellum of these parasites is involved in several functions such as flagellar motility, cell division, morphogenesis, and adhesion to target cells (De Souza 2002).

The flagellar pocket corresponds to a cellular body invagination from which the flagellum emerges and is present in the three developmental forms (Field and Carrigton 2009). There is a second membrane invagination called cytostome / cytopharynx. The cytostome is coated by some microtubules that deeply penetrate into the cell reaching the nucleus region, forming the cytopharynx / cytostome complex (Field and Carrigton 2009). This complex is present only in *T. cruzi* amastigotes and epimastigotes (Porto-Carreiro et al. 2000). The flagellar pocket and the cytostome are two surface domains that are involved in the endocytosis process. The Golgi complex and the flagellar pocket are involved in the exocytosis process. Both processes are vital for the parasite development and survival (Porto-Carreiro et al. 2000).

There are single mitochondria, which branches through the cell body. In its interior is differentiated a region known as kinetoplast, characterized by intense DNA condensation (DNA minicircles and maxicicles networks that are interconnected) (Westenberger et al. 2006). This structure characterizes the order Kinetoplastida to which the Trypanosomatidae family belongs. As previously described, the epimastigote and amastigote stages present a rodlike kinetoplast and the trypomastigote stage a round one (De Souza 2009).

The nucleus is structurally similar to that of other eukaryotic cells delimited by a nuclear membrane containing nuclear pores. In epimastigotes and amastigotes the shape is rounded while in the trypomastigotes it is elongated (De Souza 2009). The nucleolus is

generally found in the central region of the nucleus and the chromosomes do not condense during the cell division process (De Souza 2009).

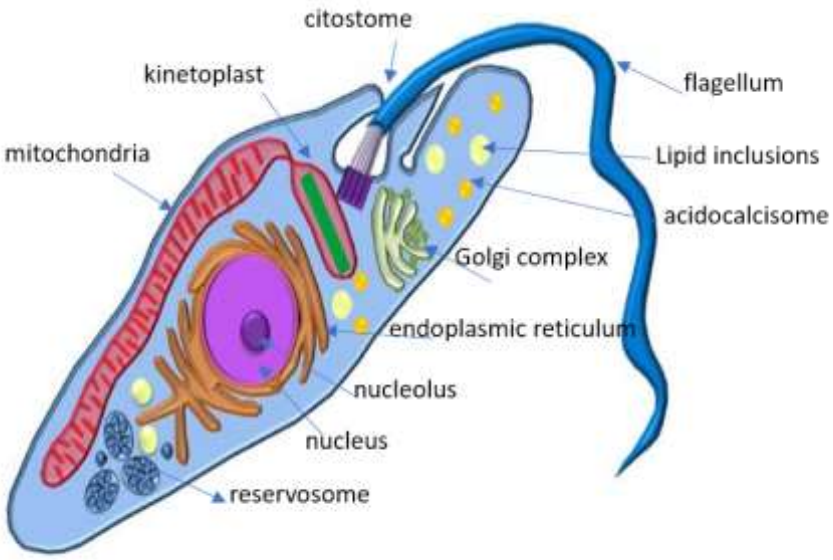
Around the nucleus and throughout the cell body there are endoplasmic reticulum profiles that are involved in the lipids and protein synthesis. The Golgi complex is composed by small stacked cisterns being in the anterior portion of epimastigotes and amastigotes and in the posterior region in trypomastigotes, participating in glycosylation processes of proteins and other important functions (De Souza 2002). Ribosomes are present and distributed throughout the cytoplasmic region (De Souza 2002).

The acidocalcisomes are organelles with spherical shape, acidified by proton pumps located in their membrane (Rohloff et al. 2004; De Souza 2009). They are present in the three developmental stages. It is considered the main ions reservoir such as sodium, calcium, magnesium and zinc (Rohloff et al. 2004). It is also involved in the pyrophosphate and polyphosphate metabolism (Rohloff et al. 2008). These organelles also participate in intracellular pH homeostasis and together with the contractile vacuole participate in osmoregulation processes (Rohloff et al. 2008).

The glycosomes are specific organelles present only in trypanosomatids (Parsons 2004; De Souza 2009). In its interior are the enzymes responsible for the initial stages of the glycolytic pathway. The glycosome is considered a specific type of peroxisome and contains enzymes that participate in other biochemical pathways such as the phosphate pentose pathway and the beta-oxidation of fatty acids (De Souza 2009).

Reservosomes are organelles similar lysosomes. Their morphology varies according to the parasite strain but are generally round (Soares 1999). They are membrane-bound and consist of an electron-dense matrix with vesicles and lipid inclusions and acid hydrolases such as cruzipain (Soares 1999). Reservosomes store secretory proteins and macromolecules and endocytosed nutrients and, therefore, it is a nutrient reserve site (Cunha-e-Silva et al.2006). These organelles were initiaally identified in the epimastigote form and their gradual disappearance in the metacyclogenesis process was described (Porto-Carreiro et al. 2000). However, structural and cytochemical analyzes have demonstrated that the other *T. cruzi* developmental stages (amastigotes and trypomastigotes) present organelles related to lysosomes known as reservosome-like (Sant'Anna et al. 2004).

Figure 4 - *Trypanosoma cruzi* epimastigote parasite schematic representation, showing internal structures.adapted from (Jessica Meloni's drawing adapted from Teixeira et al. 2011).



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9- Application of Cd - based QDs in trypanosomatids

Trypanosomatids have special characteristics what makes of these protozoan good models to study basic questions of cellular biology (De Souza 2002). Interest in taking advantage of the unique spectral properties of QDs has driven their use in biological applications in members of the Trypanosomatidae family. Some papers have already been reported. In this section it is given a brief description of each.

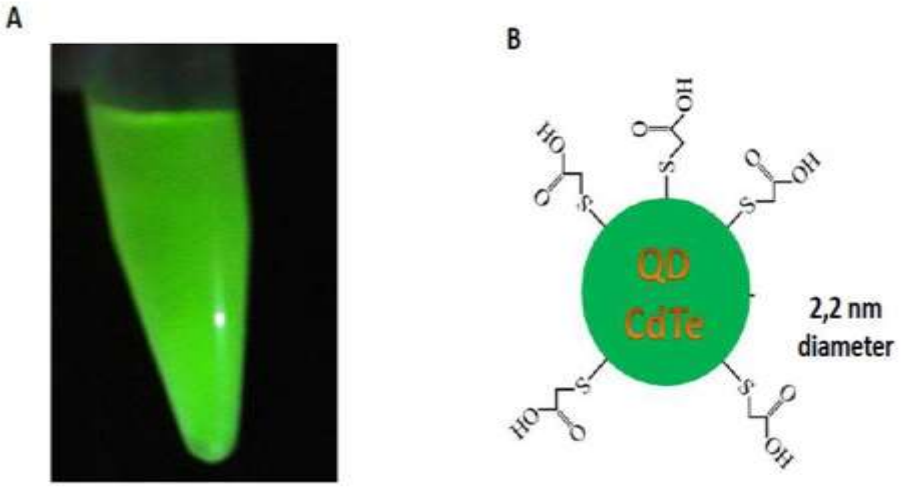
Leishmania amazonensis promastigotes (evolutionary form can be found in the insect vector digestive tube and axenic cultures) were labeled with CdS / Cd $(OH)_2$ QDs, functionalized with polyphosphate anions and / or glutaraldehyde molecules. The images obtained by confocal microscopy (CM) showed that the phosphate rich QDs interacted with DNA regions and thus evidencing a differentiated fluorescence pattern when compared to glutaraldehyde coated QDs (Santos et al. 2006).

CdS / Cd $(OH)_2$ QDs functionalized with polyethylene glycol (PEG) were used to label T. *cruzi* epimastigote cells, can be found in the insect vector digestive tube and axenic cultures. The parasites were incubated with the nanoparticles for 180 minutes at room temperature. The analysis carried out by confocal microscopy and transmission electron microscopy revealed the presence of QDs in the cystostome and in the reservosome structures

which are involved in the internalization and storage of endocited material, respectively (Chaves et al. 2008).

Feder et al. (2009) regarding the biological application of QDs was done with CdSe / CdS QDs functionalized with mercaptoacetic acid - MAA (carboxylic acid containing a thiol functional group) with fluorescence in yellow wavelength emission. They used unconjugated nanoparticles and NPs conjugated to two different lectins (glycoproteins), *Sambacus nigra* aglutinin (SNA) and Concanavalin A (ConA) to mark specific carbohydrates involved in the parasite - vector interaction (Feder et al. 2009). Epimastigote cells and the fragments of the posterior region of the midgut of *Rhodnius prolixus* were incubated with QDs for 1 hour at room temperature. The images obtained by confocal microscopy showed the parasites labeled with conjugated QDs and adhered to the perimicrovillar membrane of the insect gut. In this same study, *R. prolixus* were fed on blood containing the unconjugated nanoparticle. In this way, it has been shown that is possible to observe the non-specific labeling of the epithelial cells and symbiotic bacteria of the intestinal tract of these insects. The same authors also demonstrated that the uptake of lectin conjugated QDs by *T.cruzi* occurs via endocytosis, since it was blocked when the QD incubation was done at 4°C (Feder et al. 2009). It has been demonstrated that performed labeling assays of *T. cruzi* epimastigotes with CdTe QDs passivated and functionalized with mercaptoacetic acid-MAA. Figure 5 shows the QD colloidal suspension and the chemical structure of its surface coating.

Figure 5 - QDs for application in living cells. A) CdTe QDs colloidal suspension emitting green fluorescence, excited by ultraviolet irradiation (λ= 365nm). B) The CdTe nanocrystals were passivated and functionalized with mercaptoacetic acid- MAA (carboxylic acid containing a thiol functional group) synthetized in an aqueous medium. Each nanoparticle has a diameter of 2,2nm approximately.



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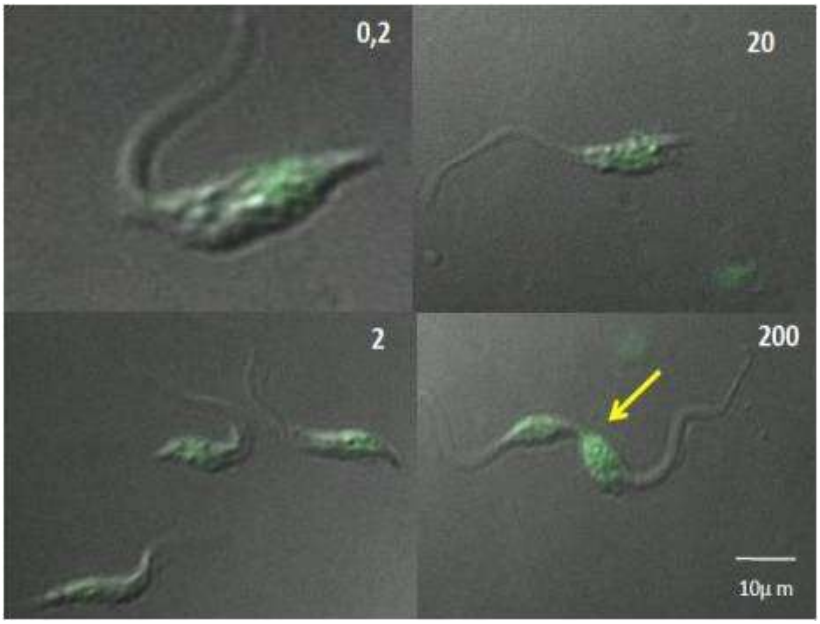
Other experiments were done showing parasites incubated with different concentrations of CdTe QDs (0.2, 2, 20 and 200 μM) for 2 hours at room temperature. The results of the biological labeling assay are shown in Figure 6. The micrographs obtained by confocal microscopy show that the QDs were internalized by the cells. At all concentrations tested we can observe non-specific labeling, because QDs functionalized with mercaptoacetic acid can bind to any protein material. We draw attention to the images that show the fluorescence inside the parasites in the process of binary division (yellow arrow).

10. Evaluation of Biological Toxicity of CdTe QDs in *T.cruzi*

Our laboratory has investigated the possible toxic effects of CdTe QDs on *Trypanosoma cruzi* epimastigote cells. We used the same QDs used for the biological labeling assays. The physical properties of these nanoparticles have been previously described (see Figure 5).

Figure 6 - Confocal microscope images of the *T. cruzi* labeled with Cadmium telluride quantum dots (CDTe- QDs). Cells with 0,2µM QD (upper left); cells with 2µM (lower left); cells with 20 µM (upper right); cells with 200µM (lower right). The arrow (yellow color) indicates binary division of epimastigotes. Bar=10µm.



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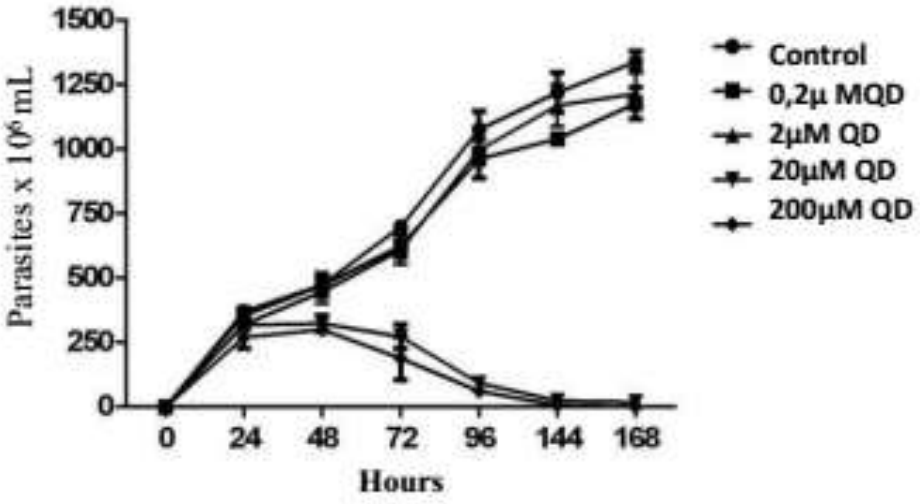
Considering that cell viability assays are one of the vital steps in toxicology to evaluate the cell response to a given substance or material (Zhang et al. 2015) we plotted a dose-growth curve. The epimastigote cells were incubated with different response concentrations and QDs at different concentrations (0.2-200µM). The cell density was observed and estimated up to 168 hours of incubation.

Based on the growth curve, it was possible to determine the concentrations of CdTe QDs that did not affect integrity (for at least seven days), proliferation and mobility of the *T. cruzi* cells (0,2 and 2 µM QD). Figure 7 shows the parasite time-scale exponential growth curve. The results indicate that the toxic effects of CdTe QDs were not time dependent but dose dependent. There was no difference in the growth rate up to 24 hours of incubation at all tested QDs concentrations. However, the cell groups incubated with the highest concentrations (20 and 200 µM QD) showed significant differences in growth rate after 72 hours, with population reduction and motility loss (data not shown). In view of the results obtained from the growth curve, the 72-hour time were standardized for tests that can clarify the toxicity mechanism of CdTe QD in *T. cruzi.*

Figure 7 - Growth curve of epimastigotes of *T. cruzi* at different concentrations of CdTe QDs in time scale (●) Control; (■) 0,2µMQD; (▼) 2µMQD; (◆) 20 µMQD; (▲)200 µM QD.



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Similarly, Fontes et al. (2012) assessed the potential toxicity of CdS / Cd $(OH)_2$ QDs in *T. cruzi* cells. The results showed that there was no significant difference in the parasite growth and viability that could be observed at 24 hours of incubation with QDs (at any tested concentration). However, the number of cells decreases in a dose-dependent manner after 48 hours (Fontes et al. 2012).

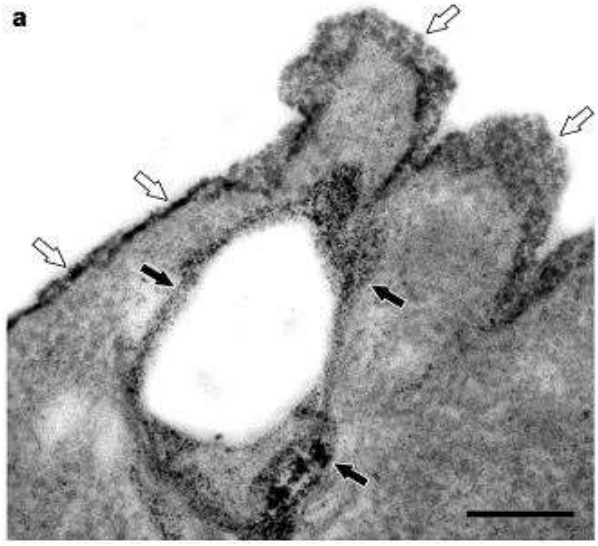
In previous studies, Vieira et al. (2011) also performed QD analyzes inside *T. cruzi* cells by transmission electron microscopy (TEM). QDs were observed in vesicles scattered in the cytoplasm and near organelles and reservosomes. These results suggest that CdTe QDs are being endocytosed via an unknown mechanism.

Others results demonstrated deposition of QDs around the plasma membrane, inside and dispersed in the cytoplasm, besides involving vacuole in *T. cruzi* labeled with 200 µM of CdTe QDs (Figure 8).

In addition, Vieira et al. (2011) had demonstrated the presence of these nanoparticles in membrane vesicles near the flagellum region as well as extensive changes in the plasma membrane near the flagellar pocket region of *T. cruzi* labeled with 200 µM of CdTe QDs. Futhermore were observed endoplasmic reticulum profiles around subcellular structures.

These results suggest the occurrence of autophagy process (Vieira et al. 2011) which was confirmed by Marques et al. 2014.

Figure 8 - Transmission electron micrograph of *T. cruzi* epimastigote labeled with 200μM of CdTe QDs. In (a): *T. cruzi* presenting deposition of QDs around the plasma membrane (large white arrow), QDs inside and dispersed in the cytoplasm, and involving vacuole (black broad arrow). Bars: in (a) 0.2 μm and in (b) 0,5 μm.



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In view of these findings**,** Marques et al., (2014) investigated whether autophagy would be a consequence of the QD toxicity in these cells. Were used the 3-methyladenine (3-MA) marker, the inhibitor of phosphatidylinositol 3-kinase (PI3K, a protein involved in early autophagy) and monodansylcadaverine (MDC), a fluorescent probe used to identify cadaverine present in autophagy vacuoles. The results showed significant differences between parasites of the control group and the parasites treated with 200 μM QDs labeled with the MDC probe. The presence of several autophagy vacuoles within the cells and the ongoing autophagy process was confirmed by the 3-MA assay.

Autophagy is a fundamental cellular pathway for maintaining cell homeostasis (Parzych and Klionsky 2013). Various research show that different cells undergo autophagic processes after exposure to nanomaterials including QDs (Yang et al. 2020).

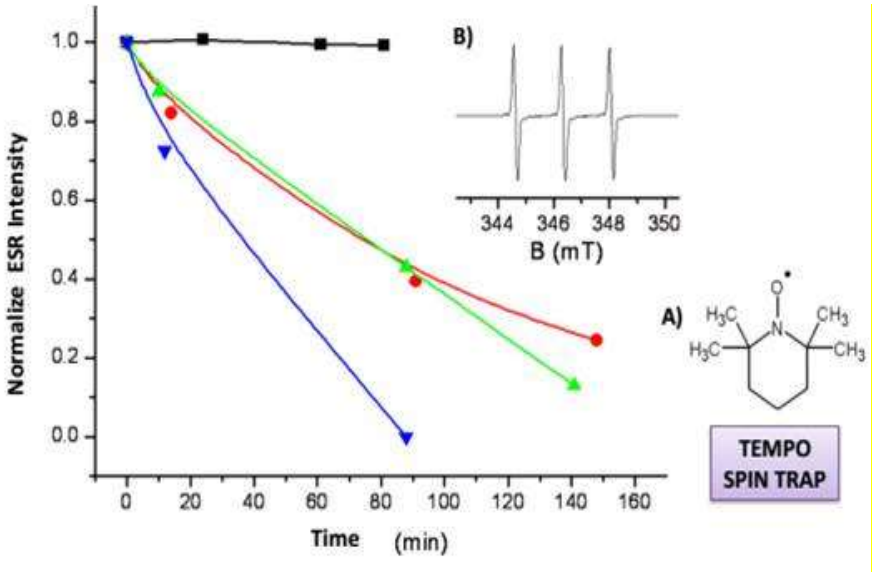
Our data show that autophagy occurs in *T. cruzi* epimastigotes when that were incubated with high concentrations. Probably autophagy occurs due to the excess of QDs into

the parasites and works as an attempt to eliminate the disturbing agent of homeostasis, in other words, a mechanism for the parasite to maintain its normal physiological conditions. It is important to highlight that, to date, the possible molecular mechanisms involved in the autophagy / QD relationship are not fully understood.

The available data in the literature indicate that one of the mechanisms of quantum dots cytotoxicity is related to ROS generation in the intracellular environment (Hu et al. 2017). It has been shown that high concentrations of QDs affect cell growth (Vieira et al. 2011). In this context, the effect of CdTe QDs on the ROS generation in *T. cruzi* was evaluated by the Electron Paramagnetic Resonance (EPR) spin trapping. The technique uses chemical species called spin traps, which react with short-lived free radicals in biology systems to form relatively stable adducts having a half-life long enough for ESR measurement (He et al. 2014).

According to Vieira et al, (2011), several damages were observed in cells labeled with 20 and 200 μM of QDs. Similar results were obtained by Marques et al., (2014). Moreover, when we compared the decay kinetics curves of the spin-marker signal, we observed that the cells labeled with 200 μM of CdTe QDs decay was faster than the other groups tested. Signal decay kinetics were completed at 145 minutes for cells incubated with culture medium alone (positive control), 140 minutes for cells labeled with 20 μM of CdTe QDs and approximately 85 minutes for cells labeled with 200 μM of QD (Figure 9).

Figure 9 - Kinetics of the decay of spin trap TEMPO. ( ) TEMPO with H2O (control); ( ) spin trap + *T.cruzi*; ( ) spin trap +*T. cruzi* labeled with QD 20µM; ( ) spin trap+ *T. cruzi* labeled with QD200µM. (A) Chemical structure of spin trap TEMPO molecule (B) The spectra of TEMPO radical showing potential intensity.



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Since decay kinetics are recorded by the ESR until all the available spin traps interact with the ROS present in the biological samples, we can indicate that *T. cruzi* exposed at 200 µM of CdTe QDs induces the highest ROS generation.

11. Final Considerations

Quantum dots (QDs) are one of the main life sciences integrated nanotechnologies**.** Since the QDs have been used as fluorescent markers for visualization of cellular images and mechanisms of different organisms. In this context, the investigation of the cells nanoparticles interactions, it becomes crucial to validate its biology application and contribute to the development of biocompatible QDs.

Thus, the objective of the present study was to present a previous review on CdTe QDs and aspects on cellular toxicity, using as a model the parasitic protozoan, *Trypanosoma cruzi*, whose tests demonstrated that cytotoxicity occurs as a function of CdTe QDs concentration.

Furthermore, our research group demonstrated that the parasites labeled with low concentrations of these nanoparticles did not affect integrity, proliferation, and mobility of the

T. cruzi cells. In contrast, QDs exposure led to a significant cytotoxicity at higher doses by affects the cellular viability and induce alterations in the subcellular structures, in the plasma membrane, autophagy processes and ROS generation. We can infer that the toxic effects of QDs in *T. cruzi* epimastigotes are dose dependent.

In summary, based in these results, we concluded that the 0.2 and 2 µM CdTe QDs (low concentrations) are suitable and could be used as bioimaging tools for alive parasites for long time.

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