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Gelatin nanoparticle fabrication and optimization of the particle size

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The biotechnology industry has recently been demanding second generation of nanoparticle bioproducts such as viruses, plasmids, virus-like particles and drug-delivery assemblies (20–300 nm). The possibility of preparing uniform nanoparticles consisting of proteins such as gelatin followed by covalent linkage of avidin was investigated. Gelatin nanoparticles were prepared by two-step desolvation. As a colloidal drug-delivery system, the essential parameters in fabrication were optimized by the Taguchi design method. However, for characterizing the nanoparticles AFM and SEM were employed. By introducing 4 factors (temperature, gelatin concentration, agitation speed and the amount of acetone) in 4 levels to the software 16 experiments were carried out and the optimum condition was gained in 50 °C, 45 mg/ml gelatin concentration, 80 ml of acetone based on reduction of the size. The produced nanoparticles size was under 174 nm. The mechanistic of the optimum conditions for preparing protein nanoparticles as well as their characterization are discussed in detail.

1 Introduction In recent years the improvement of drug therapy in terms of a more controlled body distribution to reduce side effects was focused upon. Different new drug-carrier systems in the micro- and nanometer size range were generated to overcome these problems [1–3]. Colloidal drug-delivery systems, which have been developed, include liposomes and nanopartitles. The nanometer-size ranges of these delivery systems offer certain distinct advantages for drug delivery [4–6]. Nanoparticles are solid colloidal particles ranging in size from about 10 nm to 1000 nm [4, 7].

They consist of macromolecular materials and can be used as adjuvant in vaccines, or as drug carriers, in which the active principle (drug or biologically active material) is dissolved, entrapped, encapsulated and/or to which the active principle is adsorbed or attached [4].

Conventional drug therapy requires periodic doses of therapeutic agents. For most drugs conventional methods of drug administration are effective, but some drugs are unstable or toxic and have narrow therapeutic ranges [5, 8]. Some drugs also possess a solubility problem. In such cases a method of continuous administration of therapeutic agents is desirable. To overcome these problems, controlled drug-delivery systems were introduced. The principal advantage of this technology is that the carrier polymer matrix systems allow much less active agents to be used for the desired activity [5, 9]. Polymeric nanoparticles have attractive physicochemical properties such as size, surface potential, hydrophilic–hydrophobic balance, etc. and for this reason they have been recognized as potential drug carriers for bioactive ingredients such as anticancer drugs, vaccines, oligonucleotides, peptides, etc. [5, 10]. Although various biodegradable nanoparticles of natural polymers such as starch, chitosan, liposomes, etc., are largely in use as drug carriers in controlled drug-delivery technology, gelatin nanoparticles represent a promising carrier system for controlled drug delivery [11].

Gelatin has a number of advantages as a nanoparticle material, it is a natural macromolecule, nontoxic and non-carcinogenic nature, as it possesses a relatively low anti-

genicity and it has a great deal of background for its use parental formulations. The formation of gelatin-based nanoparticles has not been extensively investigated even though its first use as a base for nanoparticles was described more than 25 years ago [12, 13].

Thus being motivated by the application potential of gelatin in biomedical and pharmaceutical fields, we, in the present paper, prepared gelatin nanoparticles in a narrow size range through a two-step desolvation method and the effective parameters on its manufacture such as temperature, gelatin concentration, agitation speed, etc., will be discussed. The efficient analysis of the complex system using statistical experimental design [14] and the Taguchi method have been performed recently. The statistical experimental design can determine the effect of the factors on characteristic properties and the optimal conditions of factors. It uses tables of orthogonal arrays and analysis of variance (ANOVA), which can estimate the effect of a factor on the characteristic properties. Conventional statistical experimental design can determine the optimum condition on the basis of the measured values of the characteristic properties; while Taguchi's experimental design (also known as a robust parameter design) does this on the basis of the variability of characteristic properties. Our goal was to consider these factors and optimize the particle size and size distribution of gelatin nanoparticles by using the Taguchi design method to obtain the optimum condition for gelatin nanoparticle fabrication.

2 Experimental

2.1 Materials Gelatin type A (from porcine skin), glutaraldehyde grade 1.25% aqueous solution, HCl and acetone were obtained from Sigma, Poole, UK. Trypsin-EDTA was purchased from Gibco (New York, NY, USA). Double-distilled water was used for all the experiments. All chemicals were of analytical grade and used as-received.

2.2 Fabrication and purification of gelatin nanoparticles Gelatin nanoparticles were prepared using a desolvation technique. Gelatin type A (1.25 g) was dissolved in distilled water (25 ml) under constant heating. Acetone (25 ml) was added to the gelatin solution as a desolvating agent to precipitate the high molecular weight (HMW) gelatin. The supernatant was discarded and the HMW gelatin re-dissolved by adding 25 ml distilled water and stirring at 600 rpm under constant heating. The pH of the gelatin solution was adjusted at 2.5. Acetone (75 ml) was added drop-wise to form nanoparticles. At the end of the process, glutaraldehyde solution (250 µl) was used for preparing nanoparticles as a crosslinking agent, and stirred for 12 h at 600 rpm. The particles were purified by three-fold centrifugation and redispersion in acetone (30%) in milliQ water. After the last redispersion, the acetone was evaporated using concentrator (speed vacuum). The resultant nanoparticles were stored at 2–8 °C. The following parameters were changed to study their effect on the characteristics of the nanoparticles: temperature, rate of agitation, concentration of gelatin, concentration of acetone and crosslinker.

Table 1 Factors and levels in the fabrication of gelatin nanobioparticles.

factors	levels			
	1	2	3	4
A) temperature (°C)	40	50	55	60
B) gelatin conc. (mg/ml)	45	50	55	60
C) acetone (ml)	60	65	75	80
D) agitation speed (rpm)	500	600	700	800

Morphological features of gelatin nanoparticles were studied using SEM (Philips, 515) and AFM (Digit 3100, England). The particle size of the resulting nanoparticles was determined by photon correlation spectroscopy (PCS), (Zetasizer 3000, England).

2.3 Data analysis As was obtained in the literature, 4 imperative factors that influence the gelatin nanoparticle size were agitation rate, concentration of gelatin, amount of acetone and temperature [11]. For consideration of the effective parameters together on nanoparticle diameter size and in order to minimize the number of experiments, automatic design and analysis of Taguchi experiments was employed through Qualitek software (version IV). Choosing these four parameters that could affect the particle size used Taguchi's orthogonal array shown in Table 1.

The orthogonal array of L_{16} type was used. L and subscript 16 means Latin square and the number of experiments, respectively. Each experiment was carried out twice.

3 Result and discussion

3.1 Fabrication of gelatin nanoparticles A method of preparing protein (e.g. gelatin) nanoparticles has been described before according to Ref. [11] and subsequently modified and new approaches introduced by our group.

According to our pervious publication [15], different synthesis parameters that most affect nanoparticle size have been investigated. It was found that the preparation of nanoparticles at low temperature was not possible due to the fact that gelatin formed a highly viscous gel. However, increasing the temperature above 50 °C increased the particle size. This might be explained by the gelling properties of gelatin. The triple helical structure begins to uncoil when the temperature greatly increases since viscosity decreases simultaneously. At 50 °C, the chains seem to be sufficiently uncoiled and the addition of the desolvating agent caused a better controlled precipitation of the macromolecules. However, the isoelectric point of gelatin is approximately 6.1 and in order to form its nanoparticles logically the pH has to be adjusted away from its isoelectric point (i.e. 2.5). The addition of the desolvating agent reduced the water available to keep the gelatin in solution, resulting in shrinkage of the hydrated gelatin chains. At a

certain point the hydration was too low and the protein chains precipitated as nanoparticles. The effects of parameters and an alternative explanation about protein nanoparticles' fabrication and purification have been introduced in our previous publications [16–18].

3.2 Physical characterization of nanoparticles

3.2.1 Analysis of SEM and AFM A range of protein nanoparticles having broadly similar particles size and anionic characters to other nanoparticles such as adenovirus and pDNA, were fabricated based on simple coacervation. A SEM gelatin nanoparticle is shown in Fig. 1, which clearly shows that smooth and spherical nanoparticles with an average diameter of 100–300 nm were produced. The photograph clearly indicates that no hairline cracks or heterogeneity appear on the nanoparticles surface. This obviously presents morphological evidence for solid and smooth nanoparticles.

3.2.2 Particle-size analysis Average particle size was calculated by PCS and the measurement was also confirmed by SEM and AFM (Table 2).

3.3 Taguchi array design A Taguchi orthogonal array design was applied to identify the optimal conditions for manufacturing the smallest diameter size of the nanobioparticles. Table 3 shows the structure of Taguchi's orthogonal array design and the results. The purpose of the analysis of variance (ANOVA) is to investigate which factors significantly affect the quality characteristic.

3.4 Determination of optimal conditions The average S/N ratio of each control factor at each level and the range of the S/N ratio of each factor (D = delta = $S/N_{max} - S/N_{min}$) are determined. The greatest variation of the S/N ratio is related to the temperature and it means that this parameter (i.e., temperature) has the most important influence on the particle diameter. The amount of acetone addition has the second effect on the nanoparticle size.

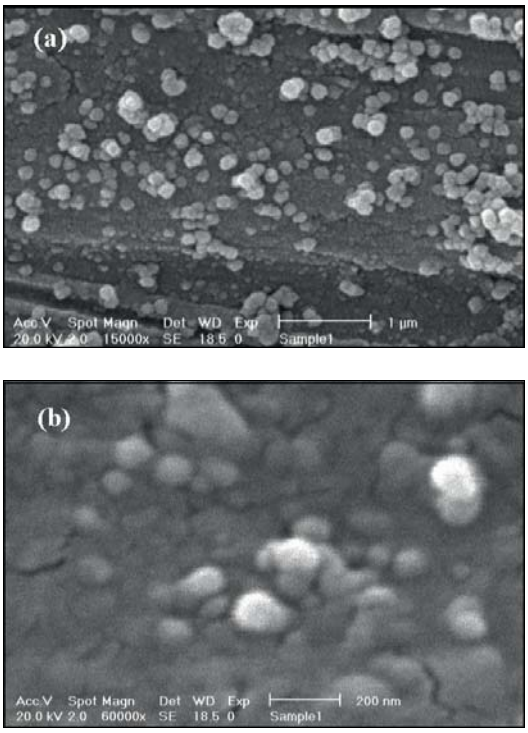

Figure 1 SEM images of gelatin nanoparticles a) 1 µm scale b) 200 nm scale, fabricated by two-step desolvation method.

Table 2 Experimental measured values for size distribution of gelatin nanoparticles.

exp. no.	polydispersity index	
	run 1	run 2
1	0.0218 ± 0.0067	0.0377 ± 0.0137
2	0.0471 ± 0.0079	0.0797 ± 0.0161
3	0.0341 ± 0.0065	0.0157 ± 0.0073
4	0.0489 ± 0.0123	0.0308 ± 0.0307
5	0.0595 ± 0.0071	0.0489 ± 0.0151
6	0.0827 ± 0.0070	0.0689 ± 0.0094
7	0.0303 ± 0.0117	0.0277 ± 0.0120
8	0.0916 ± 0.0102	0.0889 ± 0.0122
9	0.0477 ± 0.0237	0.0329 ± 0.0095
10	0.0671 ± 0.0043	0.0514 ± 0.0068
11	0.0749 ± 0.0131	0.0916 ± 0.0288
12	0.0336 ± 0.0045	0.0448 ± 0.0013
13	0.0750 ± 0.0234	0.0650 ± 0.0058
14	0.0260 ± 0.0086	0.0189 ± 0.0065
15	0.0516 ± 0.0209	0.0610 ± 0.0013
16	0.0855 ± 0.0092	0.0801 ± 0.0210

Table 3 Experimental measured values for size of gelatin nanoparticles and S/N ratio (Taguchi orthogonal array table of L_{16}).

exp. no.	experimental conditions				diameter (nm)		S/N ratio (dB)
	A	B	C	D	run 1	run 2	
1	1	1	1	1	301.1	297.5	–49.5
2	1	2	2	2	286.3	289.7	–49.1
3	1	3	3	3	290.4	306.3	–49.4
4	1	4	4	4	240.0	237.9	–47.5
5	2	1	2	3	194.0	195.6	–45.7
6	2	2	1	4	248.0	231.9	–47.6
7	2	3	4	1	209.1	215.2	–46.5
8	2	4	3	2	198.4	225.1	–46.5
9	3	1	3	4	230.3	224.6	–47.1
10	3	2	4	3	199.0	190.8	–45.7
11	3	3	1	2	206.8	201.7	–46.2
12	3	4	2	1	292.0	289.4	–49.2
13	4	1	4	2	245.2	251.3	–47.8
14	4	2	3	1	241.0	268.7	–48.1
15	4	3	2	4	298.2	295.6	–49.4
16	4	4	1	3	316.0	314.7	–49.9

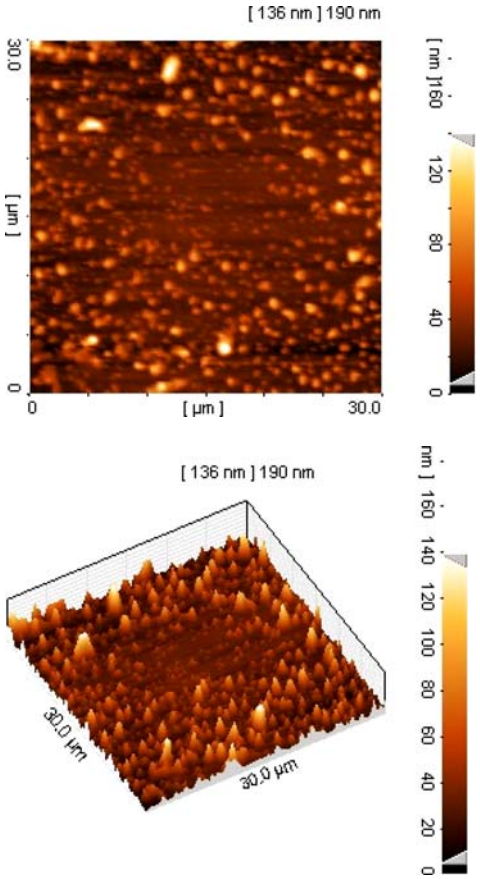

Figure 2 (online colour at: www.pss-a.com) Morphology AFM images of gelatin nanoparticles prepared with two-step desolvation.

Table 4 shows the main effects on the gelatin nanoparticle diameter size.

From Table 4 and Fig. 3 it can be seen that the temperature and amount of acetone are the significant parameters affecting the size. Therefore, based on the *S*/*N* and ANOVA analyses, the optimal parameters for nanoparticle size are the temperature at level 2, the gelatin concentration at level 1, the amount of acetone at level 4 and agitation speed at level 2. The best set of parameters for nanobioparticiples production were: temperature: 50 °C, gelatin concentration: 45 mg/ml, rate of acetone adding: 80 ml and 600 rpm agitation. Under these conditions the program estimated the gelatin nanoparticle diameter as 165.7 nm, while in the experiment 174 nm was achieved for the nanoparticle diameter. Figure 4 illustrates the gelatin

Table 4 ANOVA table of size of gelatin nanoparticles.

factors	degree of freedom	sums of squares	variance	*F* ratio
A	3	17.185	5.72*	2.87
B	3	1.341	0.44	0.22
C	3	5.448	1.81*	0.91
D	3	1.743	0.58	0.29
error	3	5.978	1.99	
total	15	31.698		

* main significant parameter

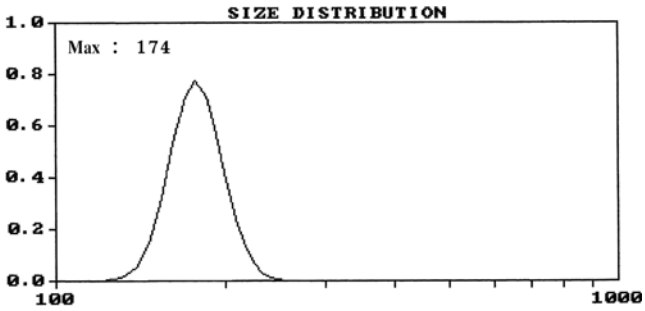

Figure 4 Size distribution and diameter size of the fabricated gelatin nanoparticles in the optimal conditions.

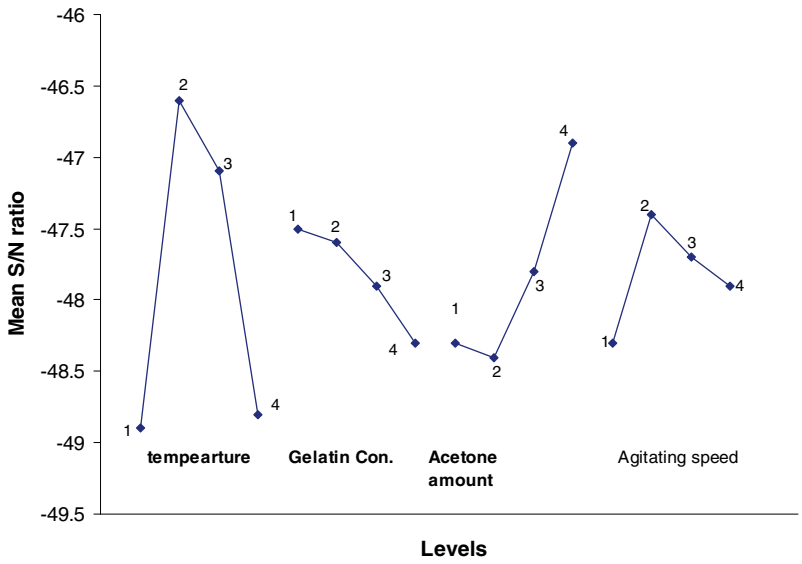

Figure 3 Response graph of *S*/*N* ratio for smaller-the-better analysis of nanoparticle size.

nanoparticle size distribution and size in the optimum condition. There is good agreement between the predicted and experimental particle size. Consequently, particle size in the fabrication of gelatin nanoparticle can be decreased through the Taguchi method approach.

4 Conclusion Our systematic investigation of the synthesis parameters shows that it is possible to prepare nanoparticles with different particle sizes and a relatively narrow size distribution. Due to the SEM and AFM analysis, the protein nanoparticle as assembled here, not only mimics the size and surface chemistry of nanoparticles such as viruses and plasmid, but also can be used as a drug-delivery vehicle in its own right. Gelatin type A was used in the two-step desolvation method for preparation of nanoparticles. The nanoparticle size fabricated here was influenced by several process variables including agitation speed, temperature and gelatin concentration, etc. The best result (minimum size of the gelatin nanoparticles) was attained at 50 °C, 45 mg/ml gelatin concentration, 80 ml acetone with 600 rpm agitating, by these conditions the gelatin nanoparticle diameter of 174 nm was achieved.

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