IN VITRO MAGNETIC TARGETED DELIVERY OF DOXORUBICIN USING IRON OXIDE NANOPARTICLES LEADS TO ENHANCED CELL DEATH IN GLIOBLASTOMA

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Iron oxide nanoparticles have been synthesized using a modified Massart method and in situ functionalized with polyethylene glycol, resulting in 8.81±1.96 nm highly crystalline nanoparticles with spinel structure. Doxorubicin was loaded for magnetic targeted delivery to the peri- nuclear areas of the U-87 MG human glioblastoma cells. Cytotoxicity was dependent on the internalized quantity of the nanoparticles, which varied for each sample.

Keywords: iron oxide nanoparticles, magnetic targeting, drug delivery, Doxorubicin, glioblastoma

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1. Introduction

Glioblastoma multiform is the most aggressive and invasive type of brain tumor, resulting in very low survival rates among patients (19% for patients with ages between 20- 44 years, 8% for 45- 54 year- old patients, respectively 5% for patients above 55 years [1]). The common treatment schemes for this type of cancer involve surgery to remove the tumor, followed by radiation therapy and/ or chemotherapy, which have low efficiency due to the high recurrence and therapy resistance [2]. In order to augment the patients' response to applied treatment and improve general wellbeing, new approaches involving nanotechnology have been proposed for the treatment of glioblastoma multiform [3, 4, 5, 6].

In this study, we have synthesized polyethylene glycol (6K molecular weight) functionalized iron oxide nanoparticles employing an *in situ* method. With the aim to propose new magnetically activated drug delivery systems for glioblastoma treatment, the chemotherapeutic substance Doxorubicin (DOX) was loaded through ad/ absorption within the Fe_3O_4@PEG 6K particles. DOX has previously been used in different clinical approaches for the management of this disease [7, 8].

The morphological and structural characterization was performed using scanning electron microscopy (SEM), high resolution transmission electron microscopy (HR-TEM) and selected area electron diffraction (SAED). The potential use of DOX-loaded Fe_3O_4@PEG 6K systems for targeted and controlled drug delivery under magnetic activation was evaluated by subjecting nanoparticle suspensions to different high amplitude alternating fields.

The *in vitro* evaluation of the nanosystems was done for U-87 MG human glioblastoma cells using the MTS tetrazolium- salt metabolic assay and microscopic characterization of the morphology. The nanosystems proved to have cytotoxic effects in targeted glioblastoma cells. The novelty of the study especially comes from the correlation of the cytotoxic effects of magnetic targeted drug-loaded nanoparticles in glioblastoma cells with high precision quantitative measurements of nanoparticle internalization.

2. Materials and methods

All chemical reagents were of analytical purity and purchased from Sigma Aldrich Chemie Gmbh, Darmstadt, Germany. The synthesis of the iron oxide nanoparticles (Fe_3O_4) was done using a modified co- precipitation method, at room temperature. For this, the precursor solution of ferrous sulfate and ferric chloride, molar ratio $[FeSO_4 \bullet 7H_2O]$:$[FeCl_3]$=1:1.6, was added drop wise into an alkaline solution of ammonia. The Fe_3O_4 nanoparticles were washed several times with ultrapure water and the final suspensions were also done in ultrapure water. The 6K polyethylene glycol (PEG with 6,000 g/mol molecular weight)

functionalized iron oxide nanoparticles (Fe_3O_4@PEG 6K) were obtained using an *in situ* conjugation method, by dissolving the polymer into the precipitation medium prior to the addition of the precursor solution [9, 10].

The chemotherapeutic drug Doxorubicin (DOX) was loaded into the Fe_3O_4@PEG 6K nanosystems through ad/ absorption from aqueous solution. For this, the Fe_3O_4@PEG 6K were immersed into a 32 μg/mL DOX solution (prepared in ultrapure water) for 24h (Fe_3O_4@PEG 6K/ DOX (24)), respectively 48h (Fe_3O_4@PEG 6K/ DOX (48)). After this time, the drug- loaded nanoparticles were magnetically separated and the supernatant was kept for spectrophotometric measurements, which was performed using a microplate reader (Tecan, Männedorf, Switzerland). The loading ability of the drug was calculated using the method described by Kamba et al [11].

The morphological characterization of synthesized nanoparticles was performed using the scanning electron microscopy method, using in this respect a SEM microscope from FEI Company (Hillsboro, OR, USA). The analysis was done for dried nanoparticle powders placed on a carbon strip and the energy of the secondary electron beam was up to 30 keV.

The structural and intimate morphological evaluation of nanoparticles was done using the transmission electron microscopy (TEM), high resolution TEM (HR-TEM) and selected area electron diffraction (SAED) analysis. The investigations were performed using a Tecnai G2 F30 S-TWIN HR-TEM (FEI Company, Hillsboro, OR, USA) equipped with SAED accessory. For analysis, the samples were prepared by making successive dilutions and sonication in ultrapure water. The final suspension was placed onto a holey carbon- coated copper grid and dried. The investigations were done in transmission mode, at 300 kV, with point resolution of 2Å and line resolution of 1Å.

In order to evaluate the magnetically activated response of the nanoparticles, volumes of 4 mL from final suspensions (3.2 mg/mL for Fe_3O_4@PEG 6K, respectively 1.6 mg/mL for Fe_3O_4@PEG 6K/ DOX) were exposed to radiofrequency magnetic fields with power of 2, 1.6, 1.2 and 0.8 kW. An Ultra Heat S Series (RF) heating system (UltraFlex Power Technologies, Sofia, Bulgaria) accessorized with a fiber optic temperature sensor (Optocon, Dresden, Germany) was used for measurements. For each sample, the thermal response was recorded up to 50^{o}C, which is considered to be the thermal limit where the apoptosis and necrosis processes of tumor cells occur [12]. The supernatants resulted from the magnetic separation were spectrophotometrically analyzed. Specific Absorption Rate (SAR) was calculated accordingly to slope of recorded temperature versus time dependence, as described in [13].

The cytotoxicity of drug-free and drug-loaded Fe_3O_4@PEG 6K nanoparticles was assessed for U-87 MG human glioblastoma cells purchased from ATCC (Mansas, VA, USA), which were cultured in Dulbecco's modified

Eagle's medium (DMEM) (Biochrom, Merck Milipore, Billerica, MA, USA), supplemented with 10% fetal bovine serum (Biochrom) and 1% Penicillin/ Streptomycin (Biochrom), in standard conditions of temperature and humidity (37±2 ^{o}C, 5±1 % CO_2 and more than 90% humidity).

The quantitative internalization of the nanoparticles in the U-87 MG cells was evaluated using the Particle Induced X-ray Emission (PIXE) technique, performed at the Romanian Center for Ion Beam Applications (RoCIBA) using a 3 MV Tandetron particle accelerator [14]. A 3 MeV proton beam was transported to the IBA chamber and extracted in air through a silicon nitride window. The samples were positioned in air, at a distance of 25mm from the Si3N4 membrane. Using this setup, a 2.83 MeV proton beam, reached the samples at normal incidence. An Amptek SiPIN detector positioned backwards at 45^{o} with respect to the beam direction was used to record characteristic X-rays from the samples (resolution 130 eV at 5.9 keV, Kα line of 55Mn). For this, 250000 cells were seeded in 6 well plates and cultured during 24h to allow attachment. Afterwards, nanoparticle treatment was applied for another 24h. At the end of the incubation time, the samples were thoroughly washed with PBS to remove the non-internalized nanoparticles and cells were collected for measurements. The spectrums were processed using the GupixWin software [15] and the Fe_3O_4 concentration per cell was calculated by normalizing the output values to the cell number.

The magnetic targeting setup was prepared as following: 5000 U-87 MG cells were seeded on 10 mm glass coverslips and cultured for 24h to allow attachment. Two of these coverslips (one representing the control area and one the targeting area) were placed into a 60 mm Petri dish which was filled with either complete DMEM either with nanoparticle suspensions in complete DMEM (concentrations of 250, 125, 62.5, respectively 31.25 μg/ mL). A 5 mm NdFeB magnet (10.8- 12.5 kOe, 1,2 kg magnetic power, Hobber) was placed under the targeting area coverslip (Fig. 1) and cells were cultured for another 24h.

In order to measure the cytotoxic effect of the nanoparticles, the MTS tetrazolium- salt based viability assay was employed (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WS, USA), according to the producer's specifications. After the incubation time, each glass coverslip was gently washed with Phosphate Buffer Saline (PBS) and was placed into a well of a 24- well plate. A 1:6 solution of MTS: complete DMEM was added in each well; the cells were incubated for another 3h in standard conditions. The cells metabolized the MTS in the solution leading to the formation of soluble formazan, which was measured at 490 nm using a Sunrise microplate reader (Tecan, Männedorf, Switzerland). Control blank samples containing only nanoparticles at the corresponding concentrations were prepared, in order to eliminate the possible interferences.

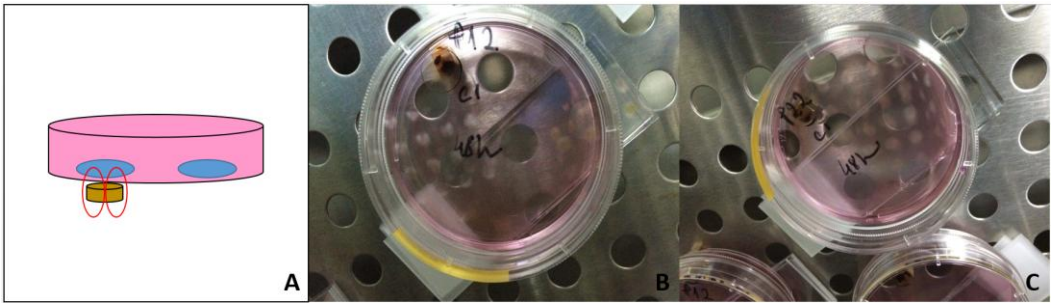

Fig. 1. Magnetic targeting of Fe_3O_4@PEG 6K (/DOX) nanoparticles experimental setup: schematic representation (A) and photographs of the experimental setup (B, C);

The qualitative evaluation of the cytotoxic effects of the nanoparticles, but also their internalization in glioblastoma cells was evaluated using optical microscopy. The glass slides containing cells from the magnetic targeting experiment were individually placed in 24 well plates and carefully washed with PBS. Fresh culture medium was placed in each well. Images of the living cells were acquired using an Olympus BX51 brightfield microscope (Tokyo, Japan).

Experiments were performed in triplicate and data was presented as mean ± SEM. The statistic evaluation was done using Student's t- test and one- way ANOVA.

3. Results and discussions

This study presents the synthesis, characterization and *in vitro* testing of polyethylene glycol functionalized iron oxide nanoparticles for the targeted magnetic delivery of Doxorubicin chemotherapeutic in glioblastoma cells. The nanoparticles were obtained using a room temperature modified chemical co-precipitation method, similar as in Popescu et al [10], where the conjugation with the polymer was done *in situ*.

The scanning electron microscopy morphological evaluation of the Fe_3O_4-based powders revealed an aggregated aspect of the ovoid nanoparticles, with physical dimensions between 5- 12 nm (data not shown). There was no noticeable difference in the aspect of the samples (Fe_3O_4, Fe_3O_4@PEG 6K, respectively Fe_3O_4@PEG 6K/ DOX (48)).

Transmission electron microscopy images confirmed the aggregated aspect and the size dispersion of the Fe_3O_4@PEG 6K nanoparticles with ovoid morphology and mean diameters of 8.81±1.96 nm (Fig. 2- a, b and Fig. 3). The high resolution TEM showed the high degree of crystallinity of the inorganic phase, the (220) crystalline plane of 0.29 nm characteristic for magnetite mineralogical phase being emphasized (Fig. 2- c, red arrows) [16]. This information was confirmed by the selected area electron diffraction spectrum,

which showed the diffraction rings of face centered magnetite phase with spinel structure: (220), (222), (400), (440), (333), (422) (Fig. 2- d) [17].

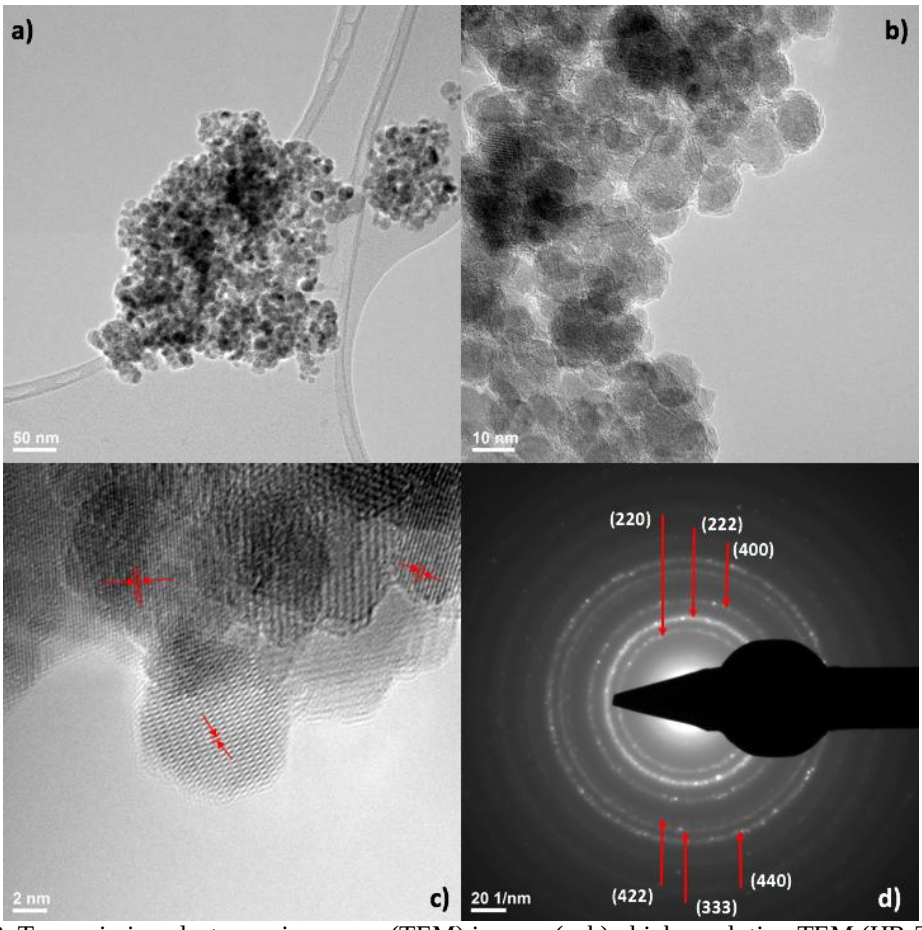

Fig. 2. Transmission electron microscopy (TEM) images (a, b), high resolution TEM (HR-TEM) (c), respectively selected area electron diffraction (SAED) spectrum (d) for Fe_3O_4@PEG 6K;

The drug loading ability of Fe_3O_4@PEG 6K was dependent on the loading time of the chemotherapeutic substance Doxorubicin. Hence, the quantity of encapsulated DOX in Fe_3O_4@PEG 6K/ DOX (24) and Fe_3O_4@PEG 6K/ DOX (48) was 0.49 wt%, respectively 0.81 wt%, with a calculated loading efficiency of 24.5%, respectively 40.95%.

Following the RF magnetic field exposure, we monitored both the thermal response of nanoparticle suspensions (occurred due to superparamagnetic properties of iron oxide nanoparticles) and the accompanying drug release (occurred due drug concentration variations within the collected supernatants). The temperature variation of nanoparticle suspensions was related to the power of applied magnetic field, in a directly proportional manner (Fig. 4) [13].

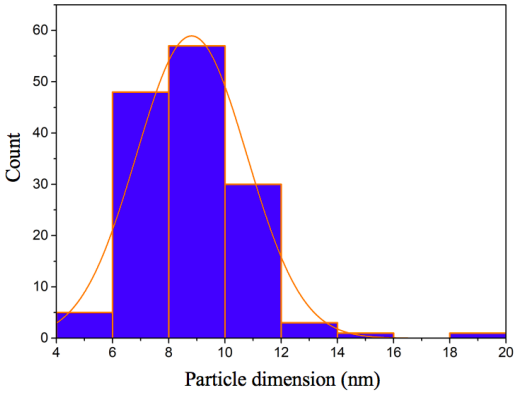

Fig. 3. Size distribution of Fe_3O_4@PEG 6K measured in TEM images;

In all nanosystems, the highest field power (2 kW) caused the most accelerated temperature increase. Interesting fact, the upper thermal limit of ~45^oC was reached faster (less than 5 min.) in the case of DOX-loaded nanoparticle suspensions. For Fe_3O_4@PEG 6K/ DOX (24) nanosystems, similar thermal response was obtained for both middle field powers in approx. 6 min. In the particular case of Fe_3O_4@PEG 6K/ DOX (48), similar thermal responses were recorded for 2 kW and 1.6 kW fields. The maximum calculated SAR value was obtained for Fe_3O_4@PEG 6K/ DOX (48) (43.68±1.25 kW/kg), followed by Fe_3O_4@PEG 6K (24.03±1.62 kW/kg), respectively Fe_3O_4@PEG 6K/ DOX (24) (37.1±0.42 kW/kg).

The UV-VIS spectra confirmed the drug release by measuring the absorbance at 480 nm (data not shown). The drug release after magnetic stimulation was proportional with the thermal variation after magnetic stimulation leading to a maximum release of 78.2% Fe_3O_4@PEG 6K/ DOX (24), respectively 72.12% for Fe_3O_4@PEG 6K/ DOX (48) (for 2 kW magnetic field).

The magnetic targeting of the nanoparticles was possible due to the native magnetic properties of Fe_3O_4 [18]. Hemery et al [19] used the magnetic properties of PEG- conjugated iron oxide nanoparticles to enhance the cytotoxicity against glioblastoma cells. Here, the magnetic targeting of Fe_3O_4@PEG 6K (DOX) nanoparticles was tested using an *in vitro* set-up similar as in [20], followed by the cytotoxicity characterization.

The MTS metabolic activity of the U-87 MG cells was evaluated after the magnetic targeting of Fe_3O_4@PEG 6K (DOX) nanoparticles, in order to provide quantitative information on their cytotoxic potential (Fig. 5- a). The viability of the cells was calculated as percent from the untreated (control) cells. Thus, unloaded Fe_3O_4@PEG 6K nanoparticles showed a biocompatible behaviour as the viability for all concentrations employed in the study was above 80% (according

to ISO 10993-12:2001(E)), even for the highest concentration of 250 μg/mL (88.25±1.06%, P<0.01) (Fig. 5- a). These results confirm previous data on the biocompatibility of PEG- functionalized iron oxide nanoparticles [21].

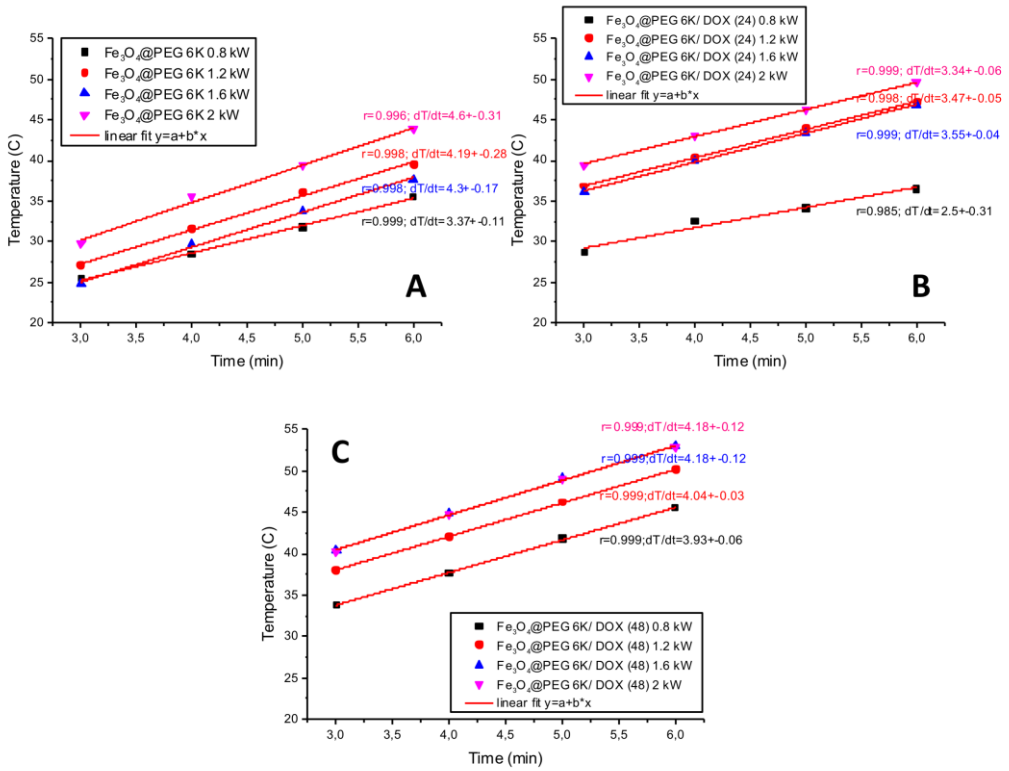

Fig. 4. Dependence of heating ability after different magnetic fields exposure: Fe3O4@PEG 6K, Fe3O4@PEG 6K (24), respectively Fe3O4@PEG 6K (48);

The loading of doxorubicin in the Fe_3O_4@PEG 6K nanoparticles increased the cytotoxicity of the nanoparticles in a manner which was dependent on the loading time of the drug (thus, the loaded quantity of DOX). The viability of glioblastoma cells after 24h of interaction with Fe_3O_4@PEG 6K/ DOX (24) decreased until 62.74±0.65% for the highest concentration employed (250 μg/mL), where P<0.001 compared to control, respectively Fe_3O_4@PEG 6K, showing an improved cytotoxic effect of the nanoparticles after loading the chemotherapeutic substance (Fig. 5- a).

The loding of the active substance for another 24h did not show any significantly statistic additional effect on the cytotoxicity of the U-87 MG cells, for none of the concentrations employed in the study, although the loading efficiency of DOX was higher for Fe_3O_4@PEG 6K/ DOX (48) compared to Fe_3O_4@PEG 6K/ DOX (24) (40.94% compared to 24.5%) (Fig. 5- a). However,

Fe_3O_4@PEG 6K/ DOX (48) showed a cytotoxic effect for all concentrations employed in the study (compared to control cells and DOX free nanoparticles) (Fig. 5- a).

These results suggest there might be a difference in the internalization of the nanoparticles in the cells after 24h interaction. To prove this, we have employed PIXE analysis to measure the quantity of internalized nanoparticles per cell. Results showed a significant difference between the internalized amout of nanoparticles per cell, in case of Fe_3O_4@PEG 6K/ DOX (24) (1325.63±164.41 pg/ cell), compared to both Fe_3O_4@PEG 6K (601.48±152.65 pg/ cell) and Fe_3O_4@PEG 6K/ DOX (48) (828.2±22.3 pg/ cell) ($P<0.05$) (Fig. 5- b).

Fig. 6 illustrates the morphology of the cells after interaction with Fe_3O_4@PEG 6K/ DOX (48) nanoparticles and their internalization. Thus, the density of the cells appeared to be reduced in case of nanoparticles- exposed U-87 MG samples, the cells having a round aspect with smaller elongations. The presence of nanoparticles was evidenced as dark aggregates located in the cytoplasm of the cells, mainly in the peri- nuclear area (Fig. 6). The localisation of the nanoparticles in this area can be explained by the high dimension of the aggregates and their localisation into the endo- lysosome compartments [11, 22].

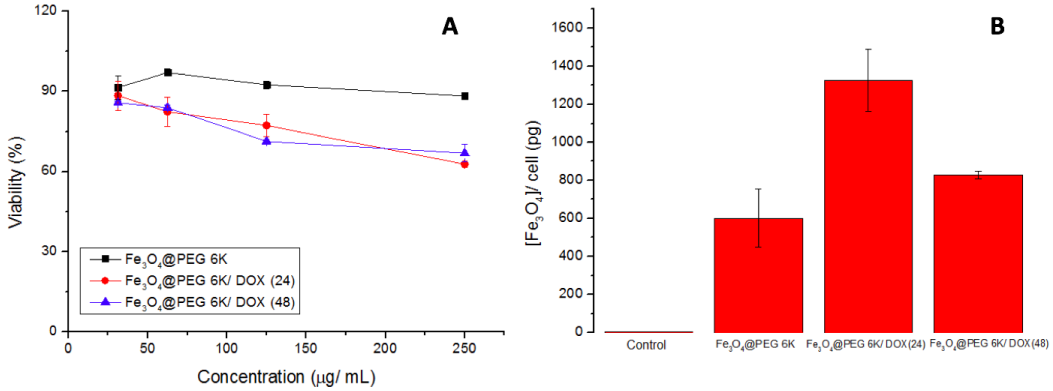

Fig. 5. (A) Viability of U-87 MG human glioblastoma cells after nanoparticle exposure and magnetic targeting during 24h; (B) quantity of internalized nanoparticles per cell; data is presented as mean±SEM;

Xia et al [23] used local magnetic field to target HeLa cells for redox delivery of doxorubicin from Fe_3O_4 loaded PEG- based micelles. Similarly, Venugopal et al [24] obtained Fe_3O_4@Au- Phytagel/ DOX to induce magnetic cell targeting and improve cytotoxicity in glioma cells. Here, we have used PEG-conjugated nanoparticles to deliver DOX using a magnetic targeted approach in glioblastoma cells, the *in vitro* cytotoxic effects being correlated with the high precision internalization measurements.

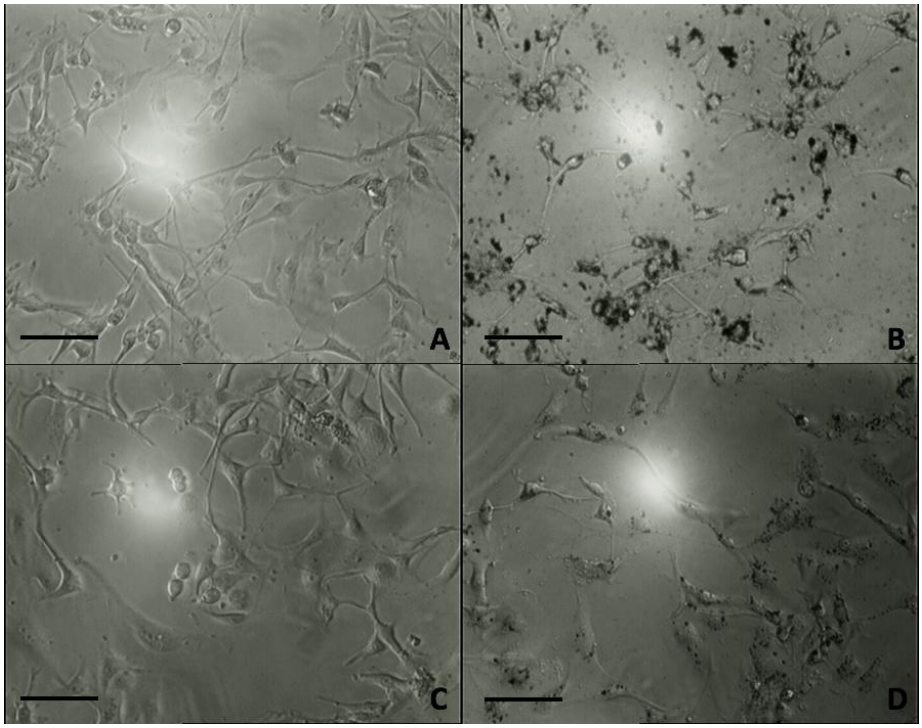

Fig. 6. U-87 MG human glioblastoma cells morphology after magnetic targeting using different concentrations of Fe3O4@PEG 6K/ DOX (48) nanoparticles (250 μg/mL A-B, respectively 125 μg/mL C-D): control areas (A, C), respectively targeted areas (B, D); measure bar 100 μm;

4. Conclusions

Iron oxide nanoparticles (Fe_3O_4) were successfully prepared and functionalized in situ with polyethylene glycol (molecular weight 6K) using a modified chemical co- precipitation method. Physical and chemical characterization revealed the obtaining of highly crystalline face centered spinel structured magnetite nanoparticles. Magnetic targeting of Fe_3O_4@PEG 6K/ (DOX) determined their intracellular internalization in the peri- nuclear areas of the aimed U-87 MG human glioblastoma cells and local doxorubicin delivery increased the cytotoxicity.

Acknowledgements

This work has been funded by the Romanian Ministry of Research and Innovation (PN 18090202/2018, PN 19060203/2019 and 36 PFE/2018) and the Operational Programme Human Capital of the Ministry of European Funds through the Financial Agreement 51668/09.07.2019, SMIS code 124705. The

structural and compositional characterizing of the materials through transmission electron microscopy was possible due to an EU-funded project [POSCCE-A2-O2.2.1-2013-1/Priority Axe 2, Project no 638/12.03.2014, ID 1970, SMIS-CSNR code 48652].

R E F E R E N C E S

[1].*** *Bristol-Myers Squibb*, "Glioblastoma at- a- glance" in https://www.bms.com/assets/bms/us/en-us/pdf/Disease-State-Info/GLIOBLASTOMA-BY-THE-NUMBERS.pdf (accessed on April 4th 2020).

[2]. *E. Ozdemir-Kaynak, A.A. Qutub, O. Yesil-Celiktas*, "Advances in Glioblastoma Multiforme Treatment: New Models for Nanoparticle Therapy", in Frontiers in Physiology, **vol.9**, 2018, pp. 170.

[3]. *** *MagForce*, "The nanomedicine company" in https://www.magforce.com/home/ (accessed on April 10th 2020).

[4]. *H. Arzani, M. Adabi, J. Mosafer, F. Dorkoosh, M. Khosravani, H. Maleki, H. Nekounam, M. Kamali*, "Preparation of curcumin-loaded PLGA nanoparticles and investigation of its cytotoxicity effects on human glioblastoma U87MG cells" in Biointerface Research in Applied Chemistry, **vol. 9**, no. 5, 2019, pp. 4225-4231.

[5]. *M. Norouzi, V. Yathindranath, J.A. Thliveris, B.M. Kopec, T.J. Siahaan, D.W. Miller*, "Doxorubicin-loaded iron oxide nanoparticles for glioblastoma therapy: a combinational approach for enhanced delivery of nanoparticles", in Scientific Reports, **vol. 10**, 2020, pp. 11292.

[6]. *F. Kazmi, K.A. Vallis, B.A. Vellayappan, A. Bandla, D. Yukun, R. Carlisle*, "Megavoltage Radiosensitizaion of Gold Nanoparticles on a Glioblastoma Cancer Cell Line Using a Clinical Platform", in International Journal of Molecular Sciences, vol. 21, 2020, pp. 429.

[7]. *** *NCT02758366 ClinicalTrials.gov* "Prolonged Exposure to Doxorubicin in Patients With Glioblastoma Multiforme and Diffuse Intrinsic Pontine Glioma" in https://clinicaltrials.gov/ct2/show/NCT02758366 (accessed on April 6th 2020).

[8]. *V. Matcovschii, D. Lisii, V. Gudumac, S. Dorosenco*, "Selective interstitial doxorubicin for recurrent glioblastoma" in Clinical Case Reports, **vol. 7**, 2019, pp. 2520– 2525.

[9]. *A.M. Grumezescu, C. Saviuc, A. Holban, R. Hristu, C. Croitoru, G. Stanciu, C. Chifiriuc, D. Mihailescu, P. Balaure, V. Lazar*, "Magnetic chitosan for drug targeting and *in vitro* drug delivery response" in Biointerface Research in Applied Chemistry, **vol. 1**, no. 5, 2011, pp. 160-165.

[10]. *R.C. Popescu, E. Andronescu, B.S. Vasile, R. Trusca, A. Boldeiu, L. Mogoanta, G.D. Mogosanu, M. Temelie, M. Radu, A.M. Grumezescu, D. Savu*, "Fabrication and Cytotoxicity of Gemcitabine- Functionalized Magnetite Nanoparticles" in Molecules, **vol. 22**, no. 7, 2017, pp. E1080.

[11]. *S.A. Kamba, M. Ismail, S.H. Hussein-Al-Ali, T.A. Ibrahim, Z.A. Zakaria*, "In vitro Delivery and Controlled Release of Doxorubicin for Targeting Osteosarcoma Bone Cancer" in Molecules, vol. 18, 2013, pp. 10580-10598.

[12]. *V. Grumezescu, O. Gherasim, I. Negrut, S. Banita, A.M. Holban, P. Florian, M. Icriverzi, G. Socol*, "Nanomagnetite-embedded PLGA Spheres for Multipurpose Medical Applications" in Materials (Basel), **vol. 12,** no. 16, pp. 2521.

[13]. *F. Reyes-Ortega, B.L. Checa Fernández, A.V. Delgado, G.R. Iglesias*, "Hyperthermia-Triggered Doxorubicin Release from Polymer-Coated Magnetic Nanorods" in Pharmaceutics, **vol. 11**, no. 10, 2019, pp. 517.

[14]. *I. Burducea, M. Straticiuc, D.G. Ghita, D.V. Mosu, C.I. Calinescu, N.C. Podaru, D.J.W. Mous, I. Ursu, N.V. Zamfir*, "A new ion beam facility based on a 3 MV Tandetron™ at IFIN-HH, Romania" in Nuclear Instruments and Methods in Physics Research Section B: Beam Interaction with Materials and Atoms, **vol. 359**, 2015, pp. 12-19.

[15]. *J.L. Campbell, N.I. Boyd, N. Grassi, P. Bonnick, J.A. Maxwell*, "The Guelph PIXE software package IV" in Nuclear Instruments and Methods in Physics Research Section B: Beam Interaction with Materials and Atoms, **vol. 268**, no. 20, 2010, pp. 3356-3363.

[16]. *B. Li, H. Cao, J. Shao, M. Qu*, "Enhanced anode performances of the Fe3O4-Carbon-rGO three dimensional composite in lithium ion batteries" in Chemical Communications, **vol. 47**, 2011, pp. 10374–10376.

[17]. *B. Thapa, D. Diaz- Diestra, J. Beltran-Huarac, B.R. Weiner, G. Morell*, "Enhanced MRI T2 relaxivity in Contrast- Probed Anchor- Free PEGylated Iron Oxide Nanoparticles" in Nanoscale Research Letters, **vol. 12**, 2017, pp. 312.

[18]. *Y. Zheng, Y. Cheng, F. Bao, Y. Wang*, "Synthesis and magnetic properties of Fe3O4 nanoparticles", in Materials Research Bulletin, **vol. 41**, no. 3, 2006, pp. 525-529.

[19]. *G. Hemery, C. Genevois, F. Couillaud, S. Lacomme, E. Gontier, E. Ibarboure, S. Lecommandoux, E. Garanger, O. Sandre*, "Monocore vs multicore magnetic iron oxide nanoparticles: uptake by glioblastoma cells and efficiency for mangetic hyperthermia" in Molecular Systems Design & Engineering, **vol. 2**, 2017, pp. 629-639.

[20]. *Y. Lu, K. Wei, C.M. Ma, S. Yang, J. Chen*, "Dual targeted delivery of doxorubicin to cancer cells using folate-conjugated magnetic multi-walled carbon nanotubes", in Colloids and Surfaces B: Biointerfaces, **vol. 89**, 2012, pp. 1-9.

[21]. *G. Yuan, Y. Yuan, K. Xu, Q. Luo*, "Biocompatible PEGylated Fe_3O_4 nanoparticles as photothermal agents for near-infrared light modulated cancer therapy" in International Journal of Molecular Sciences, **vol. 15**, no. 10, 2014, pp. 18776–18788.

[22]. *Y. Zhang, C. Yang, W. Wang, J. Liu, Q. Liu, F. Huang, L. Chu, H. Gao, C. Li, D. Kong, Q. Liu, J. Liu*, "Co-delivery of doxorubicin and curcumin by pH-sensitive prodrug nanoparticles for combination therapy of cancer" in Scientific Reports**, vol. 6,** 2016, pp. 21225.

[23]. *K. Xia, Y. Lyu, W. Yuan, G. Wang, H. Stratton, S. Zhang, J. Wu*, (2019) "Nanocarriers of Fe_3O_4 as a Novel Method for Delivery of the Antineoplastic Agent Doxorubicin Into HeLa Cells *in vitro*" in Frontiers in Oncology, **vol. 9**, 2019, pp. 250.

[24]. *I. Venugopal, S. Pernal, A. Duproz, J. Bentley, H. Engelhard, A. Linninger*, "Magnetic field-enhanced cellular uptake of doxorubicin loaded magnetic nanoparticles for tumor treatment" in Materials Research Express, **vol. 3**, 2016, pp. 095010.