Development of three-dimensional cellular culture system for testing of biological effects of radiations in tumoral and non-tumoral models

Leticia Bonfim[1(*)], Karina Oliveira Gonçalves[2], Daniel Perez Vieira[1]

1- Radiobiology Laboratory, Biotechnology Center, IPEN / CNEN-SP, Av. Lineu Prestes, 2242, São Paulo, SP, Brazil

2- Department of Physics, UNIFESP, Diadema, SP, Brazil

(*) Email: leticia.bonfim@ipen.br

ABSTRACT

Pre-clinical drug testing is currently based on based on monolayer or 2D (2D) cell cultures and, despite the large-scale use of this form of culture, there is already scientific evidence that the cellular disposition in monolayers does not adequately simulate tissue physiology, as it prevents cells from expressing their characteristics in a manner analogous to that found in the organism. For this purpose, the work aimed to produce three-dimensional structures, referred as spheroids, using magnetic levitation by adding iron nanoparticles to the cultures and with the aid of magnets. Electron microscopy showed particles with about 20nm in diameter. FTIR (Fourier-transform infrared spectroscopy) analysis showed stretches compatible with iron and amino acid (Lysine) binding. The images showed the formation of spherical bodies until the ninth day. LnCap spheroid diameter varied from (mean ± error) 434.407 ± 50.018 µm (5th day) to 264.574 ± 13.184 µm (9th day). Cultures of CHO ranged from 229.237 ± 5.278 µm to 236.719 ± 12.910 µm in the same period. Spheres generated by magnetic levitation could be measured by digital means and compared throughout the experiment. The tool can be used to test the biological effects of radiation and / or radiopharmaceuticals in culture.

Key words: Iron oxide; nanoparticles; magnetic levitation; 3D culture

RESUMO

O teste pré-clínico de medicamentos baseia-se atualmente em culturas celulares em monocamadas ou 2D (2D) e, apesar do uso em larga escala desta forma de cultura, já existe evidência científica de que a disposição celular em monocamadas não simula adequadamente a fisiologia do tecido, pois evita que as células expressem suas características de maneira análoga à encontrada no organismo. Com esta finalidade, o trabalho visou produzir estruturas tridimensionais denominadas esferóides, usando levitação magnética mediante adição de nanopartículas de ferro às culturas e com o suporte de ímãs. A microscopia eletrônica mostrou partículas com cerca de 20 nm de diâmetro. A análise FTIR (Fourier-transform infrared spectroscopy) mostrou estiramentos compatíveis com ligação de ferro e aminoácido (lisina). As imagens mostraram a formação de corpos esféricos até o nono dia. O diâmetro esferoidal LnCap variou de (média ± erro) 434,407 ± 50,018 µm (5º dia) para 264,574 ± 13,188 µm (9º dia). As culturas de CHO-KI variaram de 229,237 ± 5,278 a 236,719 ± 12,910 µm no mesmo período. As esferas geradas por levitação magnética

podem ser medidas por meios digitais e comparadas ao longo do experimento. A ferramenta pode ser usada para testar os efeitos biológicos da radiação e / ou radiofármacos em cultura.
Palavras-chave: Óxido de ferro; nanopartículas; levitação magnética; cultura 3D

1. INTRODUCTION

Currently, antitumor activity *in vitro* testing of drugs are performed in experimental models based on monolayer two-dimensional (2D) cell culture. Despite the large-scale use of this form of culture, there is already scientific evidence that the cellular disposition in monolayers do not simulate tumor physiology accurately, since it prevents cells from expressing their characteristics in a manner analogous to those found in the organism (Griffith, 2006). In addition, in the process of developing new drugs, a low percentage of success has been observed and many drugs showed significant antitumor activity in preclinical studies (Hutchinson, 2011; Hickman, 2014).

To achieve more similarity to that observed *in vivo*, it would be required a broader field of interactions between cells, what could be accomplished using three-dimensional culture techniques. This method has been receiving some attention as ways to simulate the tumor environment and promote the cell reorganization, cell-cell interaction and extracellular matrix proteins, and when cultured by this method the cells can form spheroids (Kuwashima, 1993; Song, 2004; Papusheva, 2010).

There are some methods capable of three-dimensional cell culture, being coating of culture plates with agarose one of the most used methods, which allows the formation of spheroids, which are characteristic cell aggregates with some physiological resemblance to natural conditions (Kuwashima, 1993). Another method is based on creation of three-dimensional clusters of cells with the use of magnetic levitation. For this method, iron nanoparticles are added to the cells and, with the aid of magnets, form spheroids of no more than few millimeters in diameter. Magnetic formed spheroids are cultivated suspended in the culture medium and remain viable for weeks allowing cells to produce their own extracellular matrix without inducing significant toxicity (Souza, 2010).

The intention of this work was to obtain suspended spheroids at the air-liquid interface by magnetic levitation with the addition of iron oxide nanoparticles in the cultures to stablish a test system for future assessment of effects of ionizing radiations in 3D cultures. To obtain greater stability and biocompatibility the nanoparticles were functionalized with Polyethyleneglycol (PEG) a polymer, which has been used in the literature as coating for the nanoparticles due to its high solubility and affinity with the amine group (Ali, 2016; Laurent, 2008; Babič, 2008). Adherence to cell surfaces

occurred by the addition of L-Lysine, an amino acid whose positive charge (at physiological pH) that bind to negatively charged cell surfaces (Babič, 2008).

2. MATERIALS AND METHODS

Cell Lines: LnCap (human prostate cancer) and CHO-KI (chinese hamster ovary) cells, maintained as liquid nitrogen stabilates at Center of Biotechnology (IPEN/CNEN-SP) were cultured separately with 5 mL RPMI 1640 medium, pH 7.4 (Gibco, Grand Island, USA), supplemented with 10% fetal bovine serum (Cultilab, Campinas, Brazil), 1g/L Sodium Bicarbonate (Sigma-Aldrich, Missouri, USA) and 1% penicillin and streptomycin solution (Gibco, Carlsbad, USA) in sterile plastic bottles for culture with 25cm^2 of culture area (Corning, Tewksbury, USA). Cell bottles are maintained in an incubator under controlled atmosphere (95% O_2, 5% CO_2, 37° C).

Synthesis of iron oxide nanoparticles: Particle suspensions were produced by coprecipitation using $FeCl_3.6H_2O$ and $FeSO_4.7H_2O$ as Fe^{3+} and Fe^{2+} sources, respectively. The iron ion solution was added to a flask containing NaOH (1.6 M) under sonication and constant bubbling of N_2 for 3 hours. After production, the particles were washed with deionized water. All solutions were prepared using degassed deionized water, with subsequent bubbling of N_2 for 15 minutes. The coating of the nanoparticles was obtained by the addition of PEG 4000 (22.5 mM) for 1 hour under sonication. Excess PEG was removed by washing with 100% ethanol. For functionalization, 200 μg of L-Lysine (Sigma-Aldrich, 1mg/mL) was added dropwise over 5 min under sonication. Finally, 200 μL of the nanoparticle suspension was added to the cell cultures and allowed to adhere. After 24 hours, the cells were evaluated by light microscopy and suspended in medium for experimental procedures.

Magnet array: To arrange the magnets, a suspension plate was designed using a 3D modeling software (Sketchup Pro 2017, Trimble Inc.) and printed with PLA (poly-L lactic acid). Ninety-six neodymium-iron-boron (NdFeB) magnets (3 x 8.5mm, D x L) were arranged to the plate, washed with ethanol 70% and UV sterilized (30 min in sterile environment) prior to use in cell cultures. The plate was designed to align all magnets to the center of wells of ordinary 96-well cell culture plates, without touching the surface of liquid (medium).

Plate preparation: Cell culture (96-well) plates were pre-coated using sterile agarose (1% in PBS, 80μL/well) and let to polymerize under sterile conditions for 30 minutes.

Spheroid formation: LnCap or CHO cells suspensions were prepared to provide different cell numbers (600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 4000 or 5000) in 100μL of medium. Suspensions were transferred to agarose-coated plates and covered by magnet plate. Experiments

were carried out for consecutive nine days. At 5th, 7th and 9th days, spheroids were imaged using a Nikon TS-100 inverted microscope at a 20X magnification (0.241µm/px).

Image analysis: images were analyzed using ImageJ 1.50i. Spheroid areas were measured using described µm/px equivalence.

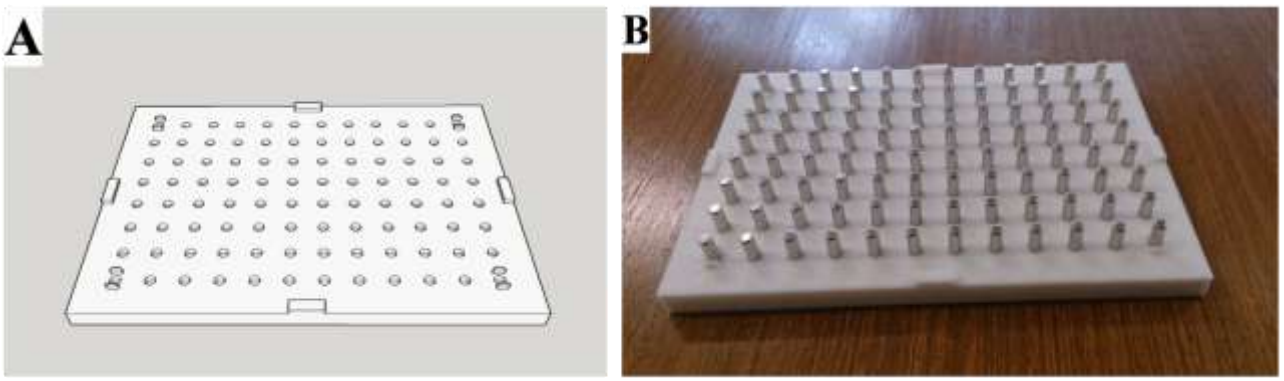
Particle characterization: The suspensions were evaluated by electron microscopy and FTIR.

Statistical analysis: Spheroid areas were compared using two-way ANOVA and Bonferroni post-testing for difference assessment from first day of measurements (5th day of experiment).

3. RESULTS AND DISCUSSION

The magnet array is shown if Figure 1. The use of a simple 3D printer with a PLA filament could produce a magnet plate with 96 magnets, with satisfactory results

FIGURE 1. 3D model of 96-magnet plate (A) and printed piece with attached magnets (B)



Electron microscopy images are shown in Figure 2. Suspensions without (A) or with (B) L-Lysine contained particles with about 20nm in diameter. It is also observed that the nanoparticles are little dispersed, because during the coprecipitation the nucleation and growth stages occur, in which the particle agglomeration may occur. L-Lysine seemed not to alter size or dispersion of particles.

The FTIR analysis is shown in Figure 3. All preparations showed a 580 cm^{-1} stretch caused by ligation of oxygen on the octahedral site of hematite (Fe-O) ligation, and 1630 and 3420cm-1 stretches that were present due to vibrational effects on O-H bonds due to water molecules on surface of particles. Main differences were observed inside 695-900, 1450-1800 and 2800-3100 cm^{-1} regions, which are characteristic of amino-containing nitro compounds, carboxylic acids and primary and secondary amines, respectively.

FIGURE 2: Electron microscopy of iron oxide nanoparticles without (A) or with (B) L-Lysine

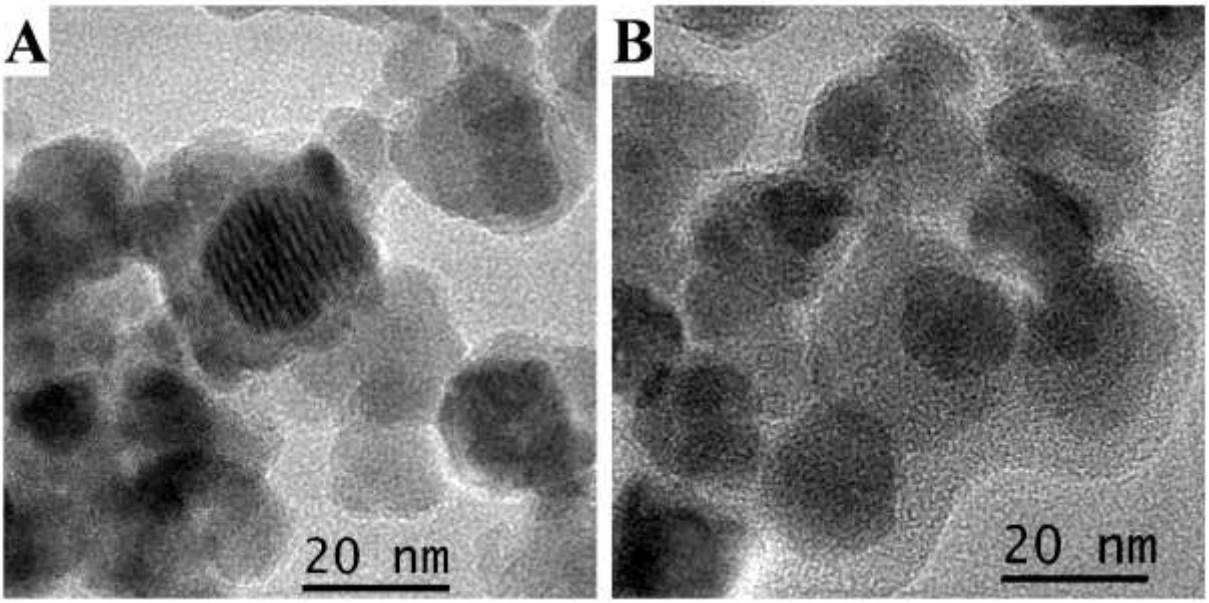
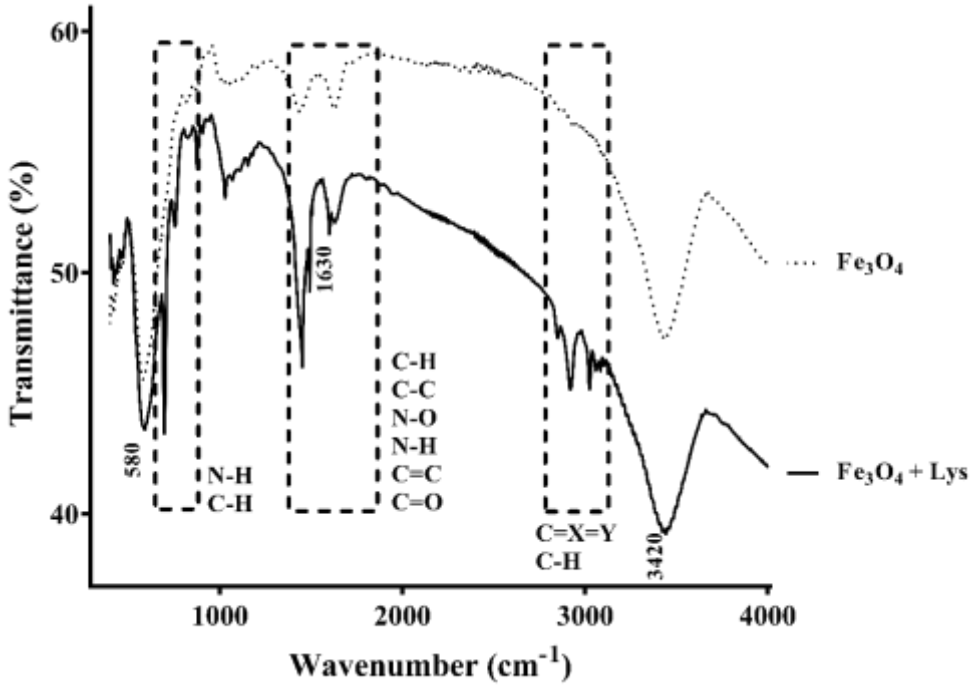


FIGURE 3. FTIR analysis of Fe_3O_4 NP with or without L-Lysine coating



Images obtained by inverted microscope and measured by software (Image J) showed the formation of the spheroids until the ninth day in culture are shown in Figure 4.

Cell spheroids were completely formed at 5th day of experiment. In this day, LnCap or CHO-KI spheroids remained integer even upon magnet removal from plates (data not shown). Measurements before this time were not possible. On the opposite hand, at 9th day the LnCap spheroids from all

tested cell densities appeared as true spheroids, not as sheet-like structures, as shown in Figure 4B. At this point, the structures were considered suitable to provide the proposed measurements. Area values were shown in Figure 5.

The mean diameter of the LnCap spheroids ranged from 434.407±50.018 µm (5th day) to 264.574±13.184 µm (9th day). Cultures of CHO-KI ranged from 229.237±5.278 to 236.719 µm in the same period. Area reduction was statistically significant when comparing LnCap spheroid from 5th to 9th day of experiment. At same time, changing from cell sheet to spheroid of these cultures was perceptible. LnCap tended to arrange first in a sheet-like epithelial structure, and further to a spheroid. Alternatively, CHO-KI readily arranged in spheric structures.

FIGURE 4. Spheroids of CHO (A) and LnCap (B) cells obtained using different cell densities per well and imaged at 5th, 7th and 9th days in culture

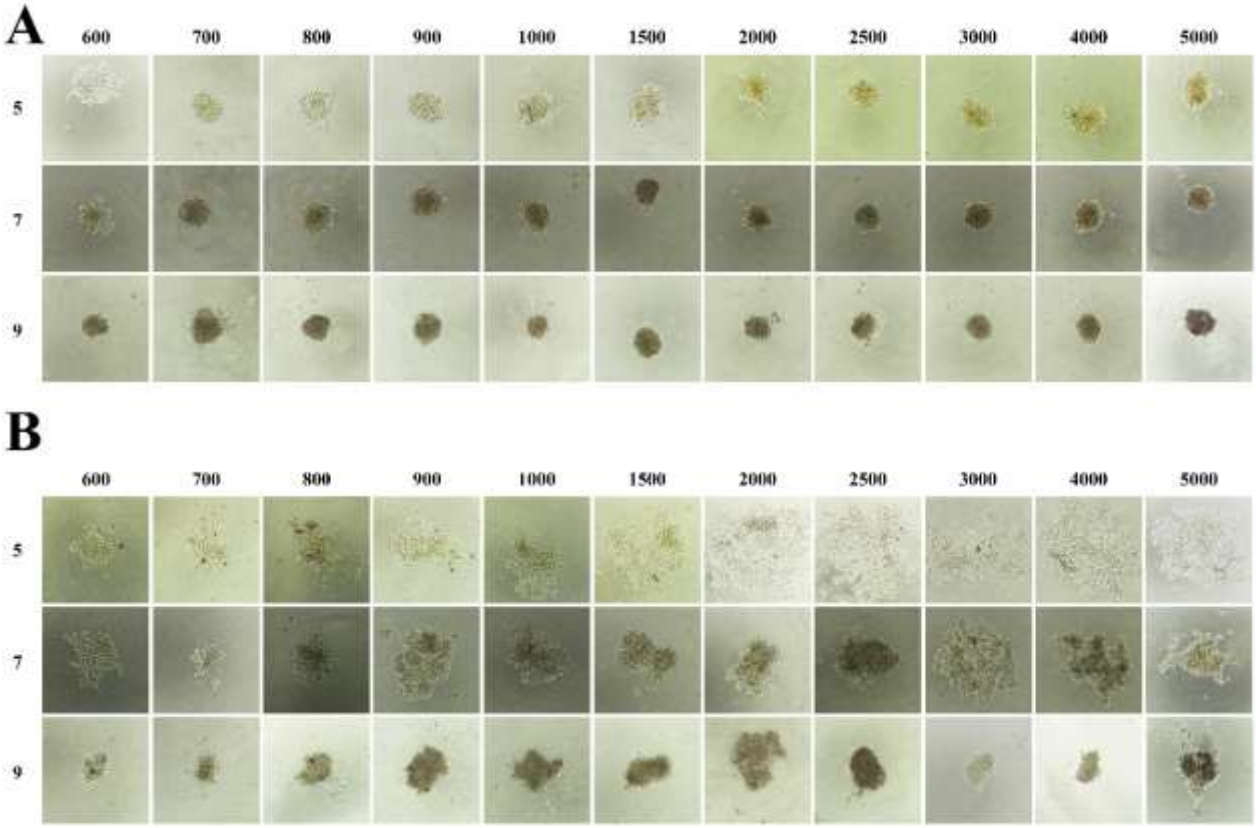
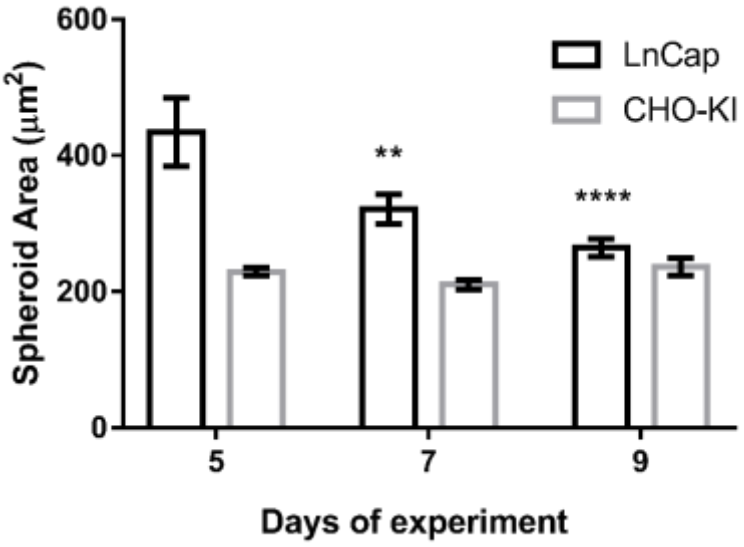


FIGURE 5. Spheroid area values (μm^2) from LnCap and CHO-KI spheroids at days 5th, 7th and 9th. Bars: SEM. (**): p<0.01. (****): p<0.0001



In-house projected and printed magnetic drive has proven to be an interesting alternative to commercial counterparts. Despite its relative success, further models with more complex design are required, in order to keep magnetic fields perfectly aligned with well centers. Agarose coating was a fairly efficient way to prevent cell adhesion to wells, but its presence decreases the work volume of media. Less restrictive approaches using polymers would be tested in further studies.

Iron oxide particles can show superparamagnetic responsiveness to magnetic fields. This characteristic is size-dependent, and can be retained as long particle size is approximately 10-20nm (Wahajuddin, 2012). The protocol used in this work could produce particles within this size range, as showed by electron microscopy.

Due to its positive charge on physiological pH (Schwaminger, 2015), L-Lysine has been chosen over another amino acids when adhesion to cell surfaces is required. Analysis of FTIR spectra showed stretches related to association of iron oxide with amino acids, with a further interpretation of L-Lys ligation to the particles. Spheroid formation and its responsiveness to magnetic field are consistent with this observation.

Study of toxicity of drugs on spheroids produced by magnetic levitation can be accomplished using magnet arrays commercially available (Tseng, 2016; Desai, 2017). Using these medium- to high-throughput methods, scalable *in vitro* approaches as calculations of IC_{50} or minimal toxicity can be reproduced. Although tridimensional cultures could be formed by other methods (Messner, 2013; Nath, 2016), there is sufficient evidence that magnetic levitation cultures, based on iron oxide nanoparticles adsorption by cells can be a suitable method of tridimensional cell culture, as showed by spheroid formation in this work. As an ultimate goal, spheroids will be further used on studies of

ionizing radiation in three-dimensional cell structures, as been currently reviewed in literature (Eke & Cordes, 2011; Hamdi, 2016; Acheva, 2014 et al.).

4. CONCLUSION

Spheres generated by magnetic levitation could be measured by digital means and compared throughout the experiment, and possibly could will be used as test system of the biological effects of radiation and / or radiopharmaceuticals in culture, for example.

ACKNOWLEDGEMENTS

The authors wish to thank Flavia R.O. Silva, Ph.D. from Microanalysis and Microscopy Lab. – Center of Material Science and Technology, IPEN/CNEN-SP for nanoparticle imaging and Caio C.S. Valeriano, M.Sc. and Leticia L.C. Rodrigues, Ph.D. for valuable help and 3D printing of magnetic plate.

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