Preparation, Characterization and Optimization of Egg Albumin Nanoparticles as Low Molecular-Weight Drug Delivery Vehicle

Elham Sadat Taheri*, **, Mohsen Jahanshahi*, Mohammad Taghi Hamed Mosavian***

(Received: 27 July 2011; in revised form: 8 June 2012; accepted: 10 June 2012)

DOI: 10.1002/ppsc.201100037

Abstract

Protein nanoparticles have been recognized as carriers to deliver low molecular-weight drugs, anticancer drug, DNA, vaccines, oligonucleotides, peptides and etc. The purpose of this research was preparation of Egg Albumin (EA) nanoparticle with suitable size/size distribution and good surface properties for drug delivery application based on simple coacervation method along with optimization of the nanoparticles by employing Taguchi method. Several synthesis parameters were examined to characterize their impacts on nanoparticle size and topography. These variables were including temperature, EA concentration, desolvating agent volume, pH value and agitation speed. In addition, size and morphology of prepared nanoparticles were analyzed by photon correlation spectroscopy (PCS) as well as atomic force microscopy (AFM). As result of Taguchi analysis in this research, desolvating agent volume and pH were most influencing factors on particle size. The minimum size of nanoparticles (~51 nm) were obtained at Temperature 55 °C, 30 mg/ml EA concentration, desolvating agent volume 50 ml, agitation speed of 500 rpm and pH 4. The mechanistic of optimum conditions for preparing protein nanoparticles from Egg Albumin for the first time and their characterization as delivering nano system are discussed.

Keywords: coacervation method, drug delivery, egg albumin, nanoparticle, taguchi method

1 Introduction

Nowadays, development of new colloidal drug delivery system for controlled and targeting release of drugs has become more interest. One of the most important aspects of ideal drug delivery system is transportation of the associated drug to its desired site of action and release drug at an optimum rate to improve the therapeutic index. Therefore, our expectations from suitable drug carriers and effective drug delivery systems are: a) improving drug bioavailability through enhancing aqueous solubility, b) increasing the residence time in the body (increasing the half-life for clearance/increasing specificity for its cognate receptor), c) targeting the drug to a specific location in the body with a concomitant reduction in the quantity of drug required and dosage toxicity, enabling the safe delivery of toxic therapeutic drugs and protection of non-target tissues and cells from severe side effects, d) degradation of carriers *in vivo* in order to not accumulate indefinitely in the tissues, e) non-toxicity of carriers [1 – 3].

Nanoparticles have been emerged as essential strategy for drug delivery. Nanoparticles have many unique properties that make them suitable as effective drug carriers. Advantages of using these particles are including [4 – 5]:

(I) Easy manipulation of particle size and surface characteristics of nanoparticles to can be prepared passive and active drug targeting after parenteral administration.

(II) In order to enhancement of drug therapeutic efficiency, nanoparticles able to control, modify and

* E. S. Taheri, M. Jahanshahi (corresponding author), Nanotechnology Research Institute, Faculty of Chemical Engineering, Babol University of Technology, Babol (Iran).
E-mail: mjahan@nit.ac.ir

** E. S. Taheri, Chemical Engineering Department, Quchan Branch, Islamic Azad University (Iran).

*** M. T. H. Mosavian, Faculty of Chemical Engineering, Ferdowsi University of Mashhad, Mashhad (Iran).

sustain release of the drug during the transportation and at the site of localization, altering organ distribution of the drug and subsequent clearance of the drug. Also they capable to reduce unwanted effects by controlled release.

(III) Selection of matrix constituents gives this possibility to modulate Controlled release and particle degradation characteristics. Drug loading is relatively high and drugs can be incorporated into the systems without any chemical reaction, this property is important for preserving the drug activity.

(IV) Site-specific was targeted by attaching targeting ligands to particles surface or use of magnetic guidance.

Nanoparticles can be fabricate from different materials consist of synthetic biodegradable polymers, natural biopolymer, lipid, polysaccharides and etc. Many factors affect on selection of nanoparticles matrix such as [6 – 7]:
(a) Size of nanoparticles required; (b) Inherent properties of the drug, e.g., aqueous solubility and stability; (c) Surface characteristics such as charge and permeability; (d) Degree of biodegradability, biocompatibility and toxicity; (e) Drug release profile desired; and (f) antigenicity of the final product.
Polymeric nanoparticles have shown a certain degree of success for the delivery of proteins and vaccines to systematic circulation and to the immune system [6]. These nanoparticles have many attractive properties for example size, surface potential, hydrophilic hydrophobic balance and etc that make them suitable as potential carriers for bioactive component such as anticancer drug, vaccine, oligonucleotides, peptides, etc. [8].
Use of natural biodegradable polymers for production of nanoparticles as drug delivery system have been developed recently [9]. Among them proteins such as albumin, gelatin, gliadin, legumin and etc are very promising and play very important role in delivery system [10]. They have many benefits than other drug carriers. Some of these advantages are discussed below [11 – 18]:
(a) Greater stability during storage and long shelf life, (b) Stability *in vivo*, (c) Ease of scale-up during manufacture, (d) Biodegradable, (e) Non-antigenic, (f) Metabolizable and can also be easily amenable for surface modification and covalent attachment of drugs and ligands, (g) Ability to deliver proteins, peptides and gene, (h) Non-toxicity, (i) Increase the stability of drugs and useful controlled released properties, (j) Synthetic protein nanostructure act as surrogate mimics such as viruses and plasmid for drug delivery system.
According to literature, albumin is an attractive protein with good and important features that is employed for nanoparticle fabrication as drug delivery system. Some of these attractive properties are consist of biodegradation into natural products, lack of toxicity and antigeniciy, maintenance of constant or nearly constant blood level, enhancement of patient compliance, ready availability [19 – 20]. Also a number of researches have reported that accumulation of albumin in solid tumors make it a potential macromolecular carrier for the site-directed delivery of antitumor drugs [21 – 22].
In this work, preparation of Egg Albumin nanoparticles in the unique range which become suitable for drug delivery application by simple coacervation method are performed. In order to find design controlling factor, the effect of various fabrication conditions such as pH value, temperature, initial protein concentration, agitation speed and amount of desolvating agent upon nanoparticles properties are examined herein. In addition, AFM is used to analysis shape and morphology of nanoparticles at different operation parameters. However, Taguchi robust design method is utilized to finally optimize the prepared EA nanoparticle size.

2 Materials and Methods

2.1 Materials

Egg albumin (very high purity) and glutaraldehyde (25 % solution) were commercially supplied by Sigma Aldrich. Acetone and all other chemicals were supplied from Merck (Germany).

2.2 Preparation of EA Nanoparticle

EA nanoparticles were prepared by a simple coacervation process. Certain concentration between 10 – 30 mg/ml of aqueous solution of EA at operation pH and temperature was stirred on magnetic stirrer and then amount of acetone (desolvating agent), was added drop-wise until the solution become just turbid. After desolvation process, 300 µl of 25 % glutaraldehyde was added as cross linking agent and stirred continuously at room temperature for 12 hr. The nanoparticle sample was purified with 4000 rpm centrifuge for 20 min then the supernatant was dialyzed.
Changes of influential synthesis parameters on particle size that were studied in our experiments are illustrate in Table 1. In order to investigate the effect of each

Table 1: Variables and their employed value in experiments.

Factors		levels		
Temperature (°C)	(A)	35	45	55
EA concentration (mg/ml)	(B)	10	20	30
Acetone volume (ml)	(C)	50	75	100
Agitation speed (rpm)	(D)	500	600	700
pH value	(E)	4	7	9

parameter on particles size, other conditions were fixed and only desired parameter was variable.

2.3 Determination of Nanoparticle Size/Size Distribution and Nanoparticle Morphology

The size distribution of the fabricated EA nanoparticles was determined by photon correlation spectroscopy (PCS). PCS is best industrially method for size analysis of particles with sub-micron size. Before samples analyzed in the PCS device, particles should be disperse well in liquid medium. In such conditions the particles are in constant random motion, referred to as Brownian motion and PCS measures the speed of this motion by passing a laser. PCS determines the average particle size and Polydispersity Index (PI) which is a range of measurement of the particle sizes within measured samples. The accurate measurement of particle size must be blow 0.7 (70 %).

Atomic force microscopy (AFM) is one of technique for analysis of nanoparticles morphology. AFM is a very high-resolution type of scanning probe microscope, with demonstrated resolution of fractions of a nanometer, more than 1000 times better than the optical diffraction limit.

2.4 Taguchi Method

Numerous experiments are needed to know effect of main and controlling parameters on particle size, therefore, it is very time and costs consuming. In order to prevent this, Taguchi method is employed for design of optimum number of experiments. Taguchi tool is most important statistical method has been developed for efficient analysis of complex system. This method supply systematic, simple and effective approach for design of experimentation to estimate optimum condition of influential factors with only a few experimental sets [23 – 25]. Signal- to-noise ratio (S/N), orthogonal array (OA) and ANOVA are most important and key analyzer in this method so that (S/N) component evaluates quality characteristics and also discrepancy from desired target is determined by this component. Signal refers to desirable and noise shows undesirable value for output characteristic. Therefore, title of smaller or bigger for (S/N) is used in analysis for better accuracy. For smaller the better type of (S/N) that is suitable in our work, this ratio is defined by following equation [26 – 28]:

$$\eta = -10\log\left(\left(\frac{1}{n}\right)\sum_{i=1}^{n} yi^{2}\right)$$

Where η is the average (S/N), 'n' is the number of experimental performed at level 'i' and 'yi' is the approved percentage of parameter y. A robust system will have a high (S/N). (S/N) should be as large as possible for higher values of approved percentages. Another major tools used in the Taguchi method is OA. This is a matrix of numbers arranged in rows and columns. Each row represents the level of factors in each run and each column represents a specific level for a factor that can be changed for each run [6]. Figure 1 explains Taguchi methodology for optimization.

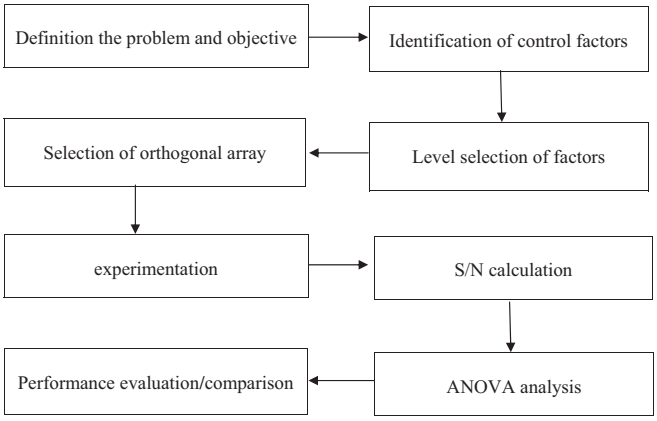


Fig. 1: Flowchart of Taguchi method for influential parameter optimization.

3 Results and Dicussions

3.1 Effect of Different Parameters on Nanoparticles Size

Size/ size distribution and surface properties of carriers are very important factors in drug delivery system so that influence drug distribution in body [29 – 30]. Result of studies and researches have been demonstrated that particles with smaller size as drug delivery vehicle have most advantage than larger size [31].

It was reported that particle size has significantly impact on cellular and tissue uptake and only submicron nanoparticles can be taken up efficiently but not the larger size microparticles in some cell lines. For example, in a Caco2cell line, nanoparticles that had 100 nm size shown 2.5 and 6 fold greater uptakes than 1 μm and 10 μm microparticles [32 – 33]. Another example is for nanoparticles that have size larger than 230 nm, these particles accumulate in the spleen due to the capillary size in this organ [34].

It was found that nanoparticles can cross the blood-brain barrier following the opening of tight junctions by hyper osmotic mannitol, which may supply sustained delivery of therapeutic agents for difficult-totreat dis-

eases like brain tumors. Tween 80 coated nanoparticles have been shown to cross the blood-brain barrier [35].
Particle size, also influence drug release. Since smaller particles have larger surface area, therefore, most of the drug associated would be at or near the particle surface, leading to fast drug release, While, larger particles have large cores which allow more drug to be encapsulated and slowly diffuse out [36].
Smaller nanoparticles show higher stability than larger size so that stability of nanoparticles as drug carriers improve with decreasing particle size in range of 100 – 200 nm and creates the chance of escaping from the vascular system via cavities in the lining of blood vessel [37].
Therefore, our purpose was fabrication of small nanoparticles with narrow size distribution to efficient as carrier for drug delivery. At first, we examined the effect of various parameters on particle size in order to find influencing parameters upon the size of nanoparticles. Results are shown Figures 2 – 6.
Figure 2 illustrates the effect of temperature on nanoparticle size. Temperature is one of the most important influential factor on particle size. As can be seen, increase of temperature from 35 to 55 °C lead to decreasing particle size. In our previous work, increasing temperature decrease size of nanoparticles for gelatin and α-lactalbumin while inverse trend were shown for BSA nanoparticles [3, 38 – 39].

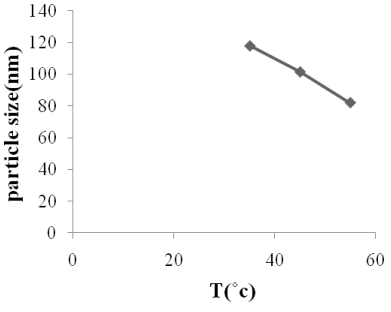


Fig. 2: Effect of temperature on nanoparticle size (other constant operation conditions are: pH = 7_ C = 10 mg/ml_ agitation speed = 600 rpm_ acetone volume = 50 ml).

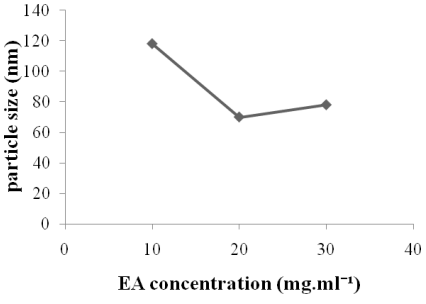


Fig. 3: Effect of initial concentration of EA on nanoparticle size (other constant operation conditions are: T = 35 °C_ pH = 7_ agitation speed = 600 rpm_ acetone volume = 50 ml).

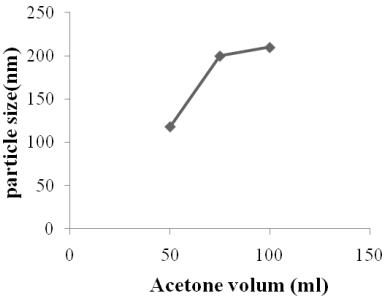


Fig. 4: Effect of acetone volume on nanoparticle size (other constant operation conditions are: T = 35°C_ C = 10 mg/ml_ agitation speed = 600 rpm_ pH = 7).

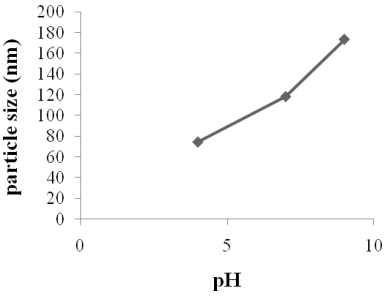


Fig. 5: Effect of pH on nanoparticle size (other constant operation conditions are: T = 35 °C_ C = 10 mg/ml_ agitation speed = 600 rpm_ acetone volume = 50 ml).

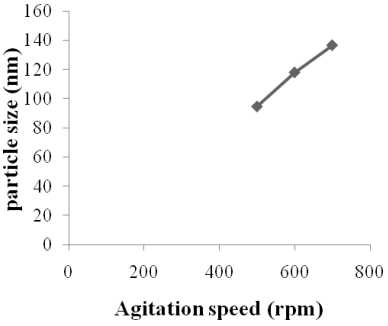


Fig. 6: Effect of agitation speed on nanoparticle size (other constant operation conditions are: T = 35 °C_ C = 10 mg/ml_ pH = 7_ acetone volume = 50 ml).

The effect of EA concentration was also investigated in this research. Figure 3 shows with increase of EA concentration, prepared nano particles have smaller size, however increase of concentration from 20 to 30 mg/ml resulting in little increasing size of particles. This trend was similar to our previous study for BSA nanoparticles. Also decreasing trend of size with increase of concentration was observed for gelatin nanoparticles but HSA nanoparticles shown decreasing and increasing trend in size with increase of concentration in our previous research [6, 38 – 39].
Another parameter that affect on particle size is amount of desolvating agent (acetone). From Figure 4 can be found that as the acetone volume increased, also the

particle size increased. This result is similar to effect of desolvating agent on size of BSA nanoparticles while inverse trend were shown for HSA. Gelatin nanoparticles also decreased with increasing of desolvating agent but at certain volume of desolvating agent, the particle size increased [6, 38 – 39].

Figure 5 explains size of particles influenced with the change of pH value. Increasing trend of nanoparticles size with increase of pH was observed for EA. Our performed studies for other proteins were which, BSA nanoparticles particle size not influenced with the change of pH since the concentration of BSA was in the low range. Of course the size of particles slightly fluctuated. Increase of pH lead to increasing size for α-lactalbumin nanoparticles in range of pH value between below and top of albumin isoelectric point but inverse trend for HSA nanoparticle was obtained [3, 6, 38].

Influence of agitation speed on particle size another factor that was evaluated. Figure 6 displays result of this effect. Generally, the size of particle is expected to reduce with increasing trend of agitation speed [40] while in this study, at 500 rpm agitation speed, minimum size was obtained. This similar trend was observed for BSA nanoparticle while inverse trend was obtained for gelatin nanoparticles in our previous work [38 – 39].

In addition, results show different effects of influential parameters on the particle size for each NP system (e.g. EA, BSA, and HSA). It is obvious that type and nature of matrix material is one of the important reasons for such difference. The initial protein materials are obtained and derived from different sources (e.g. blood plasma, skin, egg, and milk-whey). They have different physicochemical properties (e.g. size, molecular weight, iso-electric point, surface potential and hydrophilic-hydrophobic balance), different bio-molecules structure and etc. Therefore, it is clear that each studied parameter (e.g. temperature, pH, C) might have special influence on different materials.

3.2 Physical Characteristics of EA Nanoparticles

AFM method was used widely in this work to provide prepared EA nanoparticle morphology and surface information. Also effect of different production parameters on topography of nanoparticles was studied. Results are shown in Figures 7 – 11. As can be seen from figures image of shape and surface characteristic were achieved successfully, naoparticles were formed by smooth surface and their shapes are semispherical.

According to literatures, the results presented herein indicate EA nanoparticles have unique and good properties in order to be candidate as carrier for loading drug systems.

Topography of nanoparticles has been analyzed by AFM at various dimensions since the purpose was determination of morphology and surface of particles, therefore images which were clearer are shown herein.

3.3 Taguchi's Orthogonal Array Design

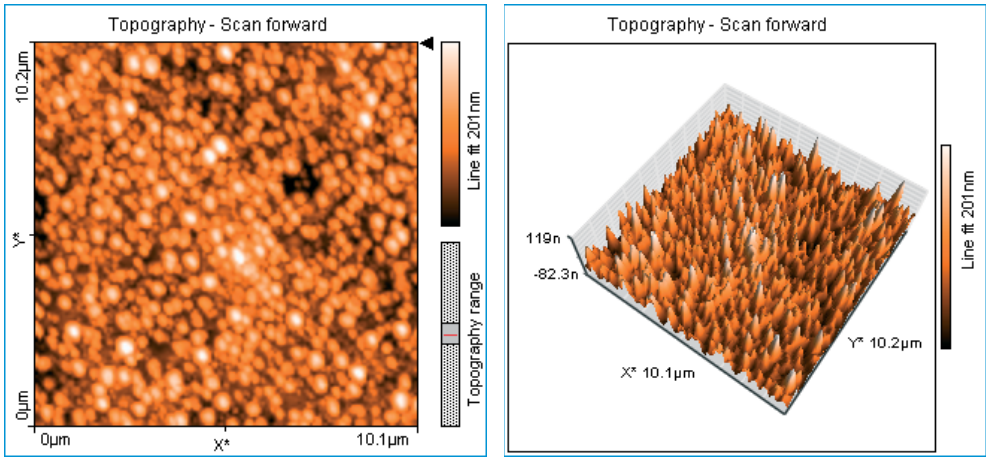
Since complex relationship exists between the mentioned influential parameters thereby optimization of these parameters for synthesis EA was performed herein.

Choice of design parameters in Taguchi optimization should be based on experiments, on the other hand, no general guideline exists for determination of them. According to experiments in the previous section, we found all of studied parameters affect on particle size, therefore all of them can be selected as design factors. Table 1 shows effective design factors and their levels used in Taguchi design method.

The number of experiments to be performed for five factors and three signal levels under full-factorial testing is 243. If 'm' effective parameters are became choice with 'n' signal levels, total number of experiments to be performed are 'n^m' [6]. According to this calculation, total number of experiments become very large, therefore, Taguchi orthogonal array suggests a serious of experiments which is selected based on the number of factors, interactions between them and the number of signal levels of each factor. Table 2 illustrates 'L_{18}' standard OA used for the five factors, each set at three signal levels.

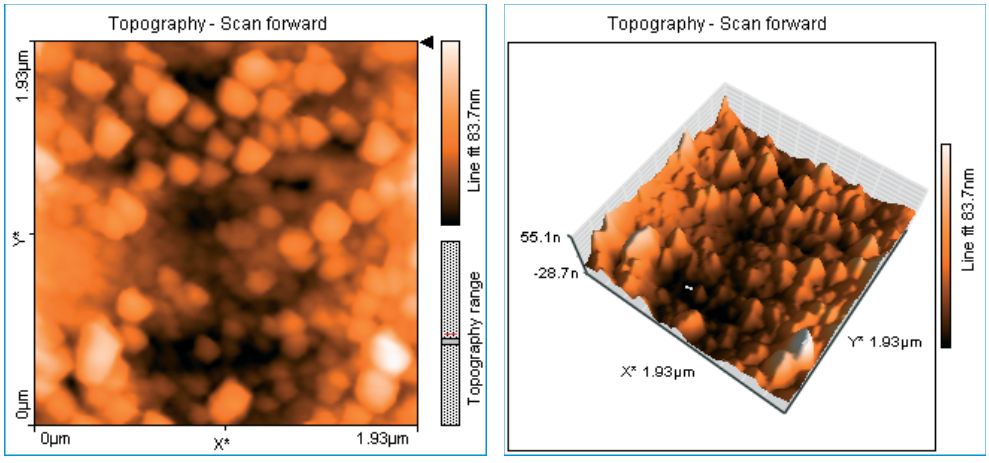
Table 2: Taguchi orthogonal array table of 'L_{18}' (experimental measured values for EA nanoparticle size and S/N ratio).

Experimental conditions							
EXP. NO.	A	B	C	D	E	Particle size (nm)	S/N Ratio (dB)
1	1	1	1	1	1	78	–37.84
2	1	2	2	2	2	141	–42.98
3	1	3	3	3	3	160	–44.08
4	2	1	1	2	2	101.5	–40.13
5	2	2	2	3	3	142.2	–43.06
6	2	3	3	1	1	102	–40.17
7	3	1	2	1	3	143.5	–43.14
8	3	2	3	2	1	99.5	–39.96
9	3	3	1	3	2	92	–39.28
10	1	1	3	3	2	181	–45.15
11	1	2	1	1	3	110	–40.83
12	1	3	2	2	1	107	–40.59
13	2	1	2	3	1	135	–42.61
14	2	2	3	1	2	117.5	–41.4
15	2	3	1	2	3	113.5	–41.1
16	3	1	3	2	3	165.5	–44.38
17	3	2	1	3	1	79.5	–38
18	3	3	2	1	2	95.5	–39.6



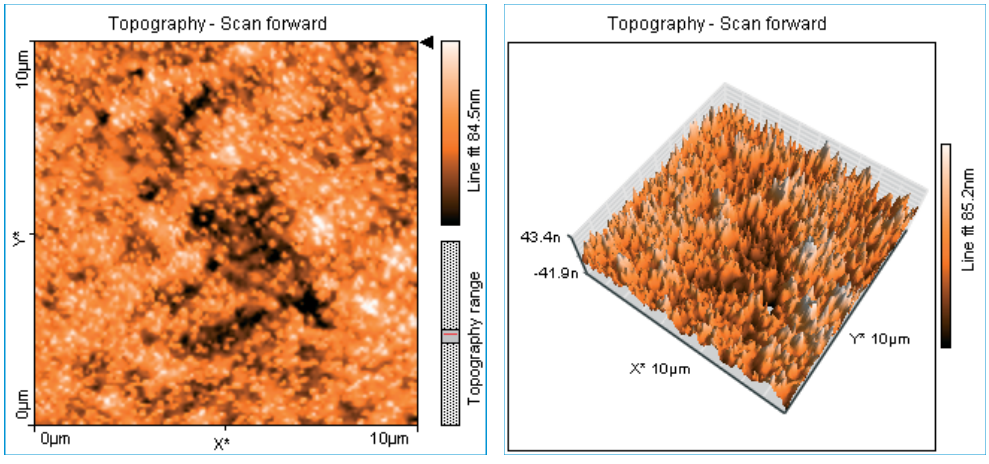
Two dimension Three dimension

(7-a) Base condition



Two dimension Three dimension

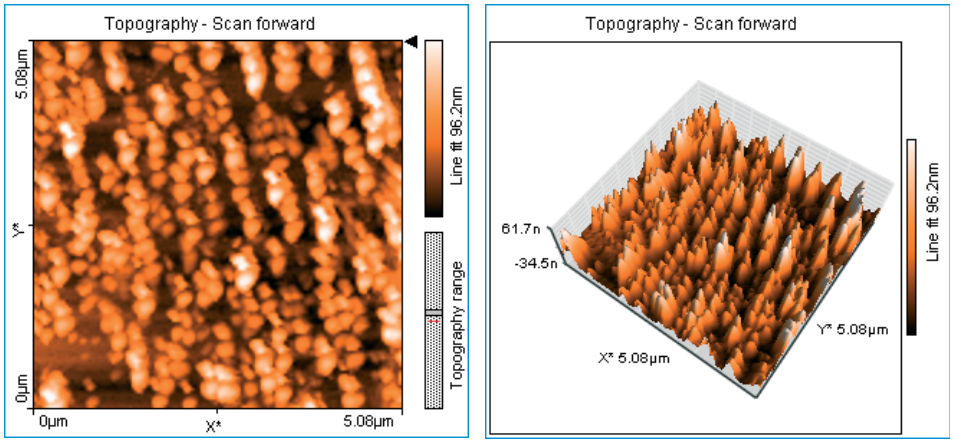
(7-b) T=45˚C



Two dimension Three dimension

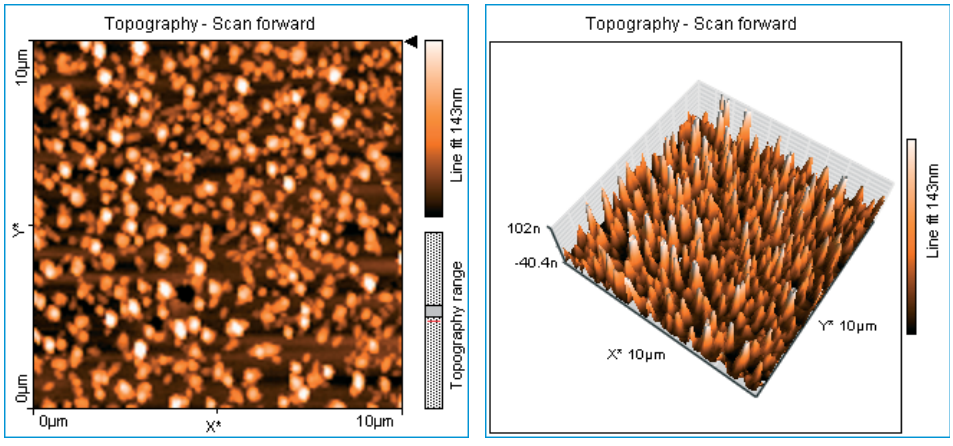
(7-c) T=55˚C

Fig. 7: Effect of temperature on topography of EA nanoparticle analysis by AFM (other constant operation conditions are: pH = 7_ C = 10 mg/ml, agitation speed = 600 rpm_ acetone volume = 50 ml).



Two dimension Three dimension

(8-a) C=20mg/ml



Two dimension Three dimension

(8-b) C=30mg/ml

Fig. 8: Effect of initial concentration on topography of EA nanoparticle analysis by AFM (other constant operation conditions are: T=35 °C_ pH=7_ agitation speed= 600 rpm_ acetone volume= 50 ml).

3.4 ANOVA Analysis and Determination of Optimal Conditions Using Taguchi Method

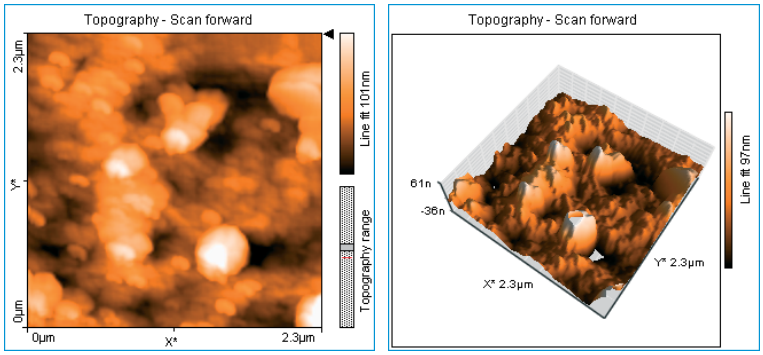
Table 3 explains ANOVA analysis for determination of significant parameters for affecting the nanoparticles size. As can be found from table 3 acetone volume has most important influence on particle size. The pH value has second most influencing parameter on the nanoparticle size also EA concentration and agitation speed has equal contribution on size. Thereby, based on ANOVA analysis, optimal factors are the temperature at level 3 (55 °C), EA concentration at level 3 (30 mg/ml), desolvating agent volume at level 1 (50 ml), agitation speed at level 1 (500 rpm), pH value at level 1 (4). Under optimal conditions, Taguchi program estimated EA nanoparticle size as 47 nm while we obtained 51 nm for particle diameter in our experiment.

Figure 12 (a – e) shows level average graph of raw data. Results explain that experimental data is in good agreement with Taguchi analysis. Size and size distribution of EA nanoparticles in the optimum condition are indi-

Table 3: The ANOVA analysis table.

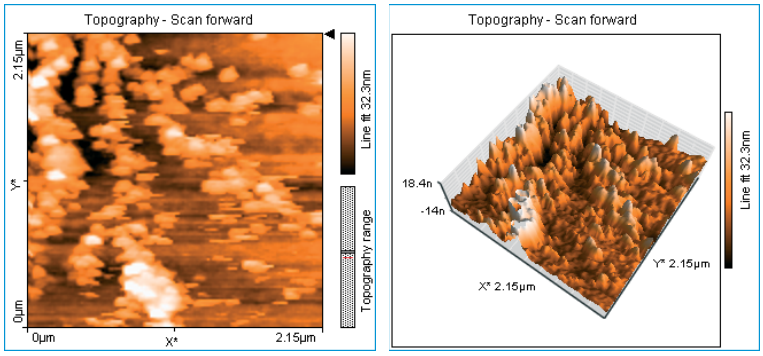
Factors	Degree of freedom	Sums of squares	variance	F-Ratio	Percent (%)
A(Temperature)	2	882.05	441.025	4.667	4.531
B(concentration)	2	1758.738	879.369	9.307	10.264
C(acetone volume)	2	5708.038	2854.019	30.207*	36.086
D(agitation speed)	2	1719.75	859.875	9.1	10.009
E(pH value)	2	4563.907	2281.953	24.152*	28.605
Error	7	661.372	94.481		10.505
Total	17	15293.859			100

*Main significant parameter



Two dimension Three dimension

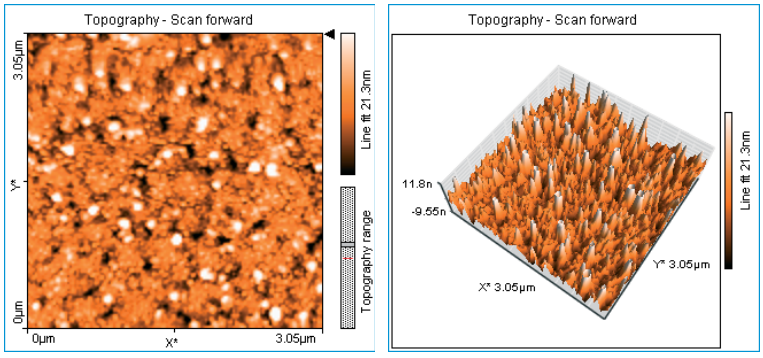
(9-a)V=75ml



Two dimension Three dimension

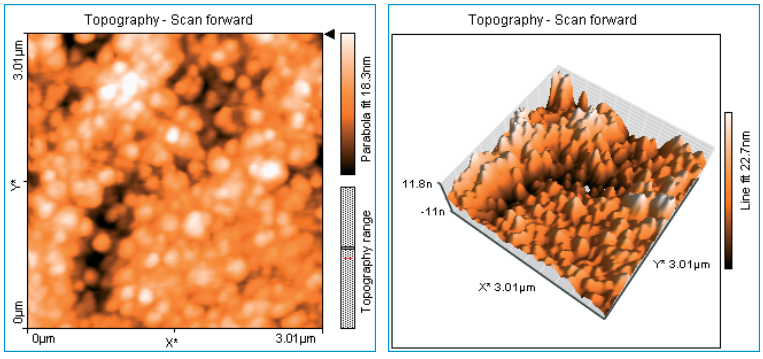
(9-b)V=100ml

Fig. 9: Effect of acetone volume on topography of EA nanoparticle analysis by AFM (other constant operation conditions are: T = 35°C_ C = 10 mg/ml_ agitation speed = 600 rpm_ pH=7).



Two dimension Three dimension

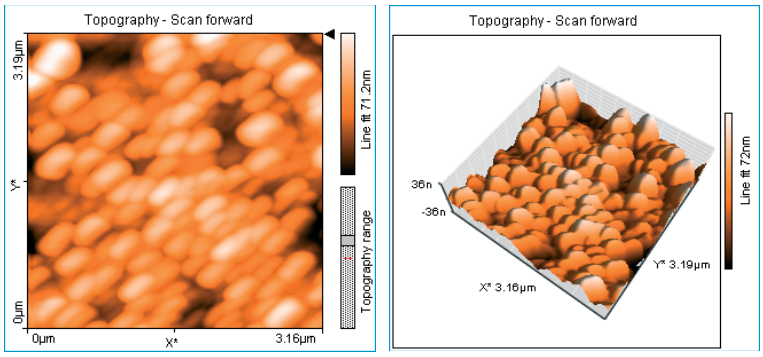
(10-a) pH=4



Two dimension Three dimension

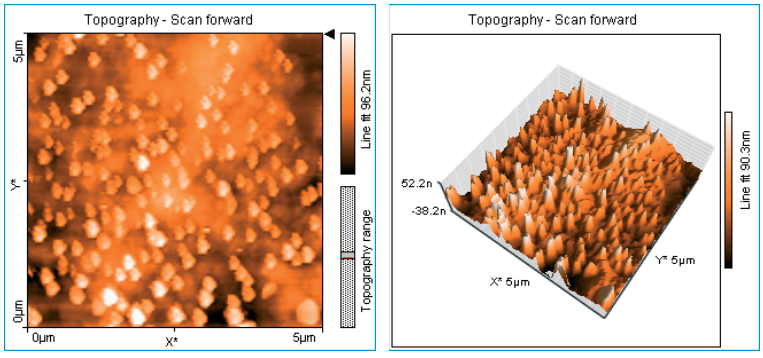
(10-b) pH=9

Fig. 10: Effect of pH on topography of EA nanoparticle analysis by AFM (other constant operation conditions are: T = 35 °C_ C = 10 mg/ml_ agitation speed = 600 rpm_ acetone volume = 50 ml).



Two dimension Three dimension

(11-a) Agitation speed=500rpm



Two dimension Three dimension

(11-b) Agitation speed=700rpm

Fig. 11: Effect of agitation speed on topography of EA nanoparticle analysis by AFM (other constant operation conditions are: T = 35 °C_ C = 10 mg/ml_ pH = 7_ acetone volume = 50 ml).

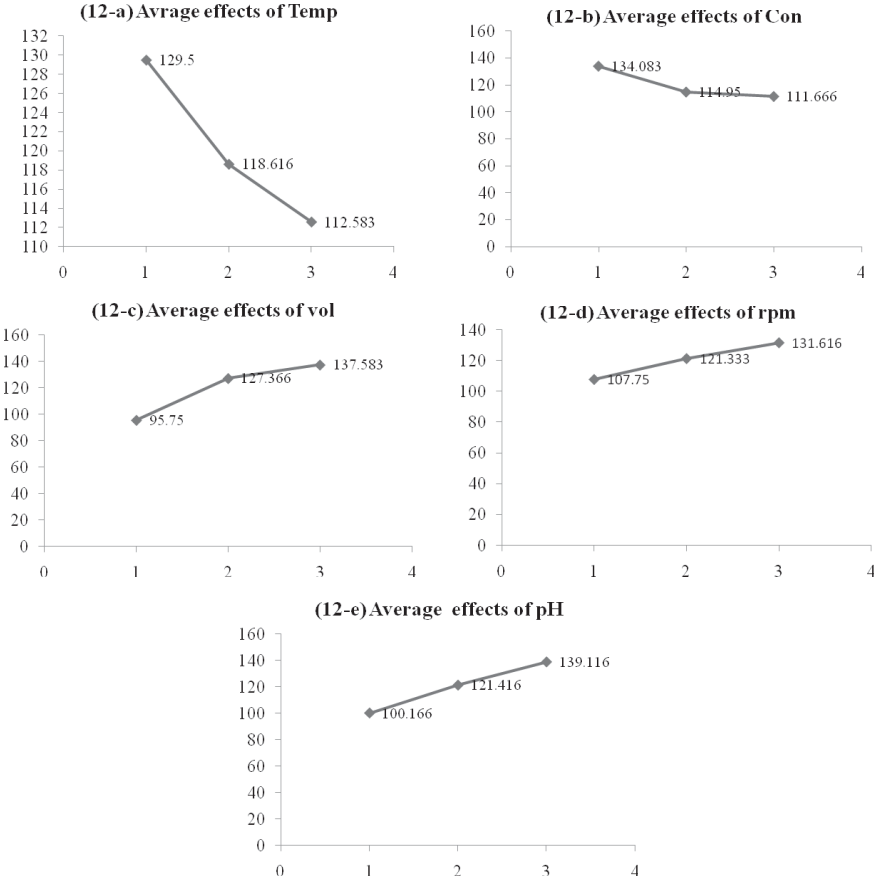


Fig. 12: (a – e) Response graph for significant parameters (The horizontal axis indicates the different levels of the each significant factor. The lines represent the trend of each factor with respect to different levels).

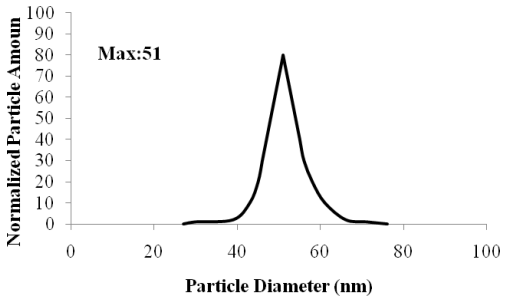


Fig. 13: Particle size distribution of EA nanoparticle in the optimal condition analysis by PCS.

cated in Figure 13. Excellent agreement between the predicted and experimental data was achieved.

4 Conclusions

Controlled drug delivery technology represents one of the frontier areas of science, which involves multidisciplinary scientific approach, contributing to human health care [41 – 43]. Nanoparticles represent many capabilities in pharmaceutical and food science [44]. According to our studies in this work, simple coacervation method is good and suitable way to prepare EA nanoparticles for drug delivery application. Nanoparticles size were manufactured herein from EA was influenced by various factors such as temperature, EA concentration, desolvating agent volume, pH value and agitation speed, So that particles with very small size were observed. Taguchi method was utilized for experiments design and analysis of experimental data. The best results (minimum size of EA) were obtained at T = 55°C, concentration = 30 mg/ml, acetone volume = 50 ml, pH = 4, agitation speed = 500 rpm. The minimum size was investigated 47 nm based on Taguchi method by software, while we attained 51 nm in our optimal experiment which is outstanding result.
Morphology study showed that shapes of particles for all of operation conditions are approximately semispherical and nanoparticles formed with smooth surface. Therefore produced EA nanoparticles are suitable enough to be used as drug carriers system. To the best of our knowledge, the current paper is the first discussing potential EA nanoparticles as delivery system and deserves further study. Loading drug on these nanoparticles will be the subject of our next publication.

Acknowledgements

The authors would like to gratefully acknowledge members of the Nanotechnology Research Institute of Babol University of Technology, Babol, Iran.

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