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Poly(DL-lactide-*co*-glycolide) Nanospheres for the Sustained Release of Folic Acid

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Biodegradable polymers have become the materials of choice for a variety of biomedical applications. In particular, poly(DL-lactide-*co*-glycolide) nanoparticles have been studied as a material for drug delivery with the controlled release. In this paper we are describing a simple method for obtaining the system for targeted and controlled delivery of folic acid in the body. Folic acid was encapsulated into the polymer matrix by means of homogenization of aqueous and organic phases. Concentration of folic acid in water was varied in order to obtain nanoparticles with different ratios of poly(DL-lactide-*co*-glycolide) and folic acid. The particles were obtained by physicochemical solvent/non-solvent method with polyvinyl pyrrolidone as a surfactant. The obtained particles are non-agglomerated, uniform and with particles size in the nanometer range. The samples were characterized using Infrared Spectroscopy (IR), Ultraviolet Spectroscopy (UV), Zeta potential measurements, Scanning Electron Microscopy (SEM) and Stereological analysis.

Keywords: Poly(DL-lactide-*co*-glycolide), Folic Acid, Nanoparticles, IR, SEM, UV-Vis, Zeta Potential Measurements, Stereological Analysis, Drug Delivery.

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1. INTRODUCTION

Poly(DL-lactide-*co*-glycolide) (PLGA or DLPLG) is a very popular copolymer used for various medical, pharmaceutical, industrial and other purposes. The most attractive application of this copolymer is, however, in medicine and pharmacy because it is a very suitable for the controlled delivery of medicaments in the body.[1] Poly(DL-lactide-*co*-glycolide)-based micro- and nanoparticles offer various advantages compared to other controlled drug delivery systems, including: the possibility to accurately control the resulting drug release kinetics over periods of days to months, complete biodegradability, good biocompatibility, easy administration into the body, etc.[2–4] Morphological characteristics of the particles, like size, size distribution and shape, are extremely important for the controlled drug delivery, and are particularly influencing the adhesion and interaction with cells (intracellular uptake).[5–9] Polymer degradation, dynamics of the release (rate and concentration) of drugs from the polymer matrices depend on the morphology of the particles (size, shape, uniformity, pore size structure, etc.).[5, 10–13]

Folic acid (pteroyl-L-glutamic acid, vitamin B_9) is a water-soluble vitamin that is essential in the human diet. It is necessary for the production and maintenance of new cells.[14] This is especially important during the periods of rapid cell division and growth such as pregnancy and infancy when it is often necessary to take specific dosages of folic acid on daily basis.[15] Folic acid is needed to replicate DNA. Thus folic acid deficiency hinders DNA synthesis and cell division. Because RNA and protein synthesis are not hindered, large red blood cells called megaloblasts are produced, resulting in megaloblastic anemia.[15] Both adults and children need folic acid to make normal red blood cells and prevent anemia. Folic acid also helps prevent changes to DNA that may lead to cancer.[16] Also, one of the most extensively studied small molecule targeting moieties for drug delivery is folic acid (folate). The high-affinity vitamin is a commonly used ligand for cancer targeting because folate receptors are frequently overexpressed in a range of tumor cells.[17–19]

In this work we followed the concept which we have already applied successfully in the encapsulation of ascorbic acid in PLGA nanoparticles.[20] Ascorbic acid (vitamin C) is also a water-soluble vitamin. The method we employed is also based on the fact that PLGA and folic

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acid (or ascorbic acid) are not miscible because PLGA is a polymer insoluble in water. With the encapsulation of folic acid into a PLGA polymer matrix, it may be possible to achieve its higher efficiency in the body.

2. MATERIALS AND METHODS

2.1. Materials

Poly(DL-lactide-*co*-glycolide) (PLGA) was purchased from Durect, Lactel, and had a lactide-to-glycolide ratio of 50:50. Molecular weight of the polymer was 40000–50000 g/mol. The time for its complete resorption in the body is 4 to 8 weeks. Molecular weight of folic acid is 441.14 g/mol (Microvit^{TM}, Adisseo). Polyvinyl pyrrolidone (povidone, PVP) was obtained from Merck Chemicals Ltd (k-25, Merck, Germany). All other chemicals and solvents were of reagent grade.

2.2. PLGA/Folic Acid Nanoparticles Formulation

Nanoparticles composed of PLGA and folic acid were synthesized following a variant of the method which has already been applied in the encapsulation of ascorbic acid in PLGA nanoparticles.[20] At room temperature, commercial granules of poly(DL-lactide-*co*-glycolide) (0.25 g) were solubilized in 20 ml of acetone during two hours (step 1). Afterwards, the aqueous solution of folic acid (5 ml) was added in PLGA solution in acetone while continuously being homogenized at 200 rpm during 30 minutes (step 2). Concentration of folic acid in water was varied in order to obtain nanoparticles with different ratio of PLGA and folic acid (PLGA/folic acid 95/5%wt, PLGA/folic acid 90/10%wt, PLGA/folic acid 85/15%wt and PLGA/folic acid 80/20%wt). Accordingly, the weights of folic acid in 5 ml of water were 13.2 mg, 27.8 mg, 44.1 mg, and 62.5 mg. During the solvation of folic acid, sodium hydrogen carbonate was added to water until a slightly alkaline environment was reached, all in order to increase the solubility of folic acid. This was followed by precipitation using 22 ml of methanol (step 3). Thus obtained solution was very slowly poured (instilled for 15 min) into 40 ml of aqueous PVP solution (0.05% w/w) while continuously stirring at 1200 rpm (step 4). Surface charge of the particles in the dispersion was determined by zeta potential measurements. After that, the solution was centrifuged at 4000 rpm for 120 min, decanted and dried (step 5, 6 and 7). The supernatant solution was stored for the analysis of folic acid content using spectrophotometry.

2.3. Percentage Yield

Particles, dried at room temperature, were weighed, and the yield was calculated in percentages using equation:

$$\text{Percentage yield} = [(\text{weight of particles})/(\text{weight of polymer} + \text{weight of folic acid})] \times 100$$

2.4. Loading Amount and Loading Efficiency

Folic acid absorbs light at the wavelength of 259 and 362 nm. Based on measuring absorbance of the solution with a known concentration of folic acid (Fig. 2(a)) at 362 nm, a calibration curve was prepared (Fig. 2(b)).

The liner relationship between light absorbance at 362 nm and folic acid concentration is shown according to the *Beer-Lambert Law*: $A = \varepsilon cl$ (where A is absorbance at sample concentration c (in this case concentration of the folic acid (mg/ml)), l is the path length of quartz cell and ε is the absortivity). By applying this standardized relationship, supernatant obtained during the synthesis was analyzed to determine the concentration and amount of non-encapsulated folic acid. Knowing the initial amount of folic acid used in PLGA/folic acid nanoparticle synthesis, the percentage of folic acid loaded into the nanoparticles

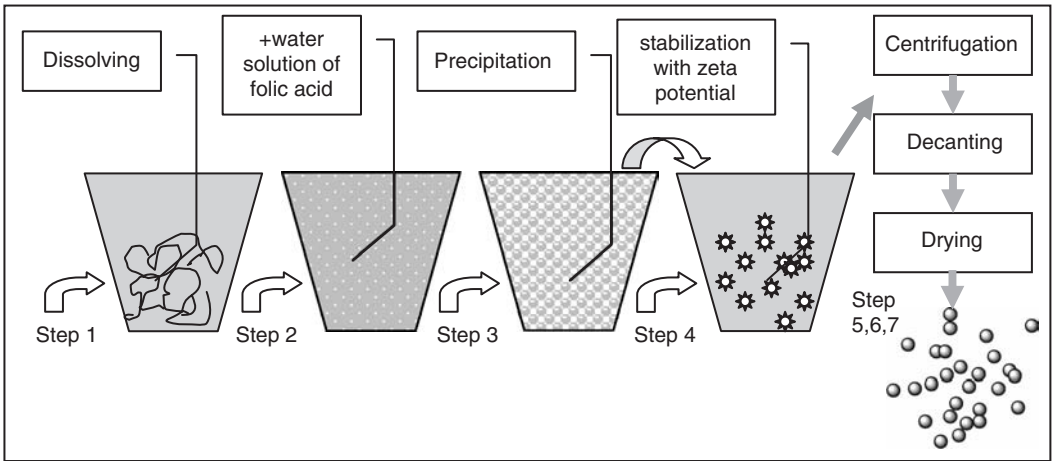

Fig. 1. Scheme of the synthesis of the PLGA/folic acid nanoparticles.

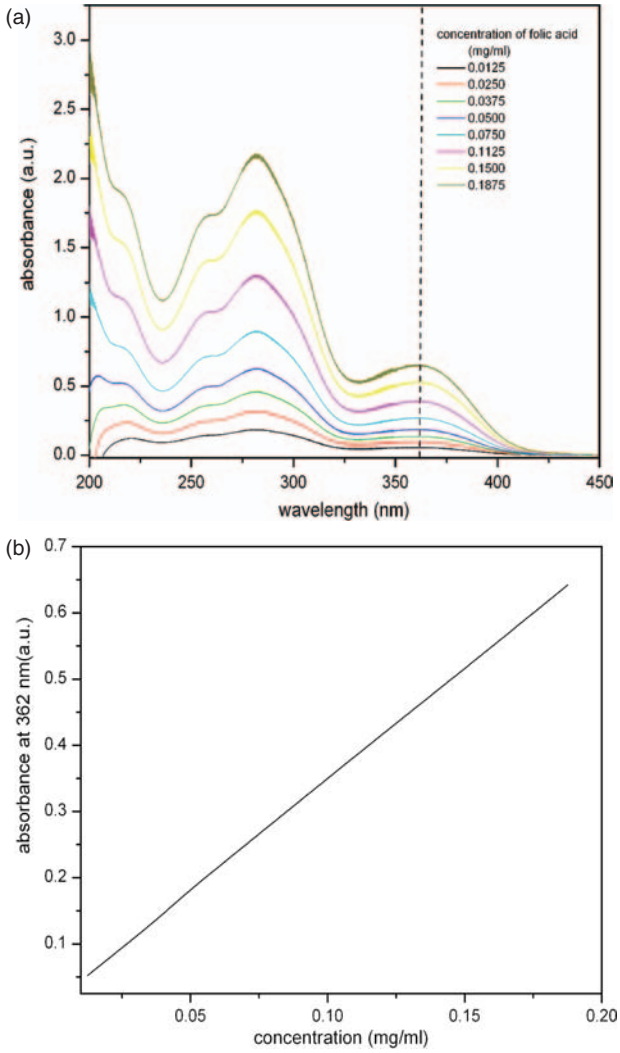

Fig. 2. (a) UV spectra of the solution with a known concentration of folic acid and (b) graph of the linear relationship between folic acid concentration (mg/mL) and absorbance at 362 nm as obtained from the samples with known folic acid concentration.

was obtained (loading efficiency). Loading amount was calculated by means of equation:

$$\text{Loading amount} = [\text{Loading efficiency}(\%)/100] \times \text{Total amount of folic acid added}$$

2.5. The Quality Analysis of the Samples

The quality analysis of the samples was performed using IR spectroscopy. IR spectra were recorded in the range of 400–4000 cm^{-1} at a MIDAC M 2000 Series Research Laboratory FTIR Spectrometer, at 4 cm^{-1} resolution. Powdered samples were dispersed in KBr and compressed into pellets.

2.6. Zeta Potential Measurements

Zeta potential was measured by Zetasizer (Nano ZS, Model ZEN3600, particles size range for zeta potential determination (5 nm–10 μm), Malvern Instruments, Malvern, UK) using the principle of electrophoretic mobility under an electric field. Zeta potential is the function of dispersion/suspension pH which determines particle stability in dispersion.

2.7. Morphology Studies

The morphology of the obtained particles of PLGA without and with folic acid was examined using a JEOL JSM-646OLV scanning electron microscope (SEM). The powder samples for SEM analysis were sputtered with gold using the physical vapor deposition (PVD) process. The samples were sputtered (SCD 005 sputter coater), using 30 mA current from the distance of 50 mm during 180 s.

2.8. Stereological Analysis

The particle size and morphology were examined using the area analysis method[21, 22] (Leica Q500MC with Leica QWin software). A few hundred particles from a representative SEM image were measured, and the following parameters were determined: area section Aa, perimeter Lp, maximal diameter of the particle Dmax, feret x and feret y.

2.9. *In Vitro* Drug Release

The concentration of folic acid in the phosphate buffered saline (PBS, one tablet dissolved in 200 mL of deionized water yields 0.137 M sodium chloride, 0.01 M phosphate buffer and 0.0027 M potassium chloride, pH is 7.4 at 25 °C, Sigma-Aldrich) as a release medium was determined with UV spectroscopy. The drug concentration in the medium was calculated using a calibration curve of the drug in the corresponding release medium at various concentrations. The *in vitro* degradation of PLGA nanoparticles loaded with folic acid was studied by dispersing nanoparticles in PBS containing 110 μl sodium azide (Sigma-Aldrich Fluka (Biochemica), 0.1 M solution NaN_3). The nanoparticle dispersions in closed ultacentrifugation tubes were kept at 37 °C ± 1 °C (Vaciotem P-Selecta), and stored in the absence of light. At different time points (0–30 days), the supernatant was taken and analyzed.

2.10. Ultraviolet Spectroscopy (UV)

The UV measurements were performed on GBC, Cintra 101 UV-Vis Spectrophotometer in the frequency interval of 200–400 nm.

3. RESULTS AND DISCUSSION

3.1. The Quality Analysis of the Samples

In order to investigate the structural characteristics of the PLGA particles with encapsulated folic acid, i.e., to

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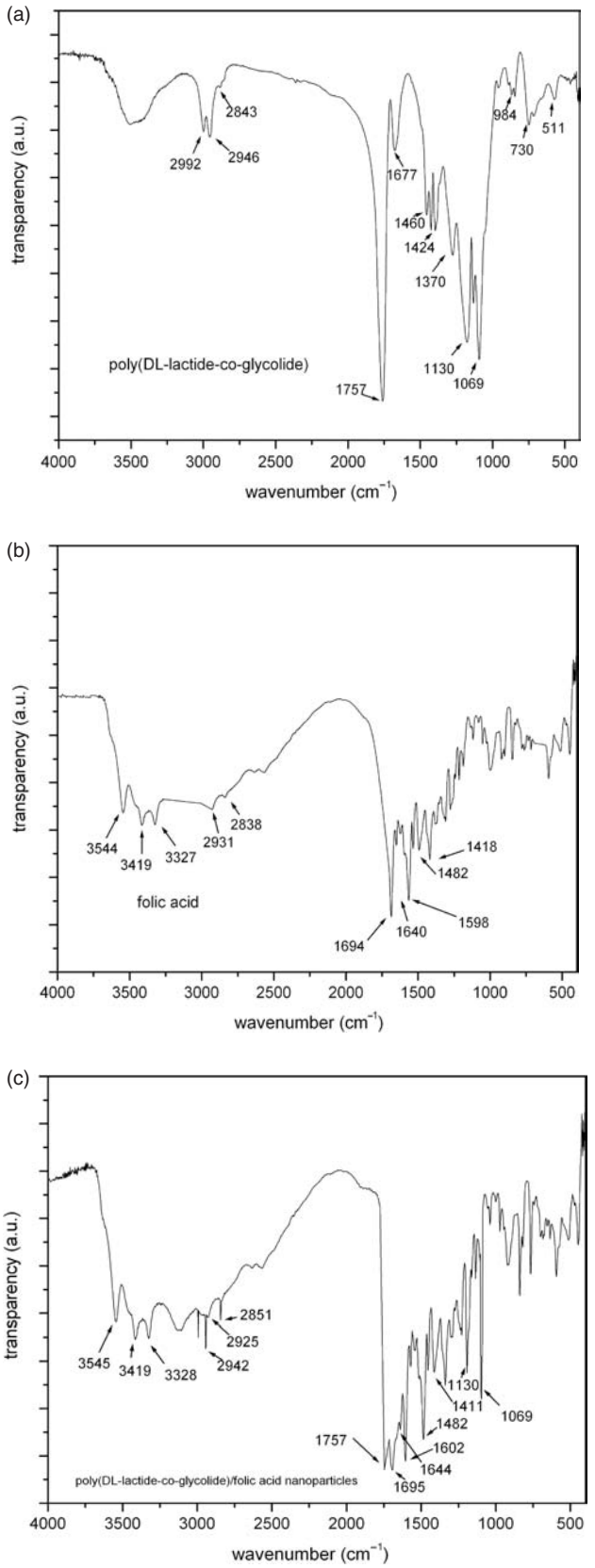

Fig. 3. IR spectra of (a) PLGA, (b) folic acid and (c) PLGA/folic acid 95/5% nanoparticles.

Table I. Percentage of yield.

PLGA/folic acid %	Yield %
95/5	53.0
90/10	51.9
85/15	51.0
80/20	52.3

confirm the qualitative composition of the samples, IR spectroscopy was used (Figs. 3(a–c)). The IR spectra of poly(DL-lactide-*co*-glycolide)/folic acid 95/5% nanoparticles are presented in Figure 3(c). Besides the characteristic groups for copolymer PLGA,[20] the spectra show all the characteristic groups for folic acid.[23] The band at 3545 cm^{-1} belongs to the hydroxyl (O-H) stretching, while the bands at 3419 and 3328 cm^{-1} are N-H stretching vibration bands.

The bands at 2942, 2925 and 2851 cm^{-1} correspond to –C-H stretching vibrations, C═O bond stretching vibration of carboxyl group appears at 1695 cm^{-1}, while the band at 1644 cm^{-1} belongs to C═O bond stretching vibration of –$CONH_2$ group. The band at 1602 cm^{-1} relates to the bending mode of N-H vibration. The band at 1482 cm^{-1} was attributed to the characteristic absorption band of phenyl ring, whereas the one at 1411 cm^{-1} corresponds to O-H deformation band of the phenyl skeleton.

3.2. Percentage of Yield in Preparation

The results of the determination of the particle yield for various PLGA/folic acid ratios were similar for each of the samples and in all cases greater than 50%, as shown in Table I.

3.3. Zeta Potential Measurements

The results of the determination of zeta potential for PLGA particles without and with different concentration of folic acid are shown in Table II. Zeta potential was reported as the average and standard deviation of measurements, with five readings taken per sample.

From the Table II we can see that all systems independently from the ratio of PLGA and folic acid posses the same zeta potential.

PVP was used as a stabilizer which creates negatively charged PLGA particles, that is, induces a specific zeta potential.[24] The potential at the slipping plane is called the zeta potential. Zeta potential is an important property of the particle in a dispersion as it has exert a significant

Table II. Zeta potential of PLGA dispersion without and with different concentration of folic acid.

PLGA/folic acid %	pH	Zeta potential (mV)
100/0	4.30	-9.6 ± 0.3
95/05	4.34	-9.3 ± 0.3
90/10	4.37	-9.0 ± 0.4

Table III. Loading efficiency and loading amount of PLGA/folic acid particles.

PLGA/folic acid %	Supernatant absorbance (362 nm)	Amount of folic acid in supernatant (mg)	Loading efficiency (%)	loading amount (mg)
95/5	0.1195	2.785	78.9	10.415
90/10	0.2641	6.394	77.0	21.406
85/15	0.4352	10.937	75.2	33.163
80/20	0.5705	14.375	77.0	48.125

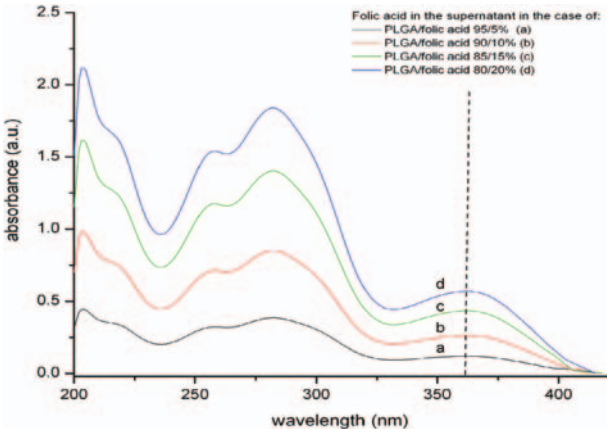

Fig. 4. UV spectra of folic acid from the supernatant.

influence on their stability. Theoretically, zeta potential stabilizes suspensions whether its value is positive or negative.[25–28] PVP reduces the agglomeration because the particles of the same charge are not attracted to each other. The concentration of PVP stabilizer is optimized in order to obtain the smallest particle dimensions for this method as well as to reduce the agglomeration to a minimum.[24, 29] A known problem addressed in the literature, which can occur with the particle stabilizer is its difficult removal from the system.[27, 30] The remains of the stabilizer are often modifying surface characteristics of the particles, thus affecting the degradation rate, distribution throughout the body, release of the medicament and biocompatibility.[28, 30] Therefore, the percentage of the used PVP is only 0.05%.

3.4. Loading Amount and Loading Efficiency

The supernatant obtained from PLGA/folic acid nanoparticle synthesis was analysed by UV spectrophotometry to determine the amount of folic acid encapsulated within the nanoparticles. The results are shown in Table III. The amount of folic acid in the supernatant has been calculated from the product of the supernatant's absorbance at 362 nm

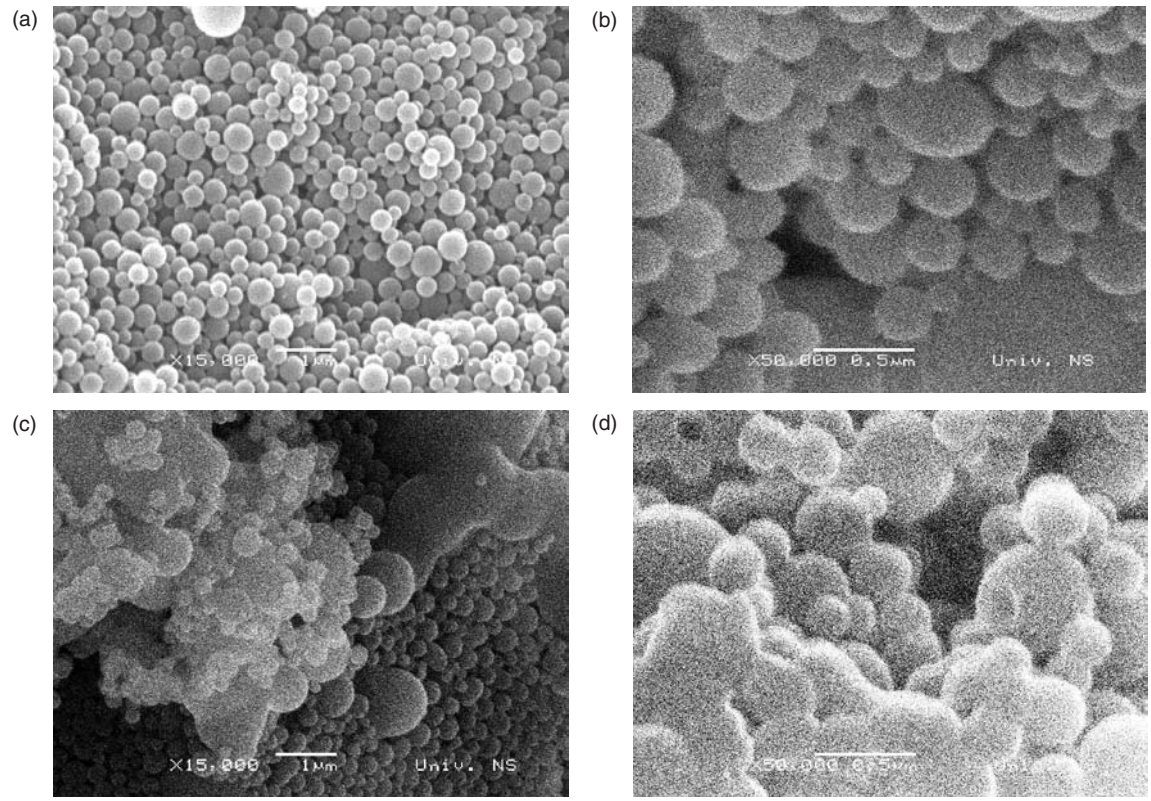

Fig. 5. SEM images of particles with different ratio of PLGA and folic acid (a) PLGA/folic acid 95/5%, (b) PLGA/folic acid 90/10%, (c) PLGA/folic acid 85/15%, (d) PLGA/folic acid 80/20%.

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(Fig. 4) and its measured volume (87 ml). Assuming that all of the folic acid concentration not found in the supernatant was encapsulated by PLGA nanospheres, the loading efficiency was determined to be greater than 75% in all ratios of PLGA/folic acid nanoparticles.

3.5. Morphology Studies

The scanning electron microscopic images of folic acid-loaded nanoparticles revealed their regular spherical shapes in the case of PLGA/folic acid 95/5% (Fig. 5(a)). Generally, their surface morphology was smooth without any noticeable pinholes or cracks within the conventional SEM resolution. The size distribution of all nanoparticles was unimodal with sizes in the total range of 140–240 nm as confirmed by the stereological analysis. The particles of the sample PLGA/folic acid 90/10% (Fig.5 (b)) also have spherical shapes, but their sizes are increased. In case of the third sample, PLGA/folic acid 85/15% (Fig. 5(c)), the uniformity is perturbed, and particles have both spherical and irregular shapes and are as well significantly agglomerated. For the fourth sample, PLGA/folic acid 80/20% (Fig. 5(d)), the particles were very much agglomerated, so that the stereological analyses could not performed.

3.6. Stereological Analysis

Based on the obtained results of the stereological analysis of PLGA/folic acid 95/5% nanoparticles, it is obvious that they are uniform: their average mean size varies from 140 to 240 nm depending on the stereological parameter taken in consideration (Dmax, feret X or feret Y) (Table III). Feret X (the projection of the particle on x axis) values range from 70 to 300 nm with the mean size at 140 nm (Fig. 6(a)). Feret Y (the projection of the particle on y axis) values range from 90 to 360 nm with the mean size at 200 nm (Fig. 6(a')). For 90/10 PLGA/folic acid particles,

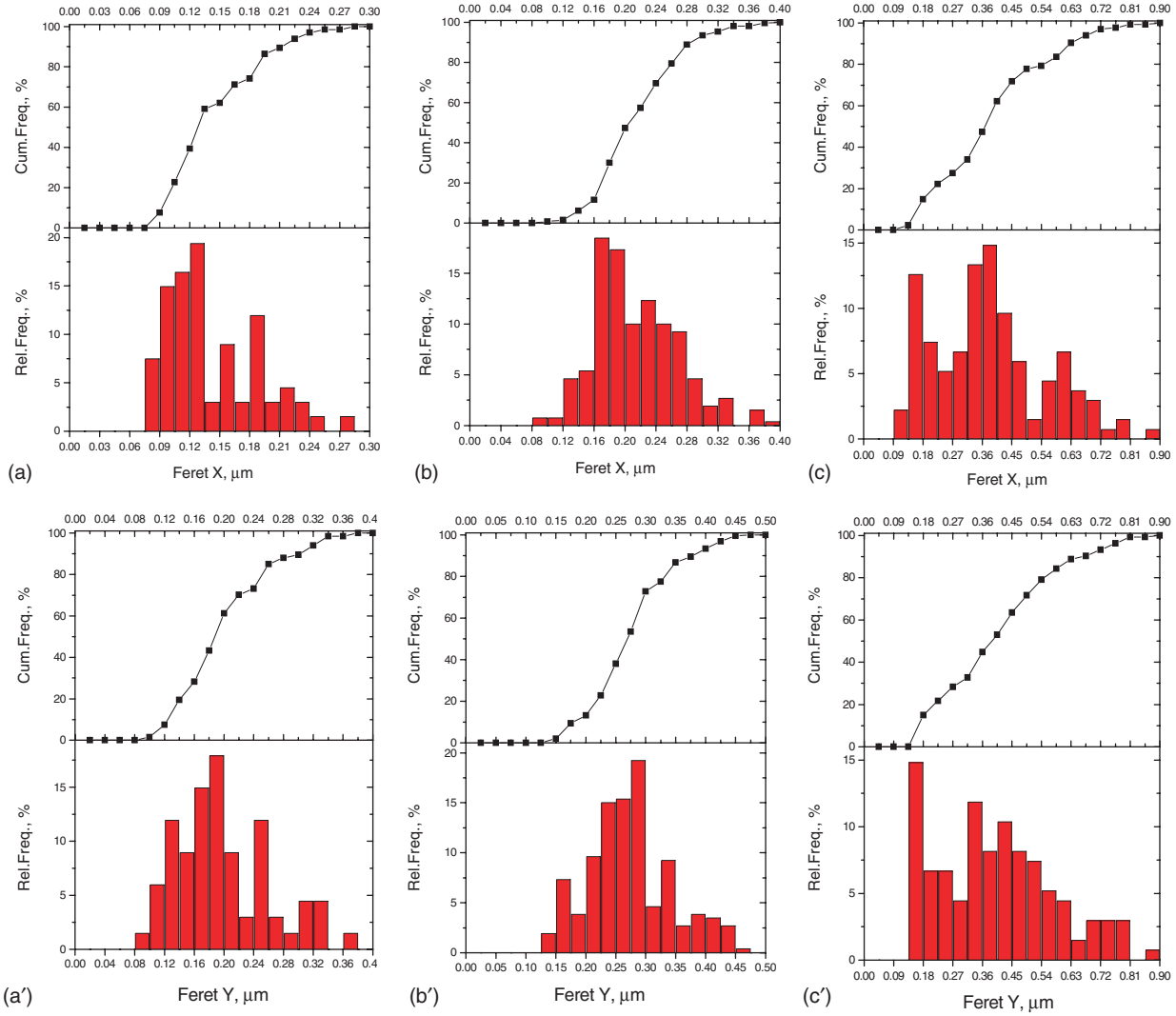

Fig. 6. Results of the stereological examining PLGA/folic acid particles based on feret X and feret Y in the case of nanoparticles aa') PLGA/folic acid 95/5%, bb') PLGA/folic acid 90/10% and cc') PLGA/folic acid 85/15%.

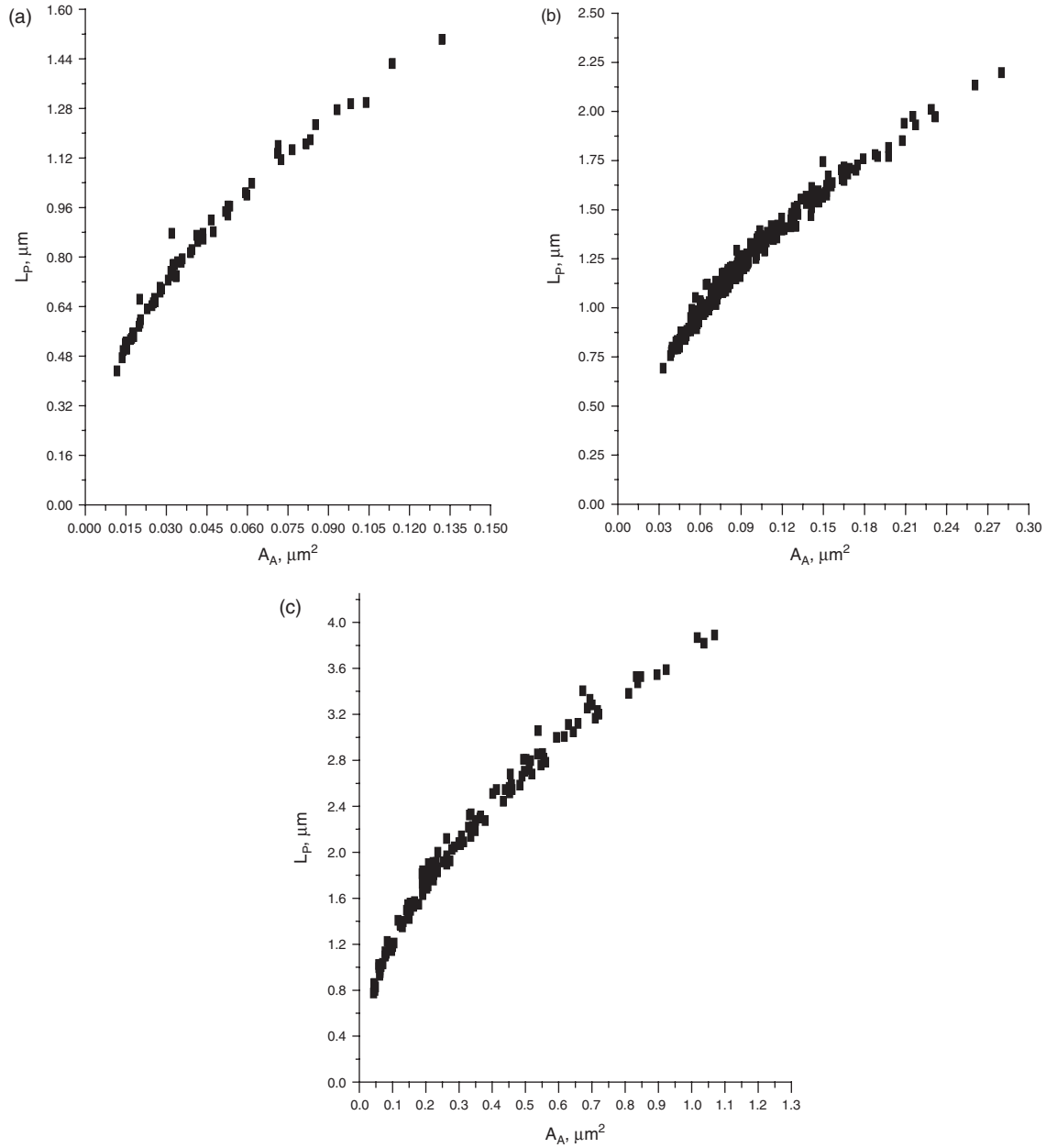

Fig. 7. Results of the stereological examining PLGA/folic acid particles based on their relation between area section A_A and perimeter L_p for aa') PLGA/folic acid 95/5% nanoparticles, bb') PLGA/folic acid 90/10% and cc') PLGA/folic acid 85/15%.

the mean value for feret X is 210 nm, while the mean value for feret Y is 270 nm (Fig. 6(bb')). For 85/15 PLGA/folic acid particles the mean value for feret X is 370 nm, while the mean value for feret Y is 400 nm (Fig. 6(cc')). Figure 7 presents comparative results for PLGA particles with different contents of folic acid based on their relation between the area section A_A and perimeter Lp.

From the comparative results of the stereological analysis based on the maximal diameter of the particle Dmax (Fig. 8, Table IV), we can see that PLGA particles without folic acid have the lowest mean value of Dmax. For PLGA particles with folic acid, it can be noted that the particles with lower content of folic acid have lower mean value of Dmax.

In our previous research, we encapsulated ascorbic acid into PLGA in PLGA/ascorbic acid ratios of 85/15%wt, 70/30%wt, and 50/50%wt.[20, 31] In the current research we are encapsulating folic acid in lesser ratios because the molecular weight of folic acid (441.1396 g/mol) is lesser than the one of ascorbic acid (176.13 g/mol).

3.7. *In Vitro* Drug Release

The rate of degradation of the PLGA nanospheres without and with the encapsulated folic acid as well as tracking

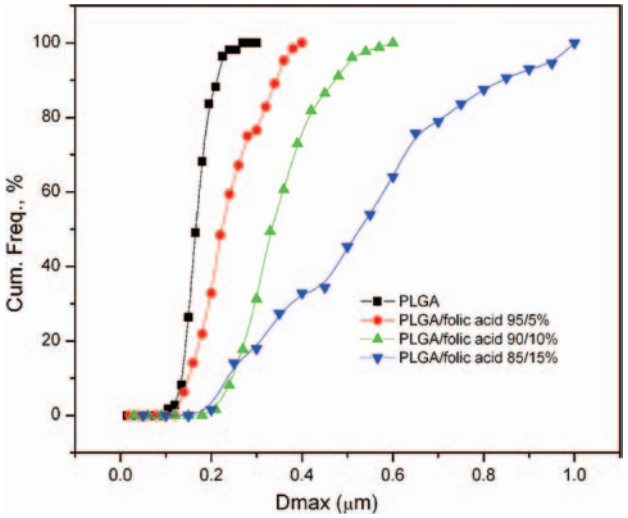

Fig. 8. Comparative results of the stereological study of (a) PLGA nanospheres and nanoparticles with different ratio of PLGA and folic acid, (b) PLGA/folic acid 95/5%, (c) PLGA/folic acid 90/10%, (d) PLGA/folic acid 85/15%, based on maximal diameter of the particle Dmax.

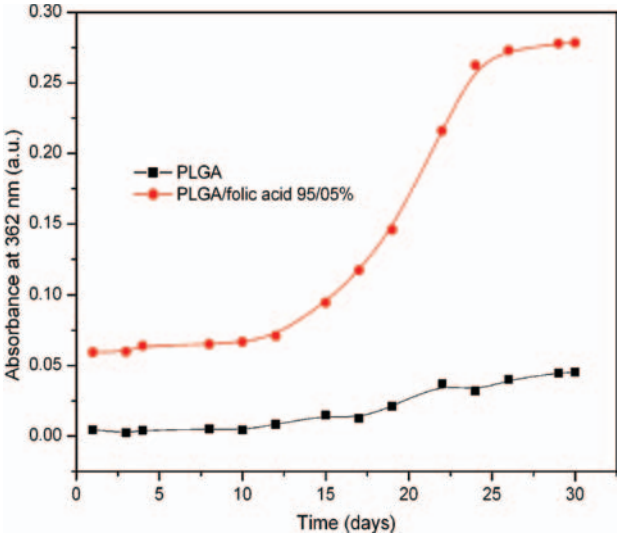

Fig. 9. Comparative curves for the dependence of the absorbance maximum ($\lambda = 362$ nm) from the time of the degradation for the PLGA without and with folic acid.

the release of folic acid from the polymeric matrix during the degradation process, have been examined with UV spectroscopy. The characteristic absorbance peak which belongs to the standard, blank PLGA sample is at 270 nm. Figure 9 shows comparative curves for the dependence of the absorbance maximum at 362 nm (the characteristic absorbance peak ascribed to folic acid) from the time of degradation for the PLGA without and with folic acid. Figure 10(a) gives a cumulative curve of the release of folic acid in percentages over the period of time of the degradation, i.e., from the first until the 30th day. Figure 10(b) also shows the relative view in percentages of the folic acid release over the period of time of the degradation.

For the folic acid release from degrading PLGA, a number profile has been observed. The first phase is a burst effect, caused by the release of the drug that was adsorbed to the outer particle surface. Initially, in the first day of degradation, 17% of folic acid was released. The second phase is characterized by a relatively slow release due to the diffusion of the drug out of the matrix (from the first until the 12th day). The third phase is a phase of an increased drug release, caused by (an extensive) polymer degradation, resulting in an increased permeability of the drug in the polymer matrix. By the end of the experiment there were no more traces of nanoparticles in the degradation medium. More than 82% of the encapsulated folic acid was released till the end of the experiment.

The particles of PLGA without and with various concentrations of folic acid, obtained with this method, can be potentially used in both passive and active transport, depending whether we want to achieve a more effective and even distribution of this important vitamin in the body throughout extended periods of time, or we want to give it other uses, e.g., research related with the cancer therapy.

The research done over the previous years has made it clear that people who do not take folic acid supplements are at increased risk for the functional folic acid deficiency, which has been proven to cause spina bifida and anencephaly, and also has been associated with many other diseases.[32–34] Polymeric nanocarriers such as poly(DL-lactide-*co*-glycolide) have shown promising pharmacokinetics at both the level of the whole body and cellular levels (passive targeting).[35–37] Folic acid may also have a role in coronary heart disease and various cancers.[38, 39] The active drug targeting is usually achieved by a chemical attachment onto a targeting component

Table IV. Results of the stereological analysis of PLGA nanospheres without and with different content of folic acid.

	Lp (μm)			Aa (μm)^2			Dmax (μm)			Feret X (μm)			Feret Y (μm)		
Ratio PLGA/folic acid	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
100/0%	0.32	0.98	0.60	0.01	0.07	0.02	0.09	0.34	0.17	0.05	0.22	0.12	0.05	0.26	0.11
95/5%	0.43	1.50	0.83	0.01	0.13	0.04	0.12	0.44	0.24	0.07	0.30	0.14	0.09	0.36	0.20
90/10%	0.69	2.19	1.24	0.03	0.28	0.10	0.18	0.65	0.34	0.09	0.39	0.21	0.14	0.54	0.27
85/15%	0.79	3.87	2.06	0.39	1.09	0.32	0.15	1.31	0.52	0.10	0.88	0.37	0.15	0.91	0.40
80/20%	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

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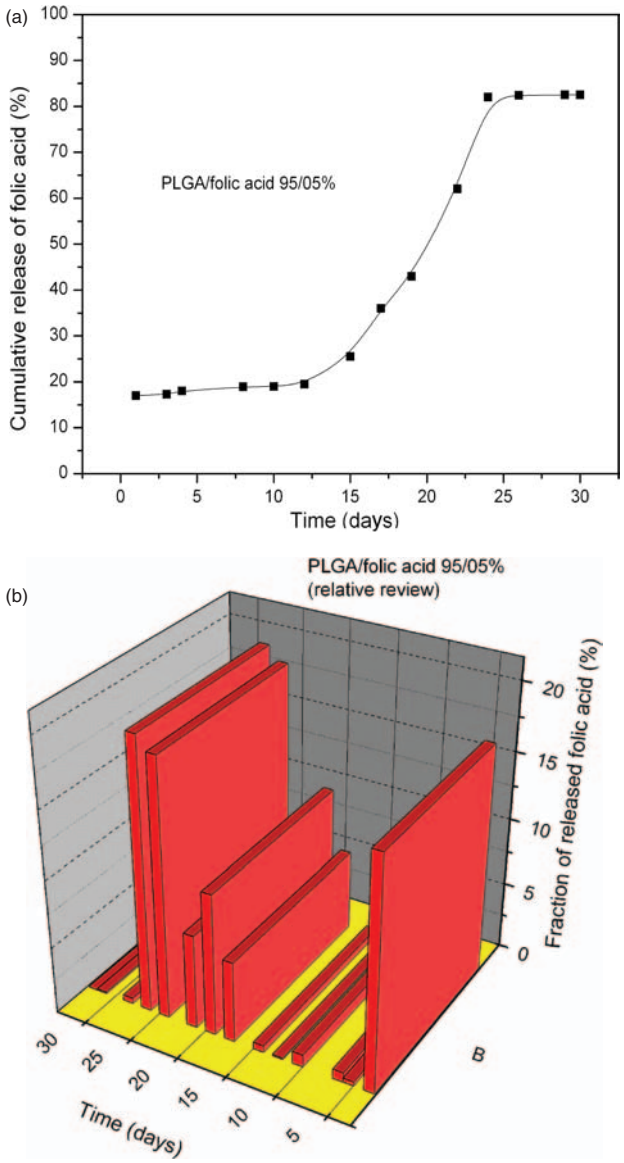

Fig. 10. (a) Cumulative curve of the release of the folic acid in percentages over the period of time of the degradation, (b) relative review in percentages of the folic acid release over the period of time of the degradation.

that strongly interacts with antigens (or receptors) displayed on the target tissue, leading to the preferential accumulation of the drug in the targeted organ, tissue, or cells.[35] In the active drug targeting, folic acid is used as a ligand to encourage intracellular uptake of drugs.[14, 40, 41] Folates (the anion form) are low molecular weight vitamins required by eukaryotic cells, and their conjugates have the ability to deliver a variety of drugs or imaging agents to pathological cells without causing harm to normal tissues.[35] Folate targeting is an interesting approach for cancer therapy because it offers several advantages over the use of monoclonal antibodies.[35, 42] More importantly, elevated levels of folate receptors are expressed on epithelial tumors of various organs such as colon, lung, prostate, ovaries, mammary glands, and brain.[43] Folate is known to be non-immunogenic, and folate-conjugated drugs and/or nanoparticles are rapidly internalized via receptor-mediated endocytosis.[23, 35, 44]

4. CONCLUSIONS

It is possible to encapsulate folic acid into PLGA particles in various concentrations, thus producing particles with different morphological characteristics. The particles of PLGA/folic acid with lesser contents of folic acid have a higher uniformity, lower levels of agglomeration, and their sizes are smaller. The percentage yields for various PLGA/folic acid ratios were similar, and in all cases greater than 50% whereas the loading efficiency was greater than 75%. The nanoparticles of PLGA/folic acid 95/5% have spherical shapes and their mean sizes are from 140 to 240 nm, depending on the stereological parameter taken in consideration (feret X, feret Y or Dmax).

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