The release of l-asparaginase from hydrogel-magnetic nanoparticles

EUGENIA TEODOR[1], GEORGIANA TRUICA[1] AND IRINA LUPESCU[2]
1 National Institute for Biological Sciences-Centre of Bioanalysis, Bucharest 6, 296, Spl. Independentei
2 Centre of Microbial Biotechnology - BIOTEHGEN, Bucharest 1, 59, Marasti

Abstract

L-asparaginase was immobilized by entrapment in hydrogel-magnetic nanoparticles obtained from magnetic nanoparticles and successive layers of chitosan and hyaluronic acid. The obtained nanostructures, with dimensions suitable for penetration cell/tissues, especially tumor tissues, were evaluated for swelling behavior, degradation behavior and the release of L-asparaginase in neutral media and slight acid media (pH 4.5). The experiments showed a good swelling behavior for all the tested samples, but a high stability of nanostructures. L-asparaginase has an initial burst from hydrogel, at two hours, and other two releases at 24 and between 50 and 72 hours.

Keywords: hydrogel-magnetic nanoparticles, L-asparaginase release, drug delivery.

Introduction

L-asparaginase (L-asparagine amido-hydrolase (E.C. 3.5.1.1.) is a chemotherapy agent, used in treatment of acute lymphoblastic leukemia (ALL), but in some cases it presents toxicity, the main side effect is an allergic or hypersensitivity reaction. Enzyme immobilization into a polymeric shell could improve their delivery and eliminate the allergic reaction [1]. The most extensively studied asparaginase is the EC-2 from *E. coli* which differs from EC-1 by its broad pH activity profile and its higher substrate affinity.

In the last decade, nanosize materials have been widely used as support for immobilization of proteins, peptides, enzymes [2-7] antibodies and nucleic acids [6], because of their unique properties [2-7]. Among these materials, magnetic nanoparticles are very popular when they are used in combination with biological materials. Magnetic nanoparticles are employed as contrast agents for magnetic resonance imaging (MRI) and as carriers for drug delivery due to them unique magnetic properties, low toxicity and the ability to function at the cellular and molecular level of biological interactions.

In previous works we presented the synthesis and characterization of hydrogel-magnetic nanoparticles, biocompatible, capable to penetrate cell and tissues and to embed active principle (protease inhibitor, L-asparaginase) [8,9]. The obtained nanostructures are nanometric dimensions (below 30 nm) and could bind or load protease inhibitor and L-asparaginase. In this work is evaluated the release of L-asparaginase from hydrogel-magnetic nanoparticles with L-asparaginase entrapped. The release was evaluated by protein assay and by L-asparaginase activity assay.

Matherials and methods

Synthesis of hydrogel-magnetic nanoparticles with immobilized L-asparaginase

Water-dispersible magnetic nanoparticles were obtained using an adapted Massart method [10]. The magnetic nanoparticles (MP) were encapsulated according to our previous work [11], using layer-by-layer technique in two different biopolymeric materials, chitosan (SIGMA) from crab shells (Chit.) and hyaluronic acid (HA) extracted by us from bovine vitreous [12]. Asparaginase was immobilized by entrapment in hydrogel layer of obtained nanostructures. L-asparaginase was obtained by biosynthesis from *Escherichia coli* using a recombinant strain of *E.coli* with improved capacity of producing isoenzyme EC 2 with anti-tumor activity (kindly supplied by BIOTEHGEN-Centre of Microbial Biotechnologies, Bucharest) [13]. The synthesized hydrogel-magnetic nanoparticles were characterized in a previous work using DLS (Dynamic Light Scattering), TEM (Transmission Electron Microscopy) and FTIR (Fourier Transformed Infrared) spectroscopy [9]. From our previous studies, the synthesized hydrogel-magnetic nanoparticles with L-asparaginase entrapped have dimensions below 30 nm, are biocompatible and relative inert to microorganisms.

Swelling test

The obtained hydrogel-magnetic nanoparticles (~ 10 mg dry weight) were suspended in tubes containing 50 mL of PBS (pH 7.2 at 20^0C). At an appropriate time interval, the excess buffer was removed carefully using a magnetic separator and the hydrogel-magnetic nanoparticles were weighted immediately. The swelling capacity was calculated according to the following equation [14]:

$$\%S = \frac{m_w - m_i}{m_i} x100 , \qquad (1)$$

where $\%S$ is swelling ratio, m_w is the weight of samples after immersion and m_i is initial weight.

In vitro degradation study

Hydrogel-magnetic nanoparticles were suspended into Falcon tubes containing 50 mL PBS (pH 7.2 at 37^0C). At predetermining time points, hydrogels were collected with a magnetic separator and excess buffer was removed from the tubes. Samples were weighted by using an analytical balance with ± 0.1 mg accuracy. After the equilibration time of swelling in PBS, the degradation ratio was calculated according to the following equations [15]:

$$\%D = \frac{m_0 - m_t}{m_0} x100 , \qquad (2)$$

where $\%D$ represent degradation ratio, m_0 is the original weight after equilibration time of swelling in PBS and m_t is the weight at time *t*.

Asparaginase release studies

0.2 g of the asparaginase-hydrogel-magnetic nanoparticles were immersed in 1 mL phosphate-buffered solution and kept at 37^0C without agitation for various time periods. The buffered solutions used were a phosphate buffered saline solution of pH 7.00 and a phosphate buffer of pH 4.5. At the end of each immersion period, 1mL of the buffered solution was removed and analyzed for asparaginase activity and protein content (Bradford, [16]), spectrophotometrically on a Jasco UV-Vis spectrophotometer. The amount of BSA was determined using a standard curve, (m=0.005, R^2=0.995).

An identical volume of fresh phosphate-buffered saline solution was added back into the container after each sample removal.

Asparaginase assay

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The residual activity of the immobilized and released 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 [17], spectrophotometrically on a Jasco UV-Vis spectrophotometer. One unit releases one micromole of ammonia per minute at 37°C and pH 5 under the specified conditions). The amount of ammonia was determined using a standard curve, (m=0.182, R^2=0.998).

Results and discussions

The hydrogel-magnetic nanoparticles were obtained from magnetic particles covered with three successive layers of different proportions of chitosan and hyaluronic acid. According to our previous studies [8,9], 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 synthesized hydrogel-magnetic nanoparticles with L-asparaginase entrapped have dimensions suitable for penetration cell or tissue, respectively below 300 nm in swelled stage and below 30 nm in dried stage, as it could see in figure 1 and in table 1. The obtained nanostructures were tested for swelling capacity, figure 2, and degradation behavior, figure 3, to evaluate the behavior of nanoparticles in biological-fluids. The hydrogel-magnetic nanoparticles had a good swelling capacity; they retain fluids up to 10 times their weight. The samples have similar composition, so they have a similar swelling behavior (figure 2).

Table 1. Composition and L-asparaginase residual activity of obtained nanostructures;

Sample	Sample Type (composition)	D (nm) (Swelled Stage)	µmoli NH_3 /sample	U/mL	Initial immobilization yield (%)
0	MP	25.21	-	-	-
1	20 ml MP +1.5 ml Chit. + 5 ml HA (and 5 mg asparaginase BIOTEHGEN) + 1.5 mL Chit.	349.5 320 **274**	0.45-0.34	1.5-1.13	43.02
2	20 ml MP + 3 ml Chit. + 3 ml HA (and 5 mg asparaginase BIOTEHGEN) + 3 ml Chit.	418.7 539.2 **261.8**	0.53-0.35	1.77-1.16	50.73
4	20 ml MP + 3 ml Chit. + 3 ml HA + 3 ml Chit. (and 5 mg asparaginase BIOTEHGEN)	768.2 835.5 **324.9**	1.13-0.95	3.76-3.17	90.76
5	20 ml MP +1.5 ml Chit. + 3 ml HA (and 5 mg asparaginase BIOTEHGEN) + 1.5 mL Chit.	304.4 676.9 **289.1**	0.64-0.55	2.13-1.83	61.05

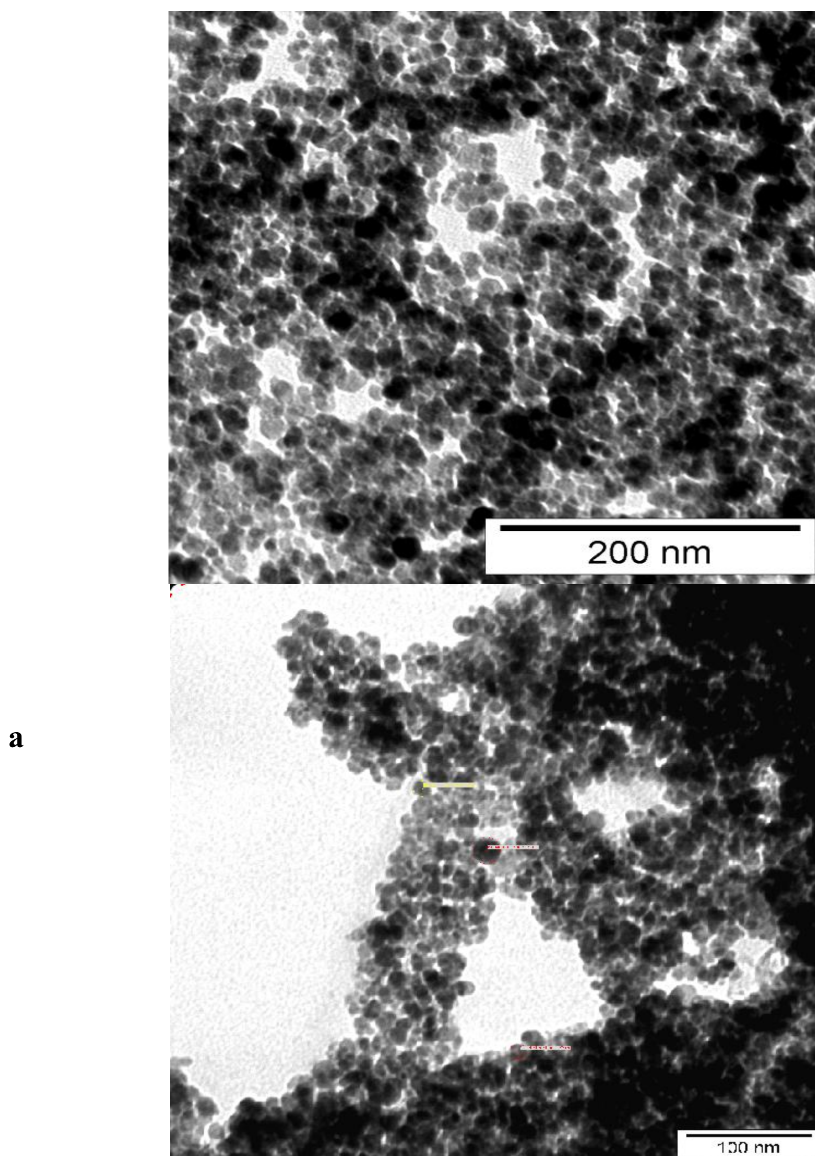

Figure 1 a) TEM images of uncovered (bare) MP (d=15.97 nm); b) MP covered with hydrogel (sample 1) (d=23.29nm)

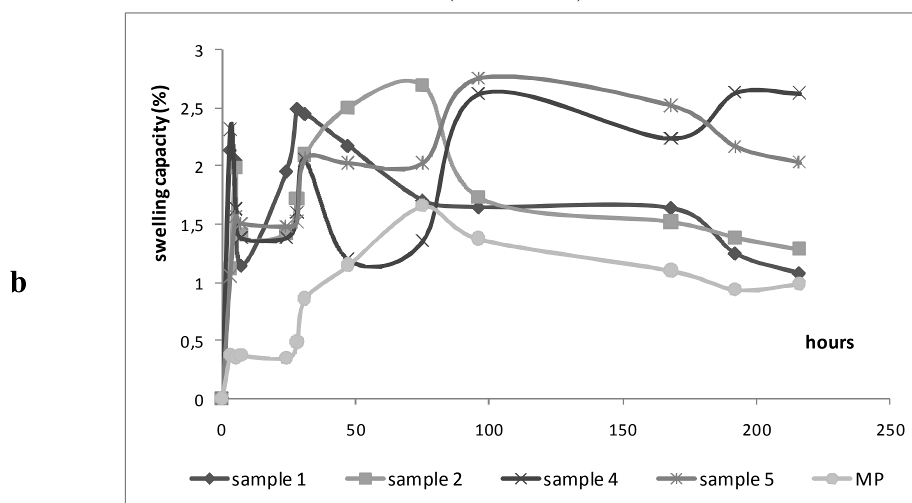

Figure 2. Swelling behavior of covered magnetic nanoparticles

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The degradation of obtained nanostructures is insignificant. Apparent degradation in first 2-3 hours is determined by the release of L-asparaginase, as it could see in figure 4. The hydrogel-magnetic nanoparticles are stable in neutral media at 37^{0}C, the degradation ratio being < 1 (figure 3). The stability of obtained nanostructures was confirmed by previous FTIR studies [9]; the spectra obtained showed that between magnetic nanoparticles and hydrogel layers of chitosan and hyaluronic acid are formed hydrogen bonds (or even covalent bonds).

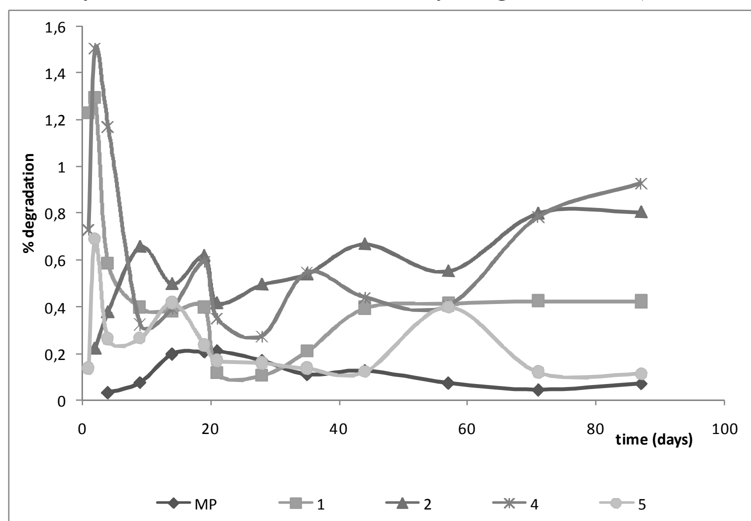

Figure 3. Degradation behavior of hydrogel-magnetic nanoparticles

The release of L-aspraginase was tested in neutral media, at pH 7, for the case of using nanoparticles in parenteral delivery and at pH 4.5 (considered the pH of tissues). The experiments were repeated two times, and the delivery of L-asparaginase was measured by two methods (protein assay and L-asparaginase activity assay). The results were similar. In figures 4 and 5 are presented results obtained by protein assay (the method is shorter and easier, so is recommended instead of enzyme activity assay). All the samples, showed a similar release of L-asparaginase. As it could see in figure 4, at pH 7, the enzyme has an initial, major burst after two hours and a second release after 24 hours and a final smaller release between 50 and 72 hours. At pH 4.5, the behavior of samples is different; the enzyme is released slowly and in reduced quantities (figure 5). All the samples obtained present L-asparaginase activity, but samples 4 and 5 had the better immobilization yield and the better stability at storing. Sample 4, which has the enzyme in external layer of hydrogel presented the best L-asparaginase activity, even after 6 months since immobilization.

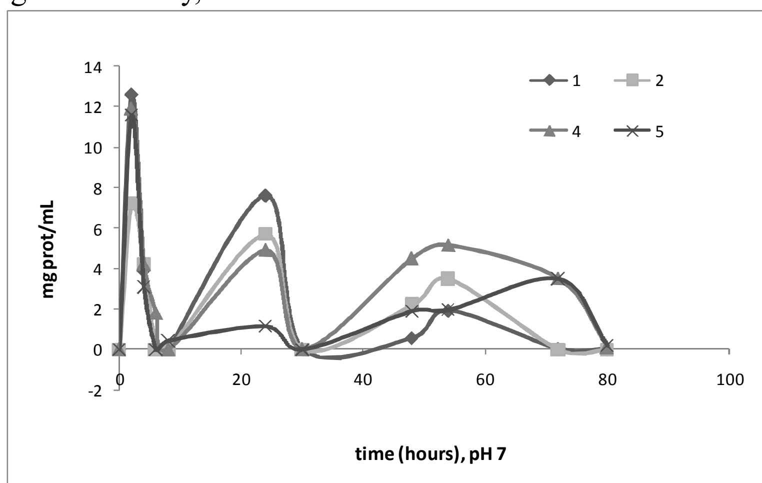

Figure 4. Release profile of L-asparaginase determined at pH 7 by protein content

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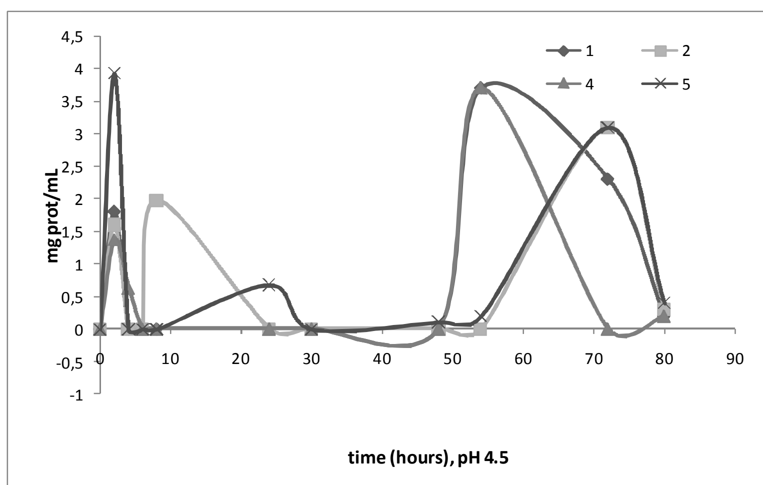

Figure 5. Release profile of L-asparaginase determined at pH 4.5 by protein content

Magnetic nanoparticles continue to receive attention from scientists. Recent studies describe an approach to synthesize ferrogels with a precise control of the opening and closure of pore configuration, which allows a burst release or no-release action of therapeutically active agent to be controlled externally and magnetically [18]. This type of ferrogel can be considered as a class of novel magnetically-tunable drug delivery system. Nanomedicines are defined as delivery systems in the nanometer size range (preferably 1 to 100 nm) containing encapsulated, dispersed, adsorbed, or conjugated drugs and imaging agents [19].

The hydrogel-magnetic nanoparticles with L-asparaginase entrapped previously obtained by us, have sizes below 300 nm in swelled stage, and below 30 nm in dried stage and are capable to penetrate cells and tissues (especially tumor tissues, which exhibit a vascular pore cutoff size between 380 and 780 nm [20]). The residual activity of the immobilized L-asparaginase remains the same after 6 months from immobilization, stored at 4^0C. The immobilization yield of the L-asparaginase in different samples was between 43-90%. The obtained nanostructures are biocompatible and could be used for delivery of L-asparaginase. The release of L-asparaginase from hydrogel-magnetic nanoparticles is greatly long-lasting compare with administration of free enzyme. In neutral conditions, L-asparaginase has an initial burst from hydrogel, at two hours, and other two releases at 24 and between 50 and 72 hours.

Conclusions

The hydrogel-magnetic nanoparticles were obtained from magnetic particles covered with three successive layers of different proportions of chitosan and hyaluronic acid. L-asparaginase was immobilized by entrapment in hyaluronic acid (middle) layer or in external chitosan layer. The obtained nanostructures, with dimensions suitable for penetration cell/tissues, especially tumor tissues, were evaluated for swelling behavior, degradation behavior and the release of L-asparaginase in neutral media and slight acid media (pH 4.5). The experiments showed a good swelling behavior for all the tested samples, but a high stability of nanostructures. L-asparaginase has an initial burst from hydrogel, at two hours, and other two releases at 24 and between 50 and 72 hours.

These results are promising for using the obtained hydrogel-magnetic nanoparticles for delivery for a wide class of therapeutic agents.

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