Journal of Magnetism and Magnetic Materials 380 (2015) 85–89

Contents lists available at ScienceDirect

Journal of Magnetism and Magnetic Materials

journal homepage: www.elsevier.com/locate/jmmm

The cytotoxicity of iron oxide nanoparticles with different modifications evaluated *in vitro*

Vlasta Zavisova [a,*], Martina Koneracka [a], Jozef Kovac [a], Martina Kubovcikova [a], Iryna Antal [a], Peter Kopcansky [a], Monika Bednarikova [b], Marta Muckova [b]

[a] Institute of Experimental Physics, Slovak Academy of Sciences, Watsonova 47, 040 01 Kosice, Slovakia
[b] hameln, rds a.s, Horna 36, 900 01 Modra, Slovakia

ARTICLE INFO

Article history:
Received 30 June 2014
Received in revised form
12 October 2014
Accepted 13 October 2014
Available online 17 October 2014

Keywords:
Magnetic fluid
Cytotoxicity
In vitro test
Melanoma
Dextran
Biocompatibility

ABSTRACT

The toxicity of magnetite nanoparticles modified with bioavailable materials such as dextran, bovine serum albumin, polyethylene glycol, and polyvinylpyrrolidone was studied in normal and cancer cells. The size distribution and magnetic properties of the modified magnetic nanoparticles were characterized by different techniques. Transmission electron microscopy showed a nearly spherical shape of the magnetite core with diameters ranging from 4 to 11 nm. Dynamic light scattering was employed to monitor the hydrodynamic size and colloidal stability of the magnetic nanoparticles: *Z*-average hydrodynamic diameter was between 53 and 69 nm and zeta potential in the range from −35 to −48 mV. Saturation magnetization of the modified nanoparticles was 55–64 emu/g_{Fe3O4}. Prepared biocompatible nanoparticles had no significant toxic effect on Chinese hamster lung fibroblast cell line V79, but they substantially affected mouse melanoma B16 cell line.

1. Introduction

Modern medicine is beginning to actively use nanotechnology in clinical diagnostics, targeted drug delivery, cancer treatment by hyperthermia and other fields [1]. Nevertheless, the toxicity of nanostructured materials is an open issue due to several factors: high reactivity, intrinsic toxicity of the material, and non-specific interactions with biological objects, that are determined by particle shape, size and structure. Biocompatibility, toxicity and ability to penetrate into cells are the main criteria that determine the effectiveness of nanoparticles in medicine [2].

One of the widely studied and currently used nanomaterials is the magnetite and magnetite-derived nanoparticles that possess stable magnetic characteristics. However, a lack of knowledge about the mechanism of magnetite (Fe_3O_4) nanoparticles penetration into tissues, organs and tumors, as well as the degree of their toxicity limits of their application [3–5].

To minimize biofouling and aggregation of magnetic nanoparticles, their escape from the reticuloendothelial system and to increase their circulation time, they are usually coated with a layer of hydrophilic and biocompatible polymers. Polymers based on poly(ethylene-*co*-vinyl acetate), polyvinylpyrrolidone (PVP), poly (lactic-*co*-glycolic acid), polyethylene glycol (PEG), poly(vinyl alcohol) are typical examples of synthetic polymeric systems [6,7]. The most commonly used natural polymers are gelatin, dextran (DEX), chitosan, and pullulan [8].

Iron oxide nanoparticles are generally coated to reduce aggregation and cytotoxicity [9]. DEX-coated iron oxide nanoparticles have been used as MRI contrast agents to investigate nanoparticle accumulation and cellular uptake in malignant neoplasms *in vivo*, and also to transform nanoparticles into active, targeted probes [10–12]. PEG is a stable, biocompatible hydrophilic polymer used in many drug and gene delivery applications [13]. In the study of Miao Yu [14], porcine aortic endothelial cells were exposed to iron oxide nanoparticles coated with either DEX or PEG. Results indicated that both coatings can reduce nanoparticle cytotoxicity, but different mechanisms may be important for different nanoparticle size. Cytotoxicity and cell uptake studies in VERO and MDCK cell lines showed low toxicity of PEG-coated superparamagnetic iron oxide nanoparticles and DEX-coated superparamagnetic iron oxide nanoparticles [15].

In our current work we focused on preparation of a stable, biocompatible magnetic fluid (MF) with low toxicity to normal cells. We also investigated the cytotoxicity of magnetite nanoparticles coated with bovine serum albumin (BSA), DEX, PVP, or PEG. We selected normal Chinese hamster lung fibroblast cell line

* Corresponding author.
E-mail address: zavisova@saske.sk (V. Zavisova).

http://dx.doi.org/10.1016/j.jmmm.2014.10.041
0304-8853/

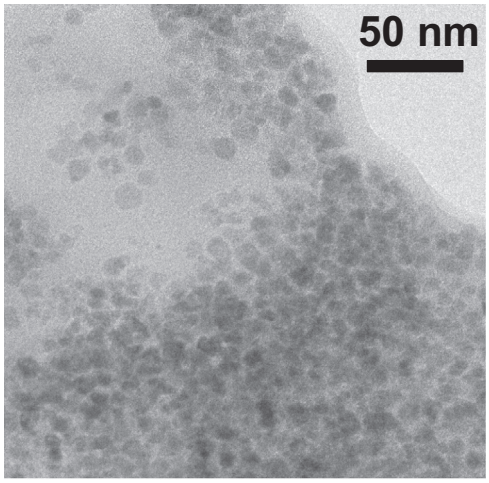

Fig. 1. TEM image of core magnetic particles in MF.

V79 and melanoma mouse cell line B16 to assess the cytotoxicity of prepared nanoparticles.

2. Materials and methods

Polyethylene glycol (average molecular weight (Mw) 400, 1000, 10,000, 20,000), dextran (average Mw 70,000), and bovine serum albumin were purchased from Sigma-Aldrich. Polyvinylpyrrolidone K30 (average Mw 40,000) was obtained from Fluka and sodium oleate from Riedel-de Haën. Typically ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) and ammonium hydroxide (NH_4OH) were used for magnetite synthesis.

Dulbecco's modified Eagles medium (DMEM) and fetal bovine serum were from BioWhittaker. MTT salt, trypsin and EDTA were obtained from Sigma-Aldrich. Streptomycin and Penicillin G antibiotics were from AppliChem and Biotika, respectively.

V79 cell line was obtained from ECACC (European Collection of Cell Cultures, UK) and B16 cell line was obtained from CRI SAS (Bratislava, Slovakia).

The co-precipitation method of ferric and ferrous salts in an alkaline aqueous medium was used to prepare spherical magnetite particles. In a typical synthesis, an aqueous solution of Fe^{3+} and Fe^{2+} (molar ratio 2:1) was prepared by dissolving in deionized water. An excess of hydroxide ions was added to the mixture of Fe^{3+} and Fe^{2+} with vigorous stirring at room temperature to form a black precipitate of magnetite nanoparticles. After washing by magnetic decantation and heating up to 50 °C, the surfactant sodium oleate ($C_{17}H_{33}COONa$) was added to the mixture to prevent agglomeration of the particles. The mixture was then stirred and heated until the boiling point was reached. The obtained oleate bilayer stabilized magnetite particles were dispersed in water. Agglomerates were removed by centrifugation at 9000 rpm for 30 min. The particles prepared by this method are referred to as MF (magnetic fluid) hereafter.

To improve biocompatibility, the MF was further modified by coating with bovine serum albumin (BSA). BSA was dissolved in water and added to the MF at weight ratio BSA/Fe_3O_4= of 2 and stirred (200 rpm at 40 °C) for six hours. The pH of the obtained colloid was adjusted to 7.4 by addition of phosphate buffer. Magnetic fluid modified by BSA (MFBSA), prepared by described procedure, is an intermediate product between MF and next biocompatible compound modification below.

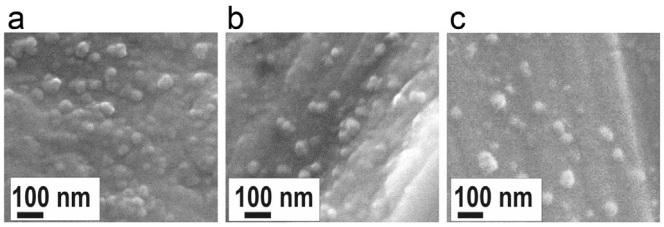

Fig. 2. SEM image of modified nanoparticles in MFDEX (a), MFPVP0.25 (b) and MFPEG1000 (c).

MFBSA was mixed with water solutions of DEX, PVP or PEG and stirred in a horizontal shaker (200 rpm and 40 °C) for 24 h to obtain magnetic fluids modified by dextran (MFDEX), PVP (MFPVP) and PEG (MFPEG). For functionalization of MFBSA by PEG, four different PEG molecular weights were used: 400, 1000, 10,000 and 20,000 g/mol at the constant PEG/Fe_3O_4 weight ratio = 1. In case of MFPVP, three samples with PVP/Fe_3O_4 weight ratios of 0.25, 0.5, 1 were prepared. DEX/Fe_3O_4 weight ratio of 3 was used to prepare MFDEX.

The prepared magnetic fluids were examined by transmission electron microscopy (TEM, JEOL-TEM 2100F microscope operated at 90 kV) under 80,000x magnification by the replication technique. Briefly, a drop of MF sample diluted in water was deposited on the 400 mesh copper grid and air dried before the picture was taken. Scanning electron microscopy (SEM, JEOL 7000F microscope) was used to evaluate the morphology and microstructure of the coated nanoparticles in the prepared MF samples. The colloidal dispersion was first diluted in water (typically $1{:}10^6$ dilution), and one droplet was deposited on an aluminum grid and dried under vacuum prior sputtering with carbon and subsequent observation.

To determine the particle size distribution the samples were measured by Dynamic light scattering (DLS) using Zetasizer Nano ZS (Malvern Instruments). The zeta potential was estimated using Laser Doppler Electrophoretic measurement technique with a scattering angle of 173° at 25 ± 0.1 °C. DLS evaluates the intensity fluctuation of scattered light reflected from nanoparticles in suspension. The fluctuation is resulting from the "Brownian motion" that keeps the particles in steady movement.

The complementary technique used to determine particle size distribution in the prepared samples was Differential Centrifugal Sedimentation (DCS). DCS enables to measure particle size by measuring the time required for the colloidal particles to settle in a density gradient in a disk centrifuge. The DC24000 UHR disk centrifuge (CPS Instruments, Inc.) was used to perform sedimentation based size distribution measurements.

Magnetic properties of the prepared samples were studied by MPMS XL-5 (Magnetic properties measuring system, SQUID magnetometer), which supplied magnetic fields with maximum intensity $\mu_0 H = 5$ T at temperature 290 K.

In vitro cytotoxicity of MFs was investigated on mouse melanoma cells B16 and Chinese hamster lung fibroblast cells V79 by colorimetric cell viability MTT assay. Cells were cultivated in DMEM supplemented with 10% fetal bovine serum, glucose (4 g/L), L-glutamine, penicillin (100 units/mL), and streptomycin (100 mg/mL) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C in sterile tissue culture dishes. Cells were treated with indicated 4–6 dilutions of MFs or buffer (untreated cells) for 24 h. MTT_{50} parameter for every tested sample is expressed as represents the Fe_3O_4 concentration (μg/mL) that reduces absorbance of MTT salt in tested cells by 50% compared to control untreated cells.

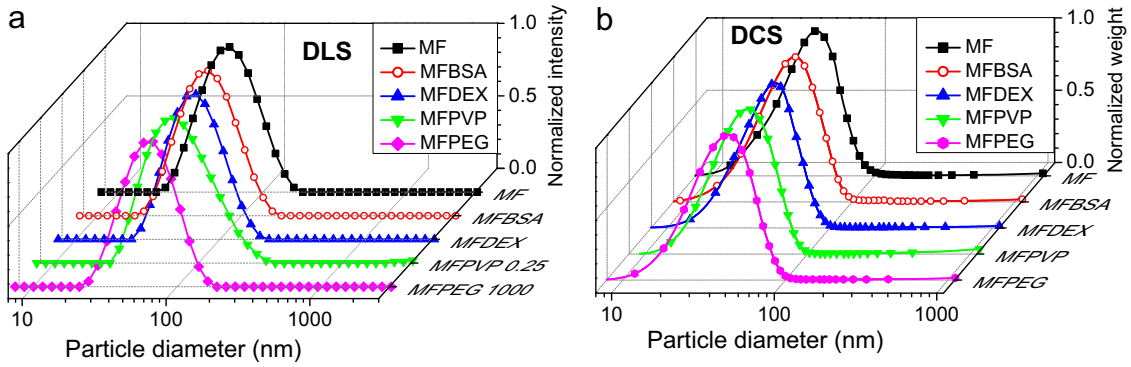

Fig. 3. Comparison of the particle size distributions of MFs determined by DLS (a) and DCS (b) method.

3. Results and discussion

Magnetic fluids containing magnetite particles stabilized by sodium oleate and modified with biocompatible substances BSA, DEX, PVP and PEG of different Mw were prepared and characterized by TEM, SEM, DLS, DCS, and SQUID magnetometer.

The magnetite cores of the prepared nanoparticles were near spherical, as shown by TEM (Fig. 1). Typical morphology of the modified nanoparticles in MFs analyzed by SEM (core with shell) is seen in Fig. 2a–c. The nanoparticles displayed sphere-like shape with a smooth surface and diameter of 28–34 nm. Nanoparticle size determined by TEM experiments was reasonably smaller than size obtained by SEM due to different sensitivities of the applied techniques.

Major requirements for nanoparticles intended for medical use are biocompatibility and suitable size. We used two different methods, DLS and DCS to measure the particle size distribution. DLS measurements reported similar size distribution for all prepared magnetic fluids (Fig. 3a), with the average hydrodynamic particle diameters D_{DLS}=53–69 nm (*Z*-average). The data are summarized in Table 1. From size distribution determined by DCS, diameter of unmodified magnetic fluid was found to be 42 nm and for modified MF it ranged from 42 to 49 nm (Fig. 3b). There was a good agreement between the particle size measured by DLS and DCS methods, although values obtained by DCS were slightly lower. The values ranged from 1 to 26 nm. This can be explained by the fact that the DLS provides information about the hydrodynamic diameter, while DCS gives us information about the actual particle diameter.

The zeta potential (*z*-potential) measurements were used to monitor modification and stability of prepared samples and the results are summarized in Table 1. The *z*-potential of initial magnetic fluid was −40.9 mV and of modified MFs was in the range from −35 to −48 mV, indicating a relative good stability of all prepared MFs.

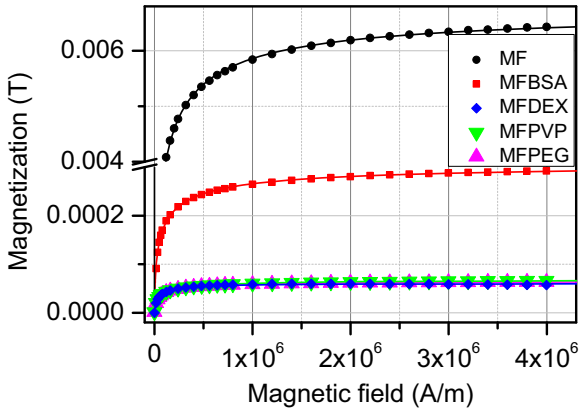

Fig. 4. Magnetization curves of MFs measured at room temperature.

The magnetization curves obtained by SQUID measurements confirmed the superparamagnetic behavior of all modified MF samples at room temperature (Fig. 4). Saturation magnetizations (Ms) in emu/$g_{Fe_3O_4}$ at 5 T as well as calculated magnetite concentrations $C_{Fe_3O_4}$ (mg/mL) of each sample are shown in Table 1.

Finally, the calculated magnetic particle core diameters obtained from magnetization curves using Langevin function [16,17] were in the range of 8–10 nm (Fig. 5). While SEM, DLS and DCS provide the diameter of particles including nonmagnetic layer along the magnetic core, diameter from magnetic measurements only depends on magnetic moment of nanoparticles and no effect from nonmagnetic layer is involved. The coating thickness, due to

Table 1
Physicochemical characteristics of different magnetic fluids and MTT_{50} parameters for two cell lines B16 melanoma mouse cells and V79 fibroblastoid Chinese hamster cells.

Sample	Ratio DEX, PVP, PEG /Fe_3O_4 (w/w)	Ms (emu/g Fe_3O_4)	C_{Fe3O4} (mg/mL)	DLS			MTT_{50} parameter	
				Z-average (nm)	PDI	*z*-potential (mV)	B16 (μg/mL)	V79 (μg/mL)
MF	0	64.35	90	54	0.11	−40.9	4.81	8.27
MFBSA	0	58.64	20	53	0.14	−43.2	159.82	123.22
MFDEX	3	56.59	10	63	0.16	−37.0	56.16	88.90
MFPVP 0.25	0.25	55.70	10	69	0.16	−37.4	57.29	113.82
MFPVP 0.5	0.5	55.90	10	65	0.15	−42.6	61.48	113.69
MFPVP 1	1	62.10	10	68	0.14	−38.6	60.15	110.74
MFPEG 400	1	58.42	10	57	0.13	−35.9	56.45	127.91
MFPEG 1000	1	58.10	10	59	0.12	−38.4	61.65	138.86
MFPEG 10,000	1	56.70	10	66	0.16	−47.6	88.39	140.42
MFPEG 20,000	1	55.60	10	61	0.16	−41.4	65.84	121.10

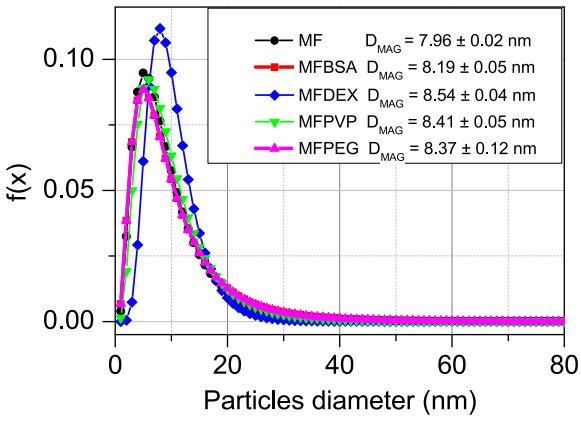

Fig. 5. Comparison of the core particle diameter obtained by fitting of MFs magnetization curves.

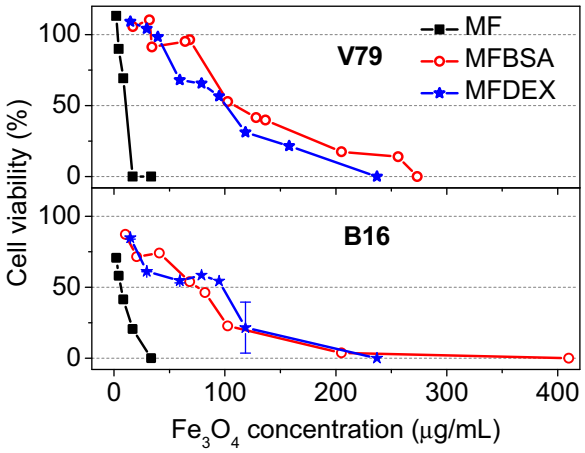

Fig. 6. *In vitro* cytotoxicity of MF, MFBSA and MFDEX tested on B16 melanoma and V79 fibroblastoid Chinese hamster cells by MTT test.

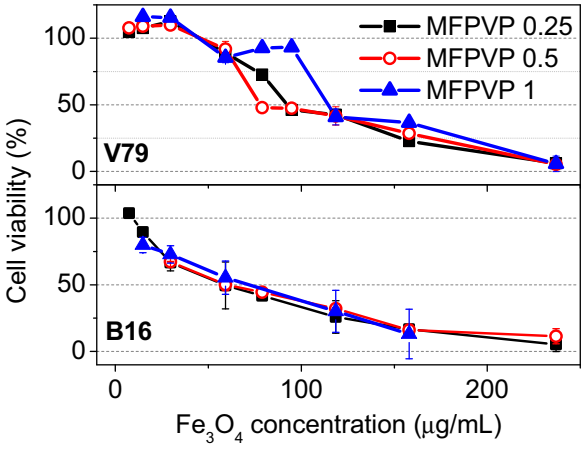

Fig. 7. *In vitro* cytotoxicity of MFPVPs tested on B16 melanoma and V79 fibroblastoid Chinese hamster cells by MTT test.

longer PEG tails, affected indirectly the steady decrease of the magnetic saturation of the MFPEGs (Table 1).

The cytotoxity of magnetic nanoparticles with different coatings was tested on V79 and B16 cells by the MTT assay. For both cell types, decreasing of cell viability with increasing magnetite concentration was observed in all tested samples (Figs. 6–8). The most significant cytotoxic effect was observed in cells treated with unmodified MF (see Fig. 6). MF coating by BSA increased viability of both cell lines 10 times.

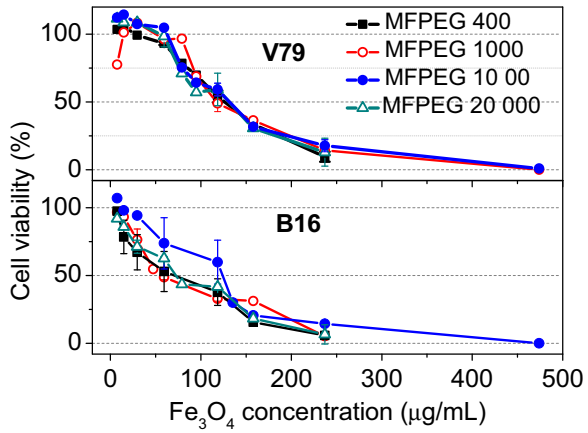

Fig. 8. *In vitro* cytotoxicity of MFPEGs tested on B16 melanoma and V79 fibroblastoid Chinese hamster cells by MTT test.

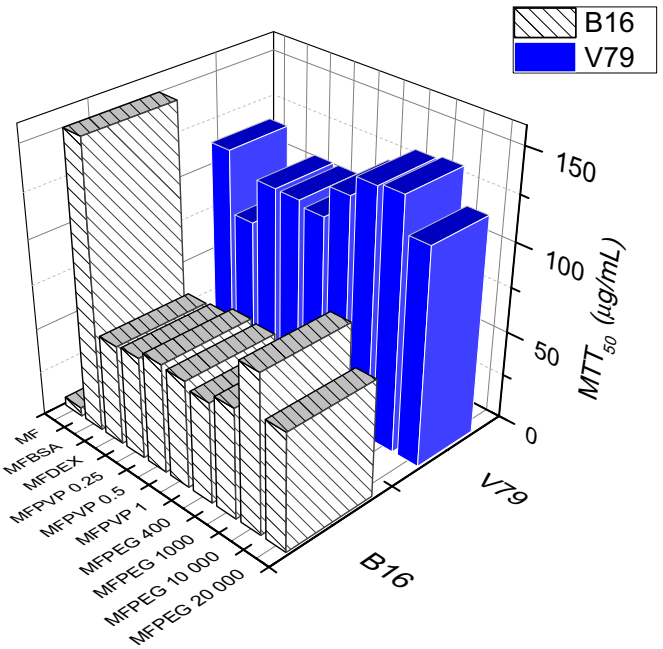

Fig. 9. Comparison of MTT_{50} parameters obtained after treatment B16 melanoma and V79 fibroblastoid Chinese hamster cells by different magnetic fluids.

To further increase the biocompatibility of MFBSA, coatings with DEX, PVP and PEG were tested. Fig. 6 illustrates that magnetic particle modifications by DEX only slightly influenced viability of V79 and B16 cells when compared to MFBSA.

Effect of PVP weight ratio to magnetite was also investigated and no significant effect on viability of V79 and B16 cells was observed (Fig. 7).

Previous studies showed that toxicity of nanoparticles was directly related to their chemical composition, surface chemistry, hydrodynamic size, and solubility in aqueous solutions [18,19].

Apart from the type of surface coating, the tail length of a magnetic nanoparticle coating (length of polyethylene oxide, PEO resp. PEG, in PEG-coated MNPs) is also important for the cytotoxicity. Häfeli et al. in paper [20] tested cytotoxicity of magnetic nanoparticles coated with the PEO with various PEO block tail length. It was found that the PEO tail block length inversely correlates with toxicity. The nanoparticles coated with the shortest 0.75 kDa tails were the most toxic, while particles coated with 15 kDa PEO tail block copolymers were the least toxic.

However, in our study, no effect of PEG tail lengths (Mw 0.4–20 kDa) on normal V79 and B16 melanoma cells was observed (Fig. 8). It could be due to the fact that the uptake and biocompatibility of nanoparticles are not only dependent on the particle size and surface but also related to the cell type [21].

Over all, DEX, PVPs and PEGs modified magnetic particles were more toxic than MFBSA to B16 cell line (Fig. 9 and Table 1). V79 cells were less sensitive to MFPEGs magnetic particles. Comparison of the MTT_{50} values revealed that MFPEG 400 and MFPEG 1000 were the most suitable for future biomedical application thanks to their low toxicity in normal V79 cells and high toxicity in melanoma B16 cells.

4. Conclusion

Cytotoxicity of iron oxide-based nanoparticles with different modifications was tested in mouse melanoma B16 cells and Chinese hamster lung fibroblast V79 cells by MTT test *in vitro*. The lowest cell viability was observed after treatment with oleate bilayer coated magnetite nanoparticles. BSA modification of magnetite nanoparticles increased cell viability in both tested cell lines more than 10 fold. Our next findings revealed that the main cytotoxicity affecting factor is the biocompatible shell coating and not the magnetite core. The biggest difference in viability of normal and cancer cells was achieved after application of MFPEG 400 and MFPEG 1000, therefore these two formulations are the most suitable for utilization in cancer treatment.

Acknowledgments

This work was supported by Ministry of Education Agency for Structural Funds of EU in frame of Projects 26220120021, 26220220005 and 26110230097, VEGA 0041, 0045, Slovak Research and Development Agency – Contract nos. APVV-0742-10 and APVV-99-026505.

References

[1] F. Watari, N. Takashi, A. Yokoyama, et al., J. R. Soc. Interface 6 (2009) 371.
[2] H. Markides, M. Rotherham, A.J. El Haj, J. Nanomater. 1 (2012).
[3] G. Liu, J. Gao, H. Ai, et al., Small 9–10 (2013) 1533.
[4] N. Singh, G.J.S. Jenkins, R. Asadi, et al., Nano Rev. 1 (2010).
[5] L.H. Reddy, J.L. Arias, J. Nicolas, et al., Chem. Rev. 112 (2012) 5818.
[6] X. Zhao, J.M. Harris, J. Pharm. Sci. 87 (11) (1998) 1450.
[7] J.M. Ruiz, J.P. Benoit, J. Control. Release 16 (1991) 177.
[8] S.P. Massia, J. Stark, D.S. Letbetter, Biomaterials 21 (2000) 2253.
[9] K. Lind, M. Kresse, N.P. Debus, et al., J. Drug Target. 10 (2002) 221.
[10] R. Weissleder, A. Bogdanov, M. Papisov, Magn. Reson. Q 8 (1992) 55.
[11] A. Moore, E. Marecos, A. Bogdanov, et al., Radiology 214 (2000) 568.
[12] D. Thorek, A. Chen, J. Czupryna, et al., Ann. Biomed. Eng. 34 (2006) 23.
[13] M. Mahmoudi, A. Simchi, M. Imani, et al., J. Phys. Chem. C 113 (2009) 8124.
[14] M. Yu, S. Huang, K. Jun Yu, et al., Int. J. Mol. Sci. 13 (2012) 5554.
[15] M.L.M. Pisciotti, E. Lima Jr., M.V. Mansilla, et al., http://dx.doi.org/10.1002/jbm.b.33068.
[16] Z. Rozynek, A. Jozefczak, K.D. Knudsen, et al., Eur. Phys. J. E 34 (2011).
[17] A. Skumiel, T. Hornowski, A. Józefczak, Int. J. Thermoph. 32 (2011) 876.
[18] I. Bhatt, B.N. Tripathi, Chemosphere 82 (2011) 3 308.
[19] M. Delay, F.H. Frimmel, Anal. Bioanal. Chem. 402 (2) (2012) 583.
[20] U.O. Häfeli, J.S. Riffle, L.H. Shekhawat, et al., Mol. Pharm. 6 (5) (2009) 1417.
[21] A. Petri-Fink, B. Steitz, A. Finka, et al., Eur. J. Pharm. Biopharm. 68 (2008) 129.