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Characterization of iron oxide nanoparticles for production of breast adenocarcinoma spheroids

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1. Introduction

The personalized treatment of breast cancer has been extensively studied for the past two decades, focusing on tumor profiling and identifying therapeutic targets and biomarkers for prognosis [1]. Tumor development involves complex interactions within the microenvironment, influencing cellular behavior in response to treatments [3]. Multicellular tumor spheroid models offer a platform for studying tumor processes efficiently, with exponential volume growth facilitating high-throughput screening. Magnetite nanoparticles (PIONS) are used for their magnetic properties, allowing directional attraction under a magnetic field while remaining inert without inherent magnetism [4]. In this study, PIONS were utilized in a magnetic aggregation/levitation system to create a three-dimensional cell culture model of breast adenocarcinoma. This model maintains cell-to-cell contact, mimicking the tumor microenvironment in vivo. The objective is to assess the effects of ionizing radiation and drug action on breast adenocarcinoma cell spheroids in terms of cell mortality and compare these effects with two-dimensional cultures [5].

2. Methodology

Cell Culture: A human breast adenocarcinoma cell line, MCF7 (ATCC® HTB-22™), was maintained in 25 cm^2 culture bottles containing RPMI-1640 medium (Gibco), supplemented with 10% (v/v) fetal bovine serum and 5% antibiotic solution (10,000 IU/mL penicillin, 10 mg/mL streptomycin, and 1 mg/mL amphotericin), in a controlled, humidified atmosphere with 5% CO_2 at 37°C.
Synthesis of iron nanoparticles: Was used co-precipitation of iron hydroxides in a microwave-assisted synthesis approach. Initially, 32.5 mM of $FeSO_4 \cdot 7H_2O$ (Sigma-Aldrich®) and 92.4 mM of $C_2H_5NO_2$ (Sigma-Aldrich®) were dissolved in a beaker containing 80 mL of deionized water under constant stirring to achieve a homogeneous solution. Subsequently, a 2 molar NaOH solution was slowly dropwise added into the mixture under continuous stirring until the pH reached 6.5. After irradiation at 950 W for 2 minutes and 30 seconds, a black precipitate was formed. The precipitate was separated, washed with deionized water, and treated with acetic acid. A solution containing $((C_6H_{14}N_2O_2)n)$ was then added dropwise under ultrasonic conditions. The product was resuspended in ultrapure water for storage.
X-Ray Diffraction (XRD) and Transmission Electron Microscopy (TEM): Stored in a microtube, 500 µL of the sterile PIONS suspension were withdrawn. With the aid of a magnet, as much liquid as possible was removed from the magnetic precipitate and subsequently washed with 1000 µL of ethyl alcohol (Alphatec). After discarding the alcohol, the microtube remained open inside a beaker in the drying oven for a period of 24 hours at 42ºC for total evaporation of the liquids present in the sample. The X-ray diffraction patterns of the powdered sample were characterized using the Rigaku diffractometer, model DMAX 2100, with

CuKα radiation (λ = 1.15416 Å) accelerated by a potential of 40 kV at a current of 20 mA. The QualX2 software was used to analyze the obtained XRD patterns, employing data comparison from COD (Crystallography Open Database) where the peak distances maintain the characteristic of the crystalline phase being observed. The powder sample of PIONS was analyzed using a high-resolution JEM-2100 microscope (JEOL) operated at 200 kV. The application of this method was associated with observing the morphology and dispersion of the NPs.

Adsorption of magnetite nanoparticles on the surface of cells and spheroid formation: The PIONS were added at a ratio of 2 µL (approximately 3 mM of iron) for every $1.5x10^6$ suspended cells. The cell-nanoparticle suspension was homogenized by pipetting and added to a 25 cm^2 culture bottle, then maintained in culture overnight at 37ºC with 5% CO_2. Cell culture plates with 96 wells were pre-treated with a solution of Pluronic® F-127 (0.5 g/L in 2-propanol, Sigma-Aldrich). This solution was heated at 60ºC for 10 minutes or until the viscosity was completely reduced, as evidenced by the absence of turbidity in the solution. Volumes of 150 µL/well were transferred to the plates. After 24 hours, the liquid was removed from the wells by suction using a vacuum pump, and the plates remained open within the sterile laminar flow hood for drying under UV radiation for 30 minutes.

Irradiation: For plate preparation prior to irradiation, as much culture medium as possible was removed from the 96-well culture plates, and 10 µL/well of PBS at room temperature was added. After 10 minutes, the plates were irradiated with doses of 2 using a ^{60}Co gamma radiation at a dose rate of 130 kGy/h. Following irradiation, the plates were washed with PBS, 100 µL/well of RPMI-1640 medium (Gibco) was added, and they were placed in the incubator at 37ºC and 5% CO_2, where they remained for analysis using the INCell Analyzer 2500 HS (Cytiva Lifesciences) op-tical microscope.

Fluorescence Microscopy Assays: The plates were stained with SYTOX® Green (10 µL/well, Thermo Scientific) and Hoechst 33342 (10 µg/mL, Sigma-Aldrich) in culture medium and kept in the incubator for 60 minutes for analysis. The experiments were conducted using the INCell Analyzer 2500 HS equipment (Cytiva Lifesciences), employing a 4X objective lens (Nikon 4X/o.20, Plan Apo, CFI/60).

Cytotoxicity assays: Cell viability studies were performed using the magnet-coupled plate on the Cell-Repellent 96-well plates (Greiner BIO-ONE). After 24 hours of incubation at 37ºC and 5% CO_2, the treatment stage was carried out using Mitomycin-C drug and control groups, where each well of the 96-well plate received 20 µL of the desired solution for analysis. For this assay, Dimethyl sulfoxide (DMSO) and Sodium Dodecyl Sulfate (SDS) were used as positive control groups; Sodium Chloride (0.045%) as the negative control, and the Mitomycin-C drug at different concentrations (1.56; 0.75; 0.375; 0.1875; 0.093 and 0.048 µg/mL). After 24 hours of application, the plate was treated with MTS solution (CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS), Promega®) and PMS (Phenazine methosulfate, Sigma-Aldrich), 40 µL/well, and returned to the incubator for an additional 2 hours until the absorbance (490 nm) was read using the Multiskan EX plate spectrophotometer (Thermo).

3. Results and Discussion

The XRD analysis showed diffraction peaks indicating an inverted spinel structure characteristic of magnetite (Fe3O4, COD: 00-153-2800). The average sizes of the crystallites obtained were calculated to be 57.574 nm using the Scherrer equation. The TEM images illustrate the distribution and shape of Fe3O4 nanoparticles, displaying quasi-cubic particles. The interaction of the high-energy electron beam with the sample reveals PIONS with minimal aggregation artifacts, observed at spatial resolutions of 5 nm and 20 nm, Figure 1.

In this work, Pluronic® F-127 in anhydrous 2-propanol was used, allowing the hydrophobic portion of the micelle to be directed towards the center of the well, preventing cellular adhesion. Figure 2: depicts the typical appearance of MCF7 spheroids after 24, 48, or 72 hours of incubation. In this experiment, the stability of spheroids after hours of incubation was assessed based on the amount of SYTOX® Green-positive cells, indicating cell death. The acquisitions reveal a reduction in the number of dead cells after 72 hours of incubation, indicating an overall increase in the total number of cells and a relative decrease in the concentration of non-viable cells over the incubation period. The experiment demonstrated that spheroids incubated for 72 hours remained intact, exhibited low friability (fewer fragments), and had few

events of cell death, making them suitable for experiments.

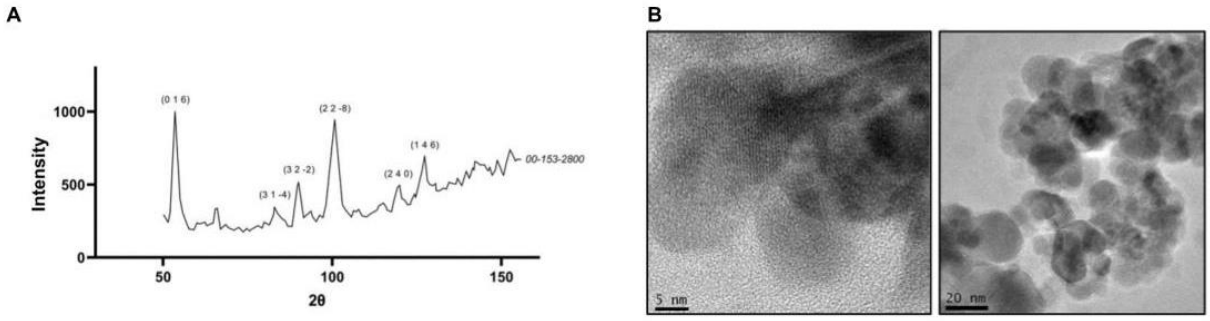

Figure 1: X-ray diffractogram of the sample of iron oxide nanoparticles synthesized with Glycine (Gly) as a surfactant and functionalized with Poly-L-lysine (PLL). The numbers above the peaks refer to the crystalline planes found in the samples (A) and Transmission Electron Microscopy (TEM) images of iron oxide nanoparticles synthesized with Glycine (Gly) as a surfactant and functionalized with Poly-L-lysine (PLL) (B).

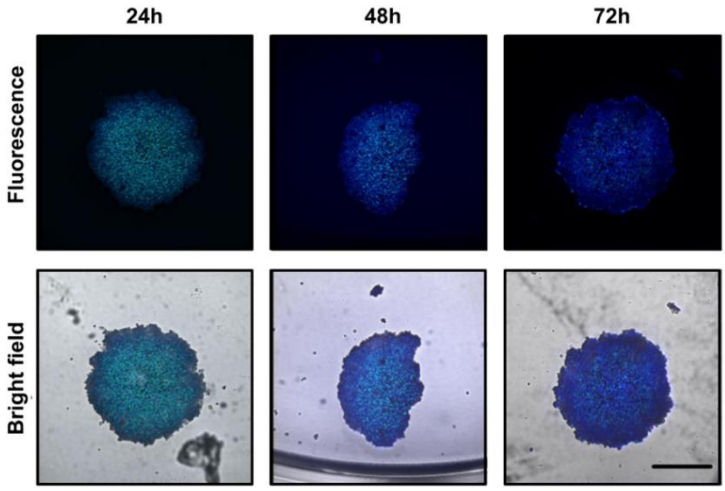

Figure 2: Evaluation of spheroid growth. Photomicrographs obtained by fluorescence microscopy using the INCell Analyzer 2500 HS (Cytiva Lifesciences) over eight days in a 96-well plate with SYTOX® Green (green) indicating dead cell nuclei and Hoechst 33342 (blue) indicating viable nuclei. Scale bar of 200 µm.

The Figure 3 depicts the quantification of dead cell nuclei (A) and spheroid volume (B) irradiated at 2 Gy in plates treated with Pluronic® F-127. As observed, the positive control group composed of Dimethyl Sulfoxide (DMSO), a universal solvent for both polar and nonpolar compounds, exhibited cytotoxicity gradually concerning the percentage amount added to the culture (C) demonstrates that percentages of 20% and 40% DMSO can be used as a positive control for cytotoxicity testing in MCF7 cells maintained in three-dimensional culture. This outcome aligns with findings in the literature [6]. The positive control group using Sodium Dodecyl Sulfate (SDS) induced a different response. Apparently, the denaturant acted upon the cells, inducing cytotoxicity at higher amounts in various concentrations, showing relevant toxicity from 625 µM onward, as depicted in (D). The three-dimensional structure allows the emergence of nutrient, waste, gas, and pH gradients across cellular layers. The pH value decreases as it penetrates the layers [7]. Mitomycin-C degrades in low pH [8], and its relatively low diffusion capacity, possibly coupled

with its pH sensitivity, aligns with the reduced efficacy reported in this study, as depicted in (E).

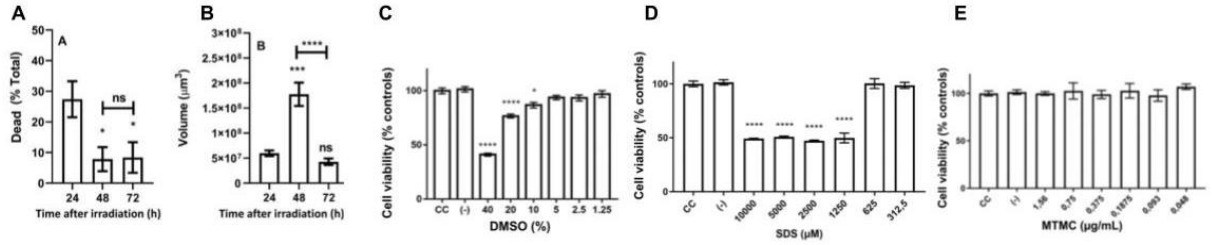

Figure 3: Quantities of dead cells (A) and spheroid volumes (B) of MCF7 irradiated at 2Gy. Cytotoxicity of DMSO concentrations in MCF7 spheroids (C). Cytotoxicity of SDS concentrations in MCF7 spheroids (D). Cytotoxicity of MTMC concentrations in MCF7 spheroids (E). Bars: represent standard errors of the means. (*): p <0.05. (**): p <0.001. (****): p <0.0001. (ns): not significant compared to control (24h). Statistical significance measured concerning the control group (CC).

4. Conclusions

The biocompatible model of three-dimensional culture, constructed through magnetic levitation as presented in this study, proved to be a viable alternative for analyzing cell viability, cytotoxicity, and morphology of a tumor spheroid. Based on the results obtained, it was possible to conclude that mitomycin C is not a favorable drug for the treatment of breast tumors, as it requires high doses to have an effect on the tissue, potentially leading to significant side effects with the use of this therapy.

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