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Liposomes radiolabeled with ^{159}Gd-DTPA-BMA: Preparation, physicochemical characterization, release profile and *in vitro* cytotoxic evaluation

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ABSTRACT

The present work describes the preparation, labeling, physicochemical characterization, and *in vitro* cytotoxic evaluation of long circulating pH-sensitive liposomes containing ^{159}Gd-DTPA-BMA. These liposomes were successfully obtained and submitted to neutron irradiation for gadolinium labeling. Their size, distribution, and homogeneity were determined by photon correlation spectroscopy, while their zeta potential was determined by laser Doppler anemometry. The morphology and structural organization were evaluated by atomic force microscopy. The stability and release profiles of Gd-DTPA-BMA in the liposomes were determined *in vitro* in Dubelco's Modified Eagle's Medium and rat serum at 70%. The results showed that liposomes remained physically stable after 8 h of irradiation and presented a low release profile of its content in two different biological mediums. The formulation of liposomes containing ^{159}Gd and its respective controls were evaluated by *in vitro* cytotoxicity against tumor cells RT2. The results showed increased cytotoxic activity of approximately 1170 fold in relation to free Gd-DTPA-BMA.

1. Introduction

In nuclear medicine, many radioisotopes have been used in diagnostic or therapeutic procedures to treat a wide range of diseases, including cancer. When used exclusively in therapeutic procedures, the radioisotopes should emit particulate radiation (auger, beta, or alpha) with energy levels between 50 and 2500 keV (Saha, 1998; Scholz, 2006). Some radioisotopes, such as Yttrium-90, Renium-186, and Samarium-153, present these characteristics, which have been applied in many clinical procedures, including the treatment of some types of cancer and chronic inflammatory processes (Bauman et al., 2005; Finlay et al., 2005; Garrean and Espat, 2005).

Gd-DTPA-BMA, a non-ionic complex containing one molecule of H_2O coordination, presents a low disassociation in aqueous solutions (Chang et al., 1992), has proven to be of great use as a dye in MRI procedures, due to its low osmolality and chemotoxicity (Fig. 1) (Meyer et al., 1990). However, Gd-DTPA-BMA has shown some adverse reactions, especially in patients suffering from chronic renal insufficiency (Thomsen, 2006). For this reason, the FDA requested that a warning be added to the product descriptions regarding the potential risk of nephrogenic systemic fibrosis (NFS) in patients with chronic renal failure (US FDA, 2010).

The gadolinium metal contains a mixture of the following isotopes: ^{152}Gd (0.20%), ^{154}Gd (2.18%), ^{155}Gd (14.80%), ^{156}Gd (20.47%), ^{157}Gd (15.65%), ^{158}Gd (24.84%), and ^{160}Gd (21.86%) (Browne et al., 1986). By submitting a natural gadolinium sample to an appropriate neutron irradiation process, the radioisotope ^{159}Gd is obtained (Alfassi, 1985). The ^{159}Gd radioisotope has a physical half-life of 18.59 h and emits negative beta particles with a maximum energy of 1.01 MeV and a characteristic gamma emission of 363.54 keV (Moralles et al., 1995). Based on these characteristics, some works demonstrate the potential use of ^{159}Gd radioisotopes in cancer treatment with some advantages over other potentially applicable radioisotopes (Bardies and Chatal, 1994; Goorley and Nikjoo, 2000). For example, in relation to ^{77}Lu, ^{159}Gd presents a longer half-life, and a negative beta emission with higher energy is particularly interesting in the treatment of solid tumors with significant mass.

Recently, our research group also demonstrated this capability in a preliminary *in vitro* study using the Ehrlich tumor model

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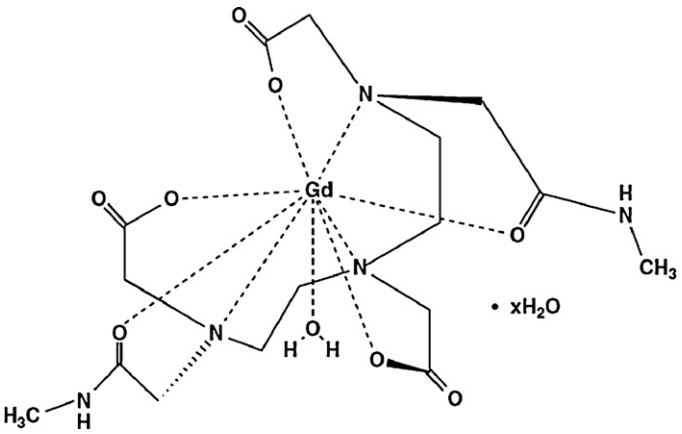

Fig. 1. Gd-DTPA-BMA (gadolinium diethylenetriaminepentaacetic acid bis(methylamide)) complex.

(Soares et al., 2010). However, the use of this type of radioisotope requires its direct application in the target organ to prevent the healthy tissue from receiving excessively high radiation doses. In this context, the use of nanostructured systems, such as liposomes or nanoparticles, allows many substances, including radioisotopes, to be delivered to target tissues in greater quantities, in turn minimizing the radiation dose within normal surrounding tissues (Vemuri and Rhodes, 1995; Tokumitsu et al., 2000). These nanostructured systems have the ability to permeate the interstitial space by passing through leaky capillaries, thus allowing for radiotracer delivery at different locations outside the mononuclear phagocyte system (MPS) (Oyen et al., 1996). Some authors have demonstrated that the mean diameter of nanoparticles has influenced biodistribution studies. Nanoparticles of larger than 300 nm and smaller than 70 nm are rapidly cleared from circulation by MPS cells (Gref et al., 1994). However, the use of a PEG (polyethyleneglycol) chain, through the addition of PEG-DSPE on the surface of the liposome, results in a decreased recognition by MPS cells, thereby increasing the half-life of their circulation in the blood (Jones, 1995; Pereira et al., 2008; Woodle, 1993). Another important factor to be considered is the size and capacity needed to reach inflammatory foci through loose junctions of the endothelium.

Prior studies have reported that the inclusion of amphipathic poly(ethyleneglycol) lipids, such as distearoylphosphoethanolamine-polyethyleneglycol 2000 (DSPE-PEG 2000), as can be seen in liposome compositions, can significantly reduce the uptake by the MPS cells, thus resulting in the liposomes' prolonged circulation (Allen et al., 1989; Garbuzenko et al., 2005; Klibanov et al., 1990; Torchilin and Papisov, 1994). The pH-sensitive liposomes are internalized in a low pH compartment within endosomal vesicles, and the encapsulated drug is released into the cytoplasm, in turn inducing cellular death. The construction of pH-sensitive liposomes takes advantage of the polymorphic phase behavior of unsaturated phosphatidylethanolamine, which generates inverted hexagonal phase two (HII) forms rather than bilayers. Liposome stabilization within bilayers can be achieved by using a titratable acid lipid, such as CHEMS (cholesteryl hemisuccinate), which is negatively charged at neutral pH (Massey, 1998). This lipid, homogenously distributed among dioleoylphosphatidylethanolamine (DOPE) molecules, provides electrostatic repulsions which decrease DOPE intermolecular interactions, thus preventing HII phase formation under physiological conditions. The protonation of CHEMS molecules in an acidic medium (e.g., endosomal vesicles), neutralizes their negative charges. In the process, the liposomes undergo destabilization and release their aqueous contents (Carvalho Júnior et al., 2007). Thus, the encapsulation of Gd-DTPA-BMA in this liposome formulation and its irradiation can lead to a higher retention of the ^{159}Gd radioisotope within liposomes and can subsequently be released in acidic mediums, such as endosomal vesicles in tumor cells. This behavior can reduce its systemic toxicity and improve the antitumor effect, considering that a higher dose of ^{159}Gd will be accumulated in the tumor.

The present work aimed to prepare and radiolabel long-circulating pH-sensitive liposomes with ^{159}Gd from Gd-DTPA-BMA. To study its physicochemical characteristics (mean size, zeta potential, and polydispersity index), morphologic stability of the system after neutron irradiation in a nuclear reactor using the atomic force microscopy (AFM) technique was applied. To investigate the release profiles of encapsulated ^{159}Gd in different physiological mediums: DMEM (Dubelco's Modified Eagle's Medium) and rat serum at 70% were administered and the cytotoxic activity of liposomes containing ^{159}Gd-DTPA-BMA to combat RT2 (Murine Glioblastoma cells) tumors cells, as wells as its controls, was evaluated.

2. Materials and methods

2.1. Materials

DOPE, DSPE-PEG, and CHEMS lipids were purchased from Lipoid GmbH (Ludwigshafen, Germany) and Sigma (St. Louis, USA), respectively. Gd-DTPA-BMA (Gadodiamide – Omniscan® – General Electric Healthcare Company) was kindly provided by FARMASA (São Paulo, Brazil). The MTT [bromide 3-(4,5-dimethyl-2-thiazolyl-2,5-diphenyl)-2H-tetrazolium] salt was purchased from Sigma (St. Louis, MO, USA), and the DMEM culture medium was purchased from Gibco BRL (Grand Island, NY, USA). All solvents used in this study were of analytical grade. All other chemicals used in this study were available commercially at a reagent grade and were used without further purification. MilliQ® water (simplicity 185, Millipore, Bedford, USA) was used throughout the study. Parafilm® was purchased from Pechiney Plastic Packaging, Inc., Chicago, USA.

2.2. Methods

2.2.1. Liposome preparation

A volume of 10 mL of liposomes was prepared according to the reversed phase evaporation method described by Szoka and Papahadjopoulos (1978). Chloroformic aliquots of DOPE, CHEMS, and DSPE-PEG, with molar ratios of 5.7/3.8/0.5, in a total lipid concentration of 40 mM, were transferred to a bottom flask where the solvent was then removed under vacuum until a lipid film on the walls of the flask had formed. An aliquot of Gd-DTPA-BMA and diethyl ether was added to the lipid solution (aqueous phase/organic phase ratio of 1/3). The film was then dissolved and shaken vigorously in a vortex. The mixture obtained was submitted to a vortex apparatus for 3 min, producing an A/O emulsion-type (water in oil).

The emulsion was submitted to evaporation under vacuum to remove the organic solvent, allowing the formation of lipid vesicles. The non-encapsulated Gd-DTPA-BMA was separated from the encapsulated Gd-DTPA-BMA in liposomes, using ultracentrifugation at 150,000 × *g*, for 1 h and a temperature of 4 °C. The precipitate was re-suspended in an attempt to obtain the same volume it had before the process of ultracentrifugation. The obtained liposomes were calibrated by passing through polycarbonate membranes of 0.4 μm, 0.2 μm, and 0.1 μm, respectively. This procedure was repeated ten times in each membrane.

2.2.2. Gd-DTPA-BMA labeling procedure

The Gd-DTPA-BMA labeling procedure which was encapsulated into pH-sensitive liposomes was conducted within polystyrene containers and performed using a TRIGA MARK-I IPR-R1 nuclear reactor at the Center for the Development of Nuclear Technology – National Commission on Nuclear Energy (CDTN-CNEN, Brazil). Liposomes samples (1 mL) containing Gd-DTPA-BMA were irradiated at 100 kW, under a thermal neutron flux of 6.6×10^{11} n cm^{-2} s^{-1} for 8 h, reaching an activity of 13.8 ± 1.4 GBq.

To verify whether or not the ^{159}Gd radioisotope had been formed, the gamma spectroscopy technique was conducted in a hyper-pure germanium detector apparatus (Canberra, France) with a resolution (FWHM) of 1.75 keV at 1332 keV, with a 15% relative efficiency. All counts were taken at a constant geometry in a position where the analyzer dead-time indicator registered less than 5%. Decay correction was conducted during the sample count. The software Genie-2000 was used for spectra processing and peak area determinations.

2.2.3. Liposome characterization

2.2.3.1. Photon correlation spectroscopy analysis. This analytical procedure allows one to determine the mean diameter of the liposomes and the polydispersity index (P.I.), which is a dimensionless measure of the broadness of the particle size distribution. The analytical procedure was conducted in a Zetasizer HS 3000 (Malvern Instruments, Malvern, UK) apparatus after its adequate dilution in ultra-pure MilliQ® water. The results are expressed as mean ± standard deviation for at least three different batches of each liposome formulation.

2.2.3.2. Zeta potential analysis. The zeta potential was determined by Laser Doppler Anemometry (LDA) in a Zetasizer HS3000 (Malvern Instruments, Malvern, UK). The samples of liposomes were analyzed following a dilution of 1/1000 in 1 mM of NaCl at a conductivity of approximately 120 ± 20 S/cm^2. The results are reported as the mean ± standard deviation of at least three different batches of each liposome formulation.

2.2.3.3. Atomic force microscopy. The atomic force microscopy (AFM) technique has been widely applied to investigate biological processes, cells, drug carriers, and other soft samples (Kasas et al., 1998). This technique allows one to visualize nanoparticles and liposomes in aqueous systems and in air, thus eliminating sample preparation (Pereira et al., 2008).

The procedure was performed in air at room temperature, using a Dimension 3100, monitored by a NanoScope IIIa controller from Digital Instruments (Santa Barbara, CA, USA). Samples of irradiated liposomes (5 μL) and control (non-irradiated) were deposited on a were deposided on a paraffin wax film (Parafilm®), spread, and partially dried with an argon stream. The images were obtained in a tapping mode using commercial silicon probes, by means of Nanosensors™, together with cantilevers at a length of 228 μm, with resonance frequencies of 75–98 kHz, spring constants of 3–7 N/m, and a nominal tip curvature radius of 5–10 nm. The scan rate used was 1 Hz. Dimensional analyses were performed using the "section analysis" program of the system. A minimum of ten images from each sample was analyzed to assure reproducible results. The values represent an average ± standard deviation of approximately 40 particle measurements.

2.2.4. Gd-DTPA-BMA encapsulation efficiency and release profile

Recently, Le and Cui (2006a,b) studied the potential use of liposomes that encapsulate gadolinium (Gd-DTPA) by means of the neutron capture therapy technique. The results of these studies showed that the addition of chloride poly-L-lysine (PLL) to the complex increased the encapsulation efficiency of Gd-DTPA while significantly limiting the release of the liposome compound. The present study investigated the encapsulation efficiency of Gd-DTPA-BMA with and without PLL in the liposomes (ratio 2/1, w/w), following the same aforementioned methodology. The separation of non-encapsulated and encapsulated Gd-DTPA-BMA in the liposomes was obtained by ultra-centrifugation at 150,000 × *g* for 1 h at 4 °C. The precipitate generated after ultracentrifugation was dispersed within a volume equal to that which had been used before the purification process together with a pH 7.4 HEPES buffer. The liposomes obtained were calibrated by being passed ten times through polycarbonate membranes of 0.4, 0.2, and 0.1 μm, respectively. Next, the liposomes containing Gd-DTPA-BMA were irradiated as mentioned above. Triplicate samples containing 5 mL of liposomes were sent for elemental analysis through the ICP-AES technique. The encapsulation efficiency was calculated by dividing the number of moles of encapsulated Gd-DTPA-BMA in liposomes and the number of total moles used in preparing the formulation.

In an attempt to simulate the physiological conditions, the study of Gd-DTPA-BMA release from liposomes was performed either in DMEM (Dulbecco's Eagle modified Medium) or in rat serum at 70%, under sink conditions, at different times. This study followed a protocol based on De Assis et al. (2007), which is briefly described below. Samples in triplicate, containing 100 μL of Gd-DTPA-BMA liposomes, with or without PLL, previously purified, and calibrated at 100 nm, were incubated in Eppendorf tubes (500 μL) within 400 μL of DMEM or rat serum at 70% for periods of 30 min, 1, 2, 3, 8, and 24 h. After each time period, the samples were ultra-filtered through polycarbonate membranes with a pore size of 30 nm (Millipore®, Brazil). After separation, the concentration of Gd-DTPA-BMA present in the supernatant was analyzed by the ICP-AES technique. The study conducted with rat serum at 70% attempted to verify the release profile of a Gd-DTPA-BMA compound of liposomes within a real biological medium. The evaluation of the stability of liposomes within a DMEM medium had the purpose of checking the stability of liposomes in common mediums used for *in vitro* studies of antitumor activity.

2.2.5. in vitro cytotoxic evaluation

The *in vitro* cytotoxic activity of samples containing the non-radioactive complex Gd-DTPA-BMA, the radioactive complex ^{159}Gd-DTPA-BMA, blank liposomes (encapsulating water only), Gd-DTPA-BMA encapsulated into liposomes (Gd-SpHL) and liposomes encapsulated with ^{159}Gd-DTPA-BMA (^{159}Gd-SpHL) were evaluated against RT2 tumor cells (Murine Glioblastoma cells) obtained from American type cell collection (ATCC). The cells were thawed and replicated, and after having reached an adequate confluence, were placed on three 96-well plates (500/well), totaling 120,000 cells. Through serial dilution, with a maximum radioactivity concentration of 650 MBq mL^{-1} well^{-1} in a volume of 100 μL, the cells were treated for 48 h within a CO_2 incubator (5% CO_2 – Pol-Eko Aparatura – ST line) in a humid atmosphere at 37 °C and in a culture medium of Dulbecco's modified eagle medium (DMEM) supplemented with sodium bicarbonate (3.7 g/L), penicillin (5000 units/mL) and 10% (w/w) sterile fetal calf serum. A total of eight replicates were used for appropriate statistical evaluation. By means of MTT assay, metabolic feasibility tests were performed to evaluate the efficacy of the treatment employed. The curve comparing the percentage of cell survival to the molar concentration of samples was obtained using the Prism 4.0 software. A non-linear regression model (sigmoidal dose–response option) was used to determine the IC_{50} values.

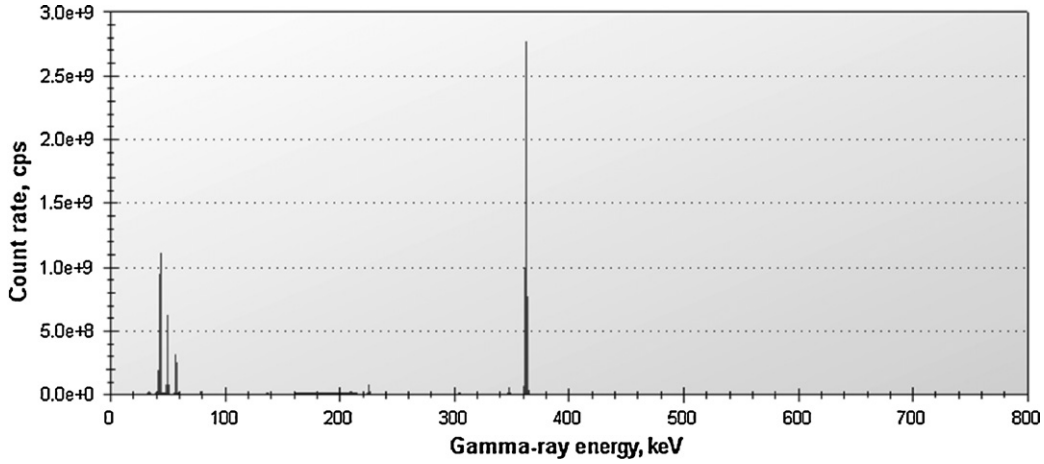

Fig. 2. Gamma spectrum of ^{159}Gd in liposome sample.

2.2.6. Statistics

All experiments were performed in triplicate and expressed as mean ± standard deviation, unless otherwise stated. Mean size and zeta potential data within each time period were compared by means of the ANOVA test, using the prism 4.0 software and considering a probability of 5% as significant.

3. Results and discussion

3.1. Liposome labeling

The isotope ^{159}Gd is not available on the market. Therefore, the only way to produce this isotope is by irradiating a natural gadolinium sample with neutron flux in a suitable nuclear reactor or from a source of ^{252}Cf (Alfassi, 1985). In a previous study, our research group irradiated the Gd-DTPA-BMA complex (Omniscan®) with neutrons to obtain the isotope ^{159}Gd-DTPA-BMA, reaching the conclusion that this complex was stable and that no significant change could be observed after 8 h of irradiation (Soares et al., 2010). The preparation of liposomes containing radioisotopes involves complex operations, which require licensing from government agencies as well as the handling of radioactive material in liquid form, which is typically performed by highly trained personnel. Faced with these difficulties, our research team formulated liposomes containing the complex Gd-DTPA-BMA by means of neutron activation, in turn obtaining a radioactive ^{159}Gd-DTPA-BMA complex.

Through the previously described procedures, Gd-DTPA-BMA encapsulated in liposomes was prepared and irradiated with neutrons for 8 h. According to Soares et al. (2010), when using the same irradiation parameters, the radioactive complex presents a specific activity of 240.8 ± 42 MBq mg^{-1}. The present study identified a specific activity of 12.6 ± 2.1 MBq mg^{-1} (value calculated and confirmed by gamma spectroscopy) for the formulation containing the radioactive complex encapsulated in liposomes. The ^{159}Gd radioisotope's characteristic peaks were determined at 58.7, 79.5, 137.5, 305.5, 348.2, and 363.5 keV (Fig. 2). The energies and their intensities are consistent with the values published by Moralles et al. (1995) and Soares et al. (2010).

3.2. Liposome characterization

3.2.1. Photon correlation spectroscopy, polydispersity index, and zeta potential analysis

The photon correlation spectroscopy (PCS) was conducted on 5 samples at different times. The samples were irradiated for 1, 2, 3, 5, and 8 h, respectively. This procedure evaluated the exposure time in which the liposomes were able to maintain their integrity. The results were calculated from three independent samples by software provided by the manufacturer, which revealed that even after 8 h of irradiation with neutrons (epithermal, thermal, and fast), the average size of the liposomes were maintained nearly constant at 100 nm (Table 1). These results show that the neutron activation process brought about no changes in the size distribution of the liposomes, thus indicating the maintenance of their physical integrity (Mumper and Jay, 1992; Ostrowsky, 1993).

The polydispersity index (P.I.) found in this study is presented in Table 1. All samples presented a P.I. of above 0.3, including the control. These results indicate that the process used in liposome preparation allows one to obtain a monodispersed system (<0.3) (De Assis et al., 2007; Lamprecht et al., 1999; Tobío et al., 1998).

The zeta potential of liposome preparation can aid in predicting the fate of *in vivo* liposomes, given that the presence of electrical charges minimizes agglomeration and fusion phenomenon (Casals et al., 2003). The zeta potential from the formulation exhibited a negative charge, with values of −53 ± 4.9 mV (Table 1), during the 3 h of neutron irradiation. The negative zeta potential conveyed by CHEMS was not masked by the presence of PEG chains. In cell biology, the concept of the zeta potential has commonly been used to study many cellular processes, such as activation, agglutination, and adhesion. Cook and Jacobson (1968) pioneered this research by finding significant differences in surface electrokinetic properties between normal and cancer cells. Recently, Zhang et al. (2008) reported a significant reduction in the zeta potential values between normal breast epithelial cells (MCF10A) and cancer breast epithelial cells (MCF7), showing an important relationship between the zeta potential and tumor malignancy. Gabizon and Papahadjopoulos (1992) demonstrated that, *in vivo*, negative charges heavily increase the clearance of liposomes due to the interaction of charged phospholipid head-groups with certain opsonizing proteins present in blood circulation. In addition, PEG-liposomes prevent clearance by preventing the opsonization of various serum proteins in the blood (Needham et al., 1992; Shimada et al., 1995).

3.2.2. Atomic force microscopy

The environment of a nuclear reactor tends to produce harmful lipid formulations, mainly due to the presence of ionizing radiation capable of promoting lipid oxidation, thereby destabilizing the formulations. The AFM technique was used in this study to com-

Table 1
Physicochemical characteristics of the liposomes under different times of neutron irradiation.

Irradiation time (h)	Mean size ± S.D. (nm) (PCS)[a]	Polydispersity index[b]	ζ Potential ± S.D. (mV)[c]
Control	100 ± 11	0.25 ± 0.01	−46 ± 8.3
1	99 ± 10	0.18 ± 0.02	−49 ± 5.5
2	102 ± 12	0.24 ± 0.05	−47 ± 6.1
3	99 ± 8	0.21 ± 0.01	−53 ± 4.9
5	100 ± 13	0.24 ± 0.04	−51 ± 7.3
8	100 ± 22	0.20 ± 0.02	−52 ± 8.1

[a] Standard deviation ($n = 3$) of the population that was reported by the instrument.
[b] Monodispersed samples (≤0.3).
[c] Measurement after 1:1000 dilution in 1 mM NaCl (conductivity, 120 ± 20 S/cm).

pare the morphology of liposomes subjected to the irradiation with neutrons to the groups that were not irradiated (control) so as to verify the physical instability of liposomes after irradiation. The irradiated and control groups underwent the AFM technique as a substrate using a paraffin wax film (Parafilm®). The results showed that the liposomes were not irradiated, isolated spherical structures with an average size of 92 ± 4 nM (Fig. 3). The size distribution of this preparation was in fact homogeneous, showing no tendency to disseminate along the substrate surface, thus proving to be a stable formulation.

The liposomes submitted to the irradiation process studied by the AFM technique revealed the same isolated spherical structures with an average diameter of 81 ± 3 nm (Fig. 4), indicating that the size of vesicles, as well as their structural integrity, had not changed during the process of neutron activation. These results aid in evaluating neutron irradiation as a new method for obtaining liposomes which have been radiolabeled directly in a nuclear reactor. Additionally, this methodology allows for a significant reduction in researcher exposure to ionizing radiation, given that there is no laboratory procedure to be conducted after the neutron irradiation process.

In this study, the AFM images were obtained after placement and drying of liposomes on a substrate surface. Liposomes, when removed from water after drying on the substrate, may undergo changes, such as losing their internal aqueous content. Fig. 4 shows that liposomes have a height of 10 nm. This may well be attributed to the height of two lipid bilayers resulting from the loss of internal content.

3.2.3. Gd-DTPA-BMA encapsulation efficiency and release profile

The encapsulation rates of different substances in the internal compartment, or in the membrane of liposomes, are important parameters that fundamentally depend on the methods of preparation and concentration of the lipid (Frézard et al., 2005). Le (2008) and Le and Cui (2006a,b) improved the encapsulation efficiency of the Gd-DTPA complex in different liposomal formulations made up of hydrogenated phosphatidylcholine (HPC), distearoylphosphocholine (DSPC), and distearoylphosphoethanolamine-methoxypolyethyleneglycol 2000 (MPEG-DSPE) in a respective molar ratio of 50/35/5, adding the poly-L-lysine (PLL) amino acid to the liposome preparation. With the same purpose, the present study also added the poly-L-lysine amino acid to the Gd-DTPA-BMA complex (1/0.25, w/w) during liposome production. However, the results obtained show no significant difference in the encapsulation efficiency between the two formulations ($p = 0.7$) (Table 2).

The release kinetics of Gd-DTPA-BMA are shown in Fig. 5 for the DMEM (Dubelco's Modified Eagle's Medium) culture medium and rat serum at 70%. It can be observed that, after 24 h, only the release of approximately 2.7% of the formulation without PLL actually occurs. For liposomes containing the PLL formulation, a release of approximately 2.2% occurred. Within 8 h of incubation, a greater tendency toward stabilizing both thermodynamic formula-

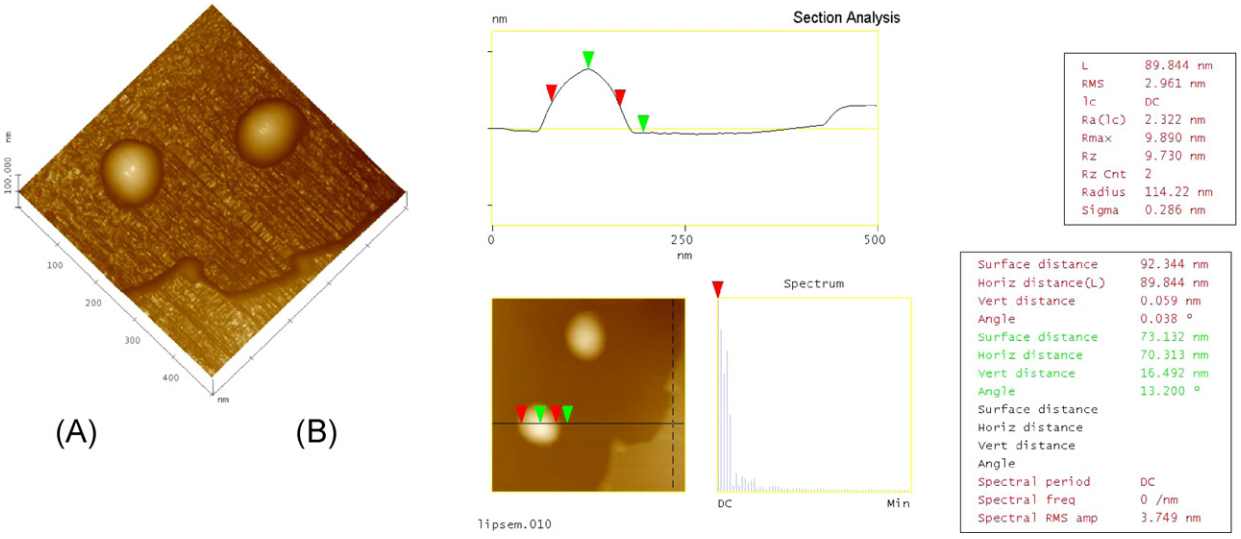

Fig. 3. Atomic force microscopy of liposomes containing Gd-DTPA-BMA (control group) (A) 3D plan and (B) top view. Using the software option "section analysis", the figure shows liposome height and diameter with green and red arrows, respectively. The measured sizes are indicated in tables with the same colors. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

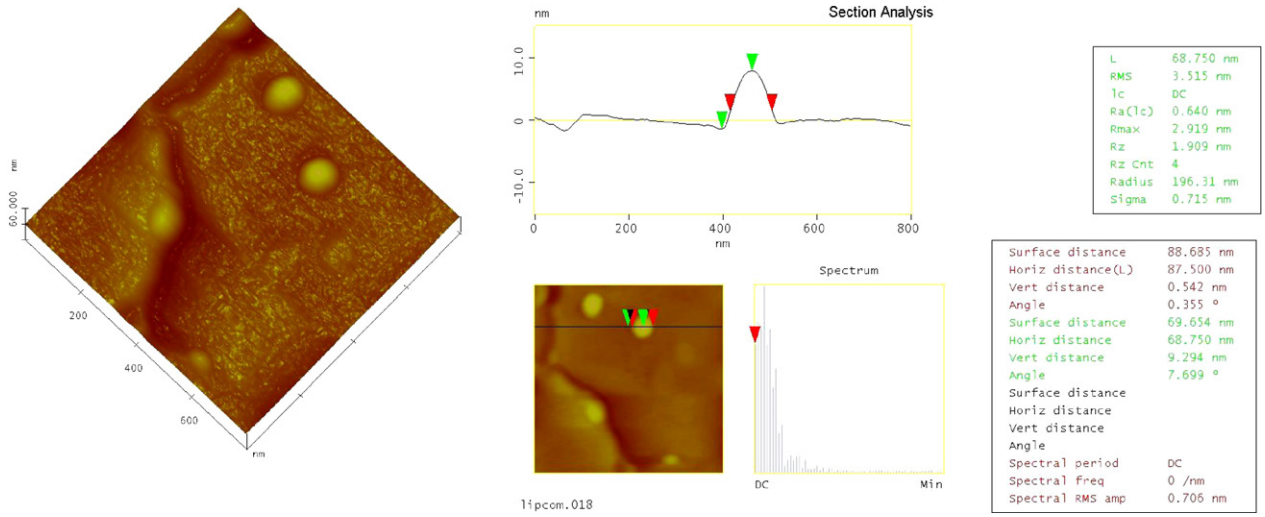

Fig. 4. Atomic force microscopy of liposomes containing ^{159}Gd-DTPA-BMA (irradiated samples) (A) 3D plan and (B) top view. Using the software option "section analysis", the figure shows liposome height and diameter with green and red arrows, respectively. The measured sizes are indicated in tables with the same colors. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

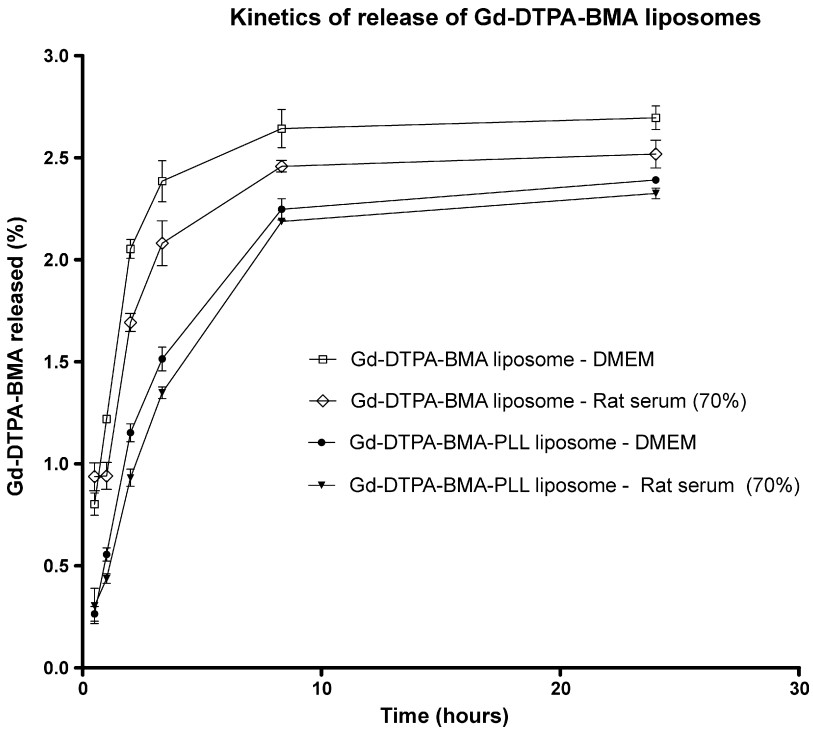

Fig. 5. Kinetics of release of Gd-DTPA-BMA liposomes (100 nM) in DMEM and rat serum at 70% ($n = 3$). The one-way ANOVA analysis followed "Bonferroni's Multiple Comparison Test" shows $p > 0.05$.

tions occurred. Therefore, it could be observed that the addition of PLL to the Gd-DTPA-BMA liposome compound did not contribute significantly to reducing the release rate of Gd-DTPA-BMA from these liposomes ($p > 0.05$).

The PLL action mechanism regarding the release of the liposome content is not well explained in the literature. According to Le (2008), the effect of PLL on the release of the gadolinium compound is due to a relative increase in the size of the Gd-DTPA-PLL compound. This finding is supported by Frézard et al. (2005), who stated that the larger the size of the substances encapsulated in liposomes,

Table 2
Gd-DTPA-BMA encapsulation efficiency in liposomes ($n = 3$).

Statistic parameter	Liposome	Poly-L-lysine liposome
Encapsulation average (%)	19.01	21.72
Standard deviation	0.92	1.87
Percentual error	4.77	8.61

Table 3
IC_{50} of control samples within RT2 cells.

IC_{50} values within RT2 cells (mM) $n = 8$				
Blank liposomes	Gd-DTPA-BMA	Gd-SpHL	^{159}Gd-DTPA-BMA	^{159}Gd-SpHL
13.8 ± 0.4	19.9 ± 0.9	0.066 ± 0.015	0.12 ± 0.01	0.017 ± 0.002

the slower its release. However, it is also emphasized that the Gd-DTPA-BMA compound is sufficiently large, which minimizes the action of poly-L-lysine.

3.2.4. In vitro cytotoxic evaluation

The cytotoxic activity of the blank liposomes, Gd-DTPA-BMA, Gd-SpHL, and ^{159}Gd-DTPA-BMA was evaluated as a control of the cytotoxic activity of the ^{159}Gd-SpHL formulation (Table 3). The results revealed an IC_{50} of 13.8 mM for blank liposomes. Moreover, lipid concentrations of below 9 mM present a cell feasibility of greater than 90%. From these results, studies aimed at evaluating the cytotoxic activity of the complex within radioactive liposomes were conducted with a lipid concentration below 9 mM. Thus, the cytotoxic activity was removed from the lipids in the formulation.

The Gd-DTPA-BMA complex has a cytotoxic activity that acts against RT2 tumor cells. However, within the liposomes, the cytotoxic activity is increased by approximately 300 fold (19.9–0.066 mM). As the Gd-DTPA-BMA complex is quite hydrophilic, its absorption in the intracellular environment has proven to be relatively insignificant, which may well explain the low cytotoxicity observed in RT2 cells. However, when encapsulated in liposomes, an increase in the cytotoxic activity of the complex could be observed. This behavior can be explained by the fact that the liposomes are structures that allow the intracelular internalization of their contents, in turn allotting greater potential to the cytotoxic effect (Miller et al., 1998; Lukyanov et al., 2004).

By means of ionizing radiation, the cytotoxic activity of the free complex is incremented approximately 166 fold. For ^{159}Gd-SpHL, an IC_{50} value of 0.017 mM (Fig. 6) could be observed, which is considered to be of the same magnitude as some cytotoxic agents used in cancer treatment. Comparing this figure to the IC_{50} of free and non-radioactive complexes it can be observed that the sum of factors – presentation form of tumor cells and radiation presence – allows for an increase of approximately 1170 times the cytotoxic activity of metal complex.

The absorbed dose rate in RT2 cells was estimated for the IC_{50} concentration using Martin's mathematic model for beta dosimetry on contaminated skin surfaces (Martin, 2006). The results showed that the absorbed dose rate value is approximately 8.5 Gy h^{-1} in RT2 cell cultures. It can therefore be concluded that the p53 activation mechanism may be used to explain the cytotoxic activity of radioactive Gd-DTPA-BMA used against RT2 cells.

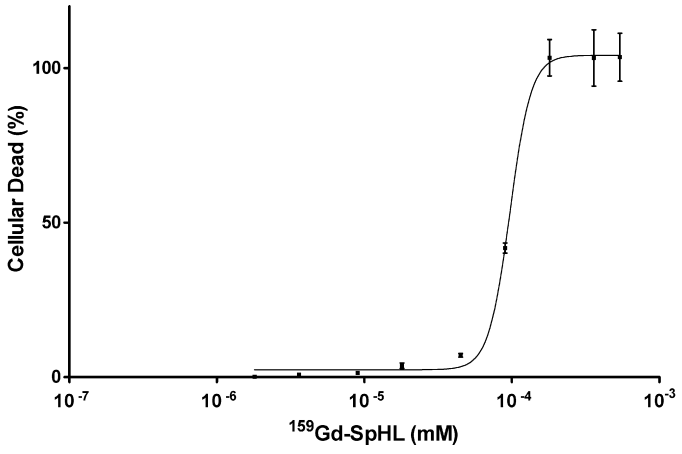

Fig. 6. Representative curves of surviving RT2 cells vs. the log of ^{159}Gd-SpHL molar concentration. The IC_{50} value was determined through non-linear regression ($n = 8$).

Prior studies have pointed out the development of nephrogenic systemic fibrosis (NSF) in groups of patients with severe chronic kidney disease, through the use of free Gd-DTPA-BMA (Omniscan®). However, no study has demonstrated the relationship between the use of Gd-DTPA-BMA encapsulated in liposomes as regards the development of NSF. Carvalho Júnior et al. (2007) demonstrated that the encapsulation of cisplatin (CDDP) in liposomes (SpHL) tends to lead to a reduction in CDDP retention via renal tissues, indicating that the SpHL-CDDP may indeed be useful in alleviating kidney damage induced by CDDP. For this reason, in patients with severe chronic kidney disease, the possible use of the ^{159}Gd-SpHL formulation, geared toward treating cancer, still warrants further investigation.

4. Conclusion

The findings in this study revealed that during and after the process of neutron irradiation (labeling process), the liposomes maintained similar physicochemical characteristics in terms of size and zeta potential. After the labeling procedure, the vesicles were also considered to be stable from a morphological point of view and showed low release kinetics in their contents' biological environment. This finding is quite suitable for systems that selectively accumulate radioisotopes within target tissues (e.g., tumor cells), in detriment of healthy tissues, which leads to the need for the safe use of this formulation in *in vivo* studies. The present results, together with further studies, may reveal a potential application of pH-sensitive liposomes containing a therapeutic radioisotope for cancer treatment.

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