ASSOCIAÇÃO BRASILEIRA DE ENERGIA NUCLEAR – ABEN
2019 International Nuclear Atlantic Conference - INAC 2019
Santos, SP, Brazil, October 21-25, 2019

Agência Brasileira do ISBN
ISBN 978-85-99141-08-3
9 788599 141083

SYNTHESIS OF PARAMAGNETIC IRON OXIDE NANOPARTICLES FOR APPLICATION IN *IN VITRO* THREE-DIMENSIONAL BIOLOGICAL MODELS THROUGH GAMMA RADIATION AND MICROWAVE REDUCTION OF IRON IONS

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ABSTRACT

Two-dimensional (2D) cell models are extensively used in biomedical research to evaluate the efficacy and safety of new drugs. However, these conventional approaches do not precisely mimic the complexity of the organ microenvironment. To overcome this obstacle, three-dimensional (3D) spheroid cell structures usually referred to as spheroids are being developed to better represent the morphological and functional similarity to the tissues. Among several techniques currently employed to produce three-dimensional cell cultures, one of the most promising is the magnetic levitation, which consists of the magnetization of the cells through adsorption of magnetic nanoparticles of iron oxide (Fe_3O_4), which are produced by the reaction of Fe^{2+} and Fe^{3+} ions in alkaline medium. This work produced paramagnetic iron oxide nanoparticles (PIONs) by coprecipitation from an Fe^{2+} source. The reduction to Fe^{3+} was obtained by the ionization caused by gamma radiation (^{60}Co) at 15 or 30 kGy radiation absorbed doses. After functionalization with poly-lysine, the nanoparticle suspensions were characterized by XRD, FTIR, zeta potential analysis, DLS and TEM which showed the successful attachment of the carboxylate groups to iron, explaining the ability of the particles to be adsorbed by the membranes. Biological assays showed that these PIONs were biocompatible and efficiently could be applied to develop prostate 3D tumor spheroids model for drug screening.

1. INTRODUCTION

Cell culture is an important tool used during the preclinical stages of safety and efficacy of new drugs. Especially in the development of cancer therapy, where the discovery of new molecular targets that affect metabolism and cell proliferation is most easily evaluated *in in vitro* cell culture models compared to *in vivo* models [1].

Traditionally, monolayer cell culture models (2D) provide important information about the structure and cellular function for studies of cancer biology and therapy. Even though this model is robust and well established, the 2D conventional model is very limited to reproduce the complexity organizational and heterogeneity *in vivo* tumors that grow in a three-dimensional (3D) network architecture [2].

3D cell culture allows the expression of components of the extracellular matrix (ECM), as well as greater interaction cell-cell and cell-matrix. In addition, the cells grow in a 3D environment with heterogeneous cell zones and gradients of nutrients, oxygen and cell proliferation that reflect more accurately the microenvironment *in vivo*. Therefore, the cultivation of cells in 3D is considered a model more representative of the actual physiology of the tumor microenvironment. These characteristics allow a better understanding of the molecular and cellular mechanisms of metabolism and toxicity screening of new drugs for cancer therapy [3].

This can be subdivided into scaffold-based or liquid-based and 3D-models. Tumor spheroids are one of the most versatile models are scaffold-free for 3D cell culture [4]. According to JAROCKYTE *et al*. (2018) [5], this model is considered a simple and low-cost approach and shows results with high pathophysiological similarity to the results of *in vivo* models during drug efficacy tests in cancer therapy.

Spheroids can be self-assembled through magnetic levitation or bioprinting cell culture in a cell-repellent surface for culturing in 3D. Both techniques employ the magnetization of cells by the application of paramagnetic iron oxide nanoparticles of iron oxide (PIONs) which electrostatically attached to the cell membranes. In this way, cells can be aggregated by the application of magnetic forces, either by levitation or bioprinting, to form three-dimensional structures in a few hours [6].

There are several methods for the synthesis of PIONs such as co-precipitation and thermal decomposition [7]. In addition, microwave irradiation [8] and ionizing irradiation (gamma irradiation) [9] are promising techniques that can favor the control of the size and shape of the nanoparticles in order to form tumor spheroids.

In this study, we synthesized PIONs by microwave coprecipitation and gamma radiation (^{60}Co) at 15 or 30 kGy radiation absorbed doses. Also, we aimed at characterizing the structural and morphological properties of PIONs and the we evaluated their biological application in self-assembled human prostate cancer 3D cellular spheroids.

2. MATERIALS AND METHODS

2.1. Materials

All chemicals used were of analytical quality and were purchased from Sigma-Aldrich® (USA). All solutions were freshly prepared before each experiment with water purified using a ultrapure water system (PURELAB® Option-Q, Elga LabWater, UK) with a resistivity of 18.2 MΩ.cm at 25°C. The water was deoxygenated by purging with nitrogen bubbling for 30 minutes before solutions were prepared.

2.2. Synthesis of PIONs

PIONs were synthesized according to the microwave co-precipitation method previously described by BOMFIM *et al*. (2019) [8]. Briefly, $Fe_2SO_4.7H_2O$ (0.905 g) and glycine (0.439 g) were mixed in ultrapure water (100 mL) and solution was slowly stirred. Next, NaOH (2 M)

was added dropwise under constant stirring until pH 12. The mixture was heated using a microwave oven (930 W) for 150 seconds. The reaction was stopped and black precipitate settled down at the bottom.

The black precipitate was washed 5 times with water. After last wash, the precipitate was resuspended in acetic acid (15 mL) and dispersed in an ultrasonic bath for 5 minutes. After this time, the PIONs were separated using an external magnet and acetic acid was discarded. The nanoparticles were slowly dispersed in poly-L-lysine solution (0.02 μg/mL, pH 7) in an ultrasonic bath for 5 minutes. Finally, PIONs were washed in sterile ultrapure water and stored at 4°C for further uses.

Aditionally, PIONs were also synthesized by gamma radiation. After the microwave oven step, the PIONs were were exposed to gamma radiation at dose rate of 3 kGy/h to reach the dose of 15 and 30 kGy using a ^{60}Co as radioactive source in the Multipurpose Gamma Irradiation Facility in the Center of Radiation Technology at the Nuclear and Energy Research Institute (IPEN) (São Paulo, BR). Afterwards, it was followed the same procedure as previously described.

2.3. Characterization of the nanoparticles

The crystal structure of the nanoparticles was characterized using X-ray diffractometry (XRD) performed at a Rigaku Miniflex II diffractometer (USA) using a graphite monochromator with Cu Kα radiation source (λ = 1.5418 Å) The polycrystalline sample patterns were scanned continuously in 2θ between 15 and 60 with a step of 0.02 and 1 s of integration time per point. The samples were prepared on a glass sample holder and measured at room temperature at the Center of Chemistry and Environment (IPEN). Peak patterns were analyzes using the QualX software [10]. The crystal size was calculated from the X-ray line broadening analysis using the Debye-Scherrer equation, $D = 0.9\lambda/\beta\cos\theta$, where D is the crystal size in nm, and λ is the X-ray wavelength, β is the half-width of the peak in rad, and θ is the corresponding diffraction angle.

Fourier transform infrared spectroscopy (FTIR) spectra were recorded in the range 400 - 4.000 cm^{-1} on a JASCO FTIR 4100 spectrophotometer (Japan) with the samples embedded in KBr pellets at the Center of Instrumentation for Research and Teaching for Federal University of São Paulo (UNIFESP) *Campus* Diadema.

The average hydrodynamic size, size uniformity and zeta (ζ) potential of PIONs were performed by using dynamic light scattering (DLS) (Litesizer^{TM} 500, Anton Paar GmbH, Austria) at the Center of Radiation Technology (IPEN). Before analyzing, the PIONs were dissolved in ultrapure water using bath sonication. The analysis was completed in triplicate at ambient temperature. The structural characteristics and average particle sizes were determined using transmission electron microscopy (TEM) by a JEM 2100 (JEOL) operated at 100 k at the Center of Materials Science and Technology (IPEN). Samples for TEM studies were prepared by placing a drop of the PIONs on a carbon-copper TEM grid.

2.3. Cell cultures

Human prostate cancer cells (LNCaP) obtained from American Type Culture Collection (ATCC® CRL-1740™) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco™,USA) supplemented with 10% fetal bovine serum (FBS) (Gibco™, USA) and 1% antibiotic solution (10,000 UI/mL penicillin and 10 mg/mL streptomycin) (Sigma-Aldrich®, USA) and maintained in a humidified 5% CO_2 incubator at 37°C in 25 or 75 cm^2 flasks with medium exchanged every other day and then sub-cultured at 80% confluence.

2.4. Cell viability assays

The analysis of the cytotoxicity of PIONs in LNCaP cells was performed using ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt)) (MTS) (CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay, Promega Corporation, USA) and phenazine methosulfate (PMS) Sigma-Aldrich®, USA) dye. 10×10^3 cells were seeded in 100 μL of media in the wells of a 96-well cell-repellent microplates (CELLSTAR® Greiner Bio-One, DE) and incubated for 24 hours before the nanoparticles were applied.

In the following day, the old medium was replaced with fresh medium containing various diluitions of PIONs (0.05, 0.1, 0.2, 0.4 and 0.8% (v/v)) and incubated for 24 hours. 200 mg/mL of latex extract solution was used as a positive control media alone without nanoparticles was a negative control. After, 20 μL/well of combined MTS/PMS solution (100 μL of PMS solution to the 2 mL of MTS solution) were added into each well of the 96-well assay plate containing the samples and again incubated for 2 hours. Cell viabilities were determined by measuring the absorbance at 490 nm using a Microplate reader (Multiskan™ spectrophotometer, Thermo Scientific™, FI).

The experiment was performed in quadruplicate. After obtaining values of absorbance, they were recalculated as percentage values of viability. Absorbance value of control group was equated to 100% and the rest of the values were calculated proportionally to control.

2.5. Spheroid formation assay

LNCaP cells grown in 2D were statically incubated overnight with PIONS, allowing for nanoparticle association with the cells at a concentration of 15 μL/2 x 10^6 cells. Once attached onto the membrane, the nanoparticles remain for 24 hours. In the following day, the cells were enzymatically detached with 0.25% Trypsin-EDTA solution (Gibco™, USA) resuspended in media, then distributed at a concentration of 50 x 10^3 cells/well into 24-well microplate covered with 1% (w/v) agarose gel. A magnetic drive was then placed bottom at the plate where cells interacted to form spheroids by bioprinting. After 6 days culture, the spheroids were formed and ready to be used.

2.6. Fluorescent staining of spheroids

After spheroid formation for 4 days, fluorescent staining was performed to assess cell morphological organization within the spheroids. Throughout staining, spheroids were anchored to the bottom of the well plate using the magnetic drive. Spheroids were first gently washed with PBS (pH 7.4). After which, a solution containing nucleic acid stain acridine orange (100 μg/mL), dead cells stain propidium iodide (50 μg/mL) (Invitrogen™, USA) was added to each well to incubate for 40 minutes. The spheroids were then washed again with PBS (pH 7.4) and imaged under a fluorescent microscope.

2.7. Statistical analysis

The results were expressed as means ± standard deviation (SD) or standard error of the mean (SEM). The statistical significance of the differences was determined using one-way ANOVA followed by Bonferroni post-hoc test. The comparisons were considered statistically significant if $p<0.05$ and very significant if $p<0.001$ using GraphPad Prism 7.0 software (GraphPad Software, USA).

3. RESULTS AND DISCUSSION

After the reaction, the black precipitates of PIONs were characterized to confirm the success of the synthesis. The XRD patterns of the PIONs synthesized only by microwave (MW), associated or not with gamma radiation 15 kGy and 30 kGy absorbed doses were basically the same and demonstrate monoclinic crystalline nature of PIONs as demonstrated in Figure 1.

The XRDs were characterized by the typical reflections (1 1 1), (2 2 0), (3 1 1), (4 0 0), (4 2 2), (5 1 1), (4 4 0), (6 2 0) and (5 3 3). Those reflections are the fingerprints of the magnetite (Fe_3O_4), which is the structure of the PIONs and they are in accordance with the data reported in the literature [11].

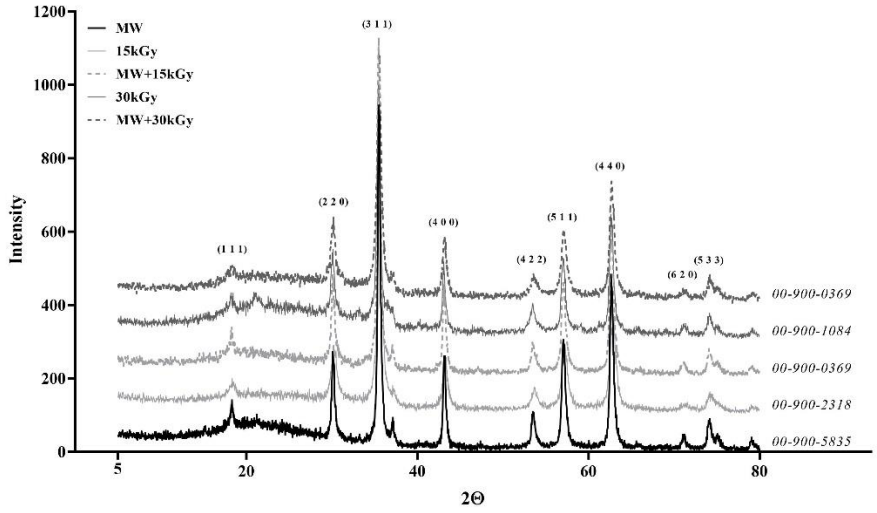

Figure 1: XRD pattern of nanoparticles obtained through different synthesis methods. Abbreviation: a.u: arbitrary unit; MW: microwave. Values on top of peaks indicate the crystalline planes. Numeric codes on right of spectra: card numbers of compounds.

The Mean Crystallite Size as computed as Debye-Scherrer equation value of MW, 15 kGy, MW+15 kGy, 30 kGy and MW+30 kGy synthesis was calculated to be 41.7, 53.0, 54.2, 49.9 and 51.0 nm, respectively (Figure 2).

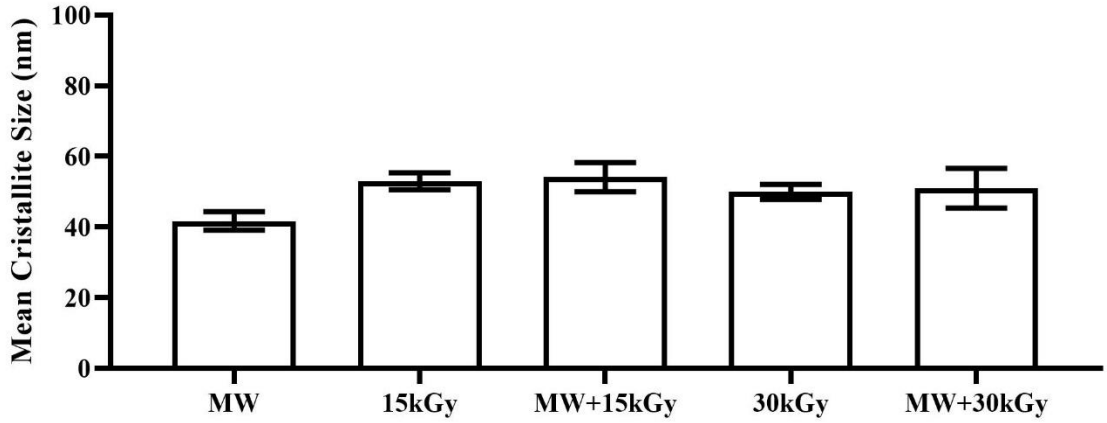

Figure 2: Mean Crystallite Size (nm) of PIONs synthetized by MW and gamma radiation obtained by Debye-Scherrer equation. Abbreviation: MW: microwave. The data represent the means ± standard error of the mean. There were no signicant differences in the size of PIONs in comparison with MW synthesis condition.

The FTIR spectrum for the five types of PIONs are shown in Figure 3. Characteristic Fe–O stretching can be observed at 585 cm^{-1}. This peak indicates the formation of magnetite nanoparticles. The absorption peaks at 3500 and 1600 cm^{-1} are attributed to stretching of the N-H and C=O amide groups, respectively.

Glycine and polylysine have the amine (NH_2) functional groups at 3500 cm^{-1} and carboxyl (–COOH) at 1600 cm^{-1} [12]. The presence of group NH_2 (~ 3500 cm^{-1}) is observed for all samples. These bands indicate the presence of predicted functional groups of glycine and polylysine strong binding on surface of the iron nanoparticles according to BOMFIM *et al*. (2019) [8].

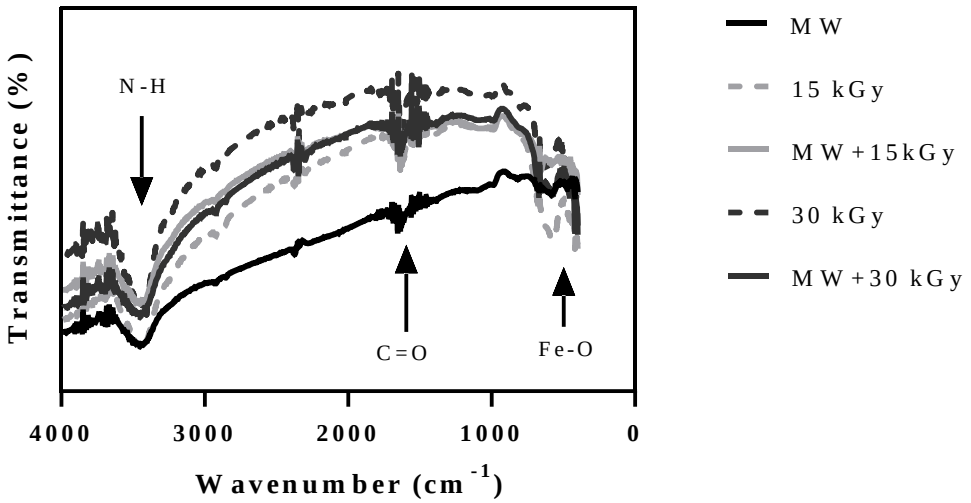

Figure 3: FTIR of PIONs. Abbreviation: MW: microwave.

The hydrodynamic size and zeta potentials of the five types of nanoparticles investigated with DLS analysis and zeta potential analyser, respectively, are presented in Figure 4 and Figure 5 The nanoparticles formulated in this study were found to be in the size range of 100-1000 nm. It is important to notice that PIONs synthesized by microwave followed by 30 kGy presented as monodisperse and with greater abundance compared to the others, which were polydisperse and with lower relative frequency in percentage.

This technique is very sensitive to evaluate dynamic aggregation and agglomeration and this relatively large size can be attributed to a high tendency in formation of clusters composed by multiple particles entrapped inside a biopolymer matrix due to Van der Waals inter particle attractions in aqueous solution as described by VIOTA *et al*. (2013) [13]. However, it is important to note that the nanoparticles were stable in the dispersion state, possessing high absolute values of zeta potential and having positive surface charges with range of 30-40 mV.

Higher absolute value of zeta potential means that nanoparticles colloidal system will be more stable. According to JUSTIN *et al*. (2018) [14], nanoparticles with zeta potential values greater than +25 mV or less than -25 mV are more stable. Besides that, positively charged nanoparticles have been shown to improve the efficacy being electrostatically attracted to the negatively charged cell membrane in order to form cell spheroids [8].

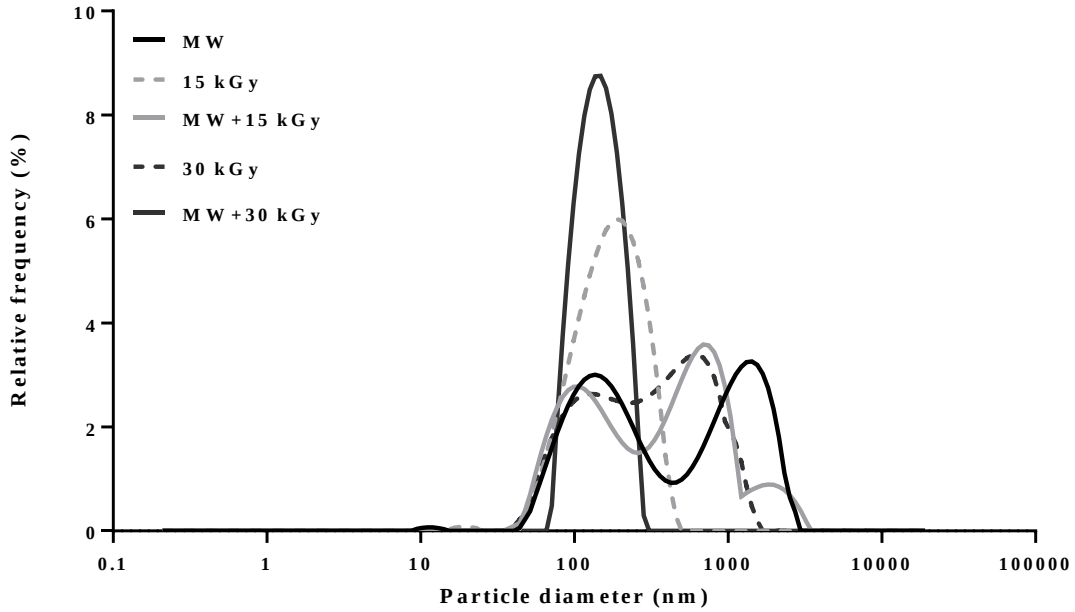

Figure 4: Hydrodynamic size distribution of PIONs. Abbreviation: MW: microwave.

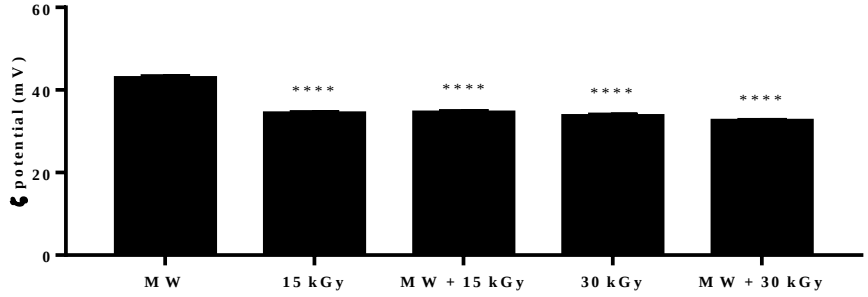

Figure 5: Zeta potential measurements of PIONs in aqueous suspension. Abbreviation: MW: microwave. The data represent the means ± standard error of means (**p<0.001 compared to the MW followed by One-way ANOVA Bonferroni post-hoc test).**

To investigate the morphological characteristics of nanoparticles, TEM analysis was carried out. Figure 6 shows the photographs of PIONs functionalized poly-L-Lysine around the iron oxide core. It could be observed that most of the synthesized nanoparticles had a quasi-cubic shape and the particle size was below 100 nm as previously described [8].

Also, the image suggests that the nanoparticles are dispersed well enough. No differences can be observed in shapes between particles obtained through syntheses with or without gamma irradiation. The average particle size of PIONs was found to be 60 nm which was nearly same as calculated using Debye-Scherrer equation X-ray diffraction.

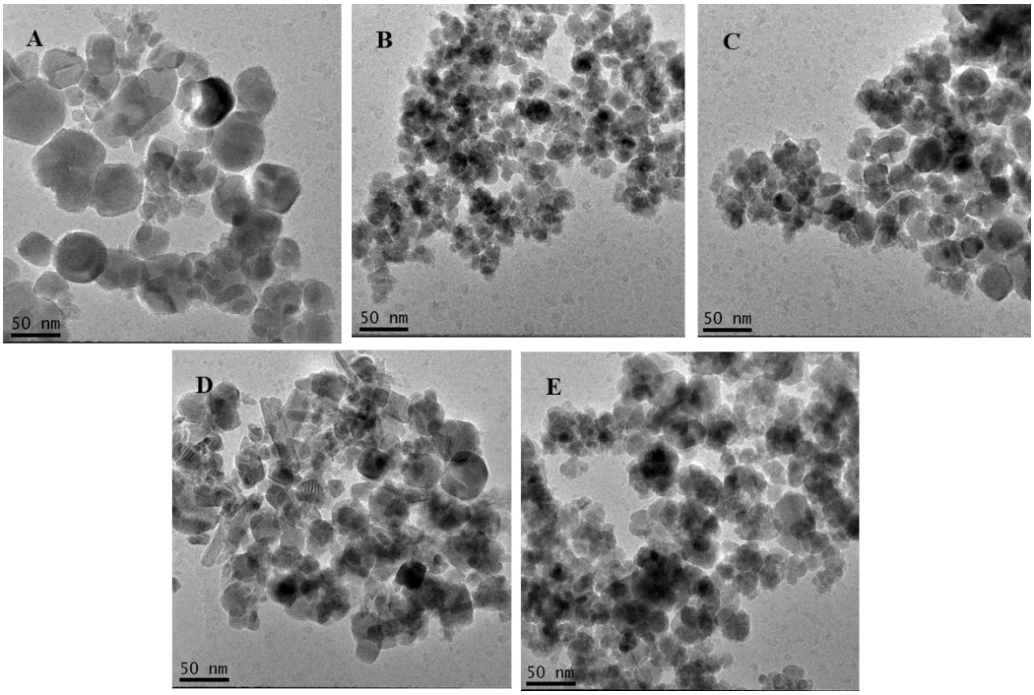

Figure 6: TEM images of PIONs. A: MW; B: 15 kGy; C: MW+15 kGy; D: 30 kGy; E: MW+30 kGy. Abbreviation: MW: microwave.

The toxicity of PIONs was studied in a prostate tumor cell line (LNCaP) as reference in *in vitro* models during pre-clinical trials in biomedical research [15]. Even though metallic nanoparticles can induce the generation of reactive oxygen species capable of damaging cellular respiration and causing damage to DNA, RNA and various structural components of the cell [16].

Figure 7 shows that PIONs synthesized by microwave or by radiation have no significant cytotoxic effects against prostate cancer LNCaP cells at all concentrations tested. Even the highest concentration at 0.8% (v/v) had no significant effect on cell metabolism. The absence of cytotoxic effects of all PIONs concentrations used in this study was in concordance with findings from previous studies using biocompatible iron oxides [8, 17].

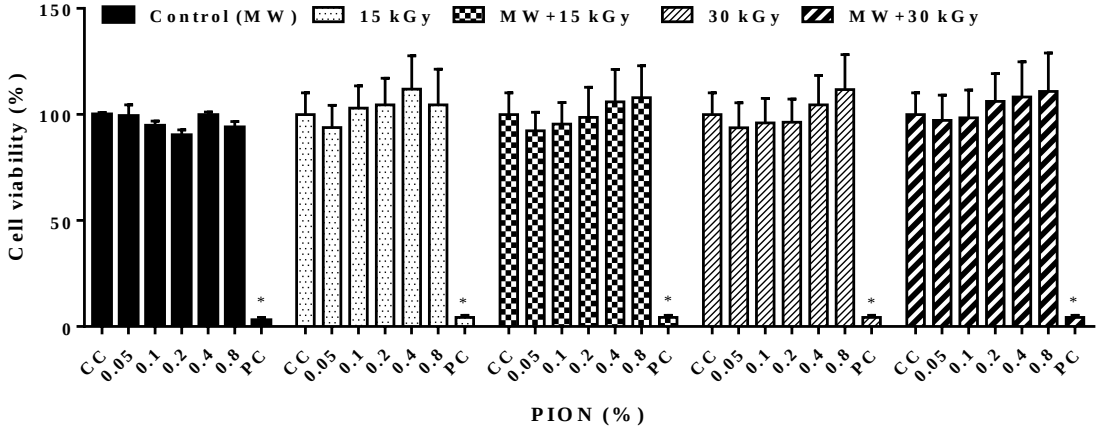

Figure 7: LNCaP cell viability after exposure to a concentration series of PIONs synthetized by microwave (MW) and gamma radiation (15 or 30 kGy) for 24 h using the MTS/PMS viability assay. Viability is given as a percentage of the control (% ± SEM; n = 4; *p<0.05 compared to the CC followed by One-way ANOVA Bonferroni post-hoc test). Abbreviation: CC: Cell Control; PC: Positive Control - latex extract solution.

Additionally, it was demonstrated that PIONs can be successfully used to develop cell spheroids (Figure 8). The surface charge of magnetite nanoparticles exerts a decisive influence on ionic interactions and the formation interaction. These nanoparticles can suitable magnetize cells by electrostatically attaching to cell membranes mainly due to the presence of cationic poly-lysine, which functions PION allowing enabling rapid formation of self-assembled spheroids [18].

Interestingly, PIONs synthesized by microwave and by radiation were able to promote the formation of homogeneous spheroids. These spheroids tended to assume a design more spherical-like design structurally and biologically representative 3D models *in vitro* mimicking more closely the complex cellular heterogeneity and interactions and tumor microenvironmental conditions [5].

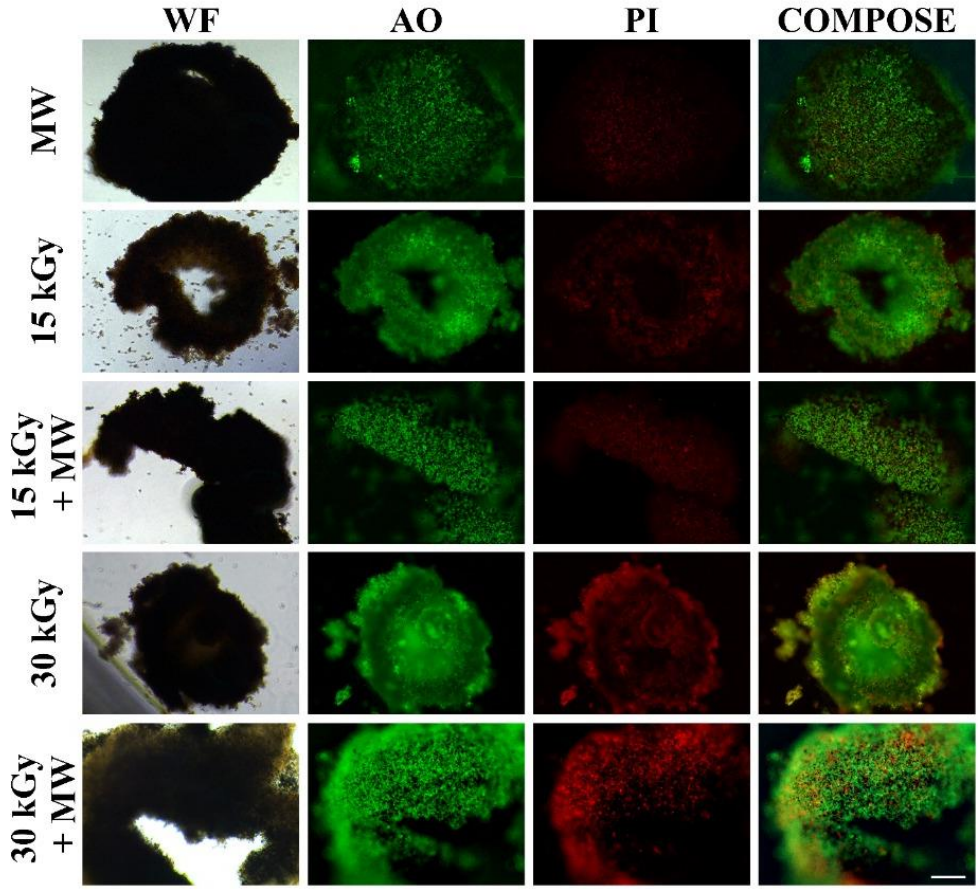

Figure 8: Formation of 3D LNCaP spheroids after 4 days of culturing. spheroids were stained with a viability cell staining acridine Orange (AO) (100 µg/mL) (green) and death cell staining propidium iodide (PI) (50 µg/mL) (red) Scale bar = 200 µm. Abbreviation: MW: microwave.

4. CONCLUSION

In summary, the results described here demonstrate that PIONs synthesized by microwaves or by gamma radiation did not present significant physicochemical and morphological differences. Besides that, these PIONs were considered biocompatible and facilitated a rapid 3D prostate cancer culture formation. Overall, magnetic levitation could be considered an easy tool to create native tissue environments *in vitro* for biomedical applications.

ACKNOWLEDGEMENTS

The authors would like to thank Professor Maria Claudia França da Cunha Felinto from the Center of Chemistry and Environment at Energy and Nuclear Research Institute for the support.

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