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

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ABSTRACT

Three-dimensional (3D) cell culture is increasingly being used in assays to assess the safety and efficacy of new drug candidates. Tumor cell spheroids can mimic with high precision the biological complexity of cellular interactions with their tumor microenvironment. Currently, several techniques can be used to construct 3D spheroids. Among them, magnetic levitation is one of the most used in biomedical research. This technique consists in the magnetization of cells through the adsorption of magnetic nanoparticles of iron oxide (Fe_3O_4) that are produced by the reaction of Fe^{2+} and Fe^{3+} ions in alkaline medium. In this work, nanoparticles of paramagnetic iron oxide (PIONS) were synthesized by coprecipitation through electron beam irradiation at 15 and 30 kGy doses. After functionalization with polar amino acids, nanoparticle suspensions were characterized by physical-chemical assays that showed the successful attachment of the carboxylate groups to the iron, explaining the ability of the particles to adsorb the membranes. Cytotoxicity assay showed that the nanoparticles synthesized by microwave (MW) and electron beam had no toxicity. Others biological assays have also shown efficient adsorption of the particles by human prostate tumor cells, allowing the *in vitro* application of a biomimetic 3D biological model with potential utilization regarding the development and evaluation of antitumor drugs and radiopharmaceuticals for the treatment of prostate cancer.

1. INTRODUCTION

Studies with 3D cell culture have been increasing for several applications in the pharmaceutical and biomedical areas, as for toxicity and drug activity evaluation [1]. Although 2D cell culture is still widely used and provides good results for pharmacological *in vitro* assays, its efficiency has been questioned because the cellular microenvironment is far from the *in vivo* environment, motivating the development of efficient 3D tissue models for testing [1, 2].

When it comes to the metastatic 2D tumor cells culture there are still more limitations, as these cells do not form narrow adhesions and as a consequence are more difficult to culture on conventional plates, turning the drug screening process more limited [3, 4]. Thus, the cell culture models in 3D became promising, since it allow the cell growth in suspension, forming spheroids by the appropriate interaction between cells and extracellular matrix (ECM) production [5].

The cell culture by magnetic levitation is one of the techniques used in the construction of spheroids. It consists of the adsorption of magnetic nanoparticles to the membrane of the cells that, when exposed to a magnetic field, allows cellular aggregation and consequent EMC formation [6]. These nanoparticles have a paramagnetic characteristic, which consists of a response behavior to a magnetic field when in the presence of a magnet. They are synthesized mostly from divalent iron sources for the formation of iron oxide (Fe_3O_4) by co-precipitation.

Paramagnetic iron oxide nanoparticles (PIONS) are the most studied, due to their biocompatibility with biological systems, but they need to be functionalized with polymers, amino acids, silica or other compounds to avoid nonspecific absorption of undesirable species and to the adequate response of the nanoparticles [7].

The purpose of this work is to compare paramagnetic iron oxide nanoparticles synthesized by microwave (MW) and electron beam irradiation in order to find the best structure for the construction of *in vitro* tumor spheroids. The process of synthesis of magnetic nanoparticles by microwave is a simple and already used process for coprecipitation, as well as processes induced by electron beam irradiation. The latter technique is a rapid, temperature independent process, without strict control of experimental conditions and high purity of the material, with the potential to obtain a more restricted particle size distribution [6, 8].

2. MATERIAL AND METHODS

2.1. Synthesis of iron nanoparticles by microwave

The nanoparticles were synthesized from the dissolution of iron sulphate II heptahydrate ($Fe_2SO_4.7H_2O$, Sigma-Aldrich) and Glycine ($C_2H_5NO_2$, Sigma-Aldrich) in ultrapure water deoxygenated by the addition of nitrogen gas (N_2). Under stirring, 2M NaOH was slowly added until pH 12 was obtained. The solution was then heated in a domestic microwave oven (930W) for 2 minutes and 30 seconds.

After heating, the solution was washed with ultrapure water with the aid of a magnet until pH ~7 and for removal of possible crystals of sodium sulfate formed in the reaction. After washing, 15 mL of acetic acid was added and the solution was dispersed in a ultrasonic bath for 5 minutes. After this time the acetic acid was withdrawn with the aid of a magnet and 0.02 μg / ml poly-L-lysine hydrobromide (Sigma-Aldrich) solution added. poly-L-lysine hydrobromide was prepared in ultrapure water at pH 7 and slowly added to the nanoparticles under ultrasonic dispersion. After this process the solution was withdrawn with the aid of a magnet and 2mL of sterile ultrapure water was added for storage in the refrigerator until use.

2.2. Synthesis of nanoparticles by electron beam irradiation

The synthesis procedure was followed as for the synthesis microwave nanoparticles. Electron beam irradiation was done after microwave heating and after pH adjustment to 12 without microwaves. The irradiation was done in the electron accelerator of the Institute of Energy and Nuclear Research (IPEN) at 15 and 30 kGy for both conditions under the radiation rate of 3 kGy/hr.

2.3. X-Ray Diffraction (XRD)

To prepare the sample for this assay an aliquot of PIONS solution was transferred to a glass vial, the water removed with the aid of a magnet and ethyl alcohol added. Thereafter the alcohol was withdrawn and the vial with the nanoparticles kept opened overnight drying at approximately 45°C. After drying, the experiment was carried out in the Orion diffractometer, model RKS-400SV-R (Capacity of 0.40 / 0.49kW, Current of 10.0 / 10.5A) at the Multi-user Center of the Chemistry and Environment Center (CQMA), IPEN / CNEN-SP. Peak patterns were compared to COD database using the QualX software [9]. The crystallite size was calculated 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. Statistical analysis was performed by One-way ANOVA followed by Bonferroni's multiple comparisons test.

2.4. Dinamic Light Scattering (DLS) and Zeta Potential

The samples were analyzed in solution by diluting the PIONS solution prepared in ultrapure water. Quartz cuvettes were used to evaluate the DLS and omega cuvettes used to evaluate the Zeta Potential. The assay was performed in the Particle Analyzer Litesizer 500 of Anton Paar, multi-user equipment of the Radiopharmacy Center, IPEN / CNEN-SP. Three analysis per sample were performed for DLS and single read analyzes for Zeta Potential. Statistical analysis for Zeta Potential was performed One-way ANOVA followed by Bonferroni's multiple comparisons test.

2.5. Fourier Transform Infrared Spectroscopy (FT-IR)

For this assay an aliquot was transferred to a microscope slide and it kept in a oven overnight at approximately 45°C. After drying the nanoparticles were evaluated in the Shimadzu IRPrestige-21 equipment at the Instrumentation Center for Research and Teaching of the Federal University of São Paulo (UNIFESP-Campus Diadema).

2.6. Cell culture

The cell line used was the LNCaP clone FGC (ATCC® CRL-1740™). For culture of this strain, RPMI-1640 culture medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin and streptomycin were used. Cells were grown under aseptic conditions, incubated at 37°C and 5% CO_2 under humid atmosphere and in 25 cm^2 culture bottles until the cells reached 80% sub confluence. For maintenance of cell culture and conduction of the experiments, the cells were removed from the culture bottles by the action of the trypsin-EDTA solution for 5 minutes. All experiments were run with cells up to 20 consecutive passages.

2.7. Adsorption to cell culture

To evaluate the adsorption of the nanoparticles, the cells were maintained in culture with the nanoparticles overnight at 37°C and 5% CO_2. The cells were maintained in culture until reaching the subconfluence of 80% of the bottle, then were trypsinized and the nanoparticles added to the cellular suspension. The suspension was homogenized to ensure better adsorption of the nanoparticles to the cell membrane and transferred to a 25 cm^2 culture bottle. The ratio of 15 μL of the nanoparticle solution to approximately 2×10^6 cells was incubated. After incubation the cells washed with PBS without EDTA, fixed with pure methanol and a 1:1 solution of 2% potassium ferrocyanide and 6% Hydrochloric Acid (HCl) added. The solution was held in contact with the cells for 20 minutes. The blue coloration of Prussia blue was obtained when in the presence of iron. The cells were photographed under an optical microscope Nikon Eclipse Ts100.

2.8. Nanoparticle cytotoxicity assay

The cells were trypsinized as described and seeded in conventional 96-well cell culture plates. 10^5 cells/mL were grown in 100 μL per well. The plate with the cells was incubated for approximately 24 hours at 37°C and 5% CO_2. After incubation the nanoparticle samples were added in 5 different proportions, based on the volume of nanoparticles standardized for use. The proportions of 8, 4, 2, 1 and 0.5% were used from the total volume obtained in the synthesis. A blank of the samples was also added to remove any interference from the nanoparticles in absorbance measure.

A negative control (non-exposed cells) and natural latex at 0.2 g/mL as a positive control was used. Positive control was prepared by incubating the latex in RPMI 1640 medium for approximately 24 hours. After incubation the latex extract was filtered on a 0.20 μm syringe filter. 100 μL of each sample was added over the cells to each well. The plate was then incubated for approximately 24 hours at 37°C and 5% CO_2.

After incubation the culture medium was removed from plates, the wells washed with PBS without EDTA and MTS solution (CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay, Promega) at 2 mg/mL in DPBS and PMS (phenazine methosulfate, Sigma-Aldrich) at 0.92 mg/mL in DPBS added, in the ratio 20:1 in RPMI-1640, as the Promega protocol. After 2 hours of incubation, as described, the absorbance reading was performed by spectrophotometry in Multiskan spectrophotometer (Thermo) equipment at the wavelength of 490 nm. Results were given as percentages of absorbance means values of cell controls. Statistical analysis performed by 2way-ANOVA followed by Bonferroni's multiple comparisons test.

2.9. Spheroids construction

After incubation, the cells with the nanoparticles were trypsinized and seeded in 96-well plates with repellency (Greiner Bio-One, Germany). 5 x 10^5 cells/ml were seeded in 100 µL per well. During plating the cells, a 96-magnet spheroid drive from Greiner Bio-one was maintained under the culture plate. The plate was incubated as described and maintained in culture for 6 days with medium exchanges every 3 days. On the 6th day the spheroids were stained with Propidium Iodide (50 µg/mL) and Hoechst (5 µg/mL) in culture medium incubated at 37°C and 5% CO_2 for 40 minutes. After incubation the spheroids were photographed under a Nikon Eclipse Ts100 fluorescence optical microscope.

3. RESULTS

3.1. X-Ray Diffraction (XRD)

After correction of the background and the narrowing of the search for mainly Fe and O compounds, the software attributed distinct peaks, which could be found as characteristics of a monoclinic structure compatible with magnetite (Fe_3O_4). Peaks and planes of diffraction are shown in Figure 1. No significant changes were found in the diffraction spectra between all the synthesized particles.

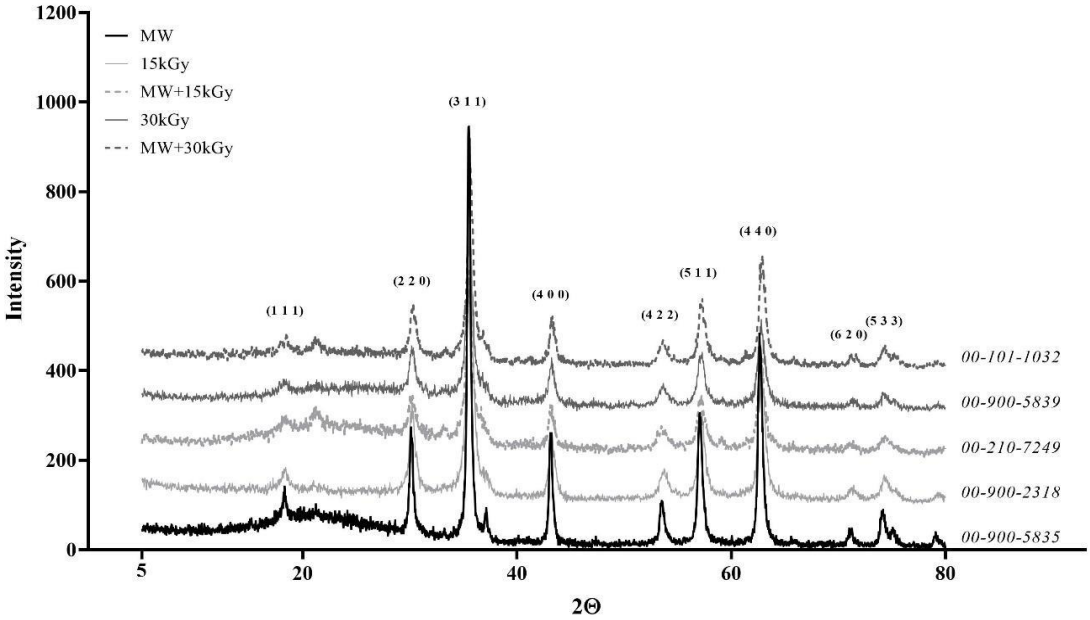

Figure 1 - X-Ray Diffraction Spectra of microwave synthesized crystallites (MW) irradiated by 15 kGy electron beam only (15kGy), synthesized in microwave and irradiated at 15 kGy (MW + 15kGy), irradiated to 30 kGy only (30kGy) and synthesized in microwaves and irradiated at 30 kGy (MW + 30kGy)

Using Scherrer's calculations, it was found that crystallites formed after electron beam irradiation, in the presence and absence of microwaves, showed a statistically significant difference compared to the microwave-formed crystallite used as standard (Figure 2). The mean particle size was 57.98 nm for particles irradiated at 15 kGy; 53.75 nm for MW + 15kGy; 53.70 nm for particles synthesized at 30 kGy; 51.22 nm for MW + 30kGy and 41.74 nm for nanoparticles synthesized only in microwaves (Figure 2).

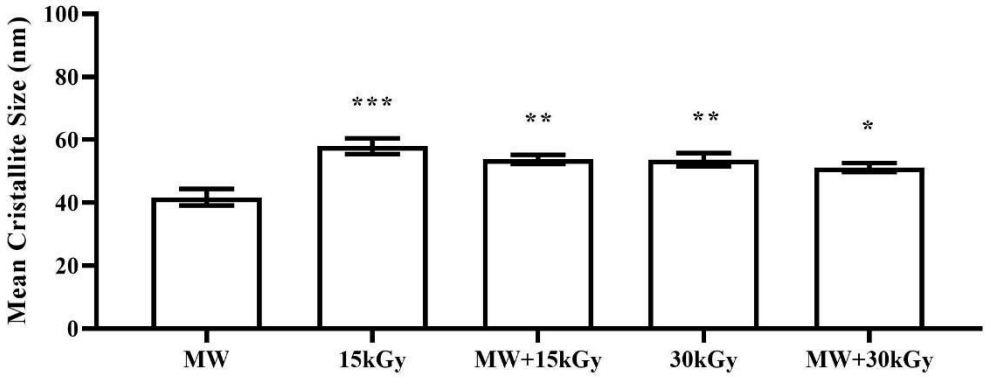

Figure 2 - Mean and standard deviation of the size of the synthesized nanoparticles. Calculation performed by the Scherrer equation

3.2. Zeta Potential

The data for the zeta potential were obtained from a single analysis. The mean values obtained were + 42.93 ± 1.90 mV for nanoparticles synthesized only in MW; + 40.20 ± 1.85 mV for 15kGy; + 35.40 ± 1.68 mV for MW + 15kGy; + 32.40 ± 1.19 mV for 30kGy and + 42.03 ± 2.33 mV for MW + 30kGy. All samples were analyzed in water.

Figure 3 shows a statistical difference in relation to the particles synthesized at 15, 30 kGy and with microwaves at 15kGy, comparing to the zeta potential of nanoparticles irradiated in microwave only. All particles showed a positive charge.

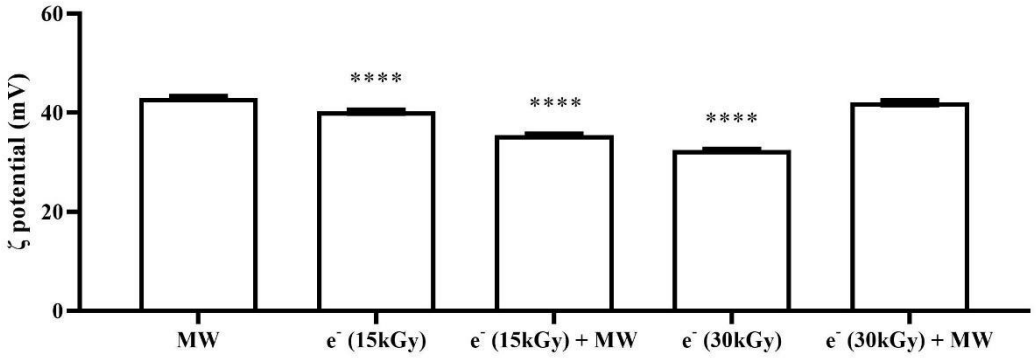

Figure 3 - Graph comparing the measurements of zeta potential obtained for all particles synthesized by electron beam and microwave

3.3. Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR spectra were obtained for all conditions of synthesized nanoparticles. Glycine and poly-L-lysine hydrobromide (Sigma-Aldrich) have the functional groups amine (NH_2) and carboxyl (-COOH). These two groups are important for establishing bonds with metals through ionic interactions. In Figure 4, the presence of the free amine band NH_2 (~ 1637 cm^{-1}) is observed for all samples, indicating that the amines are present on the surface of the iron nanoparticles. The Fe-O band presented in this spectrum can be assigned to magnetite [10].

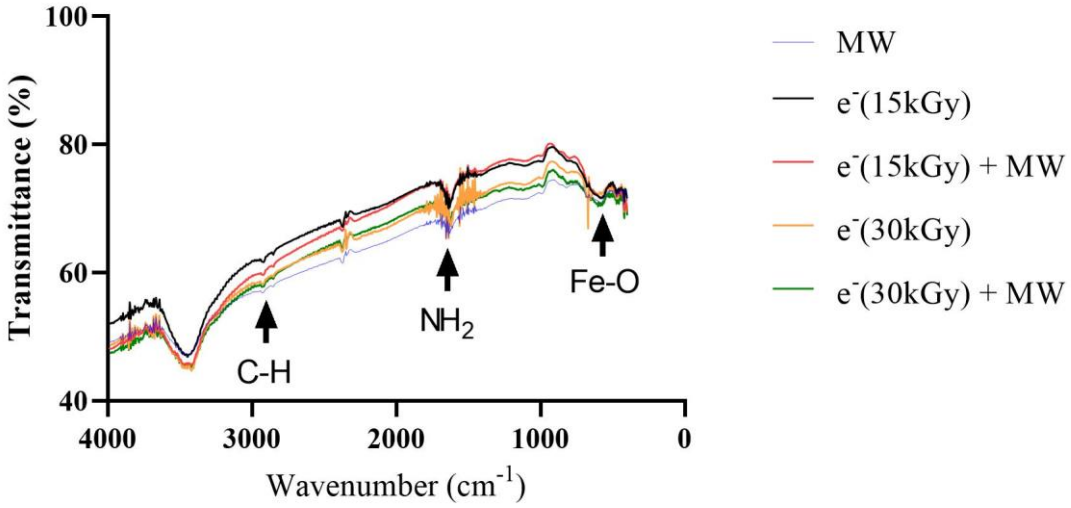

Figure 4 - FT-IR analysis for all synthesized nanoparticles, functionalized with glycine and poly-L-lysine hydrobromide

3.4. Dinamic Light Scattering (DLS)

The DLS analysis allowed to determine the hydrodynamic size of the nanoparticles and their stability from the relative frequency. As shown in Figure 5 and Table 1, the mean value of hydrodynamic size for all samples irradiated with electron beam was lower when compared to the mean value found for particles synthesized only in microwave.

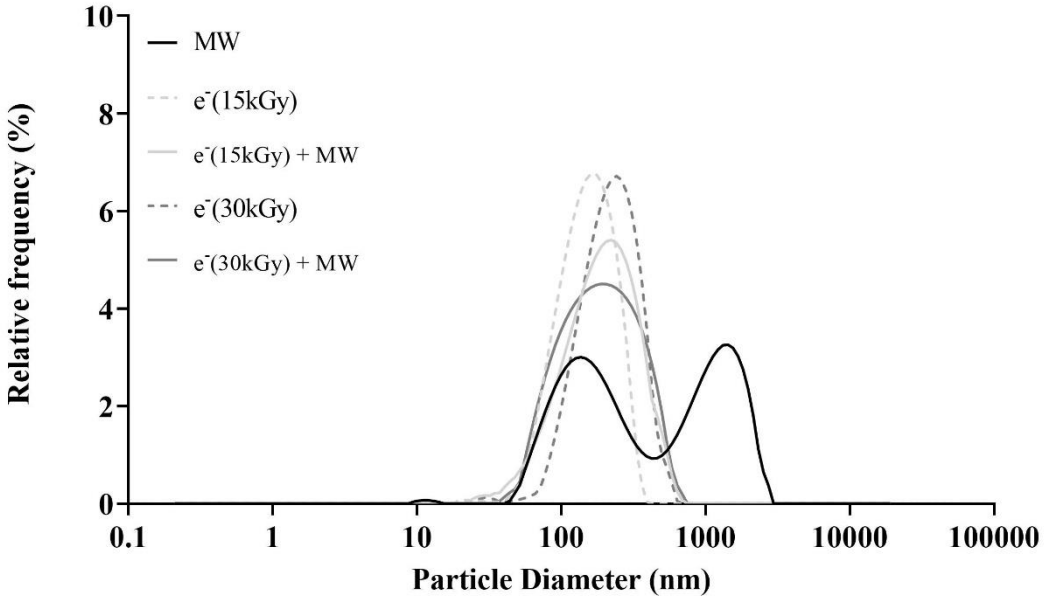

Figure 5 - Graph of the hydrodynamic size (nm) of the nanoparticles by the relative frequency (%) of the synthesized nanoparticles

Table 1 - Mean value and standard deviation of the hydrodynamic diameter of the synthesized nanoparticles for each condition

Nanoparticles	Hydrodynamic diameter (mean value) (nm)	SD (nm)
MW	511.6	99.64
15 kGy	147.50	1.60
MW + 15kGy	183.48	3.80
30 kGy	213.4	2.79
MW + 30kGy	179.45	2.15

3.5. Adsorption to cell culture

According to Figure 6, for all PIONS conditions evaluated, adsorption of the nanoparticles to the cells can be observed. The A images indicates the PIONS over the cells and in B images the blue coloration of Prussian blue indicate the presence of iron in the nanoparticles.

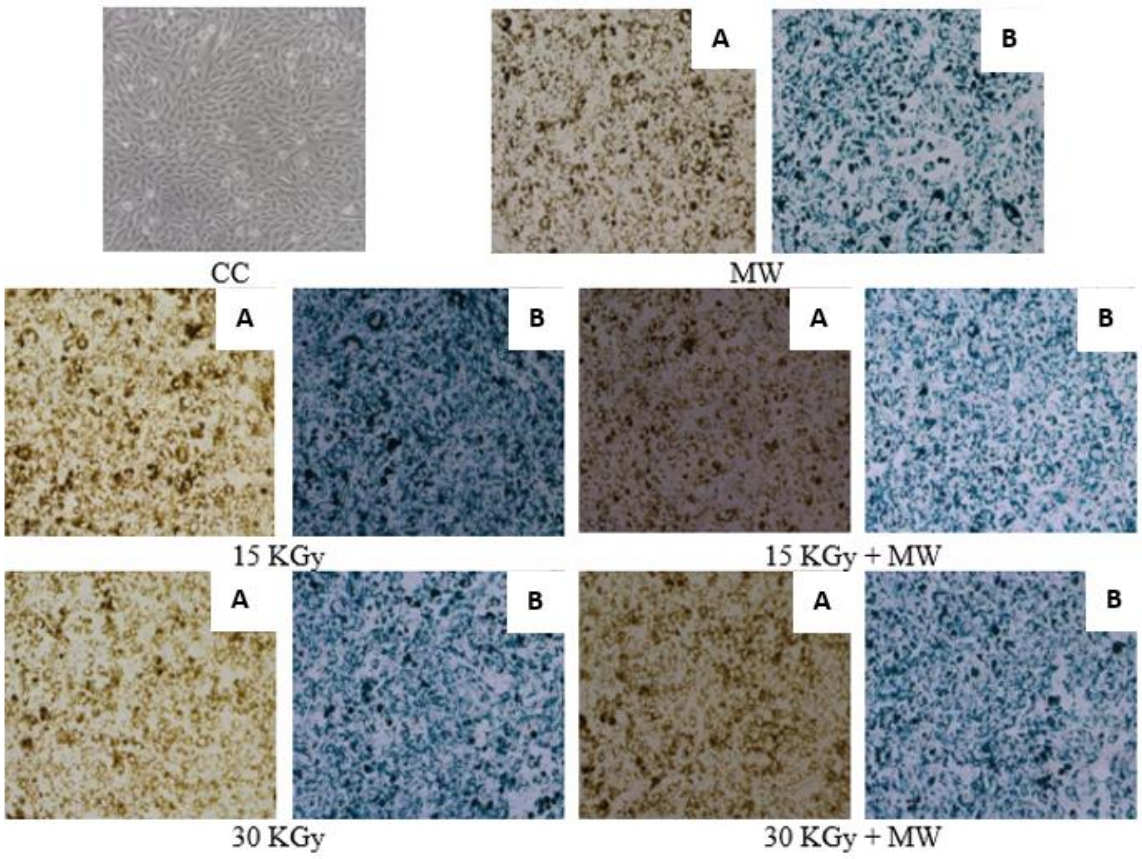

Figure 6 - LNCaP cells photomicrographs with the nanoparticles adsorbed to all nanoparticles conditions evaluated, after approximately 24 hours of incubation, without (A) and with (B) Prussian blue. CC - LNCaP cells photomicrographs at 100% confluence without adsorbed nanoparticles after approximately 24 hours of incubation. Magnification: 20x

3.6. Nanoparticles cytotoxicity assay

To evaluate whether the solution of synthesized nanoparticles could induce cytotoxicity, LNCaP cells were seeded in the 96-well plate and, after 24 h, exposed to 5 different concentrations of Fe_3O_4 nanoparticle suspensions, as described. As shown in Figure 7, the ratio used for all conditions tested did not induce cytotoxicity to the cells. The result was obtained from two independent trials.

3.7. Spheroids construction

After 6 days of incubation, the spheroids were stained with Propidium Iodide (PI) which stain all the necrotic cells in red and Hoechst stain all the cells in blue as seen in Figure 8. Then the spheroids were photographed and the incorporation of the evaluated dye.

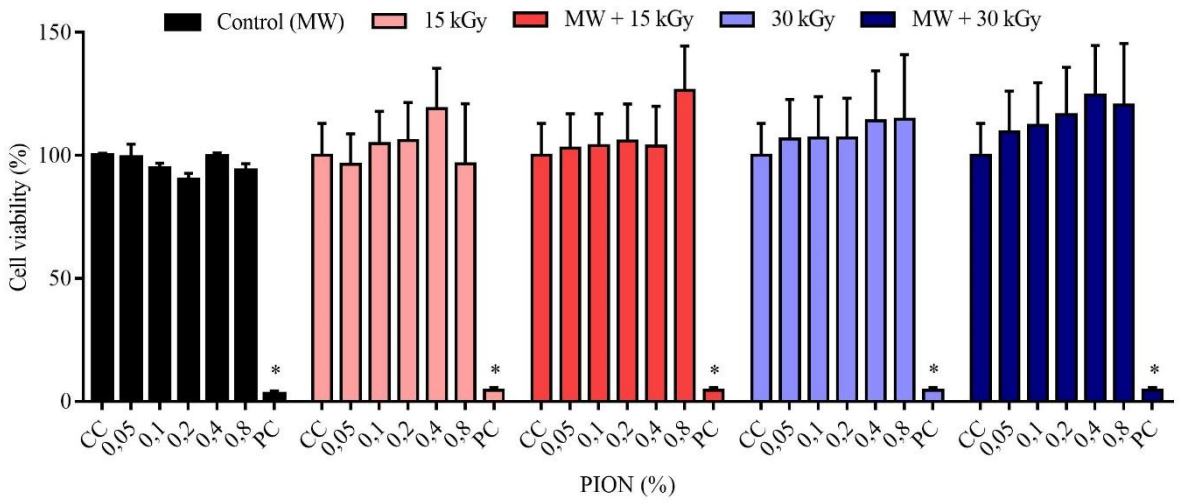

Figure 7 - Cytotoxicity assay in 2D LNCaP cells. (CC) Cell control; (PC) Positive Control - Natural latex

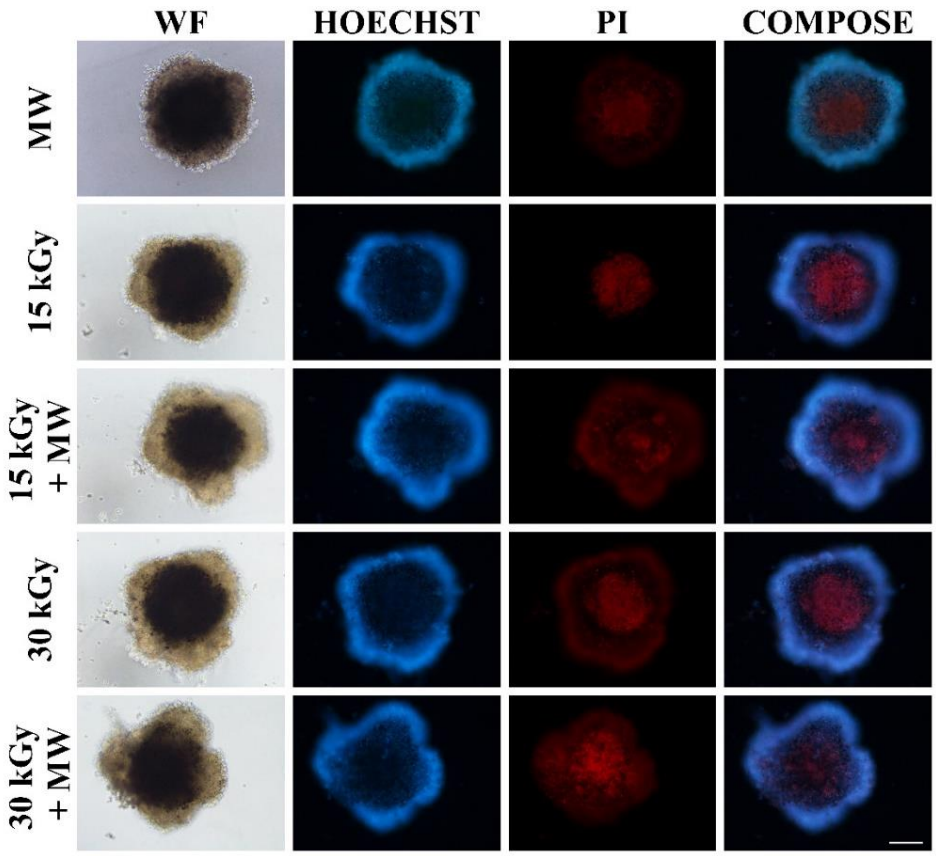

Figure 8 - Photomicrography of spheroids from 6th day culture LNCaP cells stained with Hoechst and PI for all conditions tested. Bar: 200 µm

4. DISCUSSION

In this work, paramagnetic iron oxide nanoparticles were synthesized from microwaves and electron beam irradiation to construct prostate tumor spheroids. These nanoparticles underwent a process of physico-chemical characterization and biological evaluation in prostate tumor cells regarding cytotoxicity, cell membrane adsorption and formation of tumor spheroids. In this sense, the paramagnetic iron oxide nanoparticles synthesis process was suitable for the formation of small size nanoparticles with biocompatible characteristics [11].

Interestingly, all the nanoparticles irradiated and synthesized in microwaves adsorbed to the cells due to the functionalization of the nanoparticles with poly-L-lysine hydrobromide, an acid polymer added to allow positive charge to the particles, consequent adsorption to the cell membrane (which presents negative charge) and improve its stability and biocompatibility [12]. Poly-L-lysine is a nonspecific factor for cells, useful in promoting cell adhesion to solid substrates [13].

The synthesis process in complex nanometric structures for biomedical applications can be facilitated through the use of electron beam radiation [14]. The radiation synthesis process ensures solvent radiolysis that promotes iron reduction and controlled nanoparticle precipitation [15].

The surface charge of the nanoparticles can be evaluated by the Zeta Potential analysis, for the PIONS synthesized in this work the load obtained for all the nanoparticles was greater than +30 mV indicating particles with high stability and high capacity to adsorb on the surface of the cells (Figure 3) [16].

Analysis by FT-IR (Figure 4) indicated the presence of the free NH_2 functional group in all synthesized PIONS, this functional group is present in the poly-L-lysine hydrobromide molecule, a cationic amino acid, in this way the protonation of this functional group, conducted in this work by the use of acetic acid, allows the adsorption of the nanoparticles to the cell membrane. This interaction of the nanoparticles with the cells presents enough force to maintain the nanoparticles in the membrane even after the process of trypsinization of the cells [6]. This characteristic allowed the formation of spheroids from the use of a spheroid drive with magnets, obtained from Greiner Bio-One, keeping the cells aggregated in culture until proper formation of ECM.

After 6 days of incubation, spheroids were formed for all nanoparticle conditions tested. One can observe the presence of a necrotic center characteristic of tumors, without significant differences in the construction of the spheroids between the conditions tested, as observed in Figure 8, by the reddish coloration of the PI.

As to the size of the PIONS, it can be observed that the particles that passed through electron beam irradiation presented larger size when compared to the non-irradiated sample, which indicates that nanoparticles synthesized only in microwaves allow the obtaining of smaller particles (Figure 1 and 2). Changes in the pH of synthesis may allow even smaller particles to be synthesized [6]. However, for the hydrodynamic size of the PIONS obtained by the DLS analysis, which includes the particle diameter and its diameter obtained after addition of the

amino acids, the electron beam irradiated samples presented values lower than those obtained for PIONS synthesized only in microwaves (Table 1).

5. CONCLUSION

This work describes simple methods for the synthesis of paramagnetic iron oxide nanoparticles, functionalized with glycine and poly-L-lysine hydrobromide, allowing effective adsorption of the nanoparticles to cell membrane. Among the nanoparticles synthesized by microwave and electron beam irradiated at 15 and 30 kGy it is possible to determine that the PIONS synthesized by microwave present better results compared to those irradiated. However, the prostatic tumor spheroids constructed from these nanoparticles showed stability after 6 days of culture with the expected characteristics of the tumor development.

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