ELSEVIER

Journal of Magnetism and Magnetic Materials 194 (1999) 76–82

Journal of magnetism and magnetic materials

In vitro and in vivo toxicity of magnetic microspheres

Urs O. Häfeli*, Gayle J. Pauer

The Cleveland Clinic Foundation, Radiation Oncology Department T28, 9500 Euclid Ave., Cleveland, OH 44195, USA

Abstract

The interaction of magnetic microspheres with cells was studied using an in vitro 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (=MTT) assay. Viability and metabolic activity were reduced in all examples. The MTT assay is not recommended for this application due to high variability and non-specificity. Poly(lactic acid) microspheres were further tested in vivo. Intrathecal injection in rats produced no obvious side effects over 12 months.

Keywords: Toxicity; Magnetic microspheres; Polymer; Poly(lactic acid); MTT assay; Rat; Intrathecal

1. Introduction

The therapeutic in vivo application of magnetic microspheres for cancer therapy [1], blood purification [2], lymph node imaging [3] or hyperthermia [4], just to name a few, calls for the particles to be biocompatible and non-toxic.

The important factors, which determine the biocompatibility and toxicity of magnetic microspheres, are the magnetically responsive components such as magnetite, iron, nickel, cobalt, neodymium–iron–boron or samarium–cobalt, and the size of the particles, their matrix substance and the coatings used. Iron oxide particles seem to be generally well tolerated [5] and magnetite-based MRI contrast agents have already been approved for human use [6] (Endorem® or Feridex I.V.®; Advanced Magnetics, Cambridge, Massachusetts, USA). With respect to size, in the case of nanospheres with a diameter generally less than about 100 nm, the coating is probably the most important factor and determines many of the particles' properties [7]. In the case of larger magnetic microspheres of diameters up to tens of μm, the matrix substance making up most of the sphere has a much larger influence on tissue reaction, both immediate and long-term. Large microspheres also can physically irritate the surrounding tissue or even embolize small blood vessels and capillaries, effects which must be taken into account for specific applications.

In our work, we investigated if an MTT assay [8], which quantifies cell viability, cell survival and cell growth, could be used to rapidly evaluate the toxic properties of microspheres in vitro. The verification of toxicity in vivo was attempted in an intraspinal long-term model for the magnetic

* Corresponding author. Fax: +1-216-445-4480; e-mail: hafeliu@cesmtp.ccf.org.

0304-8853/99/$ – see front matter
PII: S0304-8853(98)00560-5

microspheres made from poly(lactic acid) and magnetite.

2. Materials and methods

The in vitro toxicity of several types of magnetic microspheres was tested using a modified MTT assay [8]. The test microspheres and controls included the following types: biodegradable poly(L-lactic acid) microspheres containing 30 wt% magnetite (= PLA-MMS) [9] and non-magnetic poly(L-lactic acid) microspheres (= PLA-MS); iron–carbon (FeC) particles containing 20 wt% iron (Synmatix Corp., Southfield, Illinois, USA) and charcoal (Mallinckrodt, St. Louis, Missouri, USA); 40 wt% magnetite polystyrene microspheres PS-A (Estapor; Bangs Laboratories, Fishers, Indiana, USA), paramagnetic polystyrene latex particles PS-B (Polysciences, Warrington, Pennsylvania, USA) and non-magnetic polystyrene latex particles PS-C (Coulter, Miami, Florida, USA); and dextran-coated magnetic nanoparticles CG34 containing 30 wt% magnetite (Microcaps, Rostock, Germany).

Different microspheres were washed three times and then incubated in 3 ml of PBS pH 7.4 at 37°C for 24 h. The concentration of the magnetic microspheres was adjusted to a final iron concentration of 0.5%. The supernatant was sterilized by filtration and aliquots of 100 μl were added to 8 wells of a 96-well plate, each of which contained 5000 tumor cells in 100 μl of media. Two types of tumor cells were used: adherent human prostate cells (DU-145) growing in Eagle's minimum essential media (BioWhittaker, Walkersville, Maryland, USA) containing 10% fetal bovine serum (Fig. 1a) and murine suspension lymphoma cells (EL-4) growing in RPMI 1640 (Fig. 2a). As a control, 100 μl of PBS at pH 7.4 was added to cells in 8 of the wells. After three days of growth, 10 μl of a 5 mg/ml solution of MTT (Sigma, St. Louis, Missouri, USA) was added to each well and the plate incubated at 37°C and 5% CO_2 for another 3 h. Since living cells metabolize the MTT in their mitochondria and form blue formazan crystals

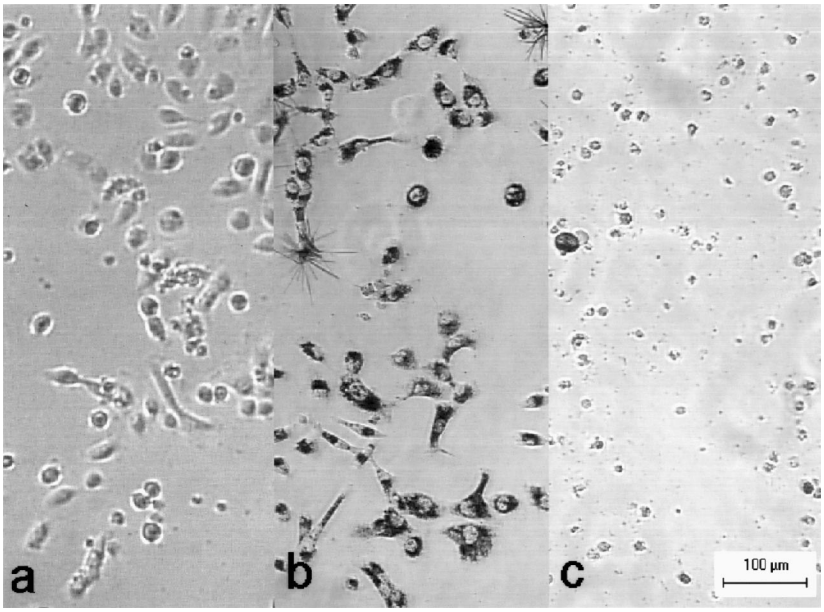

Fig. 1. Adherent human prostate cells (DU-145) growing in a 96-well plate immediately before (a) and 3 h after (b) the addition of the dye MTT. Cells in panel (c) were treated with 10 mg/ml $FeCl_3$ 3 days earlier and show the highly cytotoxic effects of such iron concentrations.

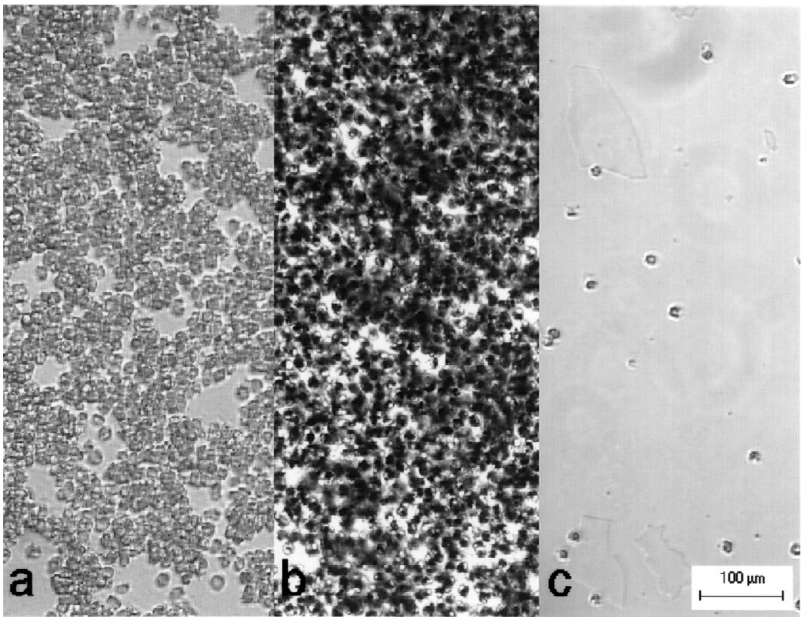

Fig. 2. Murine suspension lymphoma cells (EL-4) growing in a 96-well plate immediately before (a) and 3 h after (b) the addition of the dye MTT. Cells in panel (c) were treated with 10 mg/ml $FeCl_3$ 3 days earlier and show the highly cytotoxic effects of such iron concentrations.

(Fig. 1b and Fig. 2b), 200 µl of DMSO (Research Organics, Cleveland, Ohio, USA) was then added to each well to dissolve the crystals, followed 30 min later by 10 µl of 1M HCl (Sigma). The wells were read at 540 nm on an ELISA plate reader (Cambridge Technologies, Watertown, Massachusetts, USA) and the percent viability calculated. The average of the experimental 8 wells was subtracted from the average of 8 wells containing no cells (background) and divided by the background-corrected average of the control tumor cells. For each MTT assay, the control tumor cell viability was thus by definition 100%. The results shown in Figs. 4 and 5 are the averages and standard deviations of separate experiments, done at least one week apart.

As a separate control, 100 µl aliquots of $FeCl_3$ solutions of 0.1–10.0 mg/ml, adjusted to pH 7.4 with PBS, were tested for their toxic effects on the same cell lines in exactly the same way.

The in vivo toxicity of PLA-MMS was determined in 12 male Fischer 344 rats after intrathecal injection of 0.5 mg of the microspheres. The rats had been previously catheterized (Taconic, Germantown, New York, USA) just below the skull with a PE-10 catheter following the procedure of Kooistra [10] (Fig. 3). The microspheres ended up intraspinally in area (B), visible as black spots in the T1-weighted image. These spots represent the signal loss caused by magnetite, which shortens both the T1 and T2 times of an MR image. Every 3 months, 3 of the rats were sacrificed by CO_2 inhalation. The catheters were removed, the spinal cords harvested, fixed, embedded in paraffin, cut, stained with hematoxylin and eosin (H & E) and read by a neuropathologist.

3. Results and discussion

All the supernatants from a 24 h incubation with control and magnetic microspheres lead to the decreased viability of the two tumor cell lines tested (Fig. 4). Or in other words, the supernatants from all microspheres show toxic effects. The shape of the tumor cells was identical to that of the control

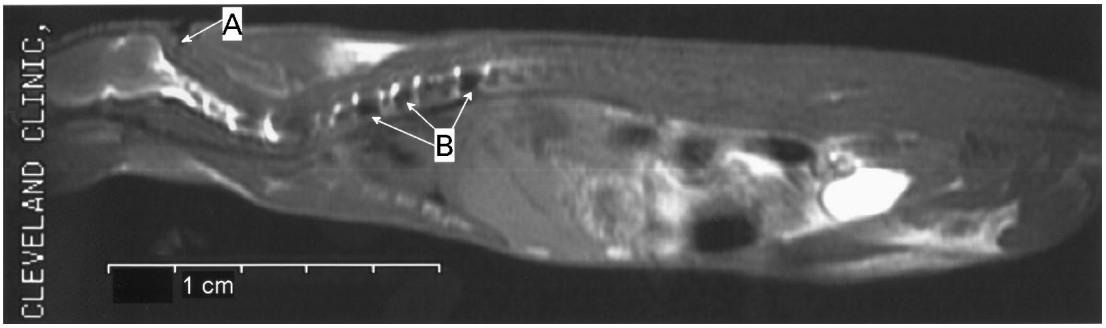

Fig. 3. MR image of a rat taken immediately after intrathecal injection of magnetic microspheres through an approximately 6 cm long catheter entering at the base of the skull (A). The microspheres ended up intraspinally in the area (B), as seen by the black spots in the T1-weighted image.

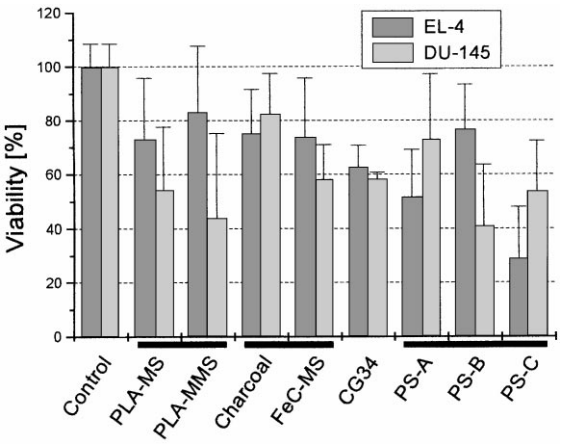

Fig. 4. Viability of the EL-4 and DU-145 cell lines after the addition of the supernatants of magnetic microspheres and their controls 24 h after incubation at 37°C. The MTT assay was measured 3 days later. PLA-MS and PLA-MMS are non-magnetic and magnetic poly(lactic acid) microspheres, charcoal is the control for the iron-carbon FeC microspheres, CG34 are dextran-coated magnetite nanospheres. PS-A and PS-B are magnetic polystyrene microspheres from different producers, with PS-C being the non-magnetic control. The experiments were done in quadruplicate (EL-4) and triplicate (DU-145).

cells, however, they seemed to proliferate more slowly. The variability was generally quite large, with the standard deviations ranging from 15 to 30%, in spite of efforts made to standardize the MTT assay.

No significant difference of viability was seen for microspheres made from materials generally considered to be biocompatible, such as the biodegradable polymer poly(lactic acid) [11] and materials not normally used in vivo, such as the polystyrene latex microspheres. The latter are generally not used in vivo due to the relatively toxic polymer precursors styrene [12]. However, our results show that the short-term toxicity of polystyrene microspheres is similar to that of the other tested microspheres. Their use as control microspheres in many in vitro cell experiments and also, for example, in the determination of microsphere toxicity to the brain [13] confirms their low acute toxicity.

Adherent DU-145 cells were as sensitive as EL-4 suspension cells to the magnetic microspheres' supernatant. The only exception were the poly(lactic acid) microspheres which seemed more toxic to the DU-145 cells. This effect, however, was only a trend and not statistically significant ($p = 0.38$ for non-magnetic PLA-MS, $p = 0.12$ for magnetic PLA-MMS; t-test).

Both magnetic and non-magnetic microspheres led to similar cell growth inhibitions with no significant viability differences in the MTT assay. It is thus unlikely that any acute toxic effects are due to the release of magnetite or iron from the magnetic microspheres. The iron oxides normally used in magnetic microspheres are inert and highly insoluble. The release of ionic iron into the medium was thus expected to be low. To confirm this we measured the release of iron into the supernatant after the autoclaving of PLA-MMS. As expected, less

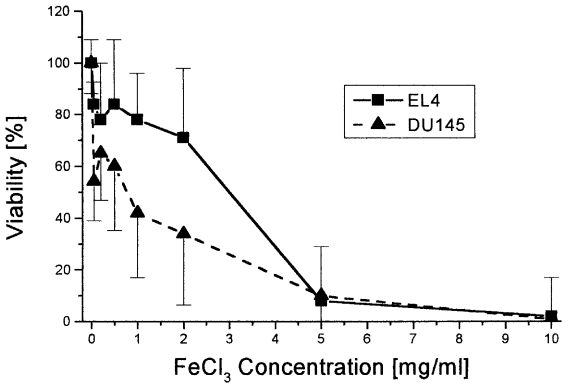

Fig. 5. Viability of the EL-4 and DU-145 cell lines as measured by the MTT assay after 3 days incubation with different concentrations of $FeCl_3$. The results are shown $\pm$ S.D. ($n = 3$).

than 50 ng/ml were released from a microsphere suspension containing 5 mg/ml magnetite.

The control incubation of the cells with $FeCl_3$ solutions showed a small effect on cell growth (Fig. 5). At concentrations below 0.005 mg/ml, no toxicity was seen. Iron concentrations between 0.01 and 2 mg/ml showed an EL-4 cell survival of about $80 \pm 20\%$. Higher iron concentrations led to complete cell kill (Fig. 1c). The DU-145 cells were more susceptible to iron than were the EL-4 cells. Their cell viability between 0.01 and 0.5 mg/ml was in the $60 \pm 20\%$ range, decreasing then rapidly to 'no survival' at the 10 mg/ml iron concentration (Fig. 2c).

It may be interesting to compare therapeutically feasible concentrations of magnetic compounds to the physiological iron levels in humans. Adults contain between 3 and 5 g of iron in the form of hemoglobin in the erythrocytes (66%) and as ferritin and hemosiderin in the storage compartments (19%) [14]. Normally, only about 4 mg of the total iron is in the form of transferrin, the only accessible iron compound in the blood. Acute iron toxicity leading to death through acidosis in humans occurs when more than 200–250 mg of iron per kilogram of body weight are given [15]. Such concentrations would never be attempted for in vivo therapy. We estimate that magnetically targeted local therapy would require just a few milligrams, and that systemic therapy should never exceed a few hundred milligrams of the magnetic compounds.

The solubility of $FeCl_3$, the iron compound used, is very low at pH 7.4 and, although it was not obvious at all test concentrations, precipitation of iron oxides took place [16]. The solubility and toxicity of different iron compounds such as the Fe(II) salts $FeSO_4 \cdot 7H_2O$ or the Fe(III) salts $Fe(NO_3)_2 \cdot 9H_2O$, $Fe(NH_4)_2(SO_4)_2$, and additionally the use of EDTA in combination with the above salts, were investigated. We found only slight improvements in solubility. The toxicity results from the different iron compounds were almost identical to those of $FeCl_3 \cdot 6H_2O$ (Fig. 5) and are thus not shown. For our experiments, the iron concentration was kept constant, but the actual species present in the wells must be described as a mixture of Fe^{2+}/Fe^{3+} ions and precipitates of iron oxide.

Since magnetic and non-magnetic microspheres showed comparable viability rates in the MTT assay, the toxic effects caused by their supernatants must have come from soluble factors present in the polymer or carbon matrix, or from the dextran coating. Soluble factors may include detergents, polymers and monomers from the microsphere preparation, or breakdown products developing during incubation. The breakdown product of the microspheres made from the biodegradable polymer poly(lactic acid) is lactic acid – and we therefore also tested lactic acid as a control. The MTT assay toxicity measured after the addition of 100 μl of 0.02 M lactic acid in PBS showed $88 \pm 2\%$ viability for the EL-4 and $59 \pm 5\%$ for the DU-145 cells. These viabilities are thus almost identical to the measured values for PLA-MS and PLA-MMS (Fig. 4). The toxic effects produced by the PLA-microspheres, however, could not be due to lactic acid, since a chemical analysis of the sterile-filtered supernatant from these microspheres resulted in a 0.0 mM lactate concentration.

The magnetic poly(lactic acid) microspheres were also tested in a long-term rat study. After direct intraspinal injection (Fig. 3), no toxic effects in the behavior of the animals were noted. The rat's weight also increased normally. None of the animals died during the 1-year follow-up. The examination of H & E stained slices of area (B) in Fig. 3 showed normal histology. We expected signs of inflammation since the breakdown product of the

microspheres is lactic acid, but none were found. Inflammatory cells were absent and the neurons and cells lining the intrathecal space all looked normal. The lack of toxic effects can be accounted for by considering the in vivo effects from polymer degradation such as local inflammation and leukocyte infiltration to be transient [17]. After 3 months, most PLA microspheres have biodegraded completely and disappeared. Histologic sections from later time points (6, 9 and 12 months) again showed no inflammatory cells, and the histology of the spinal cord appeared to be normal. The intrathecal application of small amounts of biodegradable PLA-microspheres for locally targeted radiotherapy [1] thus seems to be possible.

The dextran-coated iron oxide particles, the third type of microspheres used, tested with a viability of $60 \pm 8\%$. Kresse et al. tested the in vivo toxicity of dextran-coated iron oxide particles of 26 nm diameter in mice [18]. After intravenous injection, they found an acute LD_{50} of around 2 g/kg, calculated as iron. Similarly, Kuznetsov et al. tested dextran-coated magnetite nanospheres of 11 nm diameter (dry) and measured an LD_{50} of 5 g/kg for mice, $\sim$1 g/kg for rabbits and $\sim$0.7 g/kg for dogs, both after intraperitoneal and intravenous application [19]. These studies indicate that dextran-coated nanospheres are not very toxic in vivo, although our MTT assay gives a contradictory result with its viability of only 60%.

Our MTT assay results showed a similar sensitivity of the tumor cells towards the FeC microspheres as towards the dextran-coated CG34 microspheres. Kuznetsov et al. showed that the 100–200 nm large ferro-carbon particles had an LD_{50} of 0.7 g/kg in mice and were well tolerated below 0.5 g/kg [19]. Ferro-carbon microspheres thus were 10 times more toxic than the dextran-coated nanospheres, something that was not expected from the MTT assay measurements (Fig. 4). The reason is not yet clear, but particle size may play a major role. After intravenous application, most of the microspheres end up in organs with extensive capillary beds such as the liver, spleen, lungs and kidneys. Kuznetsov et al. reported that the death of the animals occurred between 3 and 120 h after injection of lethal doses of the microsphere suspensions, apparently due to thrombosis of the lungs' vascular systems, followed by asphyxia.

Depending on the size and the materials used to prepare the magnetic microspheres, different paths of toxicity are expected. Manganese ferrite nanoparticles of 10 nm diameter are expected to show less physical (embolizing) toxicity, but more local, chemically induced toxicity due to their higher chemical reactivity. This is exactly what Lacava et al. found with their ionic and tartrate-based magnetic fluids administered intraperitoneally to mice [20]. 0.5 ml of a 0.5 M solution showed no obvious toxicity within 30 days, however, a close look at the intraperitoneal cells showed that both magnetic fluids caused cellular death, mutagenic effects and severe inflammatory reactions.

In conclusion, even simple systems such as microspheres made from only magnetite and one matrix substance are not easily characterized in vitro for their expected in vivo behavior and biocompatibility. Chemical and physical factors interact and must be evaluated in the actual, specific in vivo situation. In this paper, we have shown that the MTT assay is not adequate for the evaluation of toxicity in magnetic microspheres. The assay's variability is too high, and since clonogenic survival cannot be distinguished easily from metabolic changes, it is also not very specific. However, the MTT assay is easy to setup and may hint at problems to come – any extreme results (30% survival or less) in more than one cell line should be taken seriously. Another in vitro cytotoxicity assay, the agar overlay technique has been used to test the toxicity of neodymium–iron–boron magnets and their coating [21]. The authors' conclusion was also that better assays are necessary. The toxicity of magnetic microspheres should thus be investigated with more specific methods in vivo with histopathological and morphological observations and cytogenetic analysis as was, for example, done by Lacava et al. [20].

Acknowledgements

We thank Dr. Thomas Bauer, Cleveland Clinic Foundation, for his reading of the stained spine sections.

References

[1] U.O. Häfeli, G.J. Pauer, W.K. Roberts et al., in: U. Häfeli, W. Schütt, J. Teller, et al., (Eds.), Scientific and Clinical Applications of Magnetic Carriers, Plenum, New York, 1997, p. 501.
[2] K. von Appen, C. Weber, U. Losert et al., Artif. Organs 20 (1996) 420.
[3] M. Kresse, S. Wagner, M. Taupitz, in: U.O. Häfeli, W. Schütt, J. Teller, et al., (Eds.), Scientific and Clinical Applications of Magnetic Carriers, Plenum Press, New York, 1997, p. 545.
[4] A. Jordan, R. Scholz, P. Wust et al., Int. J. Hyperthermia 13 (1997) 587.
[5] R. Weissleder, D.D. Stark, B.L. Engelstad et al., AJR 152 (1989) 167.
[6] P.R. Ros, P.C. Freeny, S.E. Harms et al., Radiology 196 (1995) 481.
[7] J. Kreuter, Pharm. Acta Helv. 58 (1983) 242.
[8] R. Pieters, D.R. Huismans, A. Leyva et al., Br. J. Cancer 59 (1989) 217.
[9] U.O. Häfeli, S.M. Sweeney, B.S. Beresford et al., J. Biomed. Mater. Res. 28 (1994) 901.
[10] K.L. Kooistra, M. Rodriguez, G. Powis et al., Cancer Res. 46 (1986) 317.
[11] C.C. Chu, in: D.F. Williams (Ed.), CRC Critical Reviews in Biocompatibility, CRC Press, Boca Raton, 1985, p. 261.
[12] J.A. Bond, Crit. Rev. Toxicol. 19 (1989) 227.
[13] P. Menei, A. Croue, V. Daniel et al., J. Biomed. Mater. Res. 28 (1994) 1079.
[14] National Research Council, Iron, University Park Press, Baltimore, 1979.
[15] J.J. Crotty, Clin. Toxicol. 4 (1971) 615.
[16] H. Remy, in: J. Kleinberg (Ed.), Treatise on Inorganic Chemistry, Elsevier, Amsterdam, 1956, p. 242.
[17] F.D. Anderson, D.F. Archer, S.M. Harman et al., Pharm. Res. 10 (1993) 369.
[18] M. Kresse, S. Wagner, K. Philipp et al., Proc. Int. Symp. Control. Rel. Bioact. Mater. 24 (1997) 93.
[19] O.A. Kuznetsov, N.A. Brusentsov, A.A. Kuznetsov et al., J. Magn. Magn. Mater. 194 (1999) 82.
[20] Z.G.M. Lacava, R.B. De Azevedo, L.M. Lacava et al., J. Magn. Magn. Mater. 194 (1999) 89.
[21] D. Devineni, A. Klein-Szanto, J.M. Gallo, J. Neuro-Oncol. 24 (1995) 143.