EVALUATION OF CELLULAR UPTAKE AND TOXICITY OF PLATINUM NANODENDRITES AS THERANOSTIC AGENT

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Abstract. Platinum nanodendrite (PtND) is a potential theranostic agent for improving the therapeutic index of radiotherapy. Optimally, PtND needs to be taken up efficiently by cancer cells and pose no inherent toxicity except during radiotherapy irradiation. Thus, this assessment was performed to elucidate the uptake and toxicity of PtND towards different cancer cell lines (HeLa, MCF-7, and MDA-MB-231). For the uptake study, the cells were seeded onto the glass slides until confluency and treated with 0.1 mM PtNDs overnight. The treated cells were then fixed and stained using the crystal violet staining method before being evaluated using bright field microscopy and ImageJ software. The toxicity study was completed by using the Prestoblue® cell viability assay, where the cells were first treated with PtNDs for up to 3 days before they were exposed to the Prestoblue® reagent and measured for their fluorescence values. The result shows that the PtNDs mainly reside in the cytoplasm of the cells. The particles agglomerated in cells causing the increment in particle size between 600-1200 nm on average. The cytotoxicity studies revealed that the PtNDs' toxic effect is dependent on the PtND concentration, size, treatment time and type of cells used. Overall, HeLa cells show better resistance toward PtND than MCF-7 and MDA-MB-231 cells, with non-toxic PtND concentrations of up to 0.1 mM. In conclusion, PtND shows promising pharmacokinetic properties to be used as a theranostic agent. However, their biocompatibility profiles require further verification to ensure that they are safe for theranostic applications.

Keywords: Platinum nanoparticles, theranostic, cytotoxicity, cellular uptake, agglomeration.

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Introduction

Nanoparticles are ultrafine particles between 1 and 100 nm in size [1]. The unique properties of nanomaterials sparked a lot of interest in their applications for a multitude number of fields, including biomedical, manufacturing and energy industries [1]. The combination of the field of medicine and nanotechnology for disease prevention and treatment resulted in the advancement of nanomedicine [2,3].

Nanoparticles received significant attention in the oncology field due to their ability to specifically accumulate in the tumours, especially after they were functionalized using suitable coating materials. The nanoparticle uptake mechanisms by the tissues can be summarized in two categories: a) passive, and b) active targeting. In passive targeting, the nanoparticle uptake relies on the imperfectly developed, leaky blood vessels in tumours. The leaky pores can be up to 1 μm in size, allowing nanoparticles of smaller dimensions to permeate into the tumour environment. The impaired lymphatic system in the tumour also hinders the particles larger than 4 nm to be drained out from the tumour. Eventually, a significant amount of nanoparticles can be accumulated in the tumour. This whole concept is termed as the enhanced permeability and retention effect or EPR [4].

On the other hand, active targeting employs the functionalization of the nanoparticles' surface using tumour-targeting molecules, which interacts specifically with antigen or receptors uniquely expressed by tumour cells [4]. For example, Yue et al. explored the tumour-targeting ability of HER2-targeted platinum nanoparticles (PtNPs) for breast cancer radiotherapy. They proved that the functionalized PtNPs successfully target the tumour cells and improve the efficacy of radiotherapy treatment [5]. In another study, the biocompatible human serum albumin (HSA) was used to coat the platinum nanoparticles, which successfully enhanced tumour accumulation in contrast to the normal cells [6]. The sheer amount of strategies explored for nanoparticle application proves that nanoparticles have a high potential to be used for cancer theranostics [7].

Theranostics is a term derived from 'therapeutics' and 'diagnostics' which referred to the techniques or agents that integrate diagnostic imaging and targeted therapies, which are aimed to improve the patient outcome. An ideal theranostic agent should have the following characteristics: a) deep and specific accumulations in the targeted tissues/tumours, b) possess efficient and selective mechanisms of therapeutic deliveries, c) high target-to-background contrast ratio, and d) safe and biodegradable in non-toxic products [8,9]. Based on these criteria, high-Z nanomaterials can be considered as a leading candidate to be a theranostic agent, because they have a higher mass attenuation coefficient, μ/ρ as compared to the soft tissue and iodine [10], and their nano-sized platform also contributes to their specific accumulation in the tumour. However, the toxicity of nanomaterials still becomes the main hurdle for them to breach clinical application.

In this work, a novel, high-Z nanomaterial for biomedical application, platinum nanodendrites (PtNDs) were studied. The PtNDs have been proven to give significant enhancement in both radiotherapy and diagnostic applications [11,12]. However, their cytotoxicity evaluation is still not well-documented. Thus, this study was done to elucidate the cellular toxicity and uptake of PtNDs under different treatment parameters, including the PtND size, concentration, treatment time and type of cell lines used.

Materials and Methods

Cellular Toxicity Evaluation.

The PtNDs were synthesized and characterized at the School of Materials and Mineral Resources Engineering, Nibong Tebal, Malaysia. The details of the synthesis and characterization have been discussed in previous work [11]. The cytotoxicity of PtNDs was tested on HeLa, MCF-7 and MDA-MB-231 cell lines (American Type Culture Collection (ATCC), USA) using PrestoBlue® assay. 10,000 live cells/mL of the cell lines were cultured inside the 96-well plates, supplemented with 200 µL of CM-DMEM (Gibco™, Life Technologies Corporation, USA), and left overnight to allow cell condition recovery before they were introduced with PtNDs. On the next day, the used media was removed and replaced with media containing PtNDs of size 29 nm, 36 nm, 42 nm, or 52 nm, with a maximum concentration of 0.4 mM. All the samples were triplicated. 3 plates of identical set-up were prepared, representing incubation periods of 24 hours, 36 hours, and 72 hours, respectively.

At the end of their incubation period, the used media were removed and replaced with 90 µL of fresh CM-DMEM, and 10 µL of PrestoBlue® Cell Viability Reagent (Fisher Scientific, USA). The plates were incubated again for a few hours to allow adequate reaction of PrestoBlue® reagent. The complete reaction of PrestoBlue® with healthy cell samples was identified through the colour change of the solution from blue to red, signifying that there are viable cells within the sample. Then, the PrestoBlue® reagent fluorescence readings were measured using the Varioskan Flash multimode reader (Thermo Fisher Scientific Incorporated, USA) with the reference and excitation wavelength set at 570 nm and 600 nm, respectively. The data were saved and analysed to give the result of cell survival in the function of PtND size, concentration, incubation time and cell type. In this work, the PtNDs will be deemed to have low toxicity if they can result in 80 % of cell survival and above.

Cellular Uptake Evaluation

The intracellular localization of PtNDs was tested on all three types of cells used earlier (HeLa, MCF-7 and MDA-MB-231 cells). A 22 mm × 22 mm microscope glass slide cover (Waldemar Knittel Glasbearbeitungs GmbH, Germany) was laid at the bottom of each well of a 6-well plate. 10,000 live cells/mL of the cells were seeded in each well and incubated for a few days until they reached about 80 % confluency. 0.1 mM of PtND of different sizes were introduced into their respective well, leaving one well for untreated cells (control). The plates were incubated overnight before they were fixed and stained.

In the cell fixing process, the wells were first cleared of the used media and washed carefully with 1 mL of PBS. Then, 500 µL of cold methanol was added to the cell colonies to fix them at the bottom of the wells and left for about 30 minutes. The methanol was then discarded and replaced with about 4 drops of 0.5 % crystal violet solution (Sigma-Aldrich, USA) for staining purposes, and left again for another hour. After the staining process was complete, the wells were washed gently with tap water to remove the excess staining solution.

The glass slide covers were carefully removed from the wells and left to dry in room condition for a few days. The dried slide covers were mounted onto the microscope glass slides by using the DPX mounting medium (Trajan Scientific Australia Proprietary Limited,

Australia). After the mounting medium was set, the samples were observed using the Olympus BX41 trinocular brightfield microscope (Olympus Corporation, Japan) with 100X magnification.

The cell uptake of PtNDs was assessed by analysing the microscopic images using the "Analyze Particle" macro in Image J open-source software. To do this, firstly the images were thresholded to differentiate the opaque black PtNDs from the cells. Next, a region of interest (ROI) was drawn on a selected cell by encompassing its cytoplasm border. Analyze Particle was performed on the marked cell to obtain the number of particles within the cell, as well as their mean diameter. The particle diameter was referred to the Feret's diameter, defined as the longest distance between two points of the particle's boundary [13]. At least 4 cells per sample were analysed to obtain the average number of particles taken up by the cells and their average sizes.

Results and Discussion

Cellular Toxicity Evaluation

The cellular toxicity of PtNDs was investigated for the PtND concentration, particle size, length of exposure, and the type of cell line used. The result for these parameters is presented in Figure 1. Each data was fitted using the Dose-Response curve in OriginPro 2018 software.

The study on the effect of PtND exposure on different cell types shows that different cells behaved differently upon PtND exposure. Figure 1(a) shows the cytotoxic effect of PtNDs upon three different types of cell lines, namely HeLa, MCF-7 and MDA-MB-231 cells. At low concentrations, there are no notable differences between the curve of all cells. All of them retained more than 90 % viability at PtND concentration below 0.01 mM. The vulnerability of the cells toward PtNDs was observed once the PtND concentration raised beyond 0.01 mM, with HeLa cells showing the most resistance, followed by MCF-7 and MDA-MB-231 cell lines. At 0.1 mM PtND concentration, 88.5 % of HeLa cells remained viable, as opposed to MCF-7 and MDA-MB-231 cells which have a viability of only 77.2 % and 49.1 %, respectively.

Previously, Mohammadi et al. [14] synthesized platinum nanoparticles of an average size of 36 nm with a spheroid shape and tested their cytotoxicity on two different cell lines: MCF-7 and HepG-2 cancer cell lines. Concentration-dependent cytotoxicity was observed in both cells, but the MCF-7 cells showed better resistance toward the treatment as compared to HepG-2 cells. The HepG-2 cells' viability started to be reduced significantly at 2 mg/mL platinum nanoparticle concentration (around 21 mM), but the MCF-7 cells were only significantly affected as the concentration hits 8 mg/mL (around 41 mM). Their platinum nanoparticles have better overall biocompatibility than PtNDs, which may be attributed to the properties of the particles' surface.

Fundamentally, both works highlight the significance of cell type and nanoparticle concentration in cytotoxicity assessment. The varying sensitivity of the cells toward platinum nanoparticles can be attributed to the difference between the protein expressions and the genetic mutations of the cells [15]. The cells that have more protein expression tend to have

better resistance toward drug treatment as the proteins such as proliferating cell nuclear antigen (PCNA) are responsible for regulating cellular proliferation and DNA damage repair.

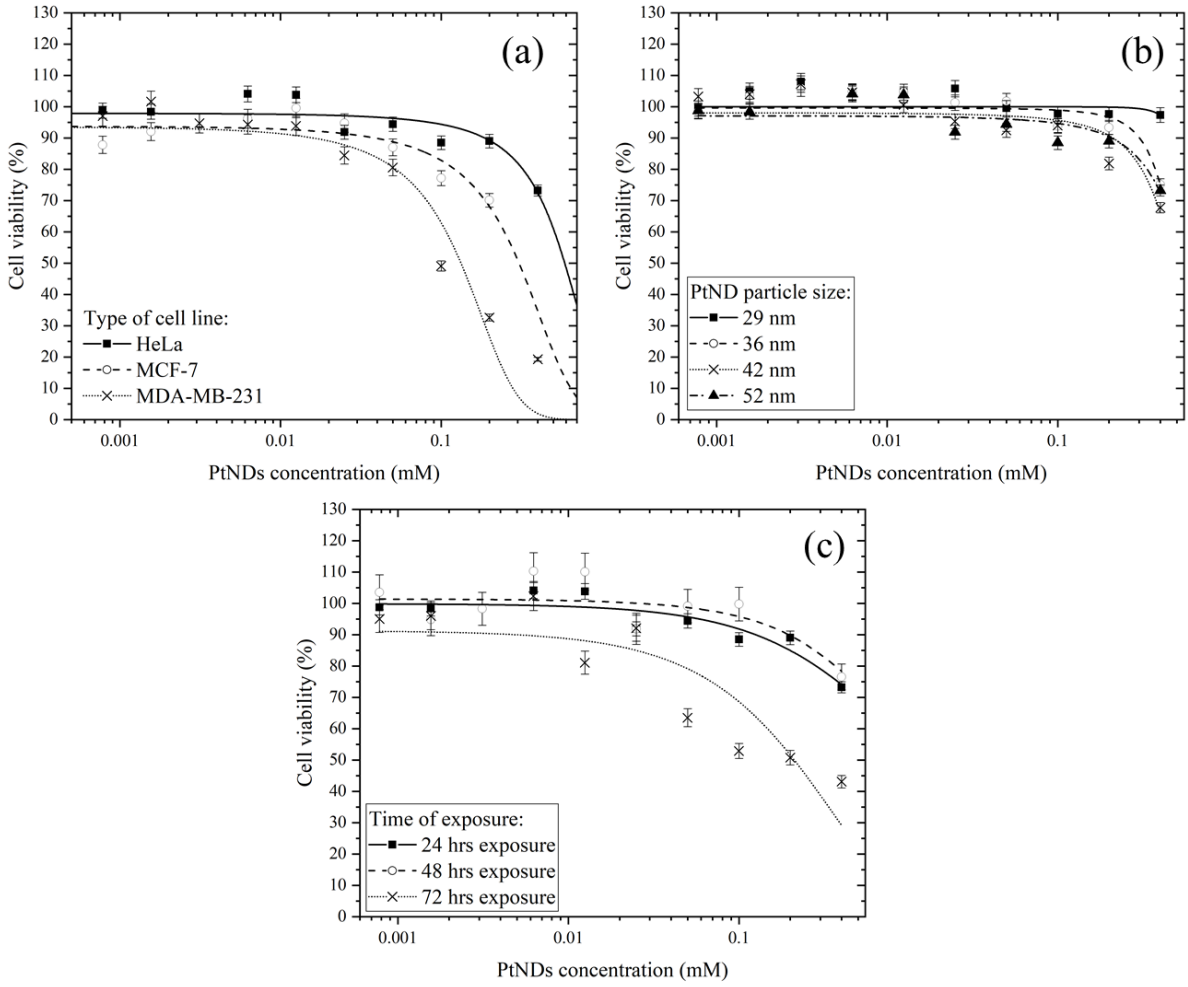

Figure 1: The dose-response curves of PtNDs for the (a) type of cell lines, (b) PtND size, and (c) length of exposure.

The data illustrated in Figure 1(b) represents the correlation between the PtND sizes and their cytotoxicity. While all PtND size depicts very low cytotoxicity at concentrations below 0.1 mM, the effect intensifies once the concentrations pass that threshold, especially for particles of size 36, 42, and 52 nm. At low concentration (0.0 to 0.2 mM), the PtNDs shows low toxicity, with the resulting cell survival of above 80 % for all PtND sizes tested. The trend continues at the higher concentrations for 29 nm PtNDs, where 97.3 % of cells remained viable at the highest PtND concentrations tested. However, 36 nm, 42 nm and 52 nm PtNDs show greater toxicity as the concentration increases, highlighting the influence of size and concentration on PtNDs' toxicity profile.

In other work, human umbilical vein endothelial cells were treated with increasing concentrations of the alginate-coated platinum (Pt@Alg) nanoparticles for 24 hours [16]. The result shows the concentration-dependent effect of the nanoparticles on cells. However, the toxicity at the highest concentration used (400 μg/mL) is still considered acceptable (75% cell viability). This is comparable to our work, where the highest PtND concentration used

only reduced the cell viability to 70%, showing that the platinum nanoparticles can exhibit a good toxicity profile at limited concentrations.

The time duration of PtND exposure on the cells also affected their biocompatibility, as presented in Figure 1(c). The exposure time of up to 48 hours does not significantly change the cell viability. However, after 72 hours, there appears a heightened cytotoxic effect of PtNDs, indicating the possible incompatibility of PtND for long-term exposure in a biological medium. Based on the dose-response curve, 0.05 mM is the maximum concentration that 80% of cells can survive after 3 days. Therefore, it is crucial to adjust the PtND concentration accordingly for an extended incubation period.

Several mechanisms of nanoparticle toxicity have been proposed, which can be categorized into direct or indirect damage. Direct damage involves direct interaction between the nanoparticles and the DNA, while indirect damage can be caused by the formation of reactive oxygen species (ROS), as well as the possible ion contamination emanating from the soluble nanoparticles [17]. The cytotoxicity might also be affected by the cellular uptake mechanisms, which will be discussed further in the next section.

Cellular Uptake Evaluation

The crystal violet staining method was used in this work to evaluate the PtND uptake and localization inside the cells. The PtNDs appear to be opaque black when they were imaged by using bright field microscopy, as demonstrated in Figure 2.

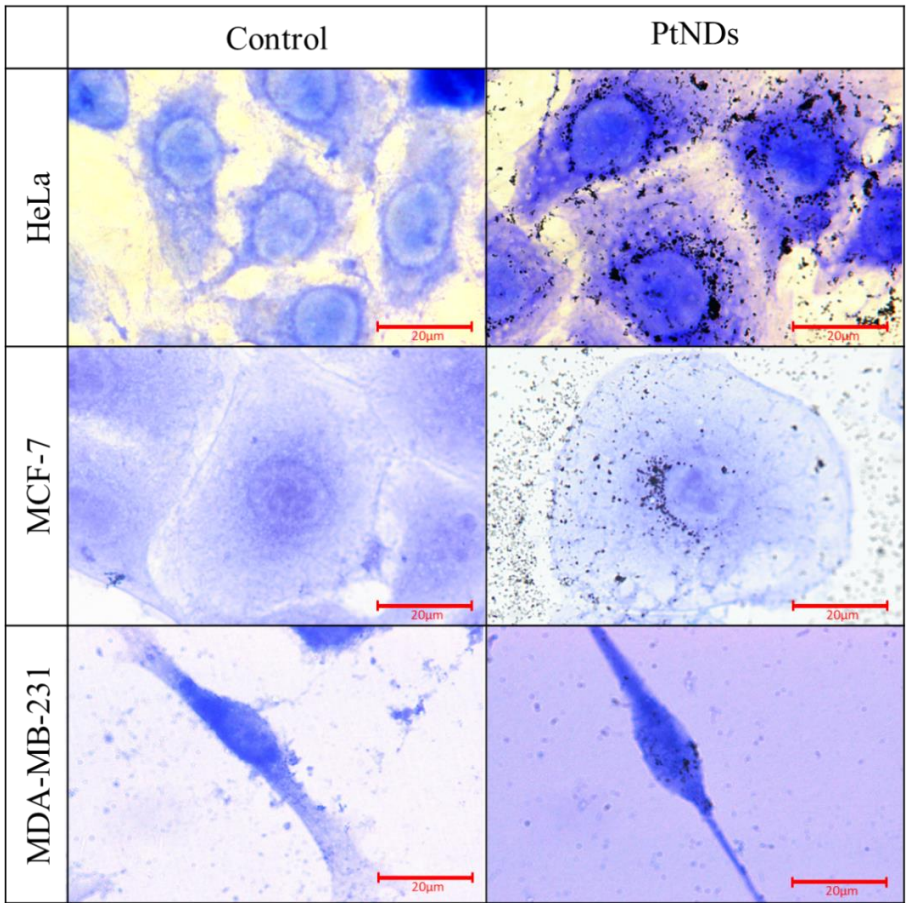

Figure 2: The control and PtND-treated HeLa, MCF-7 and MDA-MB-231 cells

Based on the microscopic images in Figure 2, it is observable that the PtND particles were taken up by the cells. The PtNDs have accumulated mostly in the cytoplasm but not into the cellular nucleus. This is coherent with previous studies, where the nanoparticles were found to be unable to be taken up by the cellular nucleus, but small nanoparticles with dimensions below 10 nm can pass the nuclear membrane through diffusion or transport via the nuclear pore complex [18]. The ImageJ's Analyze Particle was used to assess the cellular PtND uptake by HeLa cells as summarized in Figure 3 and the result is presented in Figure 4.

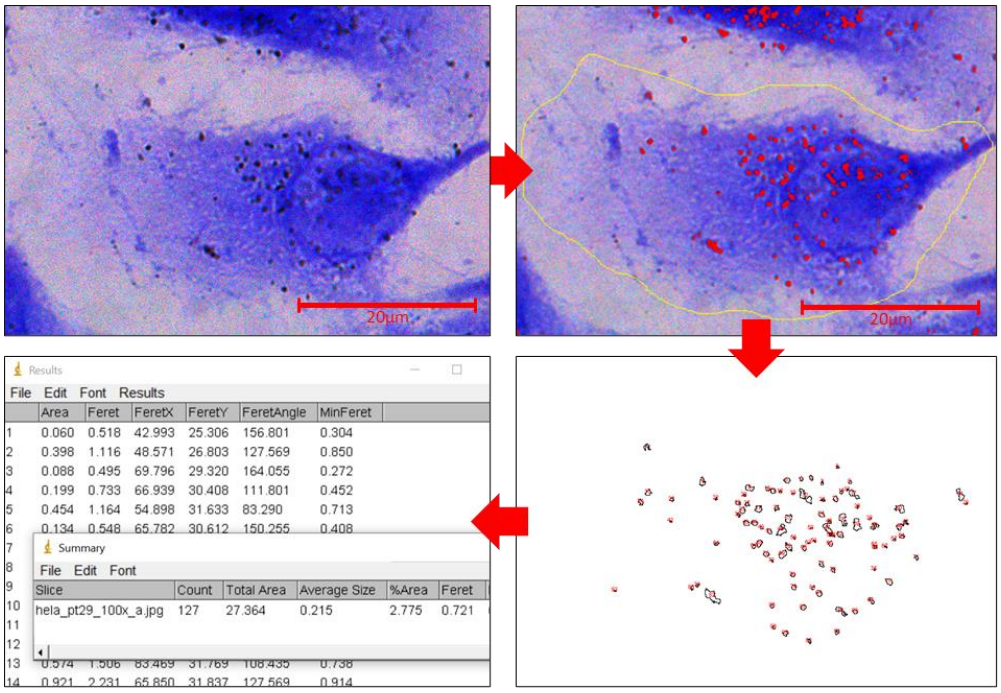

Figure 3: The cellular uptake analysis procedure using the 'Analyze Particle' macro in ImageJ software. The original image (top left) shows PtNDs accumulation in cells. The image was thresholded, resulting in red marks over the PtNDs. The yellow ROI was drawn on the thresholded image (top right). Then, Analyze Particle was performed, giving the results as shown in the bottom images

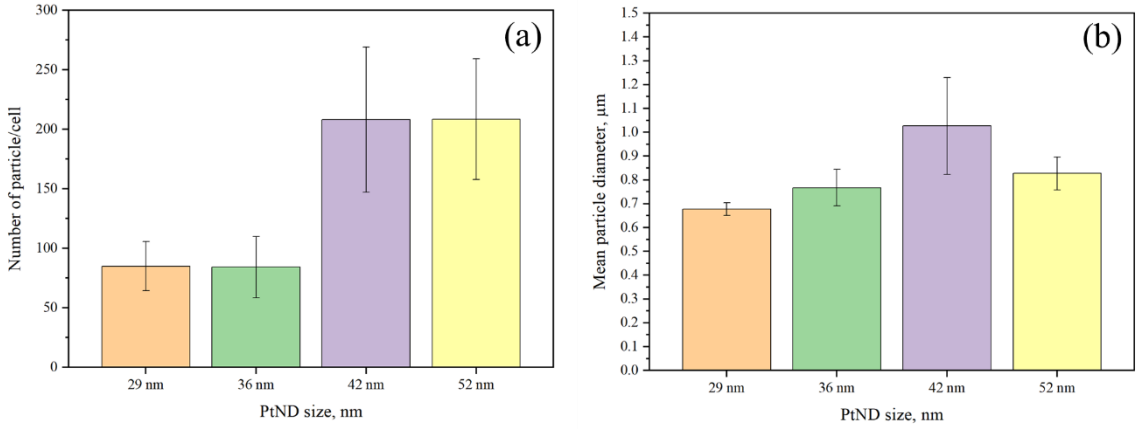

Figure 4: The results of Analyze Particle analysis in ImageJ for the prescribed PtND sizes (a) The number of particles taken up by HeLa cells and (b) the mean particle diameter of PtNDs taken up by HeLa cells

Figure 4(a) shows that the bigger PtNDs of were taken up in greater numbers by the cells compared to the smaller PtNDs. On average, around 208 particles of 42 nm or 52 nm PtNDs were taken up by HeLa cells, which is significantly higher than 29 nm and 36 nm PtNDs, where only around 85 particles were taken up by the cells ($P < 0.001$). No significant difference in the number of particles per cell can be observed between 29 nm and 36 nm PtNDs ($P = 1.000$). The same goes for the comparison between 42 nm and 52 nm PtNDs ($P = 1.000$).

However, the number of particles alone cannot represent the rate of cellular uptake of PtNDs. The particles might have agglomerated, resulting in a smaller number of particles counted, but larger particle sizes on average. Based on Figure 4(b), it is clear that the resulting PtND sizes in cells are significantly higher than their initial sizes before the administration. The particle sizes increment was between 600 and 1200 nm, where the highest particle size increment was portrayed by 42 nm PtNDs (1027 ± 203 nm), followed by 52 nm (827 ± 69 nm), 36 nm (767 ± 76 nm) and 29 nm PtNDs (678 ± 27 nm).

This result signifies that more 42 nm PtNDs were taken up by HeLa cells compared to the other PtND sizes tested. This is in line with the review by Cruje et al. [19], where 50 nm is an optimal nanoparticle size for the best rate of cellular uptake. The mechanisms behind the nanoparticle uptake by cells are mainly governed by the process of endocytosis, or specifically receptor-mediated endocytosis (RME) [20]. In this process, the nanoparticles were coated by ligands or proteins, originating from either pre-coating before the nanoparticle administration to the cells or through the adsorption of the proteins from the cell growth medium onto the surface of the nanoparticles. These proteins bind with the receptors on the cell membrane, causing the membrane to completely wrap around the nanoparticles and form endosomes. The size of nanoparticles plays a major role in this process, followed by the type of cell lines, nanoparticle shape, temperature, and the surface charge was also found to affect the rate of the particles' cellular uptake [19].

It is to be highlighted that this finding is only based on optical analysis. To obtain more accurate results, cellular uptake characterization using spectroscopy procedures is highly recommended. Nevertheless, our result verifies that the PtND particles have agglomerated in the cellular environment, and the rate of agglomeration is dependent on the cell type and the PtND sizes. The characterization of nanoparticle agglomeration state in the biological environment is highly important, as it can affect their cytotoxicity profile and dose delivery. Wills et al. emphasized that the nanoparticle doses administered in in-vitro studies are often overestimated, as most of the particles got agglomerated before even reaching the cells, and only around 1 % of the administered dose managed to reach the cells [21]. This is highly important because the efficacy and biocompatibility of nanomedicine are highly dependent on dose. Therefore, measures such as surface functionalization or coating need to be adopted to improve particle delivery and loading into the targeted cells or tissues.

Essentially, the interaction between the PtNDs and the cells used in this work is comparable with other biocompatibility studies involving platinum nanoparticles, although there are differences in terms of the platinum morphologies, fabrication methods and testing procedures. In any case, further characterization and optimization studies need to be done to improve the PtNDs' specificity and toxicity profile before it can be used as a theranostic agent.

Conclusions

The cytotoxicity of PtNDs was assessed through an *in-vitro* method, where the PtNDs of different sizes and concentrations were introduced into three different types of cell lines (HeLa, MCF-7, and MDA-MB-231). The cytotoxicity evaluations show that the PtNDs of all sizes are cytocompatible for up to 0.1 mM PtND concentration. Beyond that, the PtNDs evidenced size, cell, concentration, and time-dependent cytotoxicity. The cellular uptake study confirmed that all types of cells used in this work were able to internalize the PtNDs. However, the amount of internalized PtNDs was dependent on the type of cell and the size of PtNDs. The PtNDs also appeared to be clustered in the cellular cytoplasm, hence increasing their effective particle size to around 1 μm in the cells. No particles were found in the cellular nucleus, which is parallel to multiple previous works that addressed the nanoparticles' cellular uptake. This study evidenced a promising property of PtND to be used as a theranostic agent in radiotherapy applications.

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Author Contributions

All authors contributed to the data collection, analysis, and critically revising the manuscript. They all accept full responsibility for the whole aspect of the work.

Disclosure of Conflict of Interest

The authors have no disclosures to declare.

Compliance with Ethical Standards

The work is compliant with ethical standards.

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