



**Co-encapsulation of human serum albumin and superparamagnetic iron oxide in PLGA nanoparticles: Part II. Effect of process variables on protein model drug encapsulation efficiency**

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**Co-encapsulation of human serum albumin and superparamagnetic  
iron oxide in PLGA nanoparticles: Part II. Effect of process variables  
on protein model drug encapsulation efficiency**

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**ABSTRACT**

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This study investigates encapsulation efficiency of model drug, encapsulated by magnetic PLGA (poly D,L-lactic-co-glycolic acid) nanoparticles (NPs). This is the following part of our preceding paper, which is referred in this paper as Part I. Magnetic nanoparticles and model drug human serum albumin (HSA) loaded PLGA NPs were prepared by double emulsion solvent evaporation method. Among five important process variables, concentration of PLGA and concentration of HSA in the inner aqueous phase

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3 along with their cross-effect had the strongest influence on the encapsulation efficiency.  
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5 Encapsulation efficiency of nanoparticles ranged from 18 to 97% depending on the  
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7 process conditions. Higher encapsulation efficiencies can be achieved by using low HSA  
8  
9 and high PLGA concentrations. The optimization process, carried out by exact  
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11 mathematical tools using GAMS<sup>TM</sup>/MINOS software makes it easier to find out optimum  
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13 process conditions to achieve comparatively high encapsulation efficiency (e.g. 92.3%)  
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15 for relatively small sized PLGA NPs (e.g. 155 nm).  
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20 Keywords: encapsulation efficiency; poly(D,L-lactic-co-glycolic) acid; human serum  
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22 albumin; magnetite nanoparticles; experimental design.  
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## 33 34 INTRODUCTION

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36 Nanoparticles have been extensively investigated in biomedical and  
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38 biotechnological areas, especially in drug delivery systems for drug targeting because of  
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40 their small particle size (ranging from 10 to 1000 nm) which is one of the most important  
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42 properties for intravenous injection formulas (Mainardes et al. 2005; Allemann et al.  
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44 1998; Jeon et al. 2000; Soppimath et al. 2001). Until now, a large number of different  
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46 polymers have been investigated for formulating biodegradable nanoparticles. Among  
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48 them poly(L-lactic-acid) (PLA) and its copolymers with glycolic acid called poly(D,L-  
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50 lactic-co-glycolic acid) (PLGA) have gained popularity as vehicles for drug delivery  
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52 systems (Uhrich et al. 1999; Vert et al. 1998; Park et al. 1995; Jain et al. 2000).  
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3 Drug loaded PLGA nanoparticles have been extensively studied in recent years  
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5 due to their excellent drug loading capacity and biocompatibility. ~~Many PLGA-based~~  
6 ~~nanoformulations have reached different stages of preclinical development, although they~~  
7 ~~still present distinct challenges for researchers. The lactide/glycolide polymers chains are~~  
8 ~~cleaved by hydrolysis into natural metabolites (lactic and glycolic acids), which are~~  
9 ~~eliminated from the body by the citric acid cycle.~~ PLGA provides a wide range of  
10 degradation rates, from months to years, depending on its composition and molecular  
11 weight (Mainardes et al. 2005; Burkersroda et al. 2002; Peppas, 1995 Panyam et al. 2003)  
12 and was selected in this study.

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15 ~~Nanocapsules containing magnetic nanoparticles offer several advantages over~~  
16 ~~conventional systems; most important one is targeted delivery of medicaments. Selection~~  
17 ~~of magnetic nanoparticles for this research was a challenging task because of toxic~~  
18 ~~character of some magnetic nanoparticles. Most of the research on magnetic~~  
19 ~~nanoparticles for clinical applications has focused on iron oxide nanoparticles such as~~  
20 ~~magnetite ( $\text{Fe}_3\text{O}_4$ ) or maghemite ( $\gamma\text{-Fe}_2\text{O}_3$ ).~~ Due to their biological compatibility and  
21 FDA approval for clinical usage (Weissleder et al. 1989; Ibrahim et al. 1983; Muller et al.  
22 1996; Andujar et al. 2012), magnetite was used in this study. In this study, oleic acid-  
23 coated magnetite ( $\text{Fe}_3\text{O}_4$ ) nanoparticles were selected, because they are well dispersible  
24 in organic media.

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27 Human serum albumin (HSA) can be used to model the encapsulation behavior of  
28 a protein-type drug. It is a monomeric multi-domain macromolecule, The model drug  
29 used was HSA which is the most abundant plasma protein in the human body with a  
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3 plasma concentration of 0.6 mM (Yang et al. 2007). ~~HSA consists of 585 amino acids~~  
4 ~~that form three structurally similar  $\alpha$ -helical domains.~~

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8 Polyvinyl alcohol (PVA) ~~was used as is a very effective~~ emulsifier ~~for PLGA. It~~  
9 ~~which~~ helps to form particles of relatively small size and uniform size distribution  
10 (Scholes et al. 1993; Zambaux et al. 1998). Organic solvent ~~used was~~ dichloromethane  
11 (DCM), ~~has the ability to dissolve a wide range of organic compounds including PLGA.~~  
12 ~~DCM is volatile at room temperature and evaporates very quickly. In manufacturing the~~  
13 ~~nanoparticles, it can be removed completely by evaporation from a mixture by~~  
14 ~~mechanical stirring.~~

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25 W/O/W (i.e. water-in-oil-in-water) type double emulsion-solvent evaporation  
26 method is one of the most commonly used techniques for preparing PLGA nanoparticles  
27 (Dillen et al. 2006; Ubrich et al. 2004; Song et al. 2008). ~~Therefore, this method was used~~  
28 ~~to co-encapsulate HSA and superparamagnetic nanoparticles. Although~~  
29 ~~nanoprecipitation, phase separation (coacervation), etc. can also be considered for~~  
30 ~~formulation of drug loaded PLGA particles, double emulsion method has attracted us~~  
31 ~~because of its ease and simplicity to carry out.~~ Although single emulsion method is  
32 simpler than double emulsion method, for studying three phase systems, double emulsion  
33 method had to be applied because of their compartmentalized internal structure. ~~The~~  
34 ~~internal aqueous phase contained the model drug to be encapsulated, the intermediate~~  
35 ~~organic phase served as solvent for the polymer matrix material, while the external~~  
36 ~~aqueous phase was applied as the dispersant for the organic polymer solution~~  
37 ~~(intermediate phase).~~ The main benefit of double emulsion method is the high  
38 encapsulation efficiency of protein compounds in organic soluble polymers. In the  
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3 production of drug-loaded nanoparticles, encapsulation efficiency of the expensive drug  
4 ingredient(s) is one of the most important factors. Depending on the desired  
5 administration way, beside the encapsulation efficiency, the size of the carriers should  
6 also be optimized. If nanoparticles are intended e.g. for injection formulas, their size  
7 should preferably be lower than 220 nm, since they can be sterilized by ultra-filtration  
8 through a membrane of 220 nm cut-off (Feczko et al. 2011).  
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19 Thus, the goal of this study was to investigate the encapsulation efficiencies of  
20 prepared nanoparticles. Due to experimental design (made by STATISTICA<sup>®</sup> software),  
21 it was possible to explore precisely the influence of different parameters and their  
22 combined influences on encapsulation efficiency. GAMS<sup>™</sup>/MINOS software was used  
23 for optimization which gave precise result. Earlier no comprehensive studies were carried  
24 out on the particular effect of the studied five parameters and process conditions on the  
25 PLGA nanoparticles and no research was carried out on co-encapsulation of HSA and  
26 oleic acid coated magnetite by PLGA (the size of PLGA NPs is discussed in part I) which  
27 for sure makes this novel study not only interesting but also creates scope for further  
28 research and innovation. The outcome of this study will provide the ideal combination of  
29 variables to get comparatively high percentage of model drug loading for smallest  
30 possible size of PLGA nanoparticles which will be exploited with the real drug for further  
31 innovation in the field of targeted drug delivery.  
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## MATERIALS AND METHODS

### *Materials*

PLGA (50:50,  $M_w = 8000$ , Resomer<sup>®</sup> RG 502H) with free carboxyl end groups was supplied by Boehringer Ingelheim, Germany. HSA solution was obtained from Trigon Biotechnological Ltd., Hungary. The concentration of bulk HSA solution was 36.87 g/L. Dichloromethane (DCM) was purchased from Spektrum-3D, Hungary. PVA ( $M_w = 30,000-70,000$ ) and phosphate-buffered saline (PBS, pH 7.4) were products of Sigma-Aldrich. Micro BCA (bicinchoninic acid) protein assay kit was purchased from Pierce Biotechnology, Inc.

### *Synthesis of oleic acid-coated superparamagnetic iron oxide nanoparticles*

Neat superparamagnetic iron oxide nanoparticles were prepared by co-precipitation of Fe(II) and Fe(III) chlorides in aqueous ammonia solution by modification of an earlier published method (Horak et al. 2003). The detailed process was described in the preceding paper (Part I). The size of magnetite obtained was  $10 \pm 5$  nm.

Magnetite is coated with oleic acid to prevent agglomeration. Magnetic nanoparticles have large surface area to volume ratio. So, they have a tendency to agglomerate in order to minimize their surface energy which can be successfully prevented using oleic acid coating. Oleic acid is a naturally occurring fatty acid and have been using in pharmaceutical industries because of its low cost and toxicity. Oleic acid coating will prevent the chemical or mechanical interaction between the drug/model drug and the magnetite inside matrix type drug carriers.

### *Preparation of PLGA nanoparticles*

PLGA nanoparticles were prepared by using double emulsion-solvent evaporation method (Feczko et al. 2008; Panyam et al. 2003). The detailed process was described in Part I.

### *Determination of encapsulation efficiency*

Encapsulation efficiency of model protein drug was determined by micro BCA assay. The amount of HSA model protein drug encapsulated into the PLGA nanoparticles was determined by analyzing the protein content in the supernatant (i.e. the not encapsulated fraction of the protein introduced). The resultant encapsulation efficiency ( $EE_{HSA}$ ) was defined as the percentage of HSA model protein encapsulated into the PLGA nanoparticles relative to the total amount of HSA dissolved in the inner aqueous phase according to equation (1):

$$EE_{HSA} = \frac{m_{HSA\text{ encaps}}}{m_{HSA\text{ int}}} \times 100\% \quad (1)$$

In micro BCA assay, peptide bonds in protein reduce cupric ( $\text{Cu}^{2+}$ ) to cuprous ions ( $\text{Cu}^+$ ). Two molecules of bicinchoninic acid chelate with each  $\text{Cu}^+$  ion, forming a purple-colored product that strongly absorbs light at a wavelength of 562 nm and is analyzed spectrophotometrically (Nielsen, 2010). The amount of  $\text{Cu}^{2+}$  reduced is proportional to the amount of protein present in the solution.



### *Experimental design*

To elucidate the effect of process conditions on the mean particle size and encapsulation efficiency, and to decrease the number of the studied parameter combinations and thus the number of experiments, a  $3^{(p-1)}$  type fractional factorial experimental design was carried out using STATISTICA<sup>®</sup> (version 10.0, StatSoft Inc., USA) software, where “p” is the number of factors. The obtained experimental data were evaluated by statistical analysis, similarly to the method described by Feczko et al. (Feczko et al. 2011) for production of PLGA nanoparticles containing bovine serum albumin (BSA), and by Biró et al. (Biro et al. 2009) for chitosan microparticles.

Five process variables (also called as “factors”, denoted by F1–F5) summarized in Part I were examined as main influencing parameters for studying both the mean particle size and the encapsulation efficiency. These variables were:  $X_{\text{Fe}_3\text{O}_4}$  (% wt/wt) - the amount of iron oxide in the organic phase relative to the weight of PLGA (factor F1),  $X_{\text{PLGA}}$  (% wt/vol) - the concentration of PLGA in the organic phase (factor F2),  $X_{\text{HSA}}$  (% wt/vol) - the concentration of HSA in the inner aqueous phase (factor F3),  $X_{\text{VOLR}}$  (vol/vol) - the volume ratio of the outer aqueous phase related to the volume of organic phase (factor F4), and  $X_{\text{time}}$  (min) - the duration of the ultrasonic treatment in minutes during the second emulsification (factor F5).

To elucidate the effects of these five process variables on encapsulation efficiency, the same series of experiments were used as was done in studying the mean particle size discussed in Part I. For this, altogether  $3^{(5-1)} = 81$  experiments were needed. Additionally, 9 repetitions of experiments were also carried out to estimate the pure error. According to our experimental design 3 different levels of each variable (the lowest,

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3 highest and central values of their studied intervals) were tested. The main advantage of  
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5 experimental design is the great reduction of experimental work without remarkable loss  
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7 of the gained information. It means that instead of  $3^5 = 243$  experiments needed without  
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9 experimental design, only  $3^4 = 81$  experiments (i.e. one third of the original ones) had to  
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11 be carried out with experimental design, excluding repetitions. The experimental program  
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13 determined by the Industrial Statistics/DOE part of software package STATISTICA<sup>®</sup> was  
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15 shown in the table of the Appendix “A” in Part 1, together with the measured values of  
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17 mean particle sizes and encapsulation efficiencies obtained for each experiment.  
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### 23 *Optimization of the result*

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27 From economic point of view, the efficiency of encapsulation is extremely  
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29 important, especially when the active agent is very expensive. In certain applications,  
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31 such as production of injectable drug formulations, the smallest possible particle size  
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33 with highest possible encapsulation efficiency must be achieved which obviously  
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35 depends on the process variables. Although encapsulation efficiency is generally higher  
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37 for larger nanoparticles, they are detected and eliminated by macrophages easily and on  
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39 the other hand, their sterilization after production is difficult. Due to the high number of  
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41 variables, it was necessary to determine the optimum process conditions mathematically  
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43 to achieve higher model drug loading with the smallest PLGA capsules. For this purpose  
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45 the GAMST<sup>™</sup>/MINOS Large Scale Nonlinear Solver for Windows Ver. 5.51 (System  
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47 Optimisation Laboratory, Stanford University) software was applied which suggested the  
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49 optimum process conditions by precise mathematical tools.  
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## 56 **RESULTS AND DISCUSSION**

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As a result of the statistical analysis carried out on the measured encapsulation efficiency data, the influences of the studied process variables were characterized by the ANOVA table (see Table 1) and Pareto chart of the standardized effects. From Table 1 it is seen that linear (L) and quadratic (Q) effects of PLGA concentration (factor F2), the linear effect of HSA concentration (factor F3), and the cross-effect (linear-linear interaction) of these two factors (F2\*F3) had the strongest influences. The quadratic (Q) effect of the weight ratio of magnetite/PLGA (factor F1), and the linear-linear interactions of the PLGA concentration and the volume ratio of external aqueous and intermediate organic phases (F2\*F4), and that of the HSA concentration and volume ratio (F3\*F4) also have significant effects, respectively. From ANOVA table it is seen that all of these effects are statistically significant, since the related  $p$  values are much below the commonly accepted  $p=0.05$  criterion. Table 1 also shows that the mean square of residuals (MS) was 84.425, i.e. the mean deviation between the measured and estimated encapsulation efficiencies is  $\sqrt{84.425} = \pm 9.19\%$ , which can be considered as an acceptable deviation. The histogram of residuals (not presented here) showed almost normal distribution, therefore the estimation made by the multivariable regression applied in the STATISTICA<sup>®</sup> software was acceptable. The pure error of experimental data determined from the mean square of the deviations of the encapsulation efficiencies measured in 9 repeated runs at the central values of all variables was  $\pm 5.8\%$ , which is regarded as good accuracy.

Table 1.

The Pareto chart (Fig. 1) shows that the encapsulation efficiency was affected mostly by: PLGA concentration in the intermediate organic phase (F2 - linear (L) and quadratic (Q) effects), HSA concentration in the inner aqueous phase (F3 - linear effect (L)), and linear-linear interaction of these two factors. The iron oxide/PLGA weight ratio (F1) had lower and quadratic effect. Although the linear-linear interaction of volume ratio (F4) with PLGA concentration and also with HSA concentration was significant, they had minor effects. Among the studied five variables, the duration of sonication had no direct effect at all on the encapsulation efficiency. The explanation is given in section 3.5.

Fig. 1.

As a result of the statistical analysis, a regression equation was obtained to describe the dependence of the encapsulation efficiency  $EE_{HSA}$  on the studied process variables:

$$EE_{HSA} = 25.3189 \cdot X_{PLGA} - 4.0993 \cdot X_{PLGA}^2 - 21.4573 \cdot X_{HSA} - 0.0075 \cdot X_{Fe3O4}^2 + 4.0632 \cdot X_{PLGA} \cdot X_{HSA} - 1.1217 \cdot X_{PLGA} \cdot X_{VOLR} + 1.3810 \cdot X_{HSA} \cdot X_{VOLR} + 72.2188 \quad (2)$$

These effects are demonstrated by the diagrams in Fig. 2-5 and discussed in details below.

### ***Effect of PLGA and HSA concentrations***

Increased PLGA concentrations can generally be beneficial to achieve higher encapsulation efficiency, but this effect can be diminished under certain conditions due to the influence of other process variables. Among them, the most important one is the concentration of HSA in the inner aqueous phase. Fig. 2(a) clearly shows this effect at

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3 fixed magnetite/PLGA mass ratio (3.0% wt/wt), volume ratio of  $W_2$  and O phases (4.0  
4 vol/vol) and sonication time (3.0 min). It is seen that the encapsulation efficiency varies  
5 non-linearly with the change of PLGA concentration: the increase in the encapsulation  
6 efficiency is most rapid at the highest model drug concentration ( $X_{HSA}=3.7\%$  wt/vol),  
7 which can grow from 45.1 to 91.1% when  $X_{PLGA}$  changes from 1.0 to 4.0% (wt/vol). The  
8 increment of encapsulation efficiency is gradually decreasing with the increase in PLGA  
9 concentration for medium and low HSA concentration, i.e. rapid growth is seen around  
10  $X_{PLGA}=1.0\%$  (wt/vol), which slows down at higher PLGA concentrations, e.g. above  
11 2.5% (wt/vol). However, at low HSA concentration ( $X_{HSA}=0.7\%$  wt/vol) the effect of  
12 PLGA is much more moderate, starting from an encapsulation efficiency of about 80.1%,  
13 reaching to a maximum of about 90.1%.  
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31 Due to the cross-effect (interaction) between the PLGA and HSA, the influence of  
32 HSA concentration depends on the actual level of PLGA concentration. As is seen on  
33 Fig. 2(a), if lowest amount of PLGA is used ( $X_{PLGA}=1\%$  wt/vol) in the intermediate  
34 organic phase, the increase of  $X_{HSA}$  causes a reduction in the encapsulation efficiency  
35 from the mentioned 80.1% to about 45.1%. However, if PLGA concentration in the  
36 organic phase is high, e.g. its maximal value of 4.0% (wt/vol), the increase of HSA  
37 concentration to its maximal value ( $X_{HSA}=3.7\%$  wt/vol) results in a slight increase in the  
38 achievable encapsulation efficiency from about 90.1 to 91.1%. The explanation of this  
39 interaction lies in the fact that greater amount of PLGA matrix material can absorb more  
40 model protein drug. If the presence of PLGA is not sufficient, relatively smaller  
41 proportion of the total amount of HSA can be captured in the particles resulting lower  
42 encapsulation efficiency.  
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3 The literature data support our findings on the effect of PLGA, because it is  
4 known that the efficiency of encapsulation generally increases with the increase in the  
5 relative amount of the polymer matrices. Another effect may be that at certain conditions,  
6 particle size can also increase with the polymer concentration (Coimbra et al. 2008) and  
7 the encapsulated drug content is known to increase with the increase in particle size  
8 (Gorner et al. 1999; Budhian et al. 2007).  
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19 Fig. 2 [a,b,c,d](#).  
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22 Further explanation can be that with the increase in the concentration of polymer  
23 in the organic phase, the viscosity of the organic phase increases. The more viscous  
24 organic phase provides higher mass transfer resistance (Galindo-Rodriguez et al. 2004)  
25 and prevents protein diffusion towards the external phase, which in turns results in higher  
26 encapsulation efficiency. Increasing the PLGA concentration at a given HSA  
27 concentration will decrease the HSA/PLGA ratio in the droplets of the organic phase.  
28 This also may reduce the possibility of its escaping from these droplets. Devi Kusum et  
29 al. found that if drug (Acyclovir):polymer (PLGA) ratio increases from 1:1 to 1:2,  
30 particle size increases significantly and drug entrapment also increases (Kusum et al.  
31 2009).  
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47 As for the effect of HSA concentration, it is seen that the increase in the HSA  
48 concentration decreases the encapsulation efficiency. The high model drug loadings  
49 typically result in lower encapsulation efficiencies due to high concentration gradient  
50 causing the drug to diffuse out of the droplets (Maravajhala et al. 2009). So, the lower  
51 encapsulation efficiencies obtained with higher HSA concentrations could be explained  
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3 by the higher protein loss by diffusion towards the external aqueous phase. During the  
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5 solidification of droplets, the quantity of solvent in the dispersed phase decreases and the  
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7 drug will show the tendency to be expelled from the dispersed phase (Li et al. 2008).  
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9 Some researchers also pointed out that microspheres with high drug loadings are more  
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11 porous and have rather irregular shapes; highly porous surface is responsible for the rapid  
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13 loss of drug (Li et al. 2008). This can be also a reason for the loss of HSA from PLGA  
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15 nanospheres. Too high drug loading increases the risk of drug leakage due to the limited  
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17 space inside the nanospheres and the shrinkage of the nanospheres during its  
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19 solidification. Pamujula et al. (Pamujula et al. 2004) studied amifostine drug-loaded  
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21 PLGA microcapsules. It was found that the efficiency of encapsulation decreased with an  
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23 increase in drug loading which complies with our result.  
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31 The HSA/PLGA ratio is also an important parameter compared to the protein  
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33 concentration in the inner aqueous phase. Insufficient concentration of the PLGA can  
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35 result in more non-encapsulated HSA. In our previous studies, it was found in this respect  
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37 that if the initial protein concentration is not higher than 10% (wt/vol) of the initial PLGA  
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39 amount, more than 90% of protein can be encapsulated by using a suitable emulsifier  
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41 (Feczko et al. 2011).  
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#### 46 ***Effect of the magnetite/PLGA mass ratio***

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50 As is seen from the very small coefficient of  $X_{\text{Fe}_3\text{O}_4}$  in Eqn. 2, and also from Fig.  
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52 2(b), the presence of magnetite has only slight influence on the encapsulation of HSA  
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54 into the PLGA nanoparticles. With the increase in magnetite/PLGA ratio from 1.0 to  
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56 20.0% (wt/wt), the predicted encapsulation efficiency shows slight reduction only from  
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3  $EE_{HSA}=65.2$  to  $62.2\%$  at low ( $1.0\%$  wt/vol) PLGA concentration, and from  $EE_{HSA}=90.6$   
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6 to  $87.6\%$  at the highest studied polymer content ( $X_{PLGA}=4.0\%$  wt/vol), respectively.  
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10 **Fig. 3.**  
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13 This decrease may be due to the possibility that with the higher  $Fe_3O_4/PLGA$   
14 mass ratio, more magnetite particles can escape from the model drug loaded PLGA  
15 nanoparticles and can be dispersed into the outer aqueous phase. In this process some  
16 model protein drug may be adsorbed on the surface of the escaped magnetite  
17 nanoparticles, which will result in some protein loss and lower encapsulation efficiency.  
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#### 26 ***The interaction of the PLGA concentration and the volume ratio***

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29 It can be seen from the Pareto chart (Fig.1) and Eqn. 2 that the volume ratio of the  
30 external aqueous and the intermediate organic phases ( $X_{VOLR}$ ) does not have its own  
31 independent effect on the encapsulation efficiency, because it interacts with two other  
32 process variables. Namely, the effect of the volume ratio can be influenced by both of the  
33 PLGA and HSA concentrations, and vice versa. Fig. 2(c)4 shows certain cross-effect of  
34 the volume ratio and PLGA concentration.  
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45 **Fig. 4.**  
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49 As was seen earlier, an increase in PLGA concentration results in a rapid increase  
50 of encapsulation efficiency, especially in the lower region of the studied interval. The  
51 change of volume ratio of the  $W_2/O$  phases can slightly modify the achievable degree of  
52 encapsulation in a considerable range of PLGA concentrations. At the lower end of  
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3 polymer concentration, i.e. at around  $X_{PLGA}=1.0\%$  (wt/vol), the increase of volume ratio  
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5 from  $X_{VOLR}=2.0$  to 6.0 (vol/vol) causes a rise in the encapsulation efficiency from  
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8  $EE_{HSA}=61.9$  to 68.4% at fixed values of three other variables indicated on Fig. 2(c)4. At  
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10 the higher end of the PLGA concentration range ( $X_{PLGA}=4.0\%$  wt/vol), the effect of the  
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12 volume ratio of phases  $W_2/O$  is opposite: the increase of volume ratio from 2.0 to 6.0  
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14 (vol/vol) results in the decrease of encapsulation efficiency from  $EE_{HSA}=94.0$  to 87.1%.

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19 This phenomenon may be explained by a general principle that governs the size of  
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21 nanodroplets during shearing the given three-phase system by an external energy source.  
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23 It is known from the literature that the size of droplets is inversely proportional to the  
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25 magnitude of shear stresses (Budhian et al. 2007)]. Any change in process variables that  
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27 reduces these shear stresses will increase the nanodroplet size. In our experiments the  
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29 introduced ultrasonic energy was constant for different volume ratios. Therefore the  
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31 higher the volume ratio, the higher the liquid volume is, which in turn reduces the  
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33 available energy per unit volume, i.e. less energy density, resulting in weaker  
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35 emulsification. Weaker emulsification leads to the production of larger particles if all the  
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37 other variables are fixed. From larger droplets (or particles) less protein can escape to the  
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39 external aqueous phase.  
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46 On the other hand, at higher energy density during shearing the intermediate  
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48 organic phase into droplets (containing the already dispersed inner aqueous phase, HSA  
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50 solution) increases the probability that the tiny droplets of the HSA solution can get into  
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52 direct contact with the outer aqueous phase, which allows intermingling and their fusion,  
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54 thus causing protein loss and lower encapsulation efficiencies. This could be a reasonable  
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3 explanation of the increase of encapsulation efficiency with increasing volume ratio at the  
4 lower end of PLGA concentration. The controversial effect at high PLGA concentration  
5 is not well understood yet, but may be the consequence of higher PLGA concentration  
6 that increases the viscosity of the intermediate organic phase which may protect the inner  
7 aqueous phase from the direct contact with the external aqueous phase during shearing, or  
8 at least can diminish the fusion of the droplets of the inner aqueous phase with the outer  
9 continuous aqueous phase. The increase of encapsulation efficiency with decreasing  
10 volume ratio at higher PLGA content can be caused by reduced HSA extraction from  
11 high viscous organic phase by the relatively small amount of the external aqueous phase.  
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26 It should be noted that the cross-effect between the PLGA concentration and  
27 volume ratio, and their combined influence on the encapsulation efficiency is statistically  
28 significant and thus has to be considered, but this is not the most important effect as a  
29 whole.  
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### 36 *The interaction of HSA concentration and volume ratio*

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39 The interaction between the HSA concentration in the inner aqueous solution and  
40 the W<sub>2</sub>/O phases volume ratio is clearly seen in Fig. 2(d)5, where the magnetite to PLGA  
41 ratio, the PLGA concentration in the organic phase, and the time of sonication are fixed  
42 ( $X_{\text{Fe}_3\text{O}_4}=3.0\%$  wt/wt,  $X_{\text{PLGA}}=2.5\%$  wt/vol, and  $X_{\text{time}}=3.0$  min). Namely, at low volume  
43 ratio ( $X_{\text{VOLR}}=2.0$  vol/vol), the decrease of HSA concentration from 3.7 to 0.7% (wt/vol)  
44 results in significant increase in the encapsulation efficiency (from 72.7 to 98.2%), while  
45 for same change in HSA at high volume ratio ( $X_{\text{VOLR}}=6.0$  vol/vol) the encapsulation  
46 efficiency changes from 81.9 to 90.9%. On the other hand, the effect of volume ratio at  
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3 high HSA concentration ( $X_{\text{HSA}}=3.7\%$  wt/vol) is also significant: by increasing the  
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5 volume ratio from 2.0 to 6.0 vol/vol, the efficiency of encapsulation grows from 72.7 to  
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7 81.9%. At low HSA concentration ( $X_{\text{HSA}}=0.737\%$  wt/vol), the tendency is just opposite:  
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9 the same increase of the volume ratio leads to the decrease of the encapsulation efficiency  
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11 from 98.2 to 90.9%. The beneficial effect of the increase in volume ratio at high HSA  
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13 concentration has already been explained above by the lower energy density and small  
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15 shear stress, which leads to larger particles and minimizes protein loss. The decrease of  
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17 encapsulation efficiency with increasing volume ratio at lower HSA concentration seems  
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19 to be contradictory for the first sight, but it becomes understandable by the fact that more  
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21 external water phase can extract more HSA from the droplets of the first  $W_1/O$  emulsion  
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23 during shearing them in the external continuous  $W_2$  phase.  
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31 **Fig-5.**  
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34 ***Effect of sonication duration on the second emulsification***  
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38 It has been shown in Part I that at given values of other influencing variables the  
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40 increase in sonication time has decreased the achievable mean particle size of the  
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42 HSA/magnetite loaded nanoparticles significantly. In spite of this fact, the statistical  
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44 analysis of the data obtained for entrapping of HSA does not show any significant direct  
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46 effect of sonication time on the degree of encapsulation. This was especially interesting  
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48 because it is known from other studies that particle size greatly influences the  
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50 encapsulation efficiency, which usually increases with increasing particle size and vice  
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52 versa. Several authors have pointed out for drug-loaded particles that in general the larger  
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54 the particle size, the higher the encapsulation efficiency for drugs (Feng et al. 2001; Zhao  
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3 et al. 2007). This is the reason why the encapsulation efficiency of nanospheres is  
4 usually lower than that of microspheres (Wang et al. 1996; Sato et al. 1996). The lower  
5 encapsulation efficiencies obtained with the smaller particles was explained by the larger  
6 surface area of smaller droplets. Hence, during the emulsification step, a more direct  
7 contact between internal and external phases occurred which resulted in higher loss of  
8 model drug (HSA) by diffusion to the external medium (Dey et al. 2009). Inversely, for  
9 larger droplets, the surface area per unit mass is comparatively smaller. So, the loss of  
10 model drug will be lower and thus higher encapsulation efficiencies are obtained. Such an  
11 explanation agrees with the results of Gorner and Feng (Gorner et al. 1999; Feng et al.  
12 2001). Moreover, an increase in particle size increases the length of diffusional pathways  
13 into the aqueous phase, thereby reducing the drug loss through diffusion and increasing  
14 the drug content (Budhian et al. 2007). Dey et al. have also found that encapsulation  
15 efficiency of nanoparticles increased from about 68 to 86% with the increment of their  
16 mean diameter from 64 nm to 255 nm (Dey et al. 2009). Similar results were found by  
17 Gorner et al. (Gorner et al. 1999) where drug encapsulation efficiency grew with the  
18 increase in the particle size from about 19% for small particles to about 34% for medium  
19 and up to about 57% for large nanospheres which also complies with our earlier result  
20 (Feczko et al. 2011) and strongly supports our present finding.  
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47 Although the mean particle size was influenced by the duration of second  
48 sonication (see Part I), no direct relation was obtained between the latter and the  
49 encapsulation efficiency. The explanation of this apparent contradiction can be that,  
50 besides the duration of ultrasonic treatment, both particle size and the encapsulation  
51 efficiency were strongly influenced by other process variables as is seen in Fig. 63a and  
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63b. It is clearly seen from these plots that along the increasing sonication time both the mean particle size and the encapsulation efficiency are highly scattered due to the variation of the other influencing parameters at given sonication time. Therefore, the real effect of sonication time and particle size can only be explored by statistical analysis of the data, suitable to study the particular effects of different process variables. The effect of mean particle size on encapsulation efficiency can be explored by the optimization of the results also at various constraints, which will be discussed in details in section 3.8.

Fig. 36.

From Fig. 63a and 63b it is obvious that, depending on other process variables, at given sonication time, quite different particle sizes can be produced (Fig. 63a), and even for a given mean particle size quite different encapsulation efficiencies were achieved (Fig. 63b). However, process variables are optimized (section 3.8) in respect to get various predefined mean particle sizes with maximal encapsulation efficiency utilizing the descriptive model equations.

### *HSA loading in the particles*

The concentration of the active ingredient in the nanoparticles is also of primary importance in respect of their applications as drug preparations. Therefore the model drug loading (i.e. HSA concentration (% wt/vol) in the total mass of particles) can be calculated as:

$$X_{HSA_{encaps}} = \frac{m_{HSA_{encaps}}}{m_{PLGA} + m_{Fe_3O_4_{encaps}} + m_{HSA_{encaps}}} \times 100\% \quad (3a)$$

$$X_{HSAencaps} = \frac{V_{HSA} \cdot X_{HSA} \cdot EE_{HSA}}{V_{PLGA} \cdot X_{PLGA} \cdot (100 + \frac{X_{Fe3O4} \cdot EE_{Fe3O4}}{100}) + V_{HSA} \cdot X_{HSA} \cdot EE_{HSA}} \times 100\% \quad (3b)$$

In the planned application studies, the concentration of superparamagnetic magnetite NPs in the capsules of final product will be kept at relatively low level (few percent only). Thus for the sake of simplicity, the mass of magnetite in Eqn. 3 can be neglected.

Since the value of any protein type drug ingredient is several order of magnitude higher than the price of iron oxide, the purpose of our study was to determine the influence of process variables on the encapsulation of the model drug. The degree of iron oxide encapsulation was therefore of secondary importance, and served only to ensure a suitable magnetic behavior of particles after their administration in living organism. On the other hand, to minimize any side effects, as small amount of iron oxide nanoparticles should be present in the final drug formulation as possible, which can be realized at relatively low magnetite/PLGA mass ratios. Iron oxide content was measured by detection of iron ( $Fe^{3+}$ ) complex using a colorimetric method that is based on a prussian blue reaction and analyzed by UV/vis spectrophotometer. According to our observations, the encapsulation efficiency of magnetic nanoparticles was close to 100 %, when the initial iron oxide was 1 % (wt/wt) related to the PLGA mass; viz. no detectable amount of non-encapsulated iron oxide remained in the supernatant after the separation of PLGA nanoparticles. It should be noticed that at higher magnetite/PLGA ratio the degree of encapsulation could not quantitatively be determined because of the difficulty of

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3 separation of the encapsulated and non-encapsulated magnetic nanoparticles. The  
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5 dependence of magnetic properties of the final product as a function of the encapsulated  
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7 iron oxide nanoparticles will be the subject of further studies.  
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### 10 11 *Optimization of the process variables*

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15 In the production of drug-loaded NPs, the general goal is to achieve suitable small  
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17 particle size and at the same time, high encapsulation efficiency. From the results of our  
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19 study it was revealed that simultaneous achievement of the two requirements is not an  
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21 easy task because the effects of some process variables may be opposite (or at least  
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23 competitive) in respect of these two requirements. From the results it is seen that, low  
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25 PLGA concentration is beneficial for obtaining smaller NPs (see Part I) whereas it is just  
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27 disadvantageous in respect of the encapsulation efficiency. Other variables may help to  
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29 achieve both requirements at the same time, and there are variables which influence only  
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31 one of them: e.g. magnetite/PLGA ratio and sonication time have significant influence on  
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33 the mean particle size exclusively, but do not have significant effects on encapsulation  
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35 efficiency (from Eqn. 2,  $X_{\text{Fe}_3\text{O}_4}$  has coefficient of -0.0075 which is quite low) whereas  
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37 HSA concentration influences only the encapsulation efficiency, not the mean particle  
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39 size.  
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47 Fig. 63b shows that for given mean particle sizes quite different encapsulation  
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49 efficiencies can be achieved by varying the process conditions, which offers good  
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51 opportunity for optimization of the process. To elucidate the best conditions to obtain  
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53 suitably small NPs and high encapsulation efficiency at the same time, mathematical  
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55 optimization was carried out by GAMS<sup>TM</sup>/MINOS software package, using the  
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3 descriptive model equations: Eqn.1 in Part I and Eqn.2 in Part II, referring to the  
4 achievable mean particle size and encapsulation efficiency as a function of process  
5 variables, respectively. The reason behind the optimization was to find out suitable  
6 conditions (process variables) to get maximum encapsulation efficiency with a constraint  
7 of obtaining various (allowable) mean particle sizes. Because the required magnetic  
8 properties of the model drug loaded NPs may be different and will be influenced by the  
9 relative amount of  $\text{Fe}_3\text{O}_4$  nanoparticles applied in the organic phase, the optimization has  
10 been carried out at various magnetite/PLGA ratio. The results are shown on the  
11 composed diagrams in Fig. 47.

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26 In the bottom diagram in Fig. 47, the combined effect of PLGA concentration and  
27 magnetite/PLGA mass ratio is shown on the achievable mean particle size with maximal  
28 encapsulation efficiency (upper diagram), at fixed (optimal) values of the other three  
29 process variables. Among them, maximal sonication time ( $X_{\text{time}}=3.0$  min) was chosen,  
30 because it was the most beneficial to get the smallest achievable mean particle size, but  
31 had no significant direct effect on the encapsulation efficiency. The concentration of  
32 HSA practically had no influence on the mean particle size, but its smallest studied value,  
33  $X_{\text{HSA}}=0.737\%$  (wt/vol) offered the highest encapsulation efficiency, especially at the  
34 smallest studied volume ratio of  $X_{\text{VOLR}}=2.0$  (vol/vol) (see Fig. 2(d)5). The latter volume  
35 ratio was the best selection to obtain the smallest mean particle size (see Part I).

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51 According to the lower diagram, two independent variables, i.e. PLGA  
52 concentration and  $\text{Fe}_3\text{O}_4$ /PLGA ratio determine the achievable mean particle size at fixed  
53 values of the other three variables. An example is shown by dotted lines for  
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$X_{\text{PLGA}}=1.83\%$  (wt/vol) and  $X_{\text{Fe}_3\text{O}_4}=4.0\%$  (wt/wt), resulting in a mean diameter of about 155 nm (crossing point of the dotted lines). In the lower diagram it is also obvious that increasing the PLGA concentration at constant magnetite/PLGA ratio increases the mean particle diameter, and vice versa. Similarly, at constant PLGA concentration, the increase of magnetite/PLGA ratio enhances particle size and vice versa.

Fig. 47.

If we follow the vertical line upward to the upper diagram at a given  $\text{Fe}_3\text{O}_4$ :PLGA ratio (in Fig. 47, we have taken the example of  $X_{\text{Fe}_3\text{O}_4}=4.0\%$  wt/wt), it is seen that arriving at the point on the curve referring to the same mean particle size (155 nm in this case) will give  $\text{EE}_{\text{HSA}}=92.3\%$  which is the highest encapsulation efficiency achieved under these conditions (Fig. 47, the horizontal dotted line of the upper diagram). We can conclude that if HSA-loaded nanoparticles of 155 nm mean size should be produced (with given magnetite content determined by the  $\text{Fe}_3\text{O}_4$ /PLGA ratio), under optimal conditions as high as 92.3% encapsulation efficiency can be achieved. If smaller particles should be produced by using lower PLGA concentration (the crossing point of the dotted lines in the lower diagram of Fig. 47, that will be shifted downwards e.g. to the curve of 145 nm), the expectable encapsulation efficiency will be decreased to about 84% (the ordinate value in the upper diagram where the vertical dotted line crosses the curve of 145 nm mean size). It means the general opinion widely accepted in the literature is clearly confirmed: the larger is the particle size, the higher is the expected encapsulation efficiency and vice versa, if certain parameters such as the drug/polymer ratio in the emulsion are kept constant (at their optimal value).

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In addition to the particle size and encapsulation efficiency, several other requirements may be also important during producing PLGA nanoparticles loaded with protein type drug and magnetic NPs. Such requirements can be e.g. the concentration of active ingredient in the nanocapsules, and/or the productivity of the applied process or equipment (product yield mass per unit volume of equipment and unit time). Economical aspects, such as the cost of production per unit mass of product can be very important too. However, in this study we have dealt only with the requirements of particle size and encapsulation efficiency as optimization criteria, also taking the magnetite/polymer ratio into consideration, which may be important to achieve suitable levels of magnetic properties of the produced nanoparticles (examination of the latter will be the subject of a separate study).

Another aspect can be the concentration of the encapsulated HSA in the composite nanoparticles. It was also determined from the available experimental data according to Eqn. 3a-b, where concentration of HSA was changing from about 1.5 to 18.3% (wt/wt) depending on the applied process variables (Fig. 58). Considering these scattered values, the actual protein concentration encapsulated into the particles was primarily determined by the mass ratio of the introduced HSA and PLGA (both of them could be varied independently) and the encapsulation efficiency, influenced by the studied process variables. Fig. 58 also shows the increasing tendency of HSA concentration in particles with increasing HSA/PLGA mass ratio. It is also seen that the scattering of data also grows in this direction, mainly due to the increasing effect of other process variables on encapsulation efficiency.

Fig. 85.

In Part I, it was seen that HSA concentration in the inner aqueous phase had no significant influence on particle size. Therefore suitably small particles can be produced at relatively high HSA/PLGA mass ratio, if the PLGA concentration is not too high. However, at the same time, increasing HSA/PLGA ratio may have detrimental effect on the encapsulation efficiency (see Fig. 2(a)) resulting in relatively high amount of non encapsulated protein in the mother solution, remaining there after solidification and separation of the model drug loaded NPs, which may increase the loss of the valuable ingredient. Fig. 2(d) shows that applying low volume ratio of the external aqueous and intermediate organic phases ( $X_{VOLR}=2.0$  vol/vol) which is optimal for obtaining small particles, the achievable encapsulation efficiency steeply decreases from 98.2 to 72.7% with the increase of HSA concentration at fixed other variables. This, especially for expensive drug ingredients may extremely deteriorate the economy of the process. Therefore, the optimization of protein content in the particles will also be the subject of a separate study.

As an example, considering the conditions suitable for a high encapsulation efficiency of  $EE_{HSA}=92.3\%$ , shown by the dotted line in the upper diagram on Fig. 47, the achievable HSA content can be calculated which is about 3.6% (according to Eqn. 3). If higher protein content is needed in the particles, higher HSA/PLGA ratio should be applied which will diminish the efficiency of HSA encapsulation and thus leads to an increase in protein loss.

## CONCLUSION

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4 In this study we investigated the encapsulation efficiency of model drug HSA and  
5 magnetite loaded PLGA NPs. In Part I, the optimum conditions for small PLGA NPs  
6 were proposed and in this Part II, optimum conditions for NPs of given size with highest  
7 possible encapsulation efficiencies are proposed. For extremely expensive active  
8 ingredients, high encapsulation efficiency is of the highest importance. From economic  
9 point of view, if encapsulation efficiency is not high, the product cost will be very high or  
10 may extremely destroy the economy of the process. After performing all the 81  
11 experiments suggested by DOE along with 9 repetitions, it was found that encapsulation  
12 efficiencies can be obtained from as low as 18% to as high as 97% depending on process  
13 conditions. However, large particles are detected and eliminated by macrophages easily  
14 and on the other hand, their sterilization after production is also difficult. Thus, it is  
15 indispensable to find out optimum conditions for the formulation of relatively small NPs  
16 with highest possible encapsulation efficiencies. Due to the high number of variables, it  
17 was necessary to determine the optimum process conditions mathematically which was  
18 done successfully by using GAMS™/MINOS Large Scale Nonlinear Solver software. It  
19 was found that for 155 nm PLGA NPs, 92.3% encapsulation efficiency can be obtained  
20 which is sufficiently high. But if, there is constraint in respect of magnetite/PLGA ratio,  
21 i.e. higher iron oxide concentration is desired in PLGA NPs to achieve sufficient level of  
22 saturation magnetisation, the minimum achievable mean particle size increases but, in the  
23 worst case, it approximately remains below 160 nm even using a magnetite/PLGA ratio  
24 as high as 10.0% wt/wt (Part I). From Fig. 47, it is clear that if the size is even 165 nm,  
25 84% model drug will be encapsulated.  
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## 55 56 57 **ACKNOWLEDGEMENTS** 58 59 60

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## NOMENCLATURE

### Variables

$EE_{\text{HSA}}$	Encapsulation efficiency of HSA, % (Eqn.1 and Eqn.2)
$EE_{\text{Fe}_3\text{O}_4}$	Encapsulation efficiency of magnetite nanoparticles, % (Eqn.3a,b)
$m_{\text{Fe}_3\text{O}_4\text{int}}$	Total mass of the introduced magnetite nanoparticles, gram
$m_{\text{Fe}_3\text{O}_4\text{encaps}}$	Mass of magnetite encapsulated into PLGA NPs, gram
$m_{\text{HSAint}}$	Total mass of the introduced HSA, gram
$m_{\text{HSAencaps}}$	Mass of HSA encapsulated into the particles, gram
$m_{\text{PLGA}}$	Mass of PLGA introduced, gram
$V_{\text{HSA}}$	Volume of the introduced HSA solution (internal phase $W_1$ ), mL
$V_{\text{PLGA}}$	Volume of the introduced PLGA/magnetite solution (intermediate organic phase), mL
$V_{\text{PVA}}$	Volume of the outer aqueous phase (PVA solution), mL
$X_{\text{Fe}_3\text{O}_4}$	Relative mass of the introduced magnetite compared to the mass of

	PLGA, % (wt/wt)
$X_{PLGA}$	Concentration of PLGA in the intermediate oily phase
$X_{HSA}$	Concentration of HSA in the inner aqueous phase, % (wt/vol)
$X_{VOLR}$	Volume ratio of the outer aqueous phase to the intermediate oily phase, vol/vol
$X_{time}$	Time of the second sonication, min
Other notations and indices	
O	Oily phase (intermediate phase) of the emulsion
W ( $W_1, W_2$ )	Aqueous phase (first and second i.e. inner and external aqueous phase)
F	Result of the statistical F test on the studied variable (in ANOVA table)
p	Statistical significance level (in ANOVA table)
df	Degree of freedom (in ANOVA table)
MS	Mean square of the residuals, $nm^2$ (in ANOVA table)
SS	Sum of deviation squares, $nm^2$ (in ANOVA table)

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## Legends of Figures

Fig. 1. Pareto chart on the standardized effects of the independent process variables on the encapsulation efficiency

Fig. 2. The effect of various process variables on the encapsulation efficiency; fixed parameters: (a) PLGA and HSA concentration (b) PLGA concentration and the magnetite ratio to the mass of PLGA (c) interaction of the PLGA concentration and the volume ratio of the external and intermediate phases (d) interaction of the HSA concentration and the volume ratio of the external and intermediate phases, on the encapsulation efficiency

~~Fig. 3. The effect of the PLGA concentration and the magnetite ratio to the mass of PLGA on the encapsulation efficiency~~

~~Fig. 4. The interaction of the PLGA concentration and the volume ratio of the external and intermediate phases~~

~~Fig. 5. The interaction of the HSA concentration and the volume ratio of the external and intermediate phases~~

Fig. ~~63~~. Experimental data on the relation between the mean particle size, duration of second sonication and encapsulation efficiency, a - obtained mean particle size vs. sonication time without separation of the particular effects of different process variables, b – measured encapsulation efficiencies vs. measured mean particle sizes

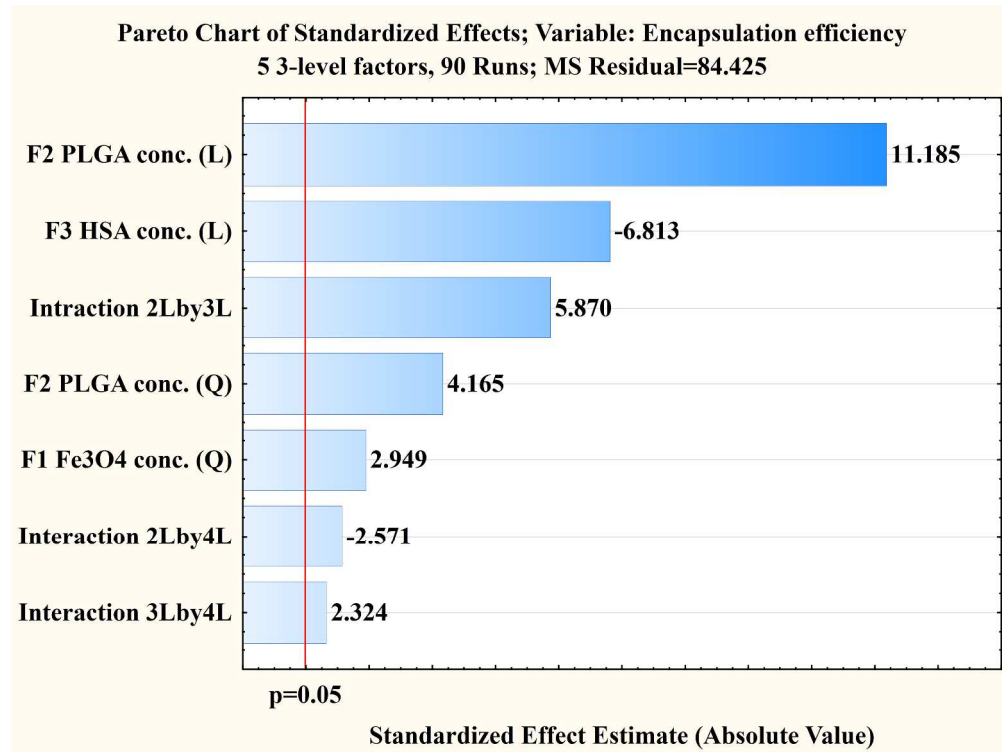
Fig. ~~74~~. Results of optimization at different magnetite/PLGA ratio. At optimal values of other process variables, PLGA concentration and magnetite/PLGA ratio determines the

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3 achievable smallest mean particle size (lower diagram) and the highest encapsulation  
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5 efficiency (upper diagram)  
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9 Fig. 58. Experimental data on the concentration of encapsulated HSA within the  
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11 composite PLGA-magnetite particles at different mass ratios of HSA and PLGA  
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13 introduced into the W/O/W double emulsion  
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### 25 **Legends of Tables**

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28 Table 1: ANOVA table obtained by statistical analysis of the measured encapsulation  
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30 efficiencies obtained with different combinations of the influencing factors  
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32 Fig. 1. Pareto chart on the standardized effects of the independent process variables on the encapsulation  
33 efficiency

34 928x696mm (96 x 96 DPI)

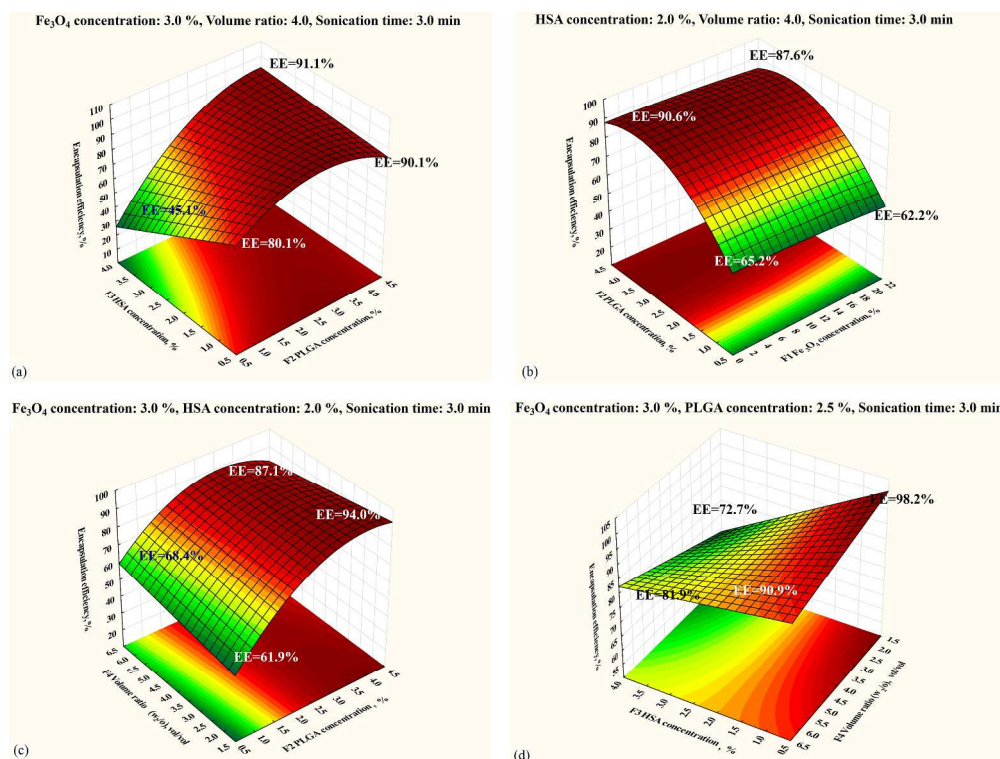


Fig. 2. The effect of various process variables on the encapsulation efficiency; fixed parameters: (a) PLGA and HSA concentration (b) PLGA concentration and the magnetite ratio to the mass of PLGA (c) interaction of the PLGA concentration and the volume ratio of the external and intermediate phases (d) interaction of the HSA concentration and the volume ratio of the external and intermediate phases  
828x624mm (96 x 96 DPI)

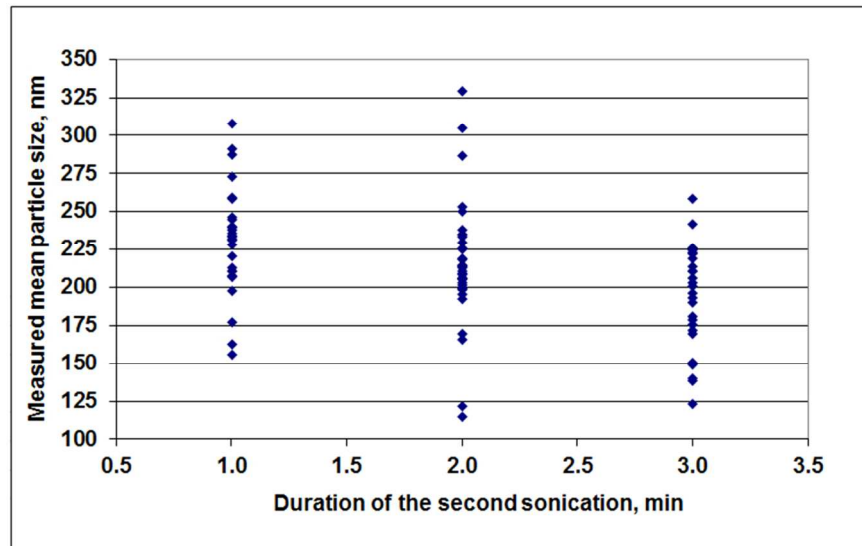
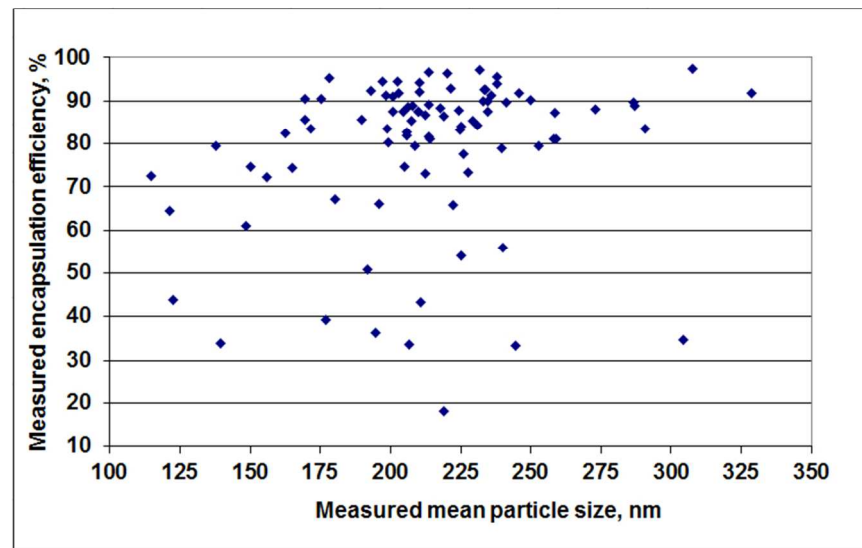
*a**b*

Fig. 3. Experimental data on the relation between the mean particle size, duration of second sonication and encapsulation efficiency, a - obtained mean particle size vs. sonication time without separation of the particular effects of different process variables, b - measured encapsulation efficiencies vs. measured mean particle sizes  
200x274mm (96 x 96 DPI)



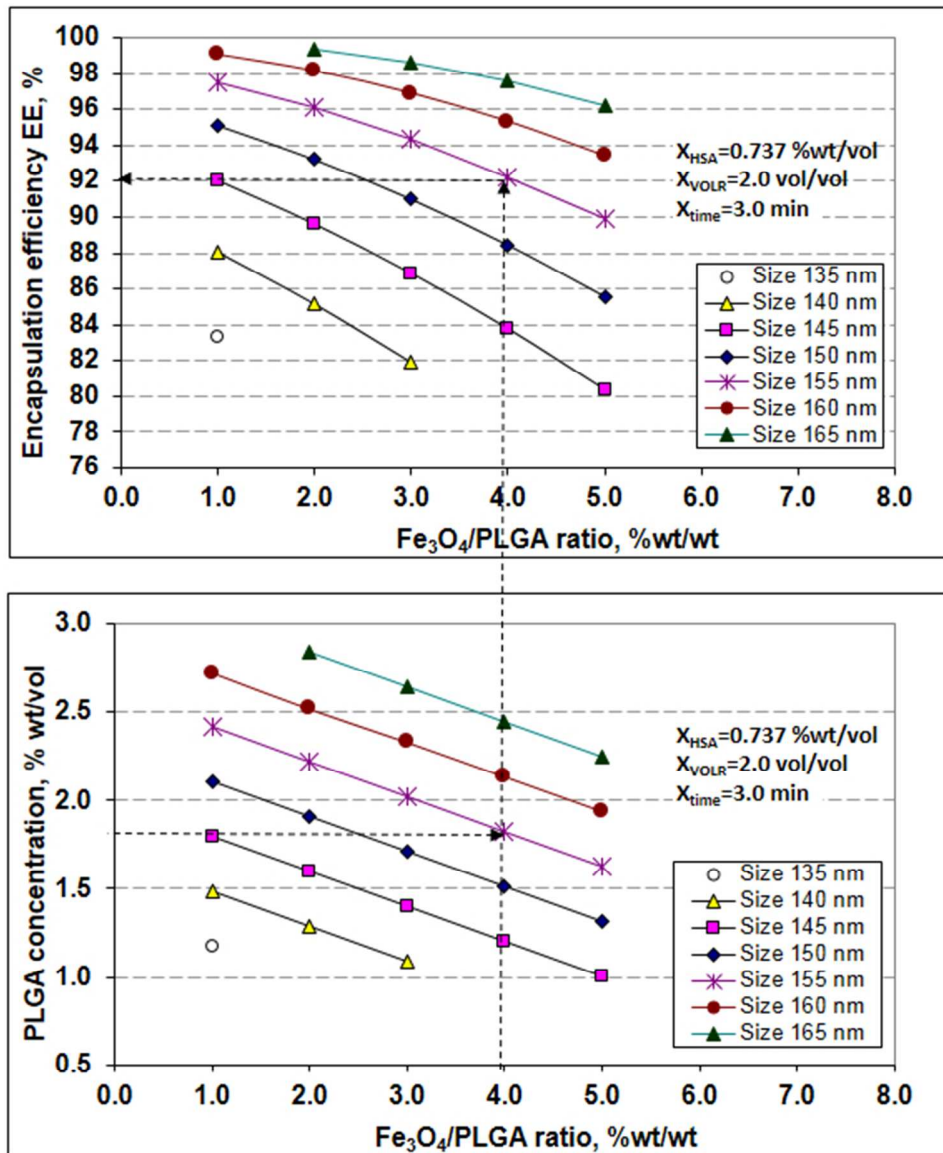


Fig. 4. Results of optimization at different magnetite/PLGA ratio. At optimal values of other process variables, PLGA concentration and magnetite/PLGA ratio determines the achievable smallest mean particle size (lower diagram) and the highest encapsulation efficiency (upper diagram) 159x190mm (96 x 96 DPI)

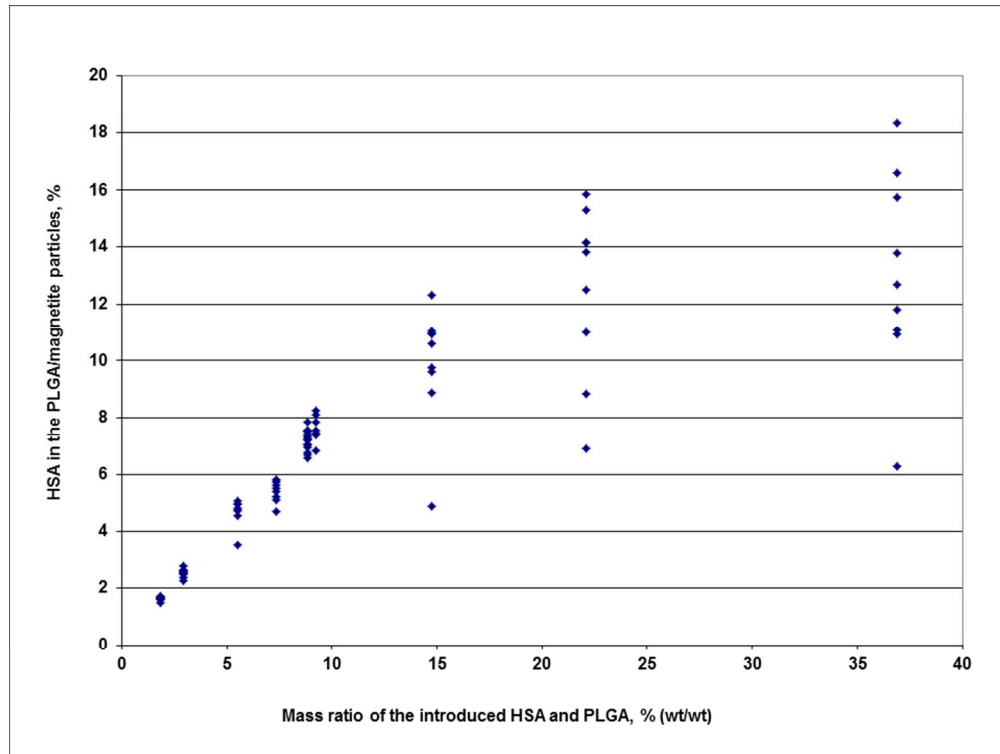


Fig. 5. Experimental data on the concentration of encapsulated HSA within the composite PLGA-magnetite particles at different mass ratios of HSA and PLGA introduced into the W/O/W double emulsion 254x190mm (96 x 96 DPI)

Table 1: ANOVA table obtained by statistical analysis of the measured encapsulation efficiencies obtained with different combinations of the influencing factors.

Factor	ANOVA; Dependent Variable: Encapsulation efficiency (HSA EE%), 5 3-level factors, 90 Runs; MS Residual=84.425				
	SS	df	MS	F	p
F1 Fe <sub>3</sub> O <sub>4</sub> conc. (Q)	734.44	1	734.436	8.69928	0.004147
F2 PLGA conc. (L+Q)	12025.95	2	6012.976	71.22274	0.000000
F3 HSA conc. (L)	3918.52	1	3918.519	46.41423	0.000000
Interaction F2*F3	2909.34	1	2909.344	34.46071	0.000000
Interaction F2*F4	558.06	1	558.062	6.61015	0.011946
Interaction F3*F4	456.04	1	456.036	5.40167	0.022592
Error	6922.85	82	84.425		
Total SS	27943.57	89			