Optimization of protein encapsulation in PLGA nanoparticles

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ABSTRACT

Influences of process parameters were investigated on the efficiency of encapsulation of bovine serum albumin (BSA) in poly(DL-lactic-*co*-glycolic acid) (PLGA) nanoparticles produced by $w_1/o/w_2$ (water-in-oil-in-water) double emulsion-solvent evaporation method. According to a 5-factorial 3-level Box–Behnken type experimental design aqueous solution of BSA was emulsified in an immiscible organic phase composed of dichloromethane and various quantities of dissolved PLGA to get water-in-oil (w_1/o) emulsion. This latter was then dispersed in a second aqueous phase (w_2) containing poly-vinyl-alcohol (PVA) surfactant as an emulsifier/stabilising agent. PLGA nanoparticles with encapsulated BSA were obtained by evaporating the dichloromethane from the w_1/o droplets. Encapsulation efficiency was determined as the weight ratio of BSA remained in the PLGA nanoparticles relative to the total weight of BSA used in the process. By statistical evaluation of the experimental results an equation was proposed to predict the encapsulation efficiency as a function of five process variables. Two optimization procedures were carried out to increase the efficiency of encapsulation, with and without constraints referring to the required mean particle size. Correlation was found between the latter and the achievable maximal encapsulation efficiency under optimal process conditions.

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1. Introduction

Among various colloidal drug delivery systems nanoparticles represent a very promising approach to control the delivery of pharmacological agents, e.g. to target given organs in the human body at therapeutically optimal rate and dose. The capability of nanoparticles to provide controlled release and protection of the protein by polymeric matrix material could be useful for drug delivery. Double emulsion technique is a promising method to encapsulate hydrophilic drugs and proteins within nano- or microparticles [1]. Poly(DL-lactic-*co*-glycolic acid) is widely utilised to manufacture nano- and microparticles due to its excellent biocompatibility, variable mechanical and biodegradability properties [2–4]. For the study reported here bovine serum albumin (BSA) was chosen as model material to investigate its encapsulation efficiency in PLGA nanoparticles.

The most important features of drug carrier nanoparticles are the size, encapsulation efficiency and release kinetics. From economic point of view the efficiency of encapsulation is extremely important, especially when the active agent is very expensive, as is typical of protein type drugs. Till now, extensive studies were

carried out worldwide to investigate the encapsulation efficiency in microparticles as a function of process parameters. Al Haushey et al. [5] analyzed the effects of 12 parameters on the size and entrapment efficiency of poly(ε-caprolactone)–BSA microparticles manufactured by double emulsion method. Blanco and Alonso [6] investigated the effect of protein and polymer properties, and co-encapsulation of surfactants on the efficiency of encapsulation and release profile.

Lamprecht et al. [7,8] studied the variation of encapsulation efficiency and the size of PLGA–BSA nanoparticles as a function of the most important process parameters, such as the concentrations of the BSA in the inner aqueous phase, the polymer concentration in the intermediate organic phase, the amount of surfactant in the external aqueous phase, the volume of the external aqueous phase, and the duration of homogenization. They analyzed the influences of these parameters one by one, keeping the other variables at fixed value. The effects of different combinations of variables were not investigated. This could be the reason that the maximal achievable encapsulation efficiency was not higher than 85%. Jimenez et al. [9] used a 3-factor 2-level full factorial design to analyze the main effect of lactic acid ratio and other circumstances on the size, zeta potential and encapsulation efficiency of methyl trypsin loaded PLGA particles.

In addition to the efficiency of encapsulation, it is also very important to know how the applied process can influence the

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stability of protein. Igartua et al. [10] found that there was no significant stability problem during the encapsulation of BSA with PLGA even at drastic conditions, i.e. in the presence of dichloromethane and the use of ultrasound energy in the first emulsification step. The results of Kang and Singh [11] were somewhat contradicting that finding, because only 72% of the initial BSA could be recovered after sonication in the presence of PLGA without any excipient. Bilati et al. [12] pointed out that the duration of sonication was of crucial importance with respect to the protein stability.

Based on a preliminary study [13] we supposed that the encapsulation efficiency in a double emulsion process could be influenced by the compositions of the inner, intermediate and external phases, the volume ratio of the external and intermediate phases, and the duration of sonication. In a recent work of Feczkó et al. [14] it was found that these circumstances also influenced the mean size of the obtained PLGA–BSA particles. Because the latter can have a crucial importance in certain pharmaceutical application the main objective of our work was to optimize the encapsulation efficiency keeping in mind the possible size requirements, too.

2. Materials and methods

2.1. Materials

PLGA (50:50, Mw = 8000, Resomer® RG 502H) with free carboxyl end groups was received from Boehringer Ingelheim, Germany. BSA was obtained from Trigon Biotechnological Ltd, Hungary. PVA (Mw = 30,000–70,000) and phosphate-buffered saline (PBS, pH 7.4) were the products of Sigma. Dichloromethane (DCM) was purchased from Spektrum-3D, Hungary.

2.2. Experimental procedure

PLGA nanoparticles containing BSA were manufactured by $w_1/o/w_2$ double emulsion-solvent evaporation technique discussed in an earlier paper in detail [13]. Briefly, BSA solution (10–50 mg/mL) in 0.5 mL phosphate-buffered saline (PBS, pH 7.4) as inner aqueous phase was added to 5 mL dichloromethane containing 50–200 mg dissolved PLGA, which served as the intermediate organic phase. The aqueous phase was emulsified in the organic PLGA solution for 60 s by a probe sonicator (Model W-220, Heat Systems-Ultrasonics, Inc.) at setting #6 (70 W electric power input) in ice bath to obtain a simple water-in-oil (w_1/o) emulsion. This first emulsion was then added into 10–30 mL aqueous solution of PVA, used as external (continuous) aqueous phase (w_2) and was emulsified by the same sonicator at setting #6 for 30–180 s in ice bath to prepare water-in-oil-in-water ($w_1/o/w_2$) double emulsion. The concentration of PVA in the external phase was changed in the range of 0.5–2.0 (w/v%). After completing the ultrasonic treatment, the dichloromethane was evaporated under magnetic stirring at 800 rpm for 2 h. The obtained particles were isolated by centrifugation (Beckman Optima Max-E) for 25 min at 30,000 × g. Size distribution was analyzed by dynamic light scattering using Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at 25 °C and was characterised by the volume mean diameter (d). The amount of BSA model protein encapsulated into the resultant PLGA nanoparticles was determined by analyzing the protein content both in the supernatant (not encapsulated fraction) and in the particles (encapsulated fraction). The protein content of particles was measured by the Biuret method [15], the supernatant was analyzed by the micro BCA (bicinchoninic acid) protein assay (Pierce Biotechnology, Inc.).

2.3. Experimental design

To elucidate the effect of process conditions on the efficiency of BSA encapsulation into PLGA nanoparticles, experiments were performed according to a measurement program determined by experimental design. This latter was carried out by STATISTICA™ Ver. 9.0 software package (StatSoft Inc, USA). The resultant experimental data were evaluated by statistical analysis using the same software package.

The following independent process variables were taken into account: the concentration of the emulsifying agent (PVA) in the external aqueous phase (F1), concentration of PLGA in the intermediate organic phase (F2), concentration of the model protein (BSA) in the inner aqueous phase (F3), volume ratio of the external aqueous phase to the intermediate organic phase (F4), and the duration of the second ultrasound treatment (sonication) applied to disperse the first water-in-oil (w_1/o) emulsion into the external aqueous media (w_2) to get water-in-oil-in-water ($w_1/o/w_2$) double emulsion. The resultant encapsulation efficiency was defined as the percentage of BSA model protein encapsulated into the PLGA particles relative to the total amount of BSA used in the process in dissolved form in the inner aqueous phase w_1 as:

$$EE\% = \frac{m_{BSA\ encapsulated}}{m_{total\ BSA\ dissolved}} \times 100 \qquad (1)$$

The experimental design was similar to that applied by Biró et al. [16] who investigated the effects of some process variables on the mean particle size of chitosan microspheres in a w/o emulsion crosslinking process. But, due to the higher number of influencing process variables, a 5-factorial 3-level (3^{K-p}) Box–Behnken type design was applied here.

For each variable 3 different levels (the lowest, highest and central values of the studied ranges) were taken into consideration to explore the possible non-linearity in their effects. One of the main advantages of applying experimental design is a considerable reduction of the experimental work, without remarkable loss of useful information. In our case only 54 runs had to be carried out including repetitions instead of all combination of the 5 independent variables at three levels, which would require $N = L^K = 3^5 = 243$ experiments. The experimental program obtained by a (3^{K-p}) Box–Behnken type design is shown in the first 54 rows of Table 1 after randomization. Encapsulation efficiencies measured in these experiments are shown in the last column. The volume mean particle sizes discussed in another paper [14] in detail are listed in the last but one column in the table. Due to the many possible effects and complexity of the studied process, altogether 14 repetitions were carried out in the central point of the studied variable intervals (denoted by C) to determine the pure error of the experiments with respect of the encapsulation efficiency.

2.4. Evaluation of the experimental data

Statistical analysis was carried out to evaluate the dependence of encapsulation efficiency on the studied process variables. In the previous paper [14] where the effects of the same process variables on the mean particle size were discussed certain experiments had to be excluded from statistical evaluation because of the extremely high particle sizes that was possibly caused by not sufficient dispersion of the droplets or agglomeration of particles. However, with respect of encapsulation all the 54 experiments proved to be adequate, thus all of them were involved in the evaluation. In a first statistical analysis the linear and quadratic effects of all variables and their two-way linear-linear interactions were taken into account, and their results were subjected to statistical tests.

Then, depending on the results of the first analysis, certain previously supposed effects that failed the $p < 0.05$ significance test have been discarded, and new statistical analysis was carried out with the remaining effects. The results are shown in Table 2, listing the

Table 1
Results of the 5-factorial 3-level $3^{(K-p)}$ type Box–Behnken experimental design after randomisation of the order of runs.

Order of implementation	Original serial number of runs	F1 PVA conc. (w/v%)	F2 PLGA conc. (w/v%)	F3 BSA conc. (w/v%)	F4 phase ratio, $r_{w2/o}$ (v/v)	F5 Duration of the 2nd sonication (min)	Measured mean particle size (nm)	Measured encapsulation efficiency (%)
Experiments scheduled by $3^{(K-p)}$ Box–Behnken experimental design								
1	3	0.50	4.00	3.00	4.00	1.75	224.8	95.5
2	4	2.00	4.00	3.00	4.00	1.75	225.9	92.0
3	50 (C)	1.25	2.50	3.00	4.00	1.75	206.7	79.5
4	22 (C)	1.25	2.50	3.00	4.00	1.75	199.3	89.3
5	10	1.25	4.00	3.00	4.00	0.50	531.5	97.3
6	8	1.25	2.50	5.00	6.00	1.75	204.7	86.5
7	5	1.25	2.50	1.00	2.00	1.75	603.8	81.2
8	9	1.25	1.00	3.00	4.00	0.50	452.8	73.2
9	7	1.25	2.50	1.00	6.00	1.75	223.1	92.1
10	1	0.50	1.00	3.00	4.00	1.75	177.1	64.6
11	6	1.25	2.50	5.00	2.00	1.75	178.7	68.0
12	12	1.25	4.00	3.00	4.00	3.00	190.2	95.7
13	20	1.25	2.50	3.00	6.00	3.00	192.1	91.2
14	49 (C)	1.25	2.50	3.00	4.00	1.75	186.9	90.0
15	17	1.25	2.50	3.00	2.00	0.50	879.0	57.9
16	11	1.25	1.00	3.00	4.00	3.00	152.2	68.6
17	18	1.25	2.50	3.00	6.00	0.50	510.6	95.7
18	13	0.50	2.50	1.00	4.00	1.75	210.0	91.5
19	21 (C)	1.25	2.50	3.00	4.00	1.75	199.1	90.4
20	16	2.00	2.50	5.00	4.00	1.75	194.1	75.8
21	47 (C)	1.25	2.50	3.00	4.00	1.75	206.7	91.7
22	48 (C)	1.25	2.50	3.00	4.00	1.75	199.4	91.4
23	15	0.50	2.50	5.00	4.00	1.75	627.9	71.6
24	19	1.25	2.50	3.00	2.00	3.00	186.9	80.5
25	14	2.00	2.50	1.00	4.00	1.75	208.7	87.4
26	2	2.00	1.00	3.00	4.00	1.75	202.6	88.8
27	23 (C)	1.25	2.50	3.00	4.00	1.75	195.8	93.4
28	52 (C)	1.25	2.50	3.00	4.00	1.75	199.3	93.0
29	54 (C)	1.25	2.50	3.00	4.00	1.75	205.7	90.5
30	37	2.00	2.50	3.00	4.00	0.50	277.9	92.3
31	51 (C)	1.25	2.50	3.00	4.00	1.75	211.6	90.7
32	25	1.25	4.00	1.00	4.00	1.75	220.4	90.3
33	28	0.50	2.50	3.00	2.00	1.75	1320.5	64.2
34	33	1.25	2.50	5.00	4.00	0.50	929.1	87.2
35	40	1.25	1.00	3.00	2.00	1.75	161.8	59.0
36	41	1.25	4.00	3.00	2.00	1.75	207.7	96.3
37	26	1.25	1.00	5.00	4.00	1.75	159.6	60.6
38	38	0.50	2.50	3.00	4.00	3.00	217.4	94.4
39	53 (C)	1.25	2.50	3.00	4.00	1.75	200.6	83.7
40	35	1.25	2.50	5.00	4.00	3.00	172.6	83.7
41	31	2.00	2.50	3.00	6.00	1.75	217.1	88.6
42	45 (C)	1.25	2.50	3.00	4.00	1.75	196.3	90.9
43	32	1.25	2.50	1.00	4.00	0.50	357.1	91.5
44	27	1.25	4.00	5.00	4.00	1.75	204.9	88.9
45	30	0.50	2.50	3.00	6.00	1.75	191.2	99.2
46	39	2.00	2.50	3.00	4.00	3.00	186.4	87.1
47	34	1.25	2.50	1.00	4.00	3.00	189.4	87.9
48	44 (C)	1.25	2.50	3.00	4.00	1.75	197.4	89.2
49	29	2.00	2.50	3.00	2.00	1.75	183.3	86.8
50	24	1.25	1.00	1.00	4.00	1.75	175.3	86.4
51	43	1.25	4.00	3.00	6.00	1.75	222.8	96.3
52	46 (C)	1.25	2.50	3.00	4.00	1.75	198.0	91.9
53	42	1.25	1.00	3.00	6.00	1.75	197.9	79.6
54	36	0.50	2.50	3.00	4.00	0.50	736.2	98.0
Additional experiments carried out to validate Eq. (2)								
55	55	1.20	0.50	5.00	2.00	2.60	142.3	28.4
56	56	1.20	1.00	5.00	2.00	2.60	163.5	39.1
57	57	1.20	1.50	5.00	2.00	2.60	168.2	51.3
58	58	1.20	2.50	5.00	2.00	2.60	188.6	66.4
59	59	1.20	4.00	5.00	2.00	2.60	207.3	85.9
60	60	1.20	1.00	5.00	2.00	0.50	819.2	33.6
61	61	1.20	1.00	5.00	2.00	0.75	175.1	44.3
62	62	1.20	1.00	5.00	2.00	1.00	172.2	47.6
63	63	1.20	1.00	5.00	2.00	1.50	167.8	38.1
64	64	1.20	1.00	5.00	2.00	2.00	160.2	28.0

combined linear and quadratic effects together that proved to be significant, and statistical indicators such as their F- and t-values, and p-levels. The pure error i.e. the mean square of deviations of the encapsulation efficiencies measured at the central points from their mean value was determined from 14 repetitions. The standardized effects are shown on a Pareto chart in Fig. 1 ranked in order of their significances. The regression coefficients were also determined by fitting a six-variable quadratic equation to the measured data.

Table 2
ANOVA table obtained by statistical evaluation of the results of 54 runs.

Factors	ANOVA; variable: encapsulation efficiency %; Rsqr = 0.82962; 5 3-level factors, 1 block, 54 runs; MS pure error = 13.845; DV: encapsulation efficiency %				
	SS	df	MS	F	p
F2 PLGA concentration (L + Q), w/v%	2018.44	2	1009.22	72.895	0.000000
F3 BSA concentration (L + Q), w/v%	662.50	2	331.25	23.926	0.000044
F4 w_2/o phase ratio (L + Q), v/v	1405.44	2	702.72	50.757	0.000001
F1 × F2 (L–L interaction between the PVA and PLGA concentrations)	192.63	1	192.63	13.913	0.002521
F1 × F4 (L–L interaction between the PVA and w_2/o phase ratio)	274.33	1	274.33	19.814	0.000653
F2 × F3 (L–L interaction between the PLGA and BSA concentrations)	149.07	1	149.07	10.767	0.005958
F2 × F4 (L–L interaction between the PLGA concentration and w_2/o phase ratio)	106.77	1	106.77	7.711	0.015706
F4 × F5 (L–L interaction between the w_2/o phase ratio and the duration of the 2nd sonication)	184.18	1	184.18	13.303	0.002952
Lack of fit	829.49	29	28.603	2.066	0.083897
Pure error	179.98	13	13.85	–	–
Total SS	5924.66	53	–	–	–
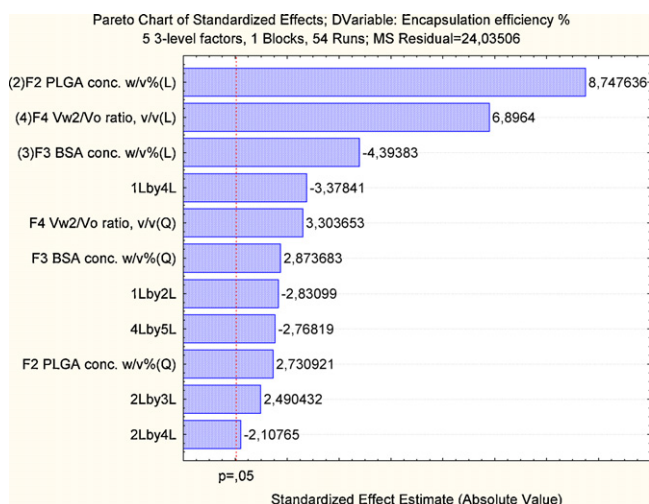
Fig. 1. Pareto chart on the standardized effects influencing the encapsulation efficiency.

3. Results and discussion

According to the results of the second statistical analysis, it was found that three independent process variables, namely PLGA concentration (F2), BSA concentration (F3), and phase ratio $r_{w2/o}$ (F4) have both linear and quadratic effects on the encapsulation efficiency. Two other variables i.e. the concentration of PVA (F1) and the duration of second sonication (F5) have no independent impact on the encapsulation: the latter can only modify the effects of other variables, due to their interactions. As is seen in Table 2, important interactions exist between the following variables: PVA and PLGA concentrations (F1 × F2), PVA concentration and phase ratio $r_{w2/o}$ (F1 × F4), PLGA and BSA concentrations (F2 × F3), PLGA concentration and phase ratio $r_{w2/o}$ (F2 × F4), and between the phase ratio and the duration of second sonication (F4 × F5). All these influences and interactions proved to be statistically significant because their p-values are well below 0.05, as is seen in Table 2. As for the pure error of experiments, the mean square of deviations determined from repetitions was 13.845, corresponding to $\sqrt{13.845} = 3.72\%$ standard deviation measured at the central point of variables, which seemed to be acceptable.

According to the Pareto chart shown in Fig. 1 it was found that the PLGA and BSA concentrations (F2(L) + F2(Q) and F3(L) + F3(Q), respectively), and the volume ratio $r_{w2/o}$ (F4(L) + F4(Q)) have the most important effects on the efficiency of encapsulation. All of them show significant non-linearity, i.e. they have both linear (L) and quadratic (Q) effects. Besides direct influences, significant interactions exist between them (denoted as 2Lby3L and 2Lby4L in the chart) and with other variables (1Lby2L, 1Lby4L, and 4Lby5L).

Fitting a regression surface to the experimental results, the following equation was obtained, applicable to predict the achievable encapsulation efficiency as a function of the studied process variables:

$$EE\% = 20.92 + 16.81 \cdot c_{PLGA} - 1.78 \cdot c_{PLGA}^2 - 1.45 \cdot c_{BSA} - 1.06 \cdot c_{BSA}^2$$
$$+ 18.53 \cdot r_{w2/o} - 1.21 \cdot r_{w2/o}^2 + 0.022 \cdot c_{PVA} \cdot c_{PLGA}$$
$$+ 0.05 \cdot c_{PVA} \cdot r_{w2/o} + 2.04 \cdot c_{PLGA} \cdot c_{BSA} - 1.72 \cdot c_{PLGA} \cdot r_{w2/o}$$
$$- 0.208 \cdot r_{w2/o} \cdot t_2 \tag{2}$$

The mean deviation between the measured and predicted encapsulation efficiencies was ±4.9%, which could be considered as reasonable. Detailed comparison is shown in Fig. 2 where circles correspond to the data of 54 experiments carried out according to the experimental program listed in the first 54 rows of Table 2, while triangles refer to the results of 10 additional experiments shown in rows 55–64, performed to validate Eq. (2). In the latter experiments we applied such combinations of variables, which were not included in the first 54 runs. In addition, the variables in the second series of experiments were selected in a way to obtain encapsulation efficiencies in a range that was not covered by the original 54 experiments. As is seen in Fig. 2, below EE = 60% efficiency no data point were obtained in the first series, but most of the data obtained by validation are in this lower region giving evidence on the validity of Eq. (2) throughout the whole studied range. The upper and lower dotted lines in Fig. 2 correspond to ±5% deviations showing acceptable agreement between the measured and predicted values.

Based on Eq. (2) the particular effects of the studied process variables were plotted in 3D diagrams and discussed in detail below.
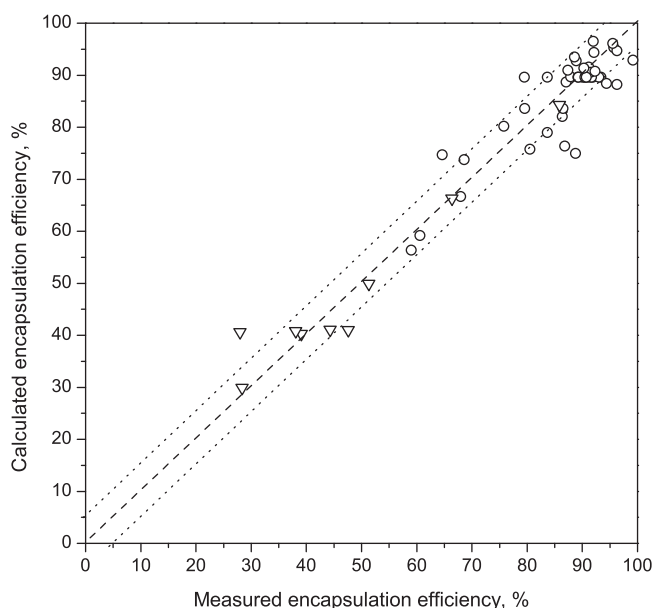
Fig. 2. Comparison of the measured and predicted encapsulation efficiencies. (○) Experiments carried out according to the 5-factorial 3-level Box–Behnken type experimental design, (▽) additional experiments to validate Eq. (2).

3.1. Effect of PVA concentration

As was shown earlier, PVA was applied as emulsifier in the external aqueous phase (w_2) to facilitate the preparation of the $w_1/o/w_2$ double emulsion. This substance was not yet present and thus could not have an effect in the first step of emulsification when essentially the capture of aqueous BSA in the PLGA containing organic droplets took place. A possible explanation of the influence of PVA on encapsulation efficiency can be that strong cavitation occurs in the complex three-phase system during the secondary sonication, which may results in further breakdown of the inner aqueous droplets containing BSA. Exploratory measurements on droplet sizes carried out by laser beam scattering both in the simple w_1/o and double $w_1/o/w_2$ emulsions gave some indications on this breakdown taking place simultaneously together with the fragmentation of the surrounding organic phase. Therefore, some part of BSA could escape from the inner aqueous phase to the external aqueous phase decreasing the efficiency of encapsulation. Concentration of PVA in the external phase could enhance the additional breakdown of the inner aqueous droplets. Eq. (2) confirms this effect, since the concentration of PVA is present in two terms of the equation showing some interactions with other variables. However, this effect is weak and under certain conditions can be neglected.

3.2. Effect of PLGA concentration

The Pareto chart in Fig. 1 shows that the amount of PLGA in the intermediate organic phase has crucial influence on the efficiency of encapsulation. Besides its high positive linear effect (F2 L) it has some negative quadratic influence (F2 Q) and interactions with three other factors (F1, F3, and F4, denoted by 1Lby2L, 2Lby3L, and 2Lby4L, respectively), resulting in non-linear relationship (see Figs. 3, 5, 6 and 8). Maximal encapsulation efficiency can be achieved in the range of PLGA concentration between 2.5 and 4.0 (w/v%) depending on other variables. Eq. (2) shows that high PLGA concentrations can generally be beneficial to achieve higher encapsulation efficiency, but due to a quadratic term and some negative interactions with other factors this effect can be lowered.
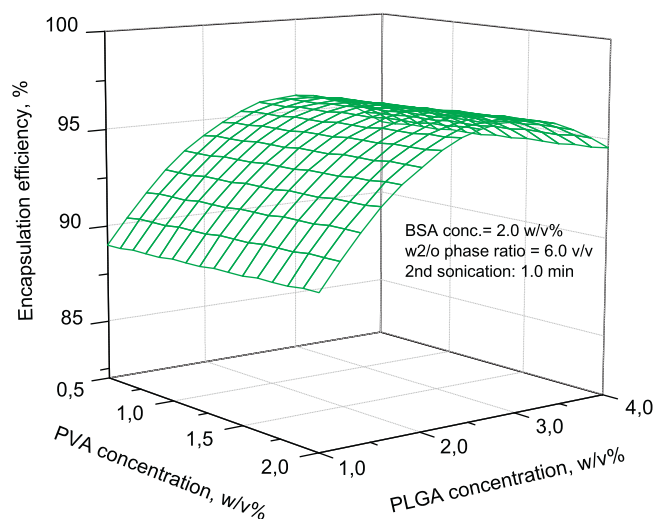

Fig. 3. Effects of the PVA and PLGA concentrations on the encapsulation efficiency at fixed other variables.

Figs. 3 and 5 show that excessively high PLGA concentration at low BSA concentration is not beneficial. However, this negative effect does not appear at high BSA concentration, probably because in this case some loss of BSA becomes relatively less significant.

3.3. Effect of BSA concentration

Eq. (2) shows a relatively high negative influence of BSA concentration indicating that under certain conditions the higher the BSA content in the inner aqueous phase the lower is the efficiency of its encapsulation. However, substantial positive interaction can be recognised with the concentration of PLGA: the efficiency of BSA encapsulation can be improved by increasing the BSA/PLGA ratio. Encapsulation efficiencies measured in 64 experiments and plotted in Fig. 4 as a function of the weight ratios BSA/PLGA confirm this suggestion. In spite of broad scattering of data points, which is due to the changes of other variables, the improving tendency of encapsulation with decreasing BSA/PLGA ratio can be recognised.

Statistical analysis gave opportunity to separate the particular effect of BSA concentration at different values of other variables shown in Figs. 5 and 7. Fig. 5 shows the special effects of c_{BSA} and c_{PLGA} also indicating their interactions. At low PLGA concentration the increase of BSA content causes considerable decrease in the encapsulation efficiency and vice versa, probably due to the limited BSA entrapment capability of the PLGA matrix. However, using higher PLGA concentrations in the organic phase, the encapsulation efficiency becomes much better, especially at high BSA concentrations, resulting in good efficiency in the range of $c_{BSA} = 3.0–4.0$ (w/v%).

3.4. Volume ratio of the external aqueous and intermediate organic phases

Fig. 6 shows the influence of the volume ratio of the external aqueous and internal organic phases and its interaction with the PLGA concentration. At low PLGA concentration strong dependence is seen, namely the reduction of volume ratio $r_{w2/o}$ diminishes the amount of BSA that can be encapsulated into the particles. Since the intensity of ultrasound treatment applied for emulsification was constant in all experiments, the explanation can be as follows: volume ratio $r_{w2/o}$ was decreased by diminishing the amount of the external aqueous phase. In this case the power density per unit volume during the second sonication was increased leading to stronger breakdown of the w_1/o droplets. This allowed escaping some BSA
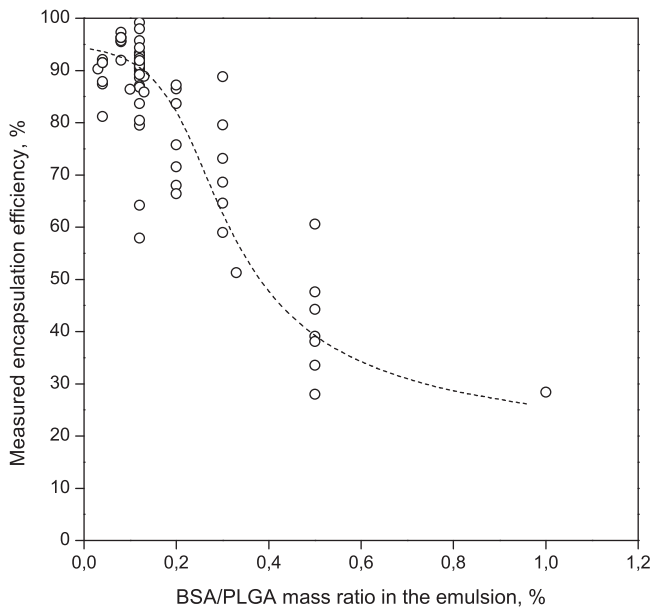
Fig. 4. Measured encapsulation efficiencies at various BSA/PLGA weight ratios.

to the external aqueous phase. However, at higher PLGA concentrations e.g. above $c_{PLGA} = 3.0$ this effect becomes smaller because of the higher encapsulation capacity of the PLGA matrix. Higher $r_{w2/o}$ volume ratios, especially at elevated PLGA concentrations, provide better encapsulation, due to the less power density per unit volume.

Fig. 7 shows significant non-linear dependence on phase ratio $r_{w2/o}$ and BSA concentration at relatively short sonication time, medium PVA and high PLGA concentrations. The arched shape of the regression surface indicates high encapsulation efficiency at about the two third of the studied intervals of variables, which gives good opportunity to optimize the process parameters.

3.5. Time of the second emulsification

Fig. 8 indicates relatively weak influence of the second sonication on the efficiency of encapsulation at different PLGA concentrations. It is seen that medium PLGA concentrations with short sonication time provides the best encapsulation results, while lower efficiency can be expected at long sonication time and small
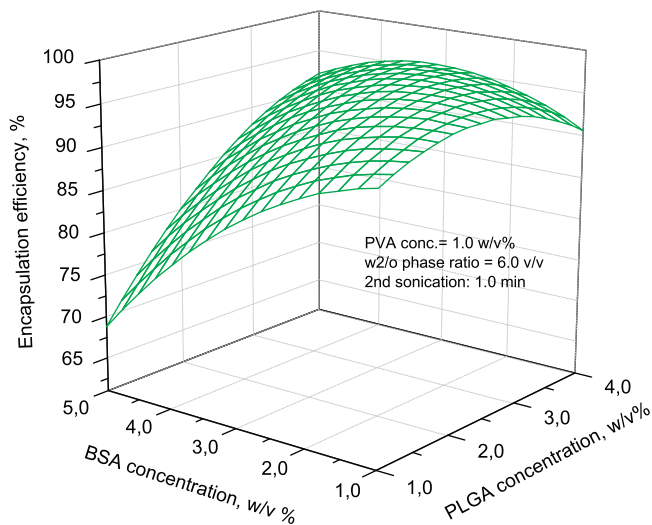

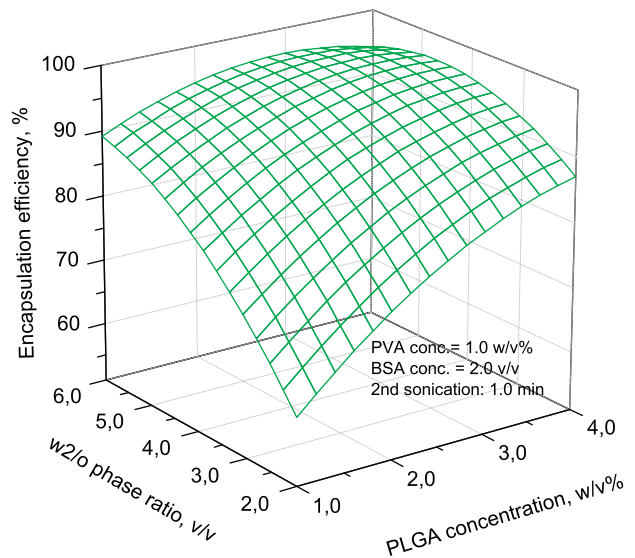**Fig. 5.** Effects of the PLGA and BSA concentrations at given combination of other variables.

Fig. 6. Effects of the w_2/o phase ratio and PLGA concentration at given combination of other variables.

PLGA concentration. A reasonable explanation can be that longer ultrasonic treatment, similarly to the increase of power density per unit volume, gives more chance for BSA to escape from the w_1/o droplets to the external aqueous phase.

3.6. Optimal conditions to maximize the encapsulation efficiency

To find the optimal combination of free decision variables where the best encapsulation efficiency can be achieved, the software package GAMS^{TM}/MINOS Large Scale Nonlinear Solver for Windows, Version 5.51 (System Optimisation Laboratory, Stanford University) was applied. According to the results of optimization, maximal PVA and PLGA concentrations ($c_{PVA} = 2.0$ (w/v%), $c_{PLGA} = 4.0$ (w/v%)), medium BSA concentration ($c_{BSA} = 3.17$ (w/v%)), relatively high w_2/o phase ratio ($r_{w2/o} = 4.8$, v/v) and short sonication time ($t_2 = 0.5$ min) offered the best encapsulation in the studied range of variables. By this combination of variables as high as 98.4%
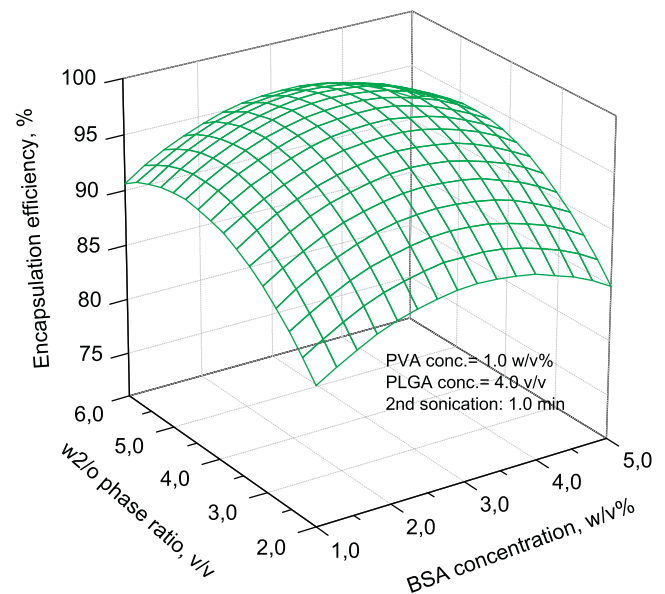

Fig. 7. Effects of the w_2/o phase ratio and BSA concentration at given combination of other variables.

Table 3
Optimum conditions calculated for different mean particle size requirements.

Required mean particle size, nm	Optimal conditions for the required mean particle size					
	F1 PVA concentration (w/v%)	F2 PLGA concentration (w/v%)	F3 BSA concentration (w/v%)	F4 $r_{w2/o}$ ratio (v/v)	F5 Time of the 2nd sonication (min)	Calculated encapsulation efficiency (%)
140	2.00	1.00	2.41	2.74	3.00	66.8
150	2.00	1.00	1.93	3.62	3.00	76.2
160	2.00	3.88	5.00	2.00	3.00	83.1
170	2.00	4.00	4.21	2.64	3.00	90.2
180	2.00	4.00	3.74	3.52	3.00	94.2
190	2.00	4.00	3.29	4.37	2.98	95.9
200	2.00	4.00	3.25	4.49	2.54	96.3
210	2.00	4.00	3.21	4.57	2.22	96.7

encapsulation efficiency could be realized, predicting significant improvement compared to other published data [7].

However, a controversial behaviour of the studied system in this respect made difficult to achieve the goal: conditions that are optimal to achieve maximal encapsulation efficiency are inappropriate to obtain particle size which is small enough. Comparing Eq. (2) to Eq. (3) proposed by Feczkó et al. [14] to predict the expectable mean particle size of PLGA–BSA nanoparticles, it can be found that some process variables have opposite effects in this respect.

$$\bar{d} = 210.87 + 9.10 \cdot c_{PVA}^2 + 22.69 \cdot c_{PLGA} - 1.95 \cdot c_{PLGA}^2$$
$$- 4.22 \cdot c_{BSA} - 2.57 \cdot r_{w2/o} - 33.31 \cdot t_2 + 10.87 \cdot t_2^2$$
$$+ 5.83 \cdot c_{PVA} \cdot r_{w2/o} - 23.42 \cdot c_{PVA} \cdot t_2 \qquad (3)$$

For example, if we substitute the variable values found as the best ones to achieve the highest encapsulation efficiency (c_{PVA} = 2.0 (w/v%), c_{PLGA} = 4.0 (w/v%), c_{BSA} = 3.17 (w/v%), $r_{w2/o}$ = 4.8 (v/v), and t_2 = 0.5 min) into Eq. (3), $\bar{d}$ = 299.36 nm mean particle size will be obtained, which is much higher than would be acceptable for certain pharmaceutical application e.g. in an injection formula. On the other hand, if we apply optimal conditions (c_{PVA} = 2.0 (w/v%), c_{PLGA} = 1.0 (w/v%), c_{BSA} = 5.0 (w/v%), $r_{w2/o}$ = 2.0 (v/v), and t_2 = 3.0 min) to achieve the smallest possible particle size, 122.4 nm in this case, according to Eq. (2) quite low encapsulation efficiency of 40.3% could be obtained, which is unacceptable because

of the high loss of active ingredient. Therefore, special optimization method had to be applied to resolve this contradiction.

3.7. Optimization of encapsulation with particle size requirements

Fortunately, the change of some variables that worsens the encapsulation efficiency moderately, influences the particle size in favourite direction, therefore suitable compromise can be found between the two concurrent demands. For example, by extending the time of sonication from 0.5 to 3.0 min, the encapsulation efficiency diminishes by few percents only (see Fig. 8), but applying proper combinations of other variables the mean particle size can be decreased from about 300 nm to 170–180 nm. Another example is shown in Fig. 3, demonstrating that PVA concentration practically does not affect the efficiency of encapsulation, but according to our earlier results [14] its increase is beneficial to decrease particle size.

To utilise this feature, optimization was carried out for various predetermined particle sizes by the GAMS software package using Eqs. (2) and (3) simultaneously. The optimal parameter combinations were determined for different required particle sizes to achieve the highest possible encapsulation efficiency as is shown in Table 3.

Analyzing these results it can be concluded that high PLGA concentration is advisable to get high encapsulation efficiency at medium BSA concentration. At given combinations of other parameters, the volume mean particle size can be reduced from 210 nm to about 160 nm by increasing the concentration of BSA from 3.2 to 5.0 (w/v%). Although this adjustment slightly reduces the efficiency of encapsulation, it remains reasonably high e.g. 90.2% and 83.1% for mean particle sizes of 170 and 160 nm, respectively. For this, the phase ratio $r_{w2/o}$ should be decreased from 4.57 to 2.64 or 2.00, respectively, which increases the ultrasonic power density causing suitable breakdown of the droplets during emulsification.

Further decrease of particle size is only possible by considerable reduction of the PLGA concentration in the organic phase, which in turn makes necessary to diminish the BSA content in the inner aqueous phase. By this, theoretically 140 and 150 nm mean particle size can be manufactured with 67 and 76% encapsulation efficiency, respectively. The elongation of ultrasonic treatment from 2.2 to 3.0 min slightly diminishes the encapsulation efficiency (Fig. 8) but reduces the achievable mean particle size considerably.

3.8. Relation between the particle size and encapsulation efficiency

The reverse effects of certain process variables on the achievable mean particle size and encapsulation efficiency resulted in a general tendency shown in Fig. 9. Circles on the diagram correspond to the results of 44 runs carried out according to the experimen-
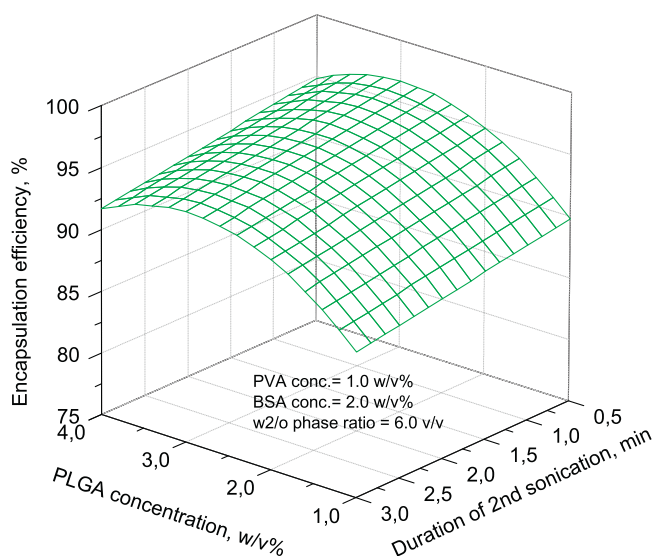

Fig. 8. Effects of the PLGA concentration and the duration of second sonication at given combination of other variables.
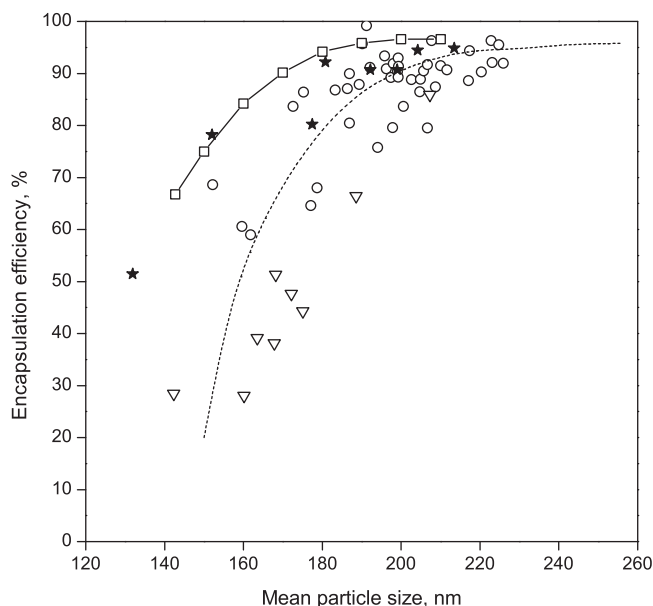
Fig. 9. Relation between the mean particle size and encapsulation efficiency. (○) Experiments carried out according to the 5-factorial 3-level Box–Behnken type experimental design, (▽) additional experiments carried out to validate Eq. (2), dotted line – general tendency of the measured data, (□) data determined theoretically at optimal conditions for maximal encapsulation efficiency, (★) experimental data obtained at optimized process variables.

tal design, omitting the erroneous particle size data [14]. Triangles refer to the results of 10 additional experiments carried out to verify Eq. (2). Dotted line indicates the tendency of the measured data. It is seen that above 180–190 nm mean particle size excellent encapsulation efficiency was achieved, but when smaller particles were manufactured much worse efficiencies were obtained, e.g. 28.4% in case of 142 nm mean size. Scattering of data points is due to the different combinations of parameters and thus the non optimal conditions applied during the experiments.

As was shown, Eqs. (2) and (3) gave opportunity to find the optimal conditions to produce particles of various mean sizes with the highest possible encapsulation efficiencies. Squares linked by solid line in Fig. 9 show these theoretical values obtained for the process variables listed in Table 3.

To justify the proposed concept, additional experiments have been carried out using the optimized combinations of variables to produce particles of various mean sizes between 140 and 210 nm. Notice that only one experiment was done for each selected particle size. The resultant data are plotted in Fig. 9 as black star symbols. Although almost all these measured encapsulation efficiencies were somewhat below the theoretically optimal results but well above the majority of data obtained in the first 64 experiments. The mean deviation of the mean particle sizes and encapsulation efficiencies measured with optimized variables from the theoretically predicted values were 9.7 nm and 6.1%, respectively.

4. Summary and conclusions

Investigation was carried out to determine the influence of process variables on the efficiency of BSA encapsulation in PLGA nanoparticles produced by $w_1/o/w_2$ (water-in-oil-in-water) double emulsion-solvent evaporation method, applying 5-factorial 3-level Box–Behnken type experimental design. By statistical evaluation of the experimental data quantitative relationship was determined between the applied process parameters and the encapsulation efficiency. Increasing the PLGA concentration in the intermediate organic phase and decreasing the BSA concentration in the inner

aqueous phase was generally beneficial to achieve better encapsulation. Higher w_2/o volume ratio was advantageous, probably due to the smaller power density during sonication, moderating the disintegration of the w_1/o emulsion droplets and thus reducing the escapement of BSA to the external aqueous phase. PVA concentration and the duration of sonication had minor influences. The proposed relationship proved to be applicable for optimization of the process conditions in order to maximize the efficiency of BSA encapsulation to PLGA nanoparticles.

Relation was found between the volume mean size of the particles and the efficiency of encapsulation. Certain changes of process variables which were suitable to improve the efficiency of encapsulation had a unfavourable effect with respect of the attainable particle size. Using the relationship proposed previously to describe the dependence of the mean particle size, optimization was carried out to determine the right conditions for maximal encapsulation efficiency, taking various particle size requirements into account.

Appendix A. Nomenclature

Variables

c_{PVA}	concentration of PVA in the external aqueous phase (w/v%)
c_{PLGA}	concentration of PLGA in the intermediate organic phase (w/v%)
c_{BSA}	concentration of BSA in the internal aqueous phase (w/v%)
$\bar{d}$	volume mean particle size (nm)
EE	encapsulation efficiency defined by Eq. (1) (%)
$m_{BSA\,encapsulated}$	weight of BSA encapsulated into the PLGA nanoparticles (mg)
$m_{total\,BSA\,dissolved}$	total weight of BSA dissolved in the inner aqueous phase (mg)
$r_{w2/o}$	volumetric ratio of the external aqueous (w_2) and intermediate organic (o) phases (mL/mL)
t_1	duration of the first sonication applied to disperse the inner aqueous phase in the intermediate organic phase (min)
t_2	duration of the second sonication applied to disperse the first water-in-oil emulsion in the external aqueous phase to get the final water-in-oil-in-water double emulsion (min)

Statistical notations

SS	sum of error squares (nm^2)
df	degree of freedom
MS	mean value of error squares (nm^2)
F	result of the statistical F test on the studied variable
p	observed significance level

Other notations, indices

C	central point of the studied intervals of all independent variables
F1, F2, F3, F4, F5	factors (corresponding to the independent variables)
$Fi \times Fj$ or iL by jL	linear-linear interactions between the ith and jth factors

K	the number of independent variables or factors
o	Intermediate organic phase
L	indication of linear effect of a variable
Q	indication of quadratic effect of a variable
w_1	inner aqueous phase
w_2	external aqueous phase

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