Research
ISSN 2413-0516

Effect of formulation parameters on the size of PLGA nanoparticles encapsulating bovine serum albumin: a response surface methodology

Tajudeen Adebileje,[a,b] Alireza Valizadeh,[b] and Amir Amani[b,c]

[a]Department of Medical Nanotechnology, School of Advanced Technologies in Medicine, International Campus, Tehran University of Medical Sciences, Tehran, Iran.
[b]Department of Medical Nanotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran.
[c]Medical Biomaterials research Center, Tehran University of Medical Sciences, Tehran, Iran.
Correspondence to: Dr. Amir Amani (e-mail: aamani@sina.tums.ac.ir).
(*Submitted: 24 July 2017 – Revised version received: 12 August 2017 – Accepted: 10 September 2017 – Published online: 26 December 2017*)

Objective Poly (lactic-co-glycolic) acid (PLGA) as a polymeric carrier for medicines has been extensively investigated because of its unique biodegradability and biocompatibility features. In this study, bovine serum Albumin (BSA), a protein which plays a key role in maintaining oncotic pressure was employed as a model protein to be encapsulated into PLGA nanoparticles (NPs). The main objective was to investigate, using Box–Behnken design, the factors affecting particle size of PLGA NPs encapsulating BSA.
Methods PLGA nanoparticles containing BSA were prepared by double emulsion solvent evaporation (DESE) method. Concentrations of PLGA, BSA and polyvinyl alcohol (PVA) as well as volume ratio (VR) of the primary emulsion to external aqueous phase in DESE were hypothesized to be the key four independent parameters affecting particle size.
Results The particle sizes obtained from 27 experiments were in the range of 266 nm to 656 nm. The model showed that as a general rule, PVA, VR and PLGA had a direct significant effect and on the particle size, while the effect of BSA was negligible.
Conclusion The independent factors appear to be affecting the size through their effects on viscosity which could be regarded as the most important factor.
Keywords double emulsion, PLGA, BSA, volume ratio, poly vinyl alcohol, Box–Behnken design

Introduction

In controlled delivery and release of therapeutic agents, nanoparticles (NPs) represent a promising approach to improve efficacy, reduce administered dosage and side effects of therapeutic agents. Biocompatible and biodegradable poly (lactic-co-glycolic) acid (PLGA), as a synthetic polymeric carrier of drugs is known to offer advantages over other types of polymers.[1]

Several techniques such as emulsion/microemulsion polymerization, precipitation polymerization, emulsion diffusion, salting out, nanoprecipitation and emulsion solvent evaporation (ESE) have been reported for preparing PLGA NPs.[2] For efficient entrapment of water soluble drugs into PLGA NPs, ESE methods have been successfully used.[3] Single emulsion solvent evaporation has been applied for this purpose, however, particle size using this technique is relatively large (in micrometer range), also entrapment of hydrophilic drugs is usually poor.[4,5] Double emulsion solvent evaporation (DESE) appears to be an interesting approach with improved ability to entrap water soluble drugs and provide sustained release of therapeutic agents in the area of pharmaceutical and cosmetic applications.[6–8] Using this method, physicochemical properties of the NPs such as particle size, drug encapsulation efficiency and drug release have been reported to be affected by processing/formulation parameters.[2,3]

Bovine serum Albumin (BSA), a water soluble protein which plays a critical role in oncotic pressure of blood by stabilizing extracellular fluids, has been addressed in literature to be loaded into the PLGA matrix by DESE method.[9,10] In a study, sizes of 320–531 nm were reported for PLGA NPs encapsulating BSA, when PLGA was dissolved in ethyl acetate.[11] Nevertheless, a careful consideration of the procedure of preparing PLGA NPs via DESE method shows several parameters which affect physicochemical properties of the prepared NPs. Concentration of PLGA and the active agent, concentration and nature of the surfactant, VR of each phase of emulsions, type of solvent, viscosity, shear stress and additives are some parameters which may affect the process.[2] Therefore, to obtain a reproducible preparation with predetermined characteristics, an analytical tool for investigating the effect of formulation/process parameters of DESE on the quality of the product is necessary. Response surface methodology is a useful tool which supersedes one factor at a time (OFAT) approach in design and understanding the effects and interactions of experimental formulation parameters. Among the different response surface methodologies proposed so far, Box–Behnken design has proved to be an efficient tool due to its ability to estimate parameters of quadratic models, use of blocks and the detection of lack of fit of a model.[12]

Examples of using Box–Behnken design have been carried out in several research studies. Among this, a study used Box–Behnken design to investigate the effect of independent process variables on the properties of NPs which includes the size, entrapment efficiency, drug loading and release of sildenafil citrate loaded into PLGA NPs.[13] Another study uses the Box–Behnken design to optimize the process variables of lorazepam loaded PLGA nanoparticles by investigating the effect of process variables on the z-average and percentage drug entrapment of PLGA NPs.[14]

Considering the multivariable process of DESE, no work so far has detailed the effect of formulation parameters simultaneously on size of BSA-loaded PLGA NPs prepared by DESE. The objective of the present study was to investigate, using Box–Behnken design, to study the effects and interactions of selected formulation parameters involved in nanoparticle formation on the size of BSA-loaded PLGA NPs prepared by DESE method. While keeping some DESE technical

parameter fixed, concentration of BSA (C_{BSA}), PLGA (C_{PLGA}) and PVA (C_{PVA}) as well as the volume ratio (VR) between primary emulsion and external aqueous phases where examined as four independent parameters affecting the NP size.

Materials and Methods

Materials

Medical grade PLGA (50:50, Mw = 50 kDa) was acquired from Shenzhen Esun Industrial Co., Ltd (Shenzhen, China). Polyvinyl alcohol (PVA) was purchased from VAM & P. VAL Co., Ltd (Tokyo, Japan). BSA was purchased from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). The organic solvent dichloromethane (DCM) was purchased from Merck Millipore.

Experimental design

The solubility of BSA (40 mg/ml) was considered and experimental independent parameters, factors and factor levels were determined in preliminary studies. The following independent formulation parameters were taken into account: C_{BSA} (%w/v) (F_1), C_{PLGA} (%w/v) (F_2), PVA concentration (%w/v) (F_3) and VR between primary emulsion (5 ml) to external aqueous phases containing PVA (F_4) (see Tables 1 and 2). A 4-factorial 3-level Box–Behnken design was applied here using STATISTICA™ Ver.12.0 software package (Stat Soft Inc., USA) to generate 27 experiments (Table 2) for each formulation variable by considering three different levels (the lowest, central and the highest values) (Table 1).

Table 1. **Independent formulation parameters (factors) designed with their lowest level (–1), center value (0) and higher level (+1)**

	Factors	Parameters	–1	0	+1
1	F_1 (%w/v)	BSA concentration at the internal aqueous phase	0.5	0.75	1
2	F_2 (%w/v)	PLGA concentration at the oil phase	0.5	1.0	1.5
3	F_3 (%w/v)	PVA concentration at the external aqueous phase	0.25	0.625	1
4	F_4 (W_1/O)	Volume Ratio (VR) between primary emulsion to external aqueous phase	2	4	6

For the VR (F_4) between primary emulsion to external aqueous phase: 2 W_1/O, 4 W_1/O and 6 W_1/O is equivalent to 10 ml, 20 ml and 30 ml of external aqueous solution of DESE containing PVA (F_3). Where W_1/O represents the total volume of the first emulsion (5 ml).

Table 2. **Experimental design and observed particle size**

Run order	Std order	F_1 C_{BSA} (%w/v)	F_2 C_{PLGA} (%w/v)	F_3 C_{PVA} (%w/v)	F_4 VR	Y Observed size (nm)	Predicted size (nm)	Residual error
1	2	1.00	0.5	0.625	4	342	291.3	50.7
2	7	0.75	1.0	0.250	6	442	431.0	11.0
3	22	1.00	1.0	1.000	4	620	652.8	–32.8
4	15	0.75	1.5	0.250	4	400	404.7	–4.7
5	1	0.50	0.5	0.625	4	359	416.3	–57.3
6	8	0.75	1.0	1.000	6	603	598.7	4.3
7	13	1.00	1.0	0.625	6	414	505.1	–91.1
8	26	0.75	1.5	0.625	6	530	515.1	14.9
9	4	1.00	1.5	0.625	4	639	576.3	62.7
10	16	0.75	0.5	1.000	4	506	482.3	23.7
11	19	0.50	1.0	0.250	4	406	415.2	–9.2
12	5	0.75	1.0	0.250	2	308	288.3	19.7
13	12	0.50	1.0	0.625	6	431	435.1	–4.1
14	20	1.00	1.0	0.250	4	285	304.2	–19.2
15	10	0.50	1.0	0.625	2	356	292.5	63.5
16	21	0.50	1.0	1.000	4	379	401.8	–22.8
17	9	0.75	1.0	0.625	4	373	398.8	–25.8
18	24	0.75	1.5	0.625	2	401	372.5	28.5
19	3	0.50	1.5	0.625	4	266	311.3	–45.3
20	27	0.75	1.0	0.625	4	364	398.8	–34.8
21	18	0.75	1.0	0.625	4	362	398.8	–36.8
22	23	0.75	0.5	0.625	2	305	282.5	22.5
23	11	1.00	1	0.625	2	317	362.5	-45.5
24	6	0.75	1	1.000	2	400	456.0	–56.0
25	17	0.75	1.5	1.000	4	656	572.3	83.7
26	14	0.75	0.5	0.250	4	317	314.7	2.3
27	25	0.75	0.5	0.625	6	523	425.1	97.9

Experimental Procedure (Preparation of NPs)

Polymeric PLGA NPs encapsulating BSA were prepared by water in oil in water (W/O/W) DESE method with PVA as a stabilizer at the external aqueous phase. In brief, the primary emulsion was obtained by dissolving BSA (25–50 mg) in 1 ml distilled water, then, emulsified with 4 ml DCM containing PLGA (25–75 mg), followed by sonication (30 s). In the second emulsification step, the primary emulsion (5 ml) was added to external phase (i.e. aqueous solution, 10 to 30 ml) containing different PVA concentrations (see Tables 1 and 2) to obtain the final double W/O/W emulsion. The final double emulsion (W/O/W) was then sonicated (60 s) and stirred for 4 hours for complete evaporation of organic solvent (DCM). To minimize diffusion of BSA to external aqueous phase and evaporation of the aqueous phase after solvent evaporation, minimum stirring time (4 h) was applied based on our preliminary studies. Subsequently, for collection of NPs, the double emulsion was centrifuged (12,000 RPM for 30 minutes) and washed two times with distilled water to remove excess PVA and non-encapsulated drug.

Particle Size Analysis

The obtained NPs were dispersed into 5 ml of distilled water. The median hydrodynamic diameter (d50) of the NPs was analyzed with dynamic light scattering (DLS < Scatteroscope-I, K-ONE LTD, South Korea).

Evaluation of Experimental Results

Effect of formulation parameters on the response (size of PLGA NPs) from 27 experiments were collected, investigated and evaluated by Box–Behnken design. Statistical analysis was carried out on two-way linear-linear interactions of the formulation parameters, at centered and scaled polynomials, certain effects that were not significant were discarded due to their *P*-value > 0.05. A new statistical analysis was performed on the remaining significant effects, and the results are shown in Table 3. Table 3 describes the analysis of variance on the formulation parameters while Table 4 describes the effect estimates of formulation parameters. The standardized effects ranked in order of their significances are also shown on a Pareto chart (Fig. 1). The regression coefficients of significant formulation parameters on the particle size were fitted into the regression equation (Equation 1) which is used for prediction of NP size, with F_1, F_2 and F_3 representing C_{BSA}, C_{PLGA} and C_{PVA}, respectively and VR being represented by F_4. At a desirability value of the independent formulation parameters (i.e. C_{BSA} = 2 %w/v, C_{PLGA} = 1 %w/v, C_{PVA} = 0.625 %w/v and VR = 4, equivalent to 20 ml of external aqueous phase), Figures 3 to 8 were generated. The figures were then employed to study the relation of the formulation parameters at a 2-dimensional space.

Equation 1:

$$Y = -471.09 + 310.83F_1 + 480F_2 + 308.89F_3 + 317.87F_3^2 + 35F_4 - 195F_1 * F_2 - 241.33F_1 * F_3$$

Table 3. **ANOVA results obtained by statistical analysis of formulation parameters on NP size**

Factor	SS	df	MS	F	*P*-value
C_{BSA} (L)	14700.0	1	14700.00	5.18031	0.034609
C_{PLGA} (L)	24300.0	1	24300.00	8.56336	0.008662
C_{PVA} (L+Q)	97656.9	2	48828.47	17.20724	0.000054
VR (L)	61061.3	1	61061.33	21.51812	0.000179
C_{BSA} * C_{PLGA}	38025.0	1	38025.00	13.40008	0.001662
C_{BSA} * C_{PVA}	32761.0	1	32761.00	11.54503	0.003019
Error	53915.7	19	2837.67		
Total SS	322420.0	26			

Q, quadratic; L, linear; df, degree of freedom; C_{BSA} * C_{PVA}, Interaction between C_{BSA} and C_{PVA}; C_{BSA} * C_{PLGA}, Interaction between C_{BSA} and C_{PLGA}.

Results

According to the results of statistical analysis evaluated by STATISTICA™ Ver.12.0 software package (Stat Soft Inc., USA), the Mean Square (MS) residual reported in this model is 2837.67 which corresponds to the highest standard error of 53.26979 (Standard error= √(MS Residual)) of interaction between BSA with PLGA and PVA (See Table 4). The applied Box–Behnken design within equally spaced level of each independent parameters sufficiently proves to fit a quadratic model (equation 1) with R^2 of 0.83278. The residual errors reported from model equation 1 for the predictions of NPs from 27

Table 4. **Effect estimate obtained by statistical analysis of formulation parameters on NP size**

Effect estimates on size (nm); R^2= 0.83278, MS Residual = 2837.67						
Factor	**Effect**	**Standard error**	***t*-value**	***P*-value**	**−95% Confidence limit**	**+95% Confidence limit**
Mean/Interc.	428.600	11.23026	38.16475	0.000000	405.095	452.1052
C_{BSA} (L)	−70.000	30.75533	−2.27603	0.034609	−67.186	−2.8142
C_{PLGA} (L)	90.000	30.75533	2.92632	0.008662	12.814	77.1858
C_{PVA} (L)	167.667	30.75533	5.45163	0.000029	51.648	116.0192
C_{PVA} (Q)	−44.700	20.63130	−2.16661	0.043185	−43.941	−0.7591
VR (L)	142.667	30.75533	4.63876	0.000179	39.148	103.5192
C_{BSA} (L) by C_{PLGA} (L)	−195.000	53.26979	−3.66061	0.001662	−153.247	−41.7525
C_{BSA} (L) by C_{PVA} (L)	−181.000	53.26979	−3.39780	0.003019	−146.247	−34.7525

Q, quadratic; L, linear; C_{BSA} * C_{PVA}, Interaction between C_{BSA} and C_{PVA}, CBSA * C_{PLGA}, Interaction between C_{BSA} and C_{PLGA}.

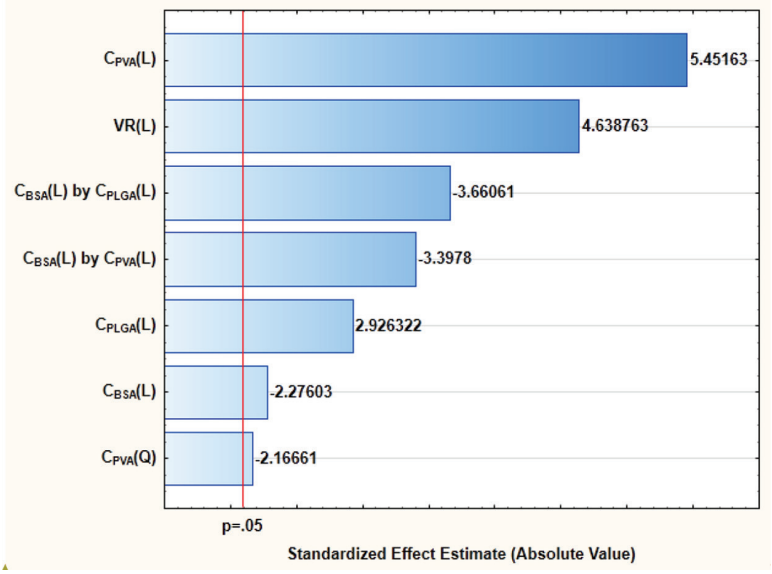

Fig. 1 **Pareto chart on the effects of independent parameters in order of their significance is shown.**

experiments carried out varies within the limits of −91.13 (Run Order 7) and +97.87 (Run Order 27). Figure 2 represents the prediction of NP size from equation 1 in comparison with the experimentally obtained values. As shown in the Pareto chart (Fig. 1) and Table 4, it was found that the independent formulation parameters (F_1, F_2, F_3 and F_4) had significant linear effects on the size of NPs. Only C_{PVA} had both linear and quadratic effects on NP size. Significant linear interactions also existed between C_{BSA} and C_{PLGA} (1L by 2L) as well as C_{BSA} and C_{PVA} (1L by 3L).

Validation

Using the software option for optimization to obtain the smallest particle size, optimum values for input variables was suggested as 0.6 (%w/v), 1 (%w/v), 0.1 (%w/v) and 6 for *F*1 to F4, respectively. The predicted size (nm) was 312.0. To validate the model, five replicates of the optimized sample were experimentally prepared and the particle size was measured as mean (SD) 298.8 (49.9 nm), showing the capability of the model to predict the size. The optimum sample showed mean (SD) encapsulation efficiency of 66.3 (1.2), using the following equation:

Encapsulation efficiency = (Amount of encapsulated BSA / Initial amount of BSA) × 100

Effect of BSA concentration

According to the Pareto chart, concentration of BSA has significant but small effect on the NP size. When either PLGA or PVA are low, increasing BSA concentration increases the size. Whereas at high values of PLGA or PVA, increasing BSA makes the size smaller (see Figs. 3 and 4). From Figure 5, the effect of BSA on particle size is observed to be negligible when compared to an increase in VR between the first emulsion and external aqueous phase.

Effect of PLGA Concentration

Statistical effect estimates of PLGA in Table 4 as well as Figures 3, 6 and 7 show that increasing PLGA at the organic phase (DCM) increases the NPs size. The only exception is when

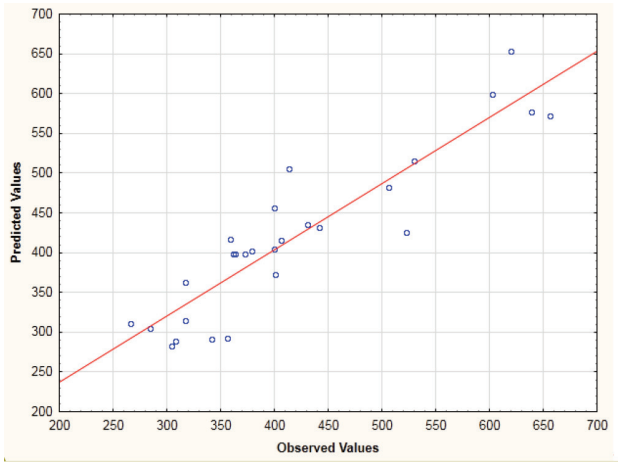

Fig. 2 **Fitted curve of observed and predicted NP size based on the regression equation (R^2= 0.83278).**

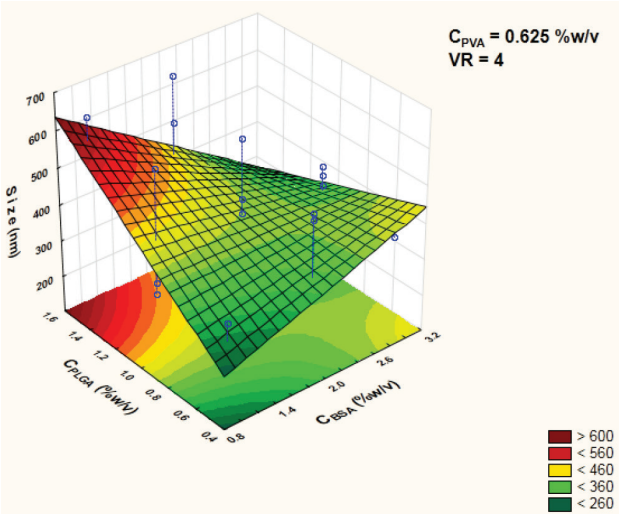

Fig. 3 **Response surface curve of BSA interacting with PLGA at a fixed concentration of PVA (0.625 %w/v) and at fixed volume ratio (4, equivalent to 20 ml of external aqueous solution containing PVA).**

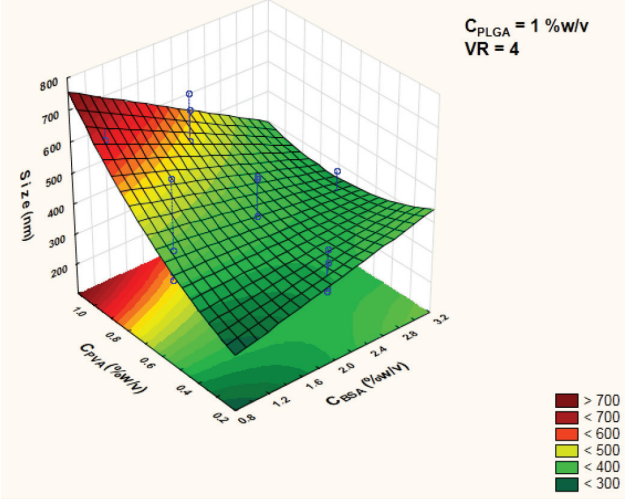

Fig. 4 **Response surface curve of BSA interacting with PVA at a fixed concentration of PLGA (1 %w/v) and at a fixed volume ratio (4, equivalent to 20 ml of external aqueous solution containing PVA).**

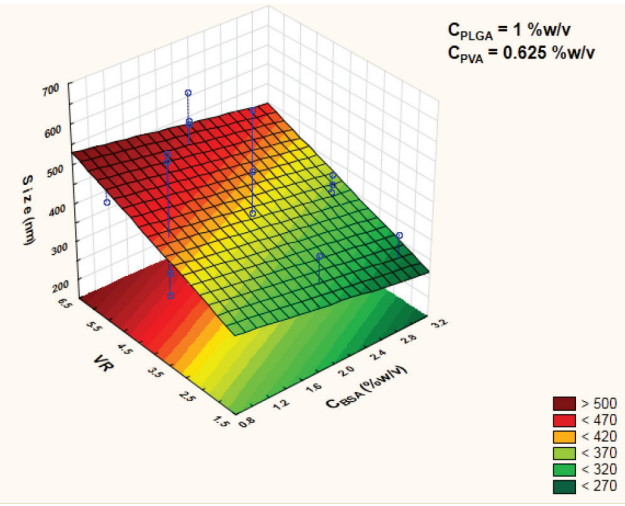

Fig. 5 **Response surface curve of BSA interacting with VR at a fixed concentrations of PLGA (1 %w/v) and PVA (0.625 %w/v).**

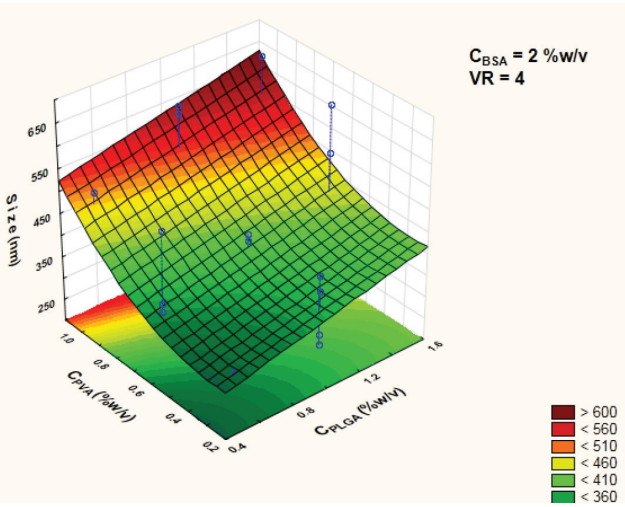

Fig. 6 **Response surface curve of PLGA interacting with PVA at a fixed concentration of BSA (2 %w/v) and VR (4, equivalent to 20 ml of external aqueous solution containing PVA).**

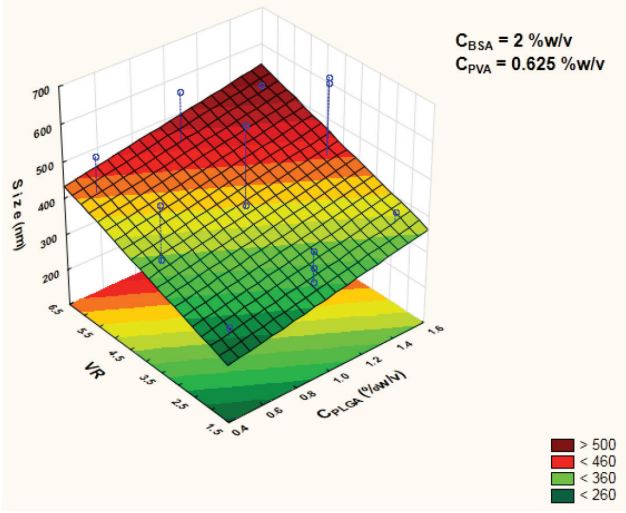

Fig. 7 **Response surface curve of PLGA interacting with VR at a fixed concentrations of BSA (2 %w/v) and PVA (0.625 %w/v).**

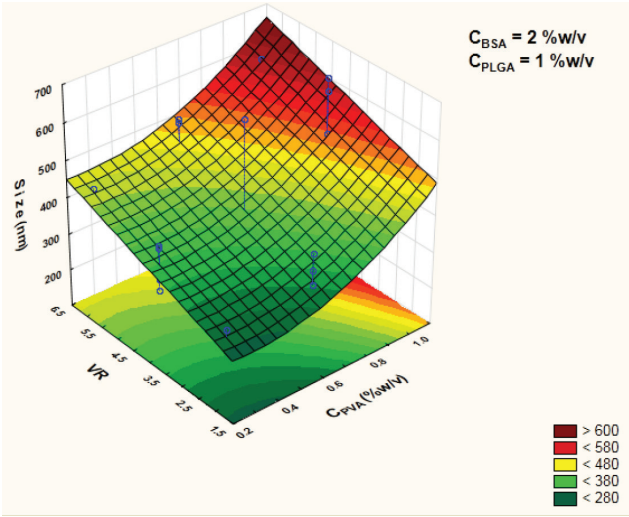

Fig. 8 **Response surface curve of PVA interacting with VR at a fixed concentrations of PLGA (1 %w/v) and BSA (2 %w/v).**

C_{BSA} is high, whereby increases in C_{PLGA} makes the NP size slightly smaller (see Fig. 3).

Effect of PVA Concentration

From the Pareto chart, the effect of PVA at the external aqueous phase was observed to be a significant parameter affecting the NP size. Such that, an increase in C_{PVA} results to production of larger particle size with an exception of small reverse effect on size when C_{PVA} is low and C_{BSA} is high. C_{PVA} appears to be the dominant parameter affecting the size in the values we studied for the four independent parameters. The exception is in Fig. 4 where at high BSA values, size appears to be nearly independent on C_{PVA}.

Effect of Volume Ratio of emulsions

The VR between the first emulsion and external aqueous phase gave rise to each experimental formulation with a total volume in the range of 15 ml to 35 ml. According to the Pareto chart, the VR phase was observed to be a significant parameter affecting particle size, such that, increasing VR increases the

NP size. At various levels of each parameters, Fig. 5, Fig. 7 and Fig. 8 shows that increasing the VR also Increase the NPs size. At a fixed VR (Fig. 6), increment in particle size was also observed while increasing both C_{PLGA} and C_{PVA}.

Discussions

BSA concentration was varied from 0.5 to 1 % (w/v) (~ 25 to 50 mg) at the inner aqueous phase. C_{BSA} was evaluated and it was observed to have a fuzzy effect on NPs size. Increasing BSA concentration has been observed to show either little or no effect on the mean particle size of PLGA NPs.[15] Since BSA is introduced into the primary emulsions and many other factors will then affect the emulsification process, it is arguable that such factors (e.g. sonication of secondary emulsion) will dominate the effect of BSA concentration.

Increasing C_{PLGA} at the organic phase increased the particle size. In a similar work that used a full factorial central composite design as a response surface methodology observed an increment in the size of PLGA NPs loaded with Insulin-lauryl sulfate increases while increasing the C_{PLGA}.[16] Another similar work also observed the production, via DESE, of larger PLGA particle size encapsulating sildenafil citrate with increasing the C_{PLGA}.[13] This is probably due to the fact that increasing PLGA concentration makes the viscosity larger.[17] Thus, the effect of sonication on breaking the particles into smaller ones become less pronounced.

From our findings, PVA showed two distinct effects on the preparation process of nanoparticles. While it plays a stabilizing role, it also increases the viscosity of the solution. Therefore, by increasing the CPVA, stability of the preparation increases which in turn decreases the size. At the same time, lesser effect is expected from sonication due to increase in viscosity. In our work, the viscosity increasing effect of PVA appears to be dominant which in turn increased the particle size. The type of surfactant and its concentration during DESE method, influences the production process and long term stability of the NPs by preventing the emulsion droplets from coagulation, coalescence and precipitation through formation of a protective layer on emulsion droplets. Several challenges have been reported regarding the optimum concentration of PVA as a surfactant/stabilizer at the external aqueous phase of the DESE.[18] It has also been observed that increase in NP size is related to excess PVA at the external aqueous phase of DESE.[15,19] In a similar study, haloperidol was loaded into PLGA NPs, the mean diameter of NPs was observed to decrease, then, gradually increased while increasing C_{PVA}.[20] In a similar study, in which hyaluronidase was encapsulated into PLGA NPs via DESE, increase in C_{PVA} was observed to increase the particle size.[21] A study also reported decrease in NPs size of PLGA encapsulating BSA when increasing C_{PVA} at the external aqueous phase from 0% to 0.4%.[22]

Increasing the VR was observed to increase particle size. A report which used fractional factorial design to study the effect of formulation variables on hyaluronidase loaded PLGA NPs also observed an increment in particle size while increasing the volume of external aqueous phase containing PVA.[21] The direct increment in particle size is suggested to be attributed to viscosity of first and final emulsion. Such that, the simultaneous increase of PLGA at the first emulsion with PVA at the external aqueous phase results to a more viscous emulsion which could not be breakdown efficiently by the available sonication and stirring parameters, then resulting to an increment in NPs size.

Conclusions

This study demonstrated that it is possible to investigate, using a Box–Behnken response surface methodology, the effect of formulation parameters on the size of PLGA NPs encapsulating proteins with BSA as a model protein. Concentrations of PLGA, PVA as well as VR showed to have a direct effect on particle size while the concentration of BSA showed a negligible effect on particle size. Generally, mechanism based on viscosity of emulsion droplets could be regarded as the dominant effect controlling the particles size.

Acknowledgments

The authors would like to thank the office of International Campus, Tehran University of Medical Sciences (TUMS) for its financial support.

Appendix

- F_1= C_{BSA} = Concentration of BSA
- F_2 = C_{PLGA} = Concentration of PLGA
- F_3= C_{PVA} = Concentration of PVA
- F_4 =VR = Volume Ratio of the primary emulsion to the external aqueous phase.
- Q = Quadratic effect
- L = Linear effect

Conflict of Interest

None.

References

1. Anderson JM, Shive MS. Biodegradation and biocompatibility of PLA and PLGA microspheres. Advanced drug delivery reviews. 2012;64:72–82.
2. Astete CE, Sabliov CM. Synthesis and characterization of PLGA nanoparticles. J Biomater Sci Polym Ed. 2006;17:247–289.
3. Jain RA. The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. Biomaterials. 2000;21:2475–2490.
4. Fong JW, Nazareno JP, Pearson JE, Maulding HV. Evaluation of biodegradable microspheres prepared by a solvent evaporation process using sodium oleate as emulsifier. J Control Release. 1986;3:119–130.
5. Bodmeier R, McGinity JW. The preparation and evaluation of drug-containing poly(dl-lactide) microspheres formed by the solvent evaporation method. Pharm Res. 1987;4:465–471.
6. Brodin AF, Kavaliunas DR, Frank SG. Prlonged drug release from multiple emulsions. Acta Pharm Suec. 1978;15:1–12.
7. Schuster D. Encyclopedia of emulsion technology: CRC Press; 1996.
8. Matsumoto S, Kohda M. The viscosity of W/O/W emulsions: an attempt to estimate the water permeation coefficient of the oil layer from the viscosity changes in diluted systems on aging under osmotic pressure gradients. J Colloid Interface Sci. 1980;73:13–20.
9. Taghipour B, Yakhchali M, Haririan I, Tamaddon AM, Samani SM. The effects of technical and compositional variables on the size and release profile of bovine serum albumin from PLGA based particulate systems. Res Pharm Sci. 2014;9:407–420.
10. Zhang JX, Zhu KJ. An improvement of double emulsion technique for preparing bovine serum albumin-loaded PLGA microspheres. J Microencapsul. 2004;21:775–785.
11. Blanco M, Alonso M. Development and characterization of protein-loaded poly (lactide-co-glycolide) nanospheres. Eur J Pharm Biopharm. 1997;43:287–294.

12. Ferreira SL, Bruns RE, Ferreira HS, Matos GD, David JM, Brandão GC, et al. Box–Behnken design:an alternative for the optimization of analytical methods. Anal Chim Acta. 2007;597:179–186.
13. Ghasemian E, Vatanara A, Najafabadi AR, Rouini MR, Gilani K, Darabi M. Preparation, characterization and optimization of sildenafil citrate loaded PLGA nanoparticles by statistical factorial design. DARU Journal of Pharmaceutical Sciences. 2013;21:1.
14. Sharma D, Maheshwari D, Philip G, Rana R, Bhatia S, Singh M, et al. Formulation and optimization of polymeric nanoparticles for intranasal delivery of lorazepam using Box–Behnken design: *in vitro* and *in vivo* evaluation. BioMed Res Int. 2014;2014:156010.
15. Azizi M, Farahmandghavi F, Joghataei M, Zandi M, Imani M, Bakhtiary M, et al. Fabrication of protein-loaded PLGA nanoparticles:effect of selected formulation variables on particle size and release profile. J Polym Res. 2013;20:110.
16. Shi K, Cui F, Yamamoto H, Kawashima Y. Optimized formulation of high-payload PLGA nanoparticles containing insulin-lauryl sulfate complex. Drug Dev Ind Pharm. 2009;35:177–184.
17. Sharma N, Madan P, Lin S. Effect of process and formulation variables on the preparation of parenteral paclitaxel-loaded biodegradable polymeric nanoparticles: A co-surfactant study. Asian J Pharm Sci. 2016;11:404–416.
18. Feczkó T, Tóth J, Gyenis J. Comparison of the preparation of PLGA–BSA nano-and microparticles by PVA, poloxamer and PVP. Colloids and Surfaces A. Chem Eng Process. 2008;319:188–195.
19. Feczkó T, Tóth J, Dósa G, Gyenis J. Influence of process conditions on the mean size of PLGA nanoparticles. Chem Eng Process. 2011;50:846–853.
20. Budhian A, Siegel SJ, Winey KI. Haloperidol-loaded PLGA nanoparticles: systematic study of particle size and drug content. Int J Pharm. 2007;336:367–375.
21. Narayanan K, Subrahmanyam V, Venkata Rao J. A fractional factorial design to study the effect of process variables on the preparation of hyaluronidase loaded plga nanoparticles. Enzyme Res. 2014;2014:162962.
22. Lamprecht A, Ubrich N, Hombreiro Pérez M, Lehr C, Hoffman M, Maincent P. Influences of process parameters on nanoparticle preparation performed by a double emulsion pressure homogenization technique. Int J Pharm. 2000;196:177–182.