

Hydrogel-magnetic nanoparticles with immobilized L-asparaginase for biomedical applications

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Abstract The association of magnetic nanoparticles, which could be controlled by a magnetic field and have dimensions which facilitate their penetration in cells/tissues, with hydrogel type biopolymeric shells confer them compatibility and the capacity to retain and deliver bioactive substances. The main objective of this work is the development of a new system based on a biocompatible polymer with organic–inorganic structure capable of vectoring support for biologic active agents (L-asparaginase, e.g.). Characterization of size and morphology of the hydrogel-magnetic nanoparticles with entrapped L-asparaginase was made using Dynamic Light Scattering method, Transmission Electron Microscopy and Confocal Microscopy. The structure of magnetic nanoparticles coated with hydrogel was characterized by Fourier Transformed Infrared Spectroscopy. The cytotoxicity of nanoparticles was evaluated and also the interactions with microorganisms. We obtained hydrogel-magnetic nanoparticles with L-asparaginase entrapped, with sizes below 30 nm in dried stage, capable to penetrate the cells and tissues.

1 Introduction

Interest in nanosized drug delivery systems has increased in the past few years, due to their biocompatibility, targeting action, and subcellular size [1, 2]. Novel nanomaterials integrated with magnetic nanoparticles have been studied broadly for applications in biology and medicine, including magnetic bioseparation, drug delivery, magnetic resonance- imaging contrast enhancement, and hyperthermia treatment of cancer due to their properties of superparamagnetism, high saturation magnetization, high-magnetic susceptibility, and low toxicity [3, 4].

Magnetic nanoparticles have been used for many years as magnetic resonance imaging (MRI) contrast agents or in drug delivery applications. Magnetic nanoparticles have been used as support material for binding of enzymes including yeast alcohol dehydrogenase [5] lipase [6] glucose oxidase [7, 8] cholesterol oxidase [9] directly via carbodiimide activation or by functionalization of magnetic nanoparticles. The immobilization is commonly accomplished through a surface coating with polymers, the use of coupling agents or crosslinking reagents, and encapsulation.

Hyaluronic acid and chitosan are two natural biopolymers with similar structure and special biological characteristics, easily to process in porous scaffolds, films and beads. They are biocompatible, biodegradable, promote cell migration and cell adhesion and electrostatic interact [10–12]. They have a great potential in the development of drug delivery systems and in tissue engineering [13–15].

L-Asparaginase is a chemotherapy agent, used in treatment of acute lymphoblastic leukaemia (ALL), but in some cases it presents toxicity, the main side effect is an allergic or hypersensitivity reaction. Enzyme immobilization into a

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polymeric shell could improve their delivery and eliminate the allergic reaction [16]. Previous works present the feasibility of intravenous PEG-asparaginase (a form a *Escherichia coli* L-asparaginase covalently linked to polyethylene glycol) administration [17], covalent immobilization of L-asparaginase on the microparticles of the natural silk sericin protein [18], or immobilization of L-asparaginase on various supports to develop a asparaginase biosensor for leukemia [19].

In our work, L-asparaginase (E.C.3.5.1.1.) synthesized and purified from *E. coli* genetic modified strain (BIOTEHGEN-Centre of Microbial Biotechnologies Bucharest, collection) [20] was immobilized by entrapment in hydrogel coated magnetic nanoparticles. Magnetic nanoparticles were obtained by co-precipitation method [21] and were covered with hydrogel-type biopolymer shell according to layer-by-layer technique using hyaluronic acid and chitosan. The residual activity of the immobilized L-asparaginase was determined spectrometrically at 450 nm with Nessler reagent method.

Characterization of size and size distribution of the hydrogel-magnetic nanoparticles with entrapped L-asparaginase was made using Dynamic Light Scattering method and transmission electron microscopy. The morphology of the particles was investigated with transmission electron microscopy and confocal microscopy. The structure of magnetic nanoparticles coated with hydrogel was characterized by Fourier transformed infrared spectroscopy (FT-IR).

Our goal was to obtain nanosize biocompatible materials, with L-asparaginase entrapped, capable to penetrate

cells/tissues and deliver L-asparaginase. In addition, the obtained nanostructures present different behaviour to adherence capacity of bacteria and yeasts and could be used in bioseparation.

2 Methods

2.1 Synthesis of hydrogel-magnetic nanoparticles

Water-dispersible magnetic nanoparticles (MP) were obtained according previous studies, using an adapted Massart method [21]. Briefly, an aqueous mixture of Fe^{2+} and Fe^{3+} salts in 1:2 molar ratio was treated with NH_4OH at 75°C . The sample was kept 30 min under stirring and heating conditions, while a black magnetic precipitate is obtained. The magnetic nanoparticles were encapsulated according to our previous work [22], using layer-by-layer technique in two different biopolymeric materials, chitosan from crab shells and hyaluronic acid extracted by us from bovine vitreous [23]. We used different proportions of 2% chitosan (chit) and 1% hyaluronic acid (HA) solutions to cover magnetic nanoparticles (Table 1).

2.2 Immobilization of L-asparaginase

L-Asparaginase was immobilized by entrapment in hydrogel layer of obtained nanostructures. L-asparaginase was obtained according previous studies [20], by biosynthesis from *Escherichia coli* using a recombinant strain of *E coli*

Table 1 Dimensions (swelled stage) and zeta potentials of hydrogel magnetic nanoparticles obtained by alternation of chitosan and hyaluronic acid layer on magnetic nanoparticles (layer-by-layer technique); L-asparaginase is entrapped in middle or external layer

Sample no.	Sample type (composition)	D media (nm)	Zeta potential (mV)
0	Magnetic nanoparticles (MP) suspension	25.21	-45.4
1	20 ml MP		
	+1.5 ml Chit.	349.5	-47.9
	+5 ml HA (and 5 mg asparaginase BIOTEHGEN)	320	-49.7
2	+1.5 ml Chit.	274	-31.7
	20 ml MP		
	+3 ml Chit.	418.7	-32.4
	+3 ml HA (and 5 mg asparaginase BIOTEHGEN)	539.2	-38
4	+3 ml Chit.	261.8	+2.44
	20 ml MP		
	+3 ml Chit.	768.2	-23
	+3 ml HA	835.5	-4.09
5	+3 ml Chit. (and 5 mg asparaginase BIOTEHGEN)	324.9	+ 8.15
	20 ml MP		
	+1.5 ml Chit.	304.4	-32.5
	+3 ml HA (and 5 mg asparaginase BIOTEHGEN)	676.9	-18.2
	+1.5 ml Chit.	289.1	+3.49

with improved capacity of producing isoenzyme EC 2 with anti-tumor activity (kindly supplied from BIOTEHGEN collection). After biosynthesis, the enzyme was purified by adsorption chromatography (bentonite), ammonium sulphate fractionation and ionic exchange chromatography (DEAE Sephadex A50). The resulted enzyme (150 U/mg) was precipitated with ethanol and dried in vacuum [20].

2.3 Asparaginase assay

The residual activity of the immobilized L-asparaginase was determined spectrometrically at 450 nm with Nessler reagent method (essentially that of Mashburn and Wriston, 1963) where the rate of hydrolysis of asparagine is determined by measuring released ammonia [24]. One unit releases one micromole of ammonia per minute at 37°C and pH 5 under the specified conditions.

2.4 Particle size analysis

Characterization of size and size distribution of the resultant magnetic nanoparticles bare and encapsulated with hydrogels was made using DLS (dynamic light scattering) and zetametry methods. The morphology of the particles was investigating with transmission electron microscopy and confocal microscopy, using a Philips EM 208 and a confocal spectral laser scanning microscope (LEICA TCS SP). The suspensions of nanoparticles were diluted, stirred and applied on grills or glass slides.

2.5 FTIR analysis

The structure of magnetic nanoparticles coated with hydrogel was characterized by FT-IR using a Bruker Tensor 27 instrument, in transmittance measurements.

2.6 Magnetic susceptibility

Magnetic properties were measured at three frequencies (976, 3904 and 15616 Hz) in a 200 A/m magnetic field with a MFK1 kappabridge (AGICO). The sensitivity of the kappabridge was 2×10^{-8} (SI).

2.7 Cytotoxicity studies

Cytotoxicity of nanostructures was determined by MTT Cell Proliferation Assay [25], a quantitative, convenient method for evaluating a cell population's response to external factors.

MTT test was done after 2 days on Vero cells (from the Vero lineage derived from epithelial cells of kidney from African green monkey, 20–25 passage), cultured with different concentrations of obtained hydrogel-magnetic

nanoparticles. Cell suspension was obtained by subconfluent culture trypsinization and was seeded into 24-well plates; each well was seeded with 5×10^4 cells/ml. Cells were cultured for 24 h in Dulbecco's modified Eagles medium/10% FBS (DMEM) and after standing overnight the culture medium was replaced with medium with hydrogel-magnetic nanoparticles.

The supernatant was replaced after 2 days of exposure of cells to magnetic nanoparticles and the cells were washed with PBS, then 500 μ l MTT solution (0.25 mg/ml) was added to each well. The cells were washed after 3 h of incubation at 37°C, the formazan crystals formed in living cells solubilized with 1 ml isopropanol, and the absorbance measured at 570 nm with a Jasco UV-Vis spectrometer. Cell viability was expressed as a percentage of control treated with different concentrations of magnetic nanostructures.

2.8 Interaction with microorganisms

The study of hydrogel-magnetic nanoparticles interactions with microorganisms was performed on Gram-positive (methicillin resistant *Staphylococcus aureus*), Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus* sp., *Klebsiella pneumoniae*, *Acinetobacter baumannii*), and yeast (*Candida albicans*).

The antimicrobial/antifungal activity was investigated by the qualitative screening of the susceptibility spectrum of different microbial strains to the tested samples by adapted variants of the diffusion method, and quantitatively by establishing the minimal inhibitory concentration (MIC) by serial decimal microdilution method [26].

The microbial adherence capacity to the inert substrate with nanoparticles was determined by a quantitative assay, i.e.: the strains were cultivated in 60 multiwell plates in the presence of different concentrations of the tested nanoparticles. The microbial biofilms developed in wells were fixed with methanol 15 min, stained with 2% violet crystal for 15 min, and re-suspended in 33% acetic acid; the absorbance of coloured suspension was measured at 490 nm.

3 Results and discussions

Magnetic nanoparticles obtained by coprecipitation method were covered with three successive layers of different proportions of chitosan and hyaluronic acid to obtain hydrogel-magnetic nanoparticles. According to our previous studies [22], the three layers of successive chitosan-hyaluronic acid-chitosan ensure the final nanometric dimensions and stability of obtained nanostructures (see Table 1). L-asparaginase was immobilized by entrapment

in hyaluronic acid (middle) layer or in external chitosan layer (Table 1). The hydrogel-magnetic nanoparticles with entrapped L-asparaginase were characterized by DLS and zetametry to determine the size, size distribution and zeta potential (Table 1).

The studies of microscopy confirm that obtained nanostructures are homogenous in shape and dimensions, the diameters of nanoparticles been around 20–30 nm in dried stage (Figs. 1, 2).

FT-IR spectrometry analyses proved the encapsulation of magnetic nanoparticles with chitosan and hyaluronic acid. As could be observed from Fig. 3 (3b, overlaid spectra) the bare nanoparticles themselves are characterized mainly of four absorption bands: first band, at

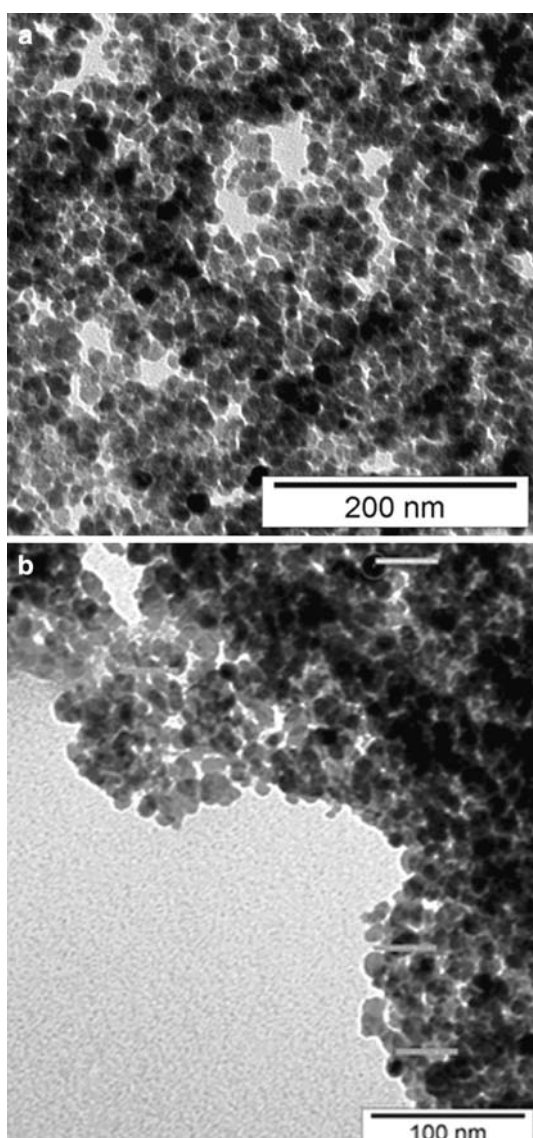


Fig. 1 **a** TEM images of uncovered (bare) MP ($d = 15.97$ nm); **b** MP covered with hydrogel (sample 5, $d = 17.51$ nm)

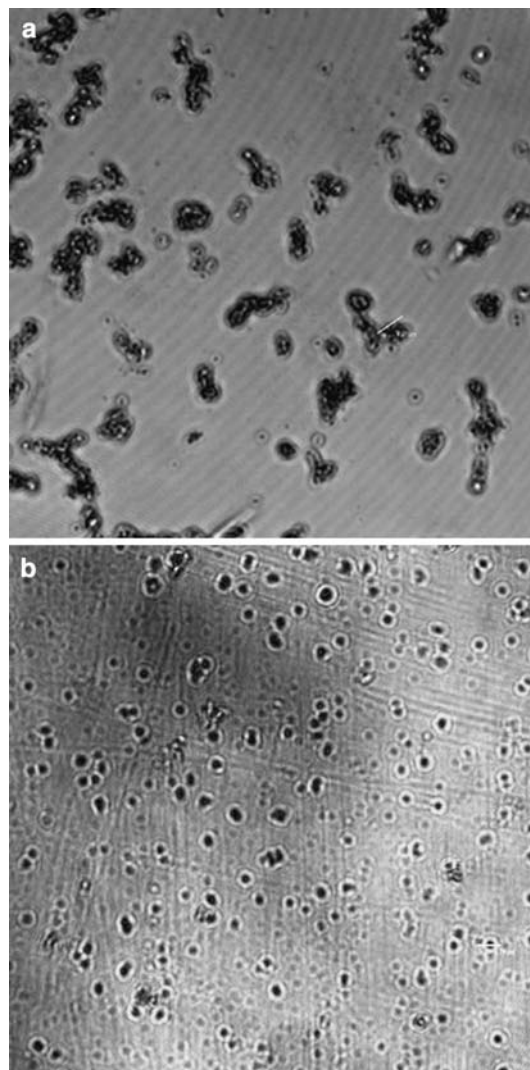


Fig. 2 **a** Confocal microscope images ($250 \times 250 \mu\text{m}$) of uncovered (bare) MP ($d_x = 90$ nm); **b** MP covered with hydrogel (sample 1) ($d_x = 169$ nm)

3125 cm^{-1} , is of medium intensity, being ascribable to free HO-groups; the second band, at about 1623 cm^{-1} is a weak, wide band, being the result of vibration frequency of NH_3^+ remained from the synthesis processes, this ascription being confirmed by the third band, that occurs at 1401 cm^{-1} , that is specific for ammonium salts. The magnetite formation is supported by the presence of a strong, wide absorption band at 580 cm^{-1} , confirmed by the weak band occurring at $400\text{--}450 \text{ cm}^{-1}$. When the layer-by-layer covering is performed using chitosan, hyaluronic acid and entrapped L-asparaginase, in all the obtained samples were noticed the specific bands of biopolymers, at the wave numbers $3,200 \text{ cm}^{-1}$, respectively $3,680 \text{ cm}^{-1}$ being confirmed on $1,790\text{--}1,520 \text{ cm}^{-1}$ region. Chitosan, respectively HA, as any macromolecular carbohydrate polymer, rises at $3,508 \text{ cm}^{-1}$ a wide band, due to

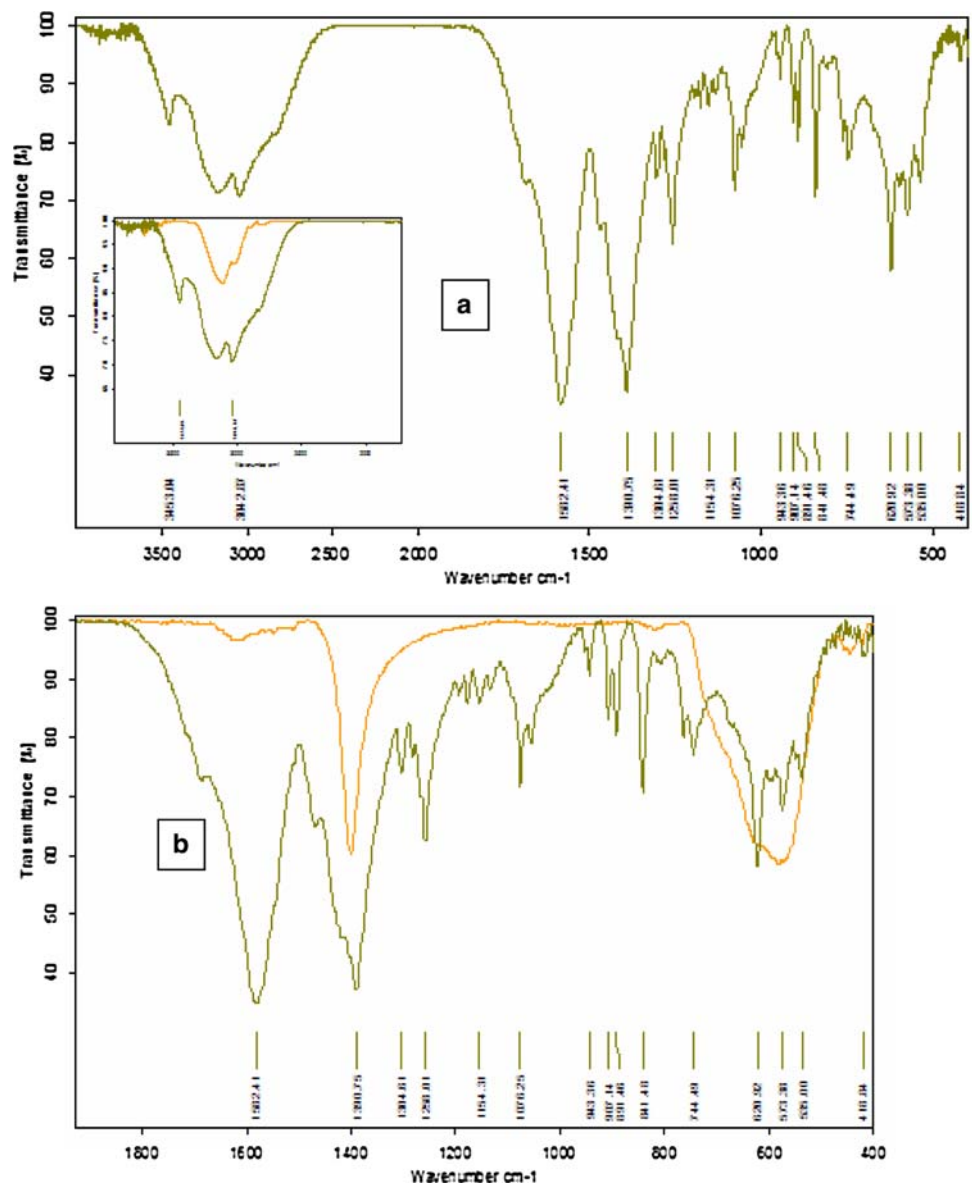
the stretching vibrations of H–O bonds, which are present even on the spectra of covered nanoparticles: These prove the presence of the chitosan and HA structural pattern in the obtained hydrogel-magnetic nanoparticles, supporting the efficiency of the covering. In addition, the changes occurred in the shape, position and intensity of 1,700–500 cm^{-1} and 3,520–3,100 cm^{-1} bands (Fig. 3a, b) allowed us to presume that H-bonds or even covalent bonds are formed between magnetic nanoparticles and hydrogel layers. It was noticed that the amide band is present, both in chitosan and in covered nanoparticles spectra, at 1,650 cm^{-1} in the chitosan case, and slightly shifted toward smaller wavenumbers in the case of covered nanoparticles, thus confirming once more the covalent link between the biocompatible polymer and the magnetic nanoparticle.

The magnetic susceptibility measurements of the synthesised samples (Fig. 4) show a decrease of magnetic susceptibility with the increase of magnetic field frequency, specific for superparamagnetic particles (<20 nm).

The residual activity of L-asparaginase entrapped in hydrogel-magnetic nanoparticles is presented in Table 2. The analysis was made in duplicates in the moment of synthesis, and at 3 and 6 months after synthesis. The residual activity remains about the same in samples 4 and 5, or is slightly diminished in sample 1 and 2.

The tests for in vitro biocompatibility were performed in triplicates on normal Vero cells cultivated with different concentrations of magnetic nanostructures (between 2 and 12 ng/cell). All the tested samples present no toxicity even at higher concentrations of nanoparticles (Fig. 5) and the phenotype of the Vero cells remains normal (Fig. 6).

Fig. 3 **a** FTIR spectra of sample 2; inset—overlay region 2,500–3,700 cm^{-1} for sample 2 and bare magnetic nanoparticles; **b** overlay region 1,900–400 cm^{-1} for sample 2 and bare magnetic nanoparticles



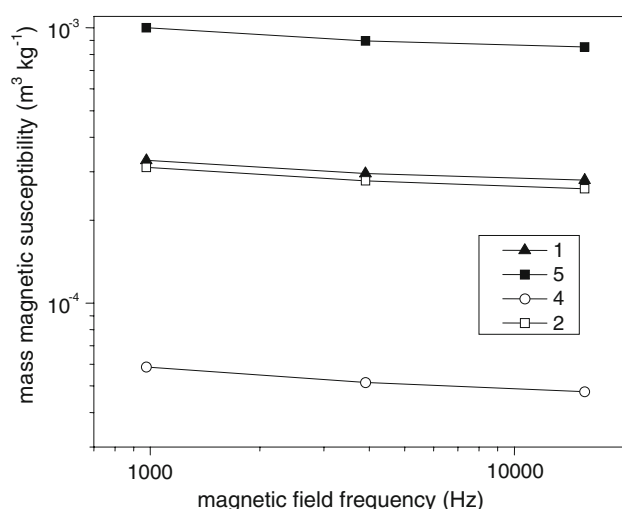


Fig. 4 Magnetic susceptibility of synthesized hydrogel magnetic nanoparticles (samples 1, 2, 4 and 5) function of the magnetic field frequency

The obtained nanostructures did not exhibit any antimicrobial or antifungal activity, but in exchange they influence the expression of bacterial adhesions, as demonstrated by the results of the adherence showing differences in the behaviour of microorganisms in the presence of magnetic nanostructures. The tested samples inhibited the adherence capacity of the majority of tested microorganisms (bacteria or fungi) excepting *Candida albicans*, stimulated by sample no. 5 (Fig. 7) and MP and *Proteus* sp. and *Klebsiella pneumoniae* stimulated by MP (Fig. 8).

Magnetic nanoparticles continue to receive attention from scientists. Recent studies describe an approach to synthesize superparamagnetic iron oxide nanoparticles in the presence of polymerized lactic acid or PVA, which can be used for developing target specific MRI contrast agents, ferrogels or drug delivery systems [27, 28] or PVP hydrogel magnetic nanospheres which exhibited passive drug release that could be exploited to enhance therapeutic efficacy [29], or in the formation of a novel hydrogel nanocomposite with superparamagnetic property [30]. Novel magnetic hybrid hydrogels were fabricated by the in situ embedding of magnetic iron oxide nanoparticles into the porous hydrogel networks, this magnetic hydrogel

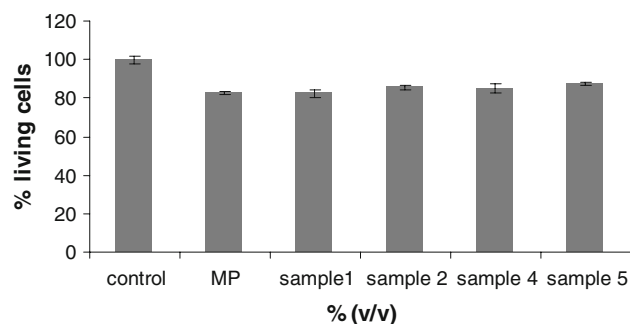


Fig. 5 Biocompatibility/cytotoxicity of obtained nanoparticles cultured 48 h with Vero cells (nanoparticles conc. was 12 ng/cell). The absorbance at 570 nm obtained for control was considered 100%. The results for the treated cells were expressed as percentage from the control, untreated culture (mean value \pm SD)

material was found to hold a potential application in magnetically assisted bioseparation [31].

Nanomedicines are defined as delivery systems in the nanometer size range (preferably 1–100 nm) containing encapsulated, dispersed, adsorbed, or conjugated drugs and imaging agents [32]

We obtained biocompatible hydrogel-magnetic nanoparticles with L-asparaginase entrapped, with sizes below 300 nm in swelled stage, and below 30 nm in dried stage, capable to penetrate the cells and tissues (especially tumor tissues). The majority of solid tumors exhibit a vascular pore cutoff size between 380 and 780 nm [33], although tumor vasculature organization may differ depending on the tumor type, its growth rate and microenvironment [33, 34].

The residual activity of the immobilized L-asparaginase remains the same after 6 months from immobilization, stored at 4°C. The immobilization yield of the L-asparaginase in different samples was between 43% and 90%. The obtained nanostructures are biocompatible and could be used for delivery of L-asparaginase in tumor tissues. A recent work presents a similar biomaterial obtained by successfully immobilization of L-asparaginase in carboxymethyl konjac glucomannan-chitosan nanocapsules prepared under mild conditions by electrostatic complexation [35]. The magnetic nanostructures described in our work are hydrogel-superparamagnetic particles with L-asparaginase entrapped and could be controlled by a magnetic field.

Table 2 L-Asparaginase residual activity of obtained nanostructures

Sample	Abs., 450 nm	$\mu\text{mol NH}_3/\text{sample}$	U/ml	Total U immobilized	Immobilization yield (%)
1	0.0965–0.0762	0.45–0.34	1.5–1.13	225	43.02
2	0.1121–0.0780	0.53–0.35	1.77–1.16	265.5	50.73
4	0.2198–0.188	1.13–0.95	3.76–3.17	475	90.76
5	0.1307–0.1147	0.64–0.55	2.13–1.83	319.5	61.05

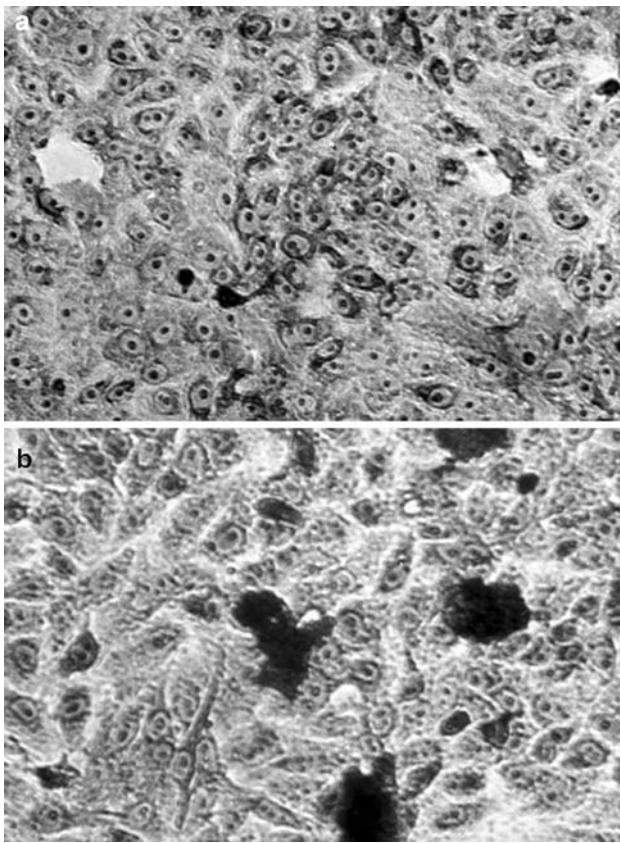


Fig. 6 a Vero cells morphology after 48 h of cultivation without or b with hydrogel-magnetic nanoparticles, 12 ng/cell (light microscopy, Giemsa stain, objective $\times 20$)

In addition, from adherence studies of microorganisms in the presence of obtained hydrogel-magnetic nanostructures, we can also use them in magnetic bioseparation, because the different capacity of bacteria or yeast to adhere, or not, to inert substrate treated with hydrogel-nanoparticles. All the tested samples inhibited the adherence of *Candida*, but could stimulate some bacteria to

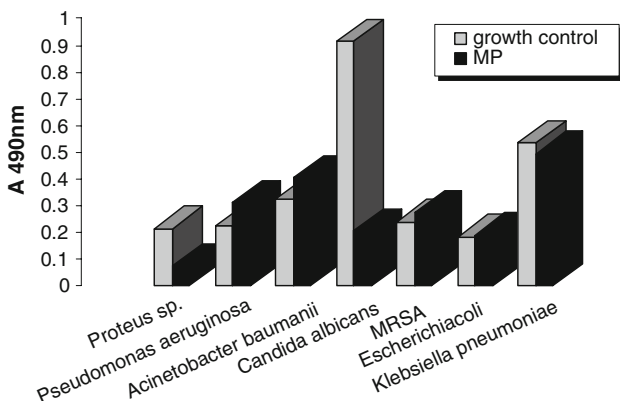


Fig. 7 The adherence capacity of some microbial strains on the inert substrate with bare magnetic nanoparticles

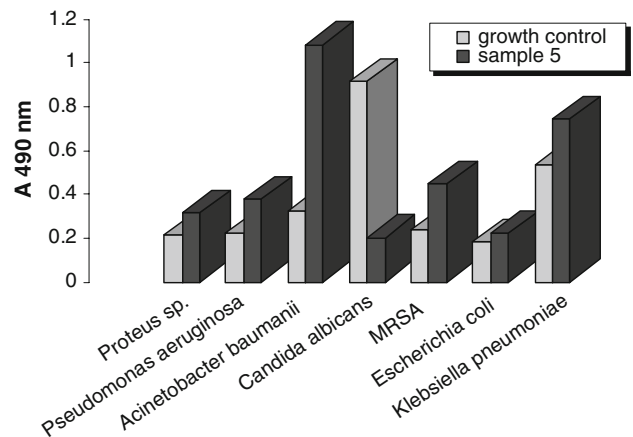


Fig. 8 The adherence capacity of some microbial strains on the inert substrate with hydrogel-magnetic nanoparticles (sample 5)

adhere (Fig. 7). The bare magnetic nanoparticles have a similar behaviour with hydrogel-magnetic nanoparticles (Fig. 8), so simple magnetic nanoparticles could be used to magnetic separation of some strains from different media.

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References

- M.N.V.R. Kumar, J. Pharm. Sci. **3**, 234 (2000)
- D. Cu, H. Gao, Biotechnol. Prog. **219**, 683 (2003). doi:10.1021/bp025791i
- P.A. Dresco, V.S. Zaitsev, R.J. Gambino, B. Chu, Langmuir **15**, 1945 (1999). doi:10.1021/la980971g
- P. Tartaj, M.P. Morales, S. Veintemillas-Verdaguer, T. Gonzalez-Carreño, C.J. Serna, J. Phys. D Appl. Phys. **36**, R182 (2003). doi:10.1088/0022-3727/36/13/202
- M.H. Liao, D.H. Chen, Biotechnol. Lett. **23**, 1723 (2001). doi:10.1023/A:1012485221802
- S.H. Huang, M.H. Liao, D.H. Chen, Biotechnol. Prog. **19**, 1095 (2003). doi:10.1021/bp025587v
- K.C. Kulla, M.D. Gooda, M.S. Thakur, N.G. Karanth, Biosens. Bioelectron. **19**, 621 (2004). doi:10.1016/S0956-5663(03)00258-6
- M. Przybyt, Mater. Sci. **21**, 398 (2003)
- G.K. Kouassi, J. Irudayaraj, G. McCarty, J. Nanobiotechnol. **3**, 1 (2005). doi:10.1186/1477-3155-3-1
- R. Masteikova, Z. Chalupova, Z. Sklupalova, Medicina (B Aires) **39**, 19 (2003)
- J.S. Mao, H.F. Liu, Y.J. Yin, K.D. Yao, Biomaterials **24**, 1621 (2003). doi:10.1016/S0142-9612(02)00549-5
- E.T. Baran, J.F. Mano, R.L. Reis, J. Mater. Sci.: Mater. Med. **18**, 759 (2004). doi:10.1023/B:JMSM.0000032815.86972.5e
- Y. Luo, K. Kirker, G. Prestwich, J. Control Release **69**, 169 (2000). doi:10.1016/S0168-3659(00)00300-X
- X.Z. Shu, K.J. Zhu, J. Microencapsul. **18**, 237 (2001). doi:10.1080/02652040010000415
- G. Abatangelo, P. Weigel, *New Frontiers in Medical Sciences: Redefining Hyaluronan* (Elsevier, Amsterdam, 2000)

16. N. Verma, K. Kumar, G. Kaur, S. Anand, *Crit. Rev. Biotechnol.* **27**, 45 (2007). doi:[10.1080/07388550601173926](https://doi.org/10.1080/07388550601173926)
17. C.H. Fu, K.M. Sakamoto, *Expert Opin. Pharmacother.* **8**, 1977 (2007). doi:[10.1517/14656566.8.12.1977](https://doi.org/10.1517/14656566.8.12.1977)
18. Y.Q. Zhang, M.L. Tao, W.D. Shen, Y.Z. Zhou, Y. Ding, Y. Ma, W.L. Zhou, *Biomaterials* **25**, 3751 (2004). doi:[10.1016/j.biomaterials.2003.10.019](https://doi.org/10.1016/j.biomaterials.2003.10.019)
19. N. Verma, K. Kumar, G. Kaur, S. Anand, *Artif. Cells Blood Substit. Immobil. Biotechnol.* **35**, 449 (2007). doi:[10.1080/10731190701460358](https://doi.org/10.1080/10731190701460358)
20. C.P. Cornea, I. Lupescu, I. Vatafu, T. Caraiani, V.G. Savoiu, G. Campeanu, I. Grebenisan, G.P. Negulescu, D. Constantinescu, *Rom. Biotechnol. Lett.* **7**, 717 (2002)
21. R. Massart, *IEEE Trans. Magn. MAG* **17**, 1247 (1981). doi:[10.1109/TMAG.1981.1061188](https://doi.org/10.1109/TMAG.1981.1061188)
22. E. Teodor, C. Petcu, M. Eremia, V. Lazar, G.A. Stanciu, S. Litescu in *Excellence Research—A way to ERA*, ed. by N. Vasiliu, L. Szabolcs (Tehnică, Braşov, 2007), section I, p. 129
23. E. Teodor, F. Cutaş, L. Moldovan, L. Tcacenco, M. Caloianu, *J. Biol. Sci.* **1**, 35 (2003)
24. L. Mashburn, J. Wriston, *Biochem. Biophys. Res. Commun.* **12**, 50 (1963). doi:[10.1016/0006-291X\(63\)90412-1](https://doi.org/10.1016/0006-291X(63)90412-1)
25. T. Mosmann, *J. Immunol. Methods* **65**, 55 (1983). doi:[10.1016/0022-1759\(83\)90303-4](https://doi.org/10.1016/0022-1759(83)90303-4)
26. V. Lazar, C. Balotescu, R. Cernat, D. Bulai, G. Nitu, L. Ilina, *Clin. Microbiol. Infect.* **9**(Suppl. 1), 227 (2003)
27. S. Liu, X. Wei, M. Chu, J. Peng, Y. Xu, *Colloids Surf. B Biointerfaces* **51**, 101 (2006). doi:[10.1016/j.colsurfb.2006.05.023](https://doi.org/10.1016/j.colsurfb.2006.05.023)
28. T.Y. Liu, S.H. Hu, K.H. Liu, D.M. Liu, S.Y. Chen, *J. Control Release* **126**, 228 (2008). doi:[10.1016/j.jconrel.2007.12.006](https://doi.org/10.1016/j.jconrel.2007.12.006)
29. D. Guowei, K. Adriane, X. Chen, C. Jie, L. Yinfeng, *Int. J. Pharm.* **328**, 78 (2007). doi:[10.1016/j.ijpharm.2006.07.042](https://doi.org/10.1016/j.ijpharm.2006.07.042)
30. D. Ma, L.M. Zhang, *J. Phys. Chem. B* **112**, 6315 (2008). doi:[10.1021/jp7115627](https://doi.org/10.1021/jp7115627)
31. Y.Y. Liang, L.M. Zhang, W. Jiang, W. Li, *ChemPhysChem* **8**, 2367 (2007). doi:[10.1002/cphc.200700359](https://doi.org/10.1002/cphc.200700359)
32. O.M. Koo, I. Rubinstein, H. Onyuksel, *Nanomedicine* **1**, 193 (2005). doi:[10.1016/j.nano.2005.06.004](https://doi.org/10.1016/j.nano.2005.06.004)
33. S.K. Hobbs, W.L. Monsky, F. Yuan, W.G. Roberts, L. Griffith, V.P. Torchilin et al., *Proc. Natl Acad. Sci. USA* **95**, 4607 (1998). doi:[10.1073/pnas.95.8.4607](https://doi.org/10.1073/pnas.95.8.4607)
34. R.K. Jain, *J. Control Release* **53**, 49 (1998). doi:[10.1016/S0168-3659\(97\)00237-X](https://doi.org/10.1016/S0168-3659(97)00237-X)
35. R. Wang, B. Xia, B.J. Li, S.L. Peng, L.S. Ding, S. Zhang *Int. J. Pharm.* (Aug):3. Epub ahead of print (2008)