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Research Article

5-Fluorouracil-Loaded BSA Nanoparticles: Formulation Optimization and *In Vitro* Release Study

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Abstract. Over the past few decades, there has been considerable interest in developing protein nanoparticles as drug delivery devices. The underlying rationale is their exceptional characteristics, namely biodegradability and nonantigenicity. Herein, phase separation method was used to prepare 5-fluorouracil-loaded bovine serum albumin (BSA) nanoparticles. Drug release was tracked by continuous flow dialysis technique. Effect of process variables on loading efficiency of 5-fluorouracil was investigated and optimized through Taguchi's M16 design with the amount of entrapped drug as response. Optimum condition was found to be 2 mg/mL of 5-fluorouracil, 3.7 mL of added ethanol, 176 μL of glutaraldehyde, drug–protein incubation time of 30 min, and pH of 8.4 for 200 mg of BSA in 2 mL drug solution. pH had the most noticeable effect on the amount of entrapped drug, but glutaraldehyde had the least. Mean diameter and zeta potential of fabricated nanoparticles under these conditions were 210 nm and −31.7 mV, respectively. Drug-loaded BSA nanoparticles suspension maintained constant release of drug for 20 h under experimental conditions, so this colloidal drug carrier is capable of releasing drug in a sustained manner.

KEY WORDS: 5-fluorouracil; BSA; drug release; nanoparticle; optimization.

INTRODUCTION

Nanoparticles are solid colloidal particles ranging in size from 10 to 1,000 nm. They may be built up from either synthetic or natural macromolecules. Natural macromolecules, and above all proteins, have gained much interest as biomaterials owing to their inherent properties of biodegradability, lack of toxicity, and nonantigenicity (1–4). Colloidal systems based on proteins might be incredibly promising as they unite the advantages of nanoparticles with the advantages of covalent protein–drug conjugates (5,6). Protein nanoparticles, due to the existence of charged groups, may also be used as a matrix in which drugs can be physically entrapped (7–9). Albumin, being widespread in nature, is an attractive macromolecular carrier and has been a subject of major interest. The potential therapeutic usefulness of albumin lies in its capacity to accommodate a wide variety of drugs in a relatively nonspecific fashion and its amenability to preparation in large batches in a simple and cost-effective manner (4).

Unlike controlled release oral formulations, no regulatory standard exists for assessing *in vitro* drug release from parenteral nanoparticulate delivery systems. In addition, the current US Pharmacopeia (USP) apparatus for *in vitro* release testing was designed mainly for oral and transdermal products and is not directly applicable for parenteral products administered subcutaneously or intramuscularly (10). Over the past decade, there have been attempts to compare *in vitro* test methods to study drug release from parenteral biodegradable nanospheres, and some noteworthy release techniques have been developed (11,12). Common methods for nanoparticulate systems fall into three broad categories: sample and separate methods, continuous flow, and dialysis (10).

Considering 5-fluorouracil (5-FU) as a typical drug, different techniques have been used to study its release from nanoparticles, heretofore. Dialysis simplicity has been exploited for tracking 5-FU release from poly(lactic-*co*-glycolic acid), chitosan, pullulan–sulfonamide, and poly(γ-benzyl-L-glutamate) nanoparticles (13–18). Some researchers used Franz cell dissolution apparatus or paddle apparatus (USP XXIII) to analyze the *in vitro* 5-FU release from polymeric nanoparticles (19,20). In addition, sampling from release medium at predetermined times and separating nanoparticles through centrifugation or ultrafiltration was used for assessing *in vitro* 5-FU release (21,22). Altogether, the variety of methods for tracking *in vitro* release profile of this model drug has made it impossible to make a reasonable comparison between various nanoparticulate systems.

In the present study, 5-FU was selected as a model drug. Due to advantages of protein nanoparticles as mentioned before, BSA nanoparticle has been chosen as a carrier for this drug. Since there is no comprehensive study in the literature concerning the influence of process variables on drug loading

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Table I. Factors and Their Levels in Taguchi's M16 Design

	Factor	Units	Level 1	Level 2	Level 3	Level 4
A	C_{drug}	mg/mL	0.5	1	1.5	2
B	$V_{ethanol}$	mL	3.5	3.6	3.7	3.8
C	$V_{glutaraldehyde}$	μL	59	118	176	235
D	$t_{incubation}$	min	0	30	60	90
E	pH		7.2	7.8	8.4	9

capacity, the effect of process variables on drug loading was investigated and optimum condition for preparing 5-FU-loaded BSA nanoparticles by coacervation method was determined using Taguchi's design of experiments. The amount of drug entrapped in the nanoparticle is of utmost important since it ultimately determines the quantity of formulation for the administration (4). Then, a simple and straightforward method has been adapted from Kostanski and Deluca (12) to track drug release.

MATERIALS AND METHODS

Materials

BSA (fraction V, minimum 98%), glutaraldehyde 8% solution, and 5-fluorouracil 99% were purchased from Sigma (Steinheim, Germany). All other reagents were purchased from Merck (Darmstadt, Germany); they were of analytical grade and used as received.

Methods

Preparation of 5-Fluorouracil-Loaded BSA Nanoparticles

5-Fluorouracil-loaded BSA nanoparticles were prepared by a desolvation method as described elsewhere (23). Briefly, 0.2 g BSA in 2.0-mL aqueous drug solution, titrated to desired pH and incubated at room temperature, was converted to nanoparticles by addition of desolvating agent, ethanol, at the rate of 1.0 mL/min and under stirring (550 rpm) at room temperature. Subsequently, 8% glutaraldehyde aqueous solution was added to induce particle cross-linking. The cross-linking process was performed under stirring of the suspension over night. Experimental values of drug concentration, pH, drug–BSA incubation time, and volumes of ethanol and glutaraldehyde were variable in optimization trials and are listed in Table I.

Optimization of the Preparation Conditions for 5-Fluorouracil-Loaded BSA Nanoparticles

Drug concentration, volume of ethanol, volume of glutaraldehyde, incubation time of drug–BSA solution, and pH value of protein solution have been considered as the most important factors for further investigation by Taguchi's method for experimental design (M16 array, five factors and four levels; Table I). Each M16 trial was performed twice. Factors such as temperature, due to the poor solubility of the drug at low temperatures and its poor stability at higher temperatures, and ethanol addition rate, since it only may affect particle size distribution (23), were neglected.

The values of levels of drug concentration were chosen according to a previous research (21) and several preliminary experiments. Ethanol levels were selected from the first signs of turbidity of solution to the first signs of protein clotting through coacervation process. Levels of glutaraldehyde and pH were assigned as per some early studies (6,23). Incubation time levels were selected according to Merodio *et al.* (7).

Separation of BSA Nanoparticles

Microparticles were precipitated by centrifugation (15,000×*g*, 2 min) and then discarded. Nanoparticles were separated by two cycles of centrifugation (25,000×*g*, 20 min) and redispersion of the pellet to the original volume in 10 mM NaCl at pH 9. Each redispersion step was carried out using ultrasonication (dr.hielscher, UP 400S, Germany) for 5 min.

Determination of Drug Content of Nanoparticles

Following separation of nanoparticles, pellets were dispersed to the original volume in phosphate buffered saline (PBS) buffer pH 7.4. Afterward, for complete liberation of

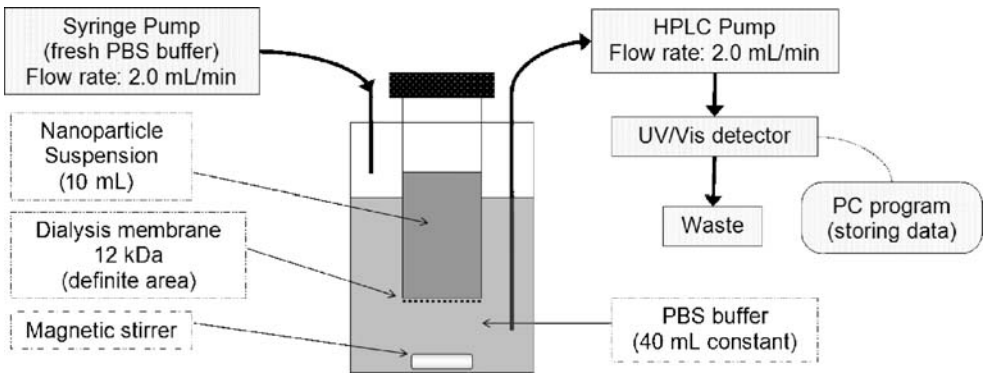

Fig. 1. Continuous flow dialysis setup used for determination of drug release profile

Table II. Variance Analysis of Data

	Factor	Degree of freedom	Sum of squares	Variance	Percent
A	C_{drug}	3	161.582	53.86	11.579
B	$V_{ethanol}$	3	199.841	66.613	14.321
C	$V_{glutaraldehyde}$	3	54.916	18.305	3.935
D	$t_{incubation}$	3	334.957	111.652	24.005
E	pH	3	644.066	214.688	46.157
	Sum	15	1,395.365		100

entrapped drug, 50 μL of trypsin solution (1 mg/mL) was added and the resulted solution was incubated at 37°C for 8 h. Later, total drug concentration was determined spectrophotometrically at 266 nm.

Determination of Particle Size and Zeta Potential

Average particle size was measured by photon correlation spectroscopy using a Malvern zetasizer 3000HS (Malvern Instruments Ltd., Malvern, UK). The samples were measured at a temperature of 25°C and a scattering angle of 90°. Zeta potential of nanoparticles was determined at 25°C and pH 7 by means of the same instrument.

In Vitro Drug Release Profile

Drug release profile was determined by using a dialysis technique adapted from Kostanski and DeLuca (12) and altered for continuous acquisition of released drug concentration. This continuous flow dialysis setup is schematically shown in Fig. 1. The setup is comprised of a 10-mL glass tube with a dialysis membrane at the bottom, which is immersed in a beaker containing 40-mL PBS buffer. Drug-loaded nanoparticles were prepared at optimum condition and after centrifugation were redispersed to the tenth of the original volume in PBS buffer (pH 7.4). This suspension was poured into the glass tube. The buffer containing released drug from nanoparticle suspension was pumped to a UV/Vis detector (266 nm) at the rate of 2.0 mL/min. Fresh buffer was added to the beaker at the same rate, in order to keep the volume constant. Dialysis setup was kept at room temperature through the experiment. Similar trial has been conducted by a drug solution with the same drug content of utilized nanoparticles.

RESULTS AND DISCUSSION

Optimization of Preparation Process

Design of experiments by Taguchi orthogonal array is a factorial-based approach, which merges statistical and engineering techniques. This approach facilitates the study of a system by a set of independent variables (factors), over a specific region of interest (levels). While traditional experimental design focuses on the average process performance characteristics, this approach concentrates on the effect of variation on the process characteristics and makes the product–process performance insensitive to variation by proper design of parameters. The degree of variation can be expressed by signal to noise (S/N) ratio. The experimental condition having the maximum S/N ratio is considered as the optimal condition. Analysis of the experimental data using analysis of variance and factor effects provides information about statistically significant factors and results in finding optimum levels of factors for design of parameters (24).

Acquired data from optimization trials were analyzed by making use of S/N ratio and considering the total amount of entrapped drug in produced nanoparticles as response. Variance analysis and optimum condition are presented in Tables II and III, respectively. Table II indicates the percentage of significance of each factor. pH is the most significant factor with about 46% of significance, but glutaraldehyde volume is the least with merely 3.9%. Table III summarizes the optimum condition, which is available from Fig. 2a–e. To validate experimental analysis results, two experiments were conducted at optimum condition. The total amounts of entrapped drug were 1.1 and 1.2 mg per 200 mg BSA, with an entrapment efficiency of 27.5% and 30%, respectively. Mean diameter, polydispersity, and zeta potential of prepared nanoparticles under optimum condition was 210 nm, 0.14, and −31.7 mV, respectively.

Figure 2 reveals the variation of response *versus* levels of factors. For better understanding, the vertical axis, i.e., S/N ratio, is converted to response unit ($Y=10^{(S/N)/20}$) (24). Figure 2a shows that the average amount of entrapped drug increases with drug concentration. It is due to the increase in quantity of adsorbed drug on BSA molecules and at the same time the increase in concentration of entrapped drug. Santhi *et al.* observed a similar trend for increasing drug to protein ratio (21). According to Fig. 2b, e, ethanol and pH graphs both reach their maximum at the third level. This observation is explained by a larger number of drug-loaded nanoparticles formed in these conditions. Through increase in the volume of cross-linking agent, nanoparticles acquire more stability–rigidity and hence mislay less drug during separation steps, which results in the rising trend seen in Fig. 2c. This means by using higher concentration of cross-linking agent more rigid nanoparticles will result. Increasing incubation time of drug–protein solution showed no

Table III. Optimum Condition for Preparation of 5-Fluorouracil-Loaded BSA Nanoparticles

	Factor	Level	Quantity
A	C_{drug}	4	2 mg/mL
B	$V_{ethanol}$	3	3.7 mL
C	$V_{glutaraldehyde}$	3	176 μL
D	$t_{incubation}$	2	30 min
E	pH	3	8.4

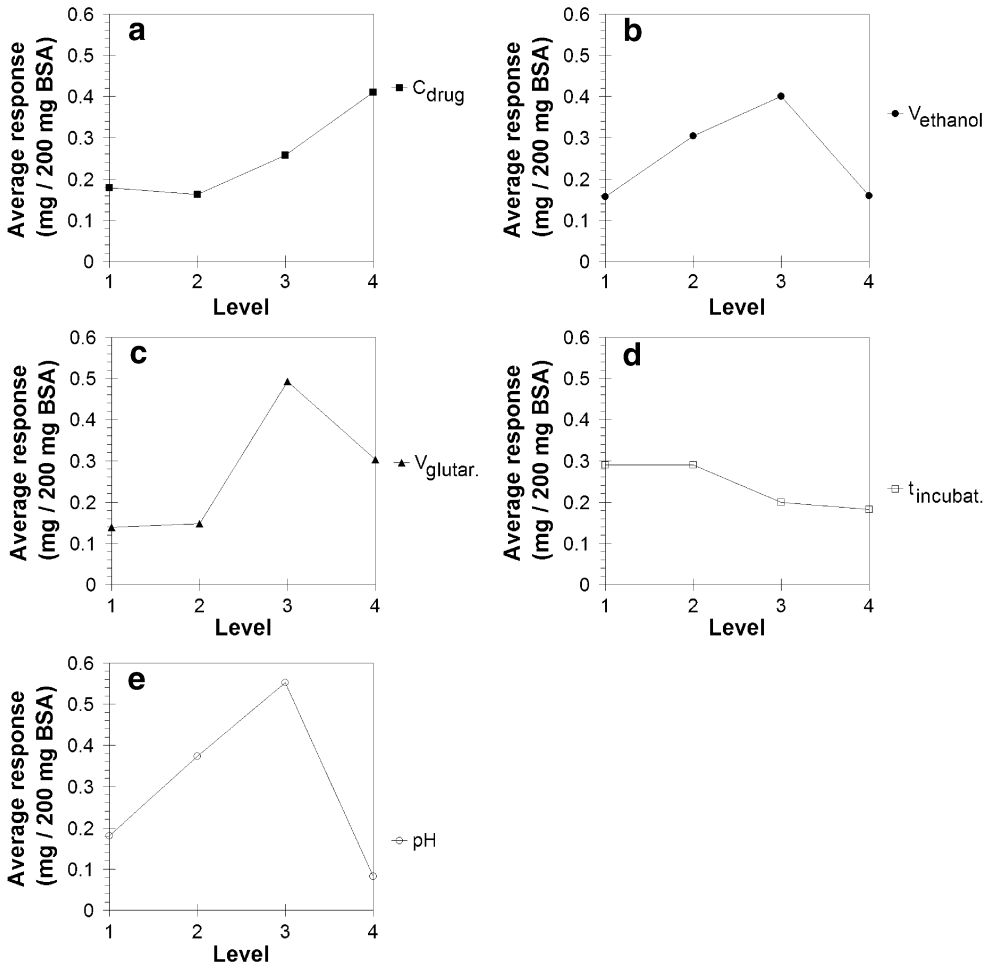

Fig. 2. Main effects plot (larger is better) for L16 array of Taguchi's experimental design; Drug concentration **a**, ethanol's volume **b**, glutaraldehyde's volume **c**, incubation time **d**, pH **e**

significant impact on amount of entrapped drug (Fig. 2d). Thus, the time required for preparation of drug–protein solution and titration of its pH to desired value is enough for the complete interaction between drug and protein.

In Vitro Drug Release

To assess drug release, a method was adapted from Kostanski and DeLuca (12). Some essential points were considered in adapting this method. The dialysis membrane molecular weight cutoff was selected much greater than drug, allowing the method to show burst effect phenomenon, if any. Membrane surface area was constant in all experiments. The volume of buffer was maintained constant through addition of fresh buffer to glass beaker.

Drug release from produced nanoparticles under optimum condition was followed using above-mentioned method. Figure 3 is the result of continuous acquisition of drug concentration in effluent. In this figure, *y*-axis is converted from voltage to concentration unit by a standard curve and

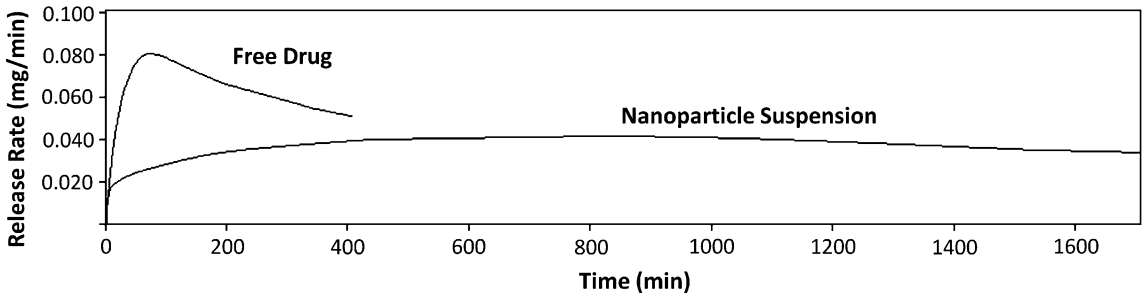

Fig. 3. Comparison of 5-fluorouracil release rate profile from dialysis setup in free form and loaded in BSA nanoparticles

then to release rate with its multiplication by flow rate (2.0 mL/min). As this figure represents, nanoparticle suspension has maintained a constant concentration of drug in effluent for about 20 h. This is because of constant rate release of the drug into the release medium. After this time, effluent concentration starts to decline gradually. In contrast, free drug solution, because of burst diffusion of drug through dialysis membrane, produced a peak-like profile. This also confirms that the utilized method for assessing drug release is able to reflect burst release of 5-FU. Thus, 5-fluorouracil-loaded BSA nanoparticles are capable of sustained release of drug without any burst release.

CONCLUSION

Preparation process for 5-fluorouracil-loaded BSA nanoparticles was studied by M16 array of Taguchi's experimental design. Optimum condition for the production of 5-FU-loaded BSA nanoparticles was as follows for 200 mg BSA in 2-mL drug solution: 2 mg/mL of 5-FU, 3.7 mL of added ethanol, 176 μL of glutaraldehyde, incubation time of 30 min, and pH of 8.4. This leads to production of nanoparticles with mean size of 210 nm. The total amount of entrapped drug rises with drug concentration and volume of glutaraldehyde but reaches its maximum at the third level in the case of ethanol volume and pH. Drug–BSA incubation time graph shows a quasilinear trend. Hence, the time spent for preparation of drug–protein solution and titration of its pH is enough for full interaction between drug and protein. Comparison between release profiles of free drug and nanoparticle suspension confirms that this colloidal drug carrier is capable of releasing drug in a controlled manner. The adapted method for assessing drug release has the quality of being a standard technique for nanoparticulate drug delivery systems since it combines reliability of data and simplicity with the ability of revealing burst release by proper selection of membrane molecular weight cutoff.

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REFERENCES

1. F. DeJaeghere, E. Doelker, and R. Gurny. Nanoparticles. In E. Mathiowitz (ed.), *Encyclopedia of Controlled Drug Delivery*, Wiley, Hoboken, 1999, pp. 641–664.
2. S. M. Moghimi, A. C. Hunter, and J. C. Murray. Long-circulating and target-specific nanoparticles: theory to practice. *Pharmacol. Rev.* **53**:283–318 (2001).
3. B. G. Muller, H. Leuenberger, and T. Kissel. Albumin nanospheres as carriers for passive drug targeting: an optimized manufacturing technique. *Pharm. Res.* **13**:32–37 (1996).
4. G. V. Patil. Biopolymer albumin for diagnosis and in drug delivery. *Drug Dev. Res.* **58**:219–247 (2003).
5. M. Michaelis, K. Langer, S. Arnold, H. Doerr, J. Kreuter, and J. Cinatl. Pharmacological activity of DTPA linked to protein-based drug carrier systems. *Biochem. Bioph. Res. Co.* **323**:1236–1240 (2004).
6. C. Weber, C. Coester, J. Kreuter, and K. Langer. Desolvation process and surface characterization of protein nanoparticles. *Int. J. Pharm.* **194**:91–102 (2000).
7. M. Merodio, A. Arnedo, M. J. Renedo, and J. M. Irache. Ganciclovir-loaded albumin nanoparticles: characterization and *in vitro* release properties. *Eur. J. Pharm. Sci.* **12**:251–259 (2001).
8. M. Merodio, J. M. Irache, F. Valamanesh, and M. Mirshahi. Ocular disposition and tolerance of ganciclovir-loaded albumin nanoparticles after intravitreal injection in rats. *Biomaterials*. **23**:1587–1594 (2002).
9. W. Lin, M. C. Garnett, S. S. Davis, E. Schacht, P. Ferruti, and L. Illum. Preparation and characterization of rose Bengal-loaded surface-modified albumin nanoparticles. *J. Control. Rel.* **71**:117–126 (2001).
10. S. S. D'Souza, and P. P. DeLuca. Methods to assess *in vitro* drug release from injectable polymeric particulate systems. *Pharm. Res.* **23**:460–474 (2006).
11. S. S. D'Souza, and P. P. DeLuca. Development of a dialysis *in vitro* release method for biodegradable microspheres. *AAPS PharmSciTech*. **6**2:E323–E328 (2005).
12. J. W. Kostanski, and P. P. DeLuca. A novel *in vitro* release technique for peptide-containing biodegradable microspheres. *AAPS PharmSciTech*. **11**:4 (2000).
13. M. Boisdron-Celle, P. Menei, and J. P. Benoit. Preparation and characterization of 5-fluorouracil-loaded microparticles as biodegradable anticancer drug carriers. *J. Pharm. Pharmacol.* **47**:108–114 (1995).
14. N. Faisant, J. Siepmann, and J. P. Benoit. PLGA-based microparticles: elucidation of mechanisms and a new, simple mathematical model quantifying drug release. *Eur. J. Pharm. Sci.* **15**:355–366 (2002).
15. A. Bozkir, and O. M. Saka. Formulation and investigation of 5-FU nanoparticles with factorial design-based studies. *Il Farmaco*. **60**:840–846 (2005).
16. Y. Zheng, W. Yang, C. Wang, J. Hu, S. Fu, L. Dong, L. Wu, and X. Shen. Nanoparticles based on the complex of chitosan and polyaspartic acid sodium salt: preparation, characterization and the use for 5-fluorouracil delivery. *Eur. J. Pharm. Biopharm.* **67**:621–631 (2007).
17. L. Liu, P. Jin, M. Cheng, G. Zhang, and F. Zhang. 5-fluorouracil-loaded self-assembled pH-sensitive nanoparticles as novel drug carrier for treatment of malignant tumors. *Chin. J. Chem. Eng.* **14**:377–382 (2006).
18. S. Li, A. Wang, W. Jiang, and Z. Guan. Pharmacokinetic characteristics and anticancer effects of 5-fluorouracil loaded nanoparticles. *BMC Cancer.* **8**:103 (2008).
19. P. A. McCarron, A. D. Woolfson, and S. M. Keating. Sustained release of 5-fluorouracil from polymeric nanoparticles. *J. Pharm. Pharmacol.* **52**:1451–1459 (2000).
20. A. Lamprecht, H. Yamamoto, H. Takeuchi, and Y. Kawashima. Microsphere design for the colonic delivery of 5-fluorouracil. *J. Control. Rel.* **90**:313–322 (2003).
21. K. Santhi, S. A. Dhanaraj, V. Joseph, S. Ponnusankar, and B. Suresh. A study on the preparation and anti-tumor efficacy of bovine serum albumin nanospheres containing 5-fluorouracil. *Drug Dev. Ind. Pharm.* **28**:1171–1179 (2002).
22. C. Lo, K. Lin, and G. Hsiue. Preparation and characterization of intelligent core-shell nanoparticles based on poly(*d*,*l*-lactide)-*g*-poly(*N*-isopropyl acrylamide-*co*-methacrylic acid). *J. Control. Rel.* **104**:477–488 (2005).
23. K. Langer, S. Balthasar, V. Vogel, N. Dinauer, H. von Briesen, and D. Schubert. Optimization of the preparation process for human serum albumin (HSA) nanoparticles. *Int. J. Pharm.* **257**:169–180 (2003).
24. R. K. Roy. *A Primer on the Taguchi Method*, Van Nostrand Reinhold, New York, 1990.